

miR-22-5p revealed as a potential biomarker involved in the acute phase of myocardial infarction via profiling of circulating microRNAs

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Abstract. Acute myocardial infarction (AMI) is a life-threatening episode of coronary artery disease. Recently, circulating myocardial-derived microRNAs (miRNAs) have been reported as potential biomarkers of infarction. The present study aimed to identify differentially expressed miRNAs in patients with ST-segment elevation myocardial infarction that could be potentially dysregulated in response to early myocardial damage. miRNA expression profile analysis was performed using the Serum/Plasma Focus miRNA Polymerase Chain Reaction (PCR) panel of Exiqon A/S (Vedbaek, Denmark) on plasma samples of patients on the first day of AMI (admission) and on samples from the identical patients collected six months following AMI. Selected miRNAs were validated by reverse transcription-quantitative PCR (RT-qPCR) using independent patients with AMI and a control group of patients with a stable coronary artery disease. Thirty-two species of plasma

miRNA were differentially expressed ($P < 0.05$) on admission compared with six months following AMI. Subsequent validation in an independent patient group confirmed that miR-133b and miR-22-5p were significantly up-regulated in the serum of patients with AMI. The receiver operating characteristic (ROC) curve analysis demonstrated a diagnostic utility for miR-22-5p, which has not previously been reported to be associated with AMI. Among the selected miRNAs, miR-22-5p represents a novel promising biomarker for the diagnosis of AMI.

Introduction

Acute myocardial infarction (AMI) is a life-threatening episode of coronary artery disease, and an as yet unresolved clinical issue with high morbidity and mortality. Insufficient blood supply and oxidative stress result in necrosis of cardiac tissue, pathological remodeling and left ventricular dysfunction (1-3). An early and accurate diagnosis of AMI is essential for an optimal treatment outcome. Therefore, new approaches that are able to complement and improve current strategies for AMI diagnosis are urgently needed.

Recent discoveries have revealed the existence of stable cardiomyocyte-enriched microRNAs (miRNAs) circulating in human blood cells or plasma/serum (4,5). miRNAs are small, non-coding RNA molecules, 20-25 nucleotides long, which inhibit gene expression by promoting messenger RNA (mRNA) degradation or inhibiting translation (6-8). It is noteworthy that numerous studies have revealed that some fraction of the circulating miRNAs is secreted from healthy or damaged cells (9). The fact that these miRNAs are able to be detected in peripheral blood and are relatively stable in serum, plasma and other biofluids makes them potentially useful in aiding diagnosis or guiding therapy through rapid and easy tests eliminating the necessity of performing an invasive procedure (10,11).

The present study aimed to compare the miRNA profiles in plasma samples of patients on the first day of AMI (admission)

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Abbreviations: AMI, acute myocardial infarction; BNP, B-type natriuretic peptide; CAD, coronary artery disease; CI, confidence interval; IPA, ingenuity pathway analysis; miRNA, microRNA; NT-proBNP, N-terminal pro B-type natriuretic peptide; PCA, principal component analysis; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; STEMI, ST-segment elevation myocardial infarction

Key words: circulating microRNAs, microRNA expression profiles, miR-133b, miR-22-5p, acute myocardial infarction

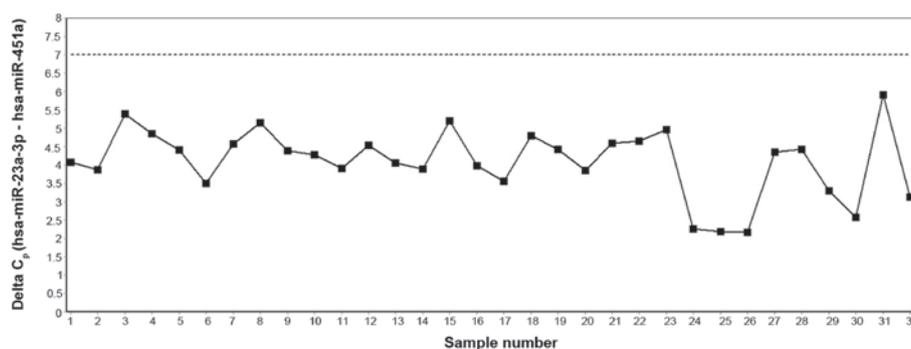


Figure 1. Hemolysis test of plasma samples. miR-451a and miR-23a-3p were determined by RT-qPCR in plasma samples from patients on the first day and six months following MI. The Cp value of miR-23a-3p minus the Cp value of miR-451a is plotted. The difference does not reach 7 (horizontal dotted line), set as indicative of hemolysis, in any of the samples. RT-qPCR, quantitative reverse transcription-polymerase chain reaction; MI, myocardial infarction.

with those from the identical patients collected six months after AMI (stable phase) in order to identify differentially expressed miRNAs that could be potentially dysregulated in response to early myocardial damage. The most promising miRNAs were additionally studied using a set of AMI serum samples from a second independent cohort and a control group of patients with a stable coronary artery disease (CAD).

Materials and methods

Patients. Sixteen patients for the study group and fourteen patients for the validation group, diagnosed with ST-segment elevation myocardial infarction (STEMI), were randomly selected from our previously described cohorts of patients admitted to the Medical University of Warsaw and the Medical University of Białystok (12). The control group comprised seven age- and sex-matched individuals selected from a cohort of patients with a stable CAD and no history of myocardial infarction (MI). The whole cohort of CAD patients has been characterized in our previous study (12).

The design and conduct of this study complied with the Declaration of Helsinki. The protocol of the study was approved by the Ethics Committees of the Medical University of Warsaw and the Medical University of Białystok. Written informant consent was obtained from all patients.

Plasma and serum collection, and hemolysis assessment. Venous whole blood samples (4–8 ml) were drawn from the patients diagnosed with STEMI at two time points: On the first day of AMI (admission), and six months following AMI using standard phlebotomy techniques. For the study group, plasma was isolated using BD Vacutainer® CPT™ glass tubes with sodium citrate (BD Biosciences, Franklin Lakes, NJ, USA), following the manufacturer's protocol. For the validation and control groups, blood samples were drawn into serum separator tubes (Profilab s.c., Warsaw, Poland) according to the manufacturer's instructions. The plasma and serum were transferred into fresh tubes and stored at -80°C prior to subsequent analysis.

Oxyhemoglobin was assayed in all plasma and serum samples analyzed from the study, validation and control groups. Absorbance at $\lambda=414$ nm was measured spectrophotometrically (NanoDrop ND-1000; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Additionally, at the profiling stage, hemolysis in the

plasma samples was assessed using two miRNAs available on the Serum/Plasma Focus miRNA Polymerase Chain Reaction (PCR) panel: miRNA-451a, which is specific to erythrocytes, and miRNA-23a-3p, which is unaffected by hemolysis. The ΔCp (crossing point) values for (miR-23a-3p - miR-451a) were calculated. Samples with a ΔCp value >7.0 were likely to have undergone hemolysis. Both the absorbance measurements at $\lambda=414$ nm (data not shown) and the ΔCp data (Fig. 1) indicated that no serum and plasma sample was affected by hemolysis.

miRNA isolation, complementary DNA (cDNA) synthesis and quality controls. Total RNA was extracted from 200 μl plasma/serum using an miRCURY™ RNA Isolation kit - Biofluids (Exiqon A/S, Vedbaek, Denmark). To improve the yield and reproducibility between isolations, 1.25 $\mu\text{g/ml}$ MS2 bacteriophage RNA carrier (Roche Diagnostics GmbH, Mannheim, Germany) was added at the beginning of the procedure. To control isolation efficiency and yield, three synthetic RNA spike-ins (UniSp2, UniSp4 and UniSp5; Exiqon A/S) were added to the samples at concentrations recommended by the manufacturer. Total RNA was eluted with 50 μl ribonuclease-free water and stored at -80°C prior to analysis. cDNA was synthesized from purified miRNA using the miRCURY™ LNA™ microRNA PCR, Polyadenylation and cDNA synthesis kit II from Exiqon A/S, according to the manufacturer's protocol. During cDNA synthesis, two spike-ins (UniSp6 and cel-miR-39; Exiqon A/S) were added to detect the presence of potential inhibitors in the cDNA synthesis process or in reverse transcription-quantitative PCR (RT-qPCR). The quality control analysis was performed according to the protocol provided in the manual (<http://www.exiqon.com/ls/Documents/Scientific/QC-PCR-Panel-Manual.pdf>). All samples passed the criteria and were included in further studies.

miRNA profiling. For initial screening, quantification of the miRNA levels in samples taken from patients with AMI [both on the first day of AMI (admission) and six months following AMI] was performed by using the Serum/Plasma Focus microRNA PCR panel, Version V3 (Exiqon A/S) in a 96-well format, which was designed to detect the 179 most expressed miRNAs in human serum/plasma. RT-qPCR reactions were performed using ExiLent SYBR® Green master mix (Exiqon A/S) according to the protocol provided by the manufacturer. Negative controls (no template) were performed

Table I. Clinical characteristics of patients from study and validation groups.

Characteristic	Study group (n=16)	Validation group (n=14)	P-value
Gender (female/male)	3/13 (18.8%/81.2%)	0/14 (0.0%/100.0%)	0.200
Age (years)	54.9±11.3	58.2±11.1	0.422
BMI (kg/m ²)	26.8±2.3	28.0±4.3	0.345
Smoking	7 (43.8%)	8 (57.1%)	0.715
Hypertension	4 (25.0%)	10 (71.4%)	0.026
Diabetes	2 (12.5%)	2 (14.3%)	>0.999
Hypercholesterolemia	9 (56.2%)	10 (71.4%)	0.466
Previous MI	0 (0.0%)	0 (0.0%)	NA
Anterior MI	9 (60.0%)	5 (35.7%)	0.272
Previous revascularization	0 (0.0%)	0 (0.0%)	NA
Non-coronary atherosclerosis	0 (0.0%)	1 (7.1%)	0.467
WBC (x10 ³ /μl)	12.2±3.3	13.0±3.6	0.506
NT-proBNP (pg/ml)	1,052.4 (458.1-1,504.3)	784.1 (514.5-1,640.0)	0.861
LVEF (%)	49.9±11.3	41.0±10.0	0.039
Medication			
Aspirin	16 (100.0%)	14 (100.0%)	NA
Clopidogrel	15 (93.8%)	14 (100.0%)	>0.999
Beta blockers	16 (100.0%)	13 (92.9%)	0.467
ACE inhibitors	16 (100.0%)	14 (100.0%)	NA
Statins	16 (100.0%)	14 (100.0%)	NA
Diuretics	6 (37.5%)	6 (42.9%)	>0.999

Data at admission (on the first day of AMI) are presented as the mean value ± standard deviation or median (first quartile - third quartile) for continuous variables, and the number or percentage of patients for categorical variables. P<0.05 was considered to indicate a statistically significant value. ACE, angiotensin-converting enzyme; BMI, body mass index; LVEF, left ventricular ejection fraction; (A)MI, (acute) myocardial infarction; NA, not applicable; NT-proBNP, N-terminal pro-B-type natriuretic peptide; WBC, white blood cells.

and profiled in an identical manner as for the samples. The amplification was performed in a LightCycler[®] 480 Real-Time PCR system (Roche Diagnostics, Basel, Switzerland). The amplification curves were analyzed using the Roche LC software (version 1.5), both for determination of the Cp values (by the second derivative method) and for melting curve analysis.

Quantification of individual miRNAs. Selected miRNAs whose levels were found to differ between patients on admission and six months following AMI were subjected to a subsequent validation step by RT-qPCR. The specific microRNA LNA[™] PCR primer sets and ExiLENT SYBR[®] Green Master Mix (Exiqon A/S) were used to assess the presence of individual miRNAs in serum samples according to the manufacturer's protocol.

miRNA RT-qPCR data analysis. RT-qPCR results were analyzed using the GenEx software, version 6.0 (MultiD Analyses AB, Göthenburg, Sweden). Data obtained for the negative control plate were subtracted from the data for the miRNA PCR panels. Only miRNA species with a Cp value <37 and at least 5 points below the negative control Cp value were included in the data analysis. For the profiling study, the expression data were normalized to a global mean. A logarithmic transformation (log₂) was used to normalize the expression data in the profiling stage. The geNorm and NormFinder algorithms (Exiqon A/S software) were used to

select the reference gene for the validation studies. The data were normalized to miR-19b-3p as a stable endogenous reference gene, and UniSp2 as a stable exogenous reference gene.

Prediction and functional analysis of miRNA targets. Ingenuity Pathway Analysis (IPA; www.ingenuity.com; Qiagen, Inc., Valencia, CA, USA) was used to search mRNA targets for dysregulated miRNAs. To avoid exceeding the maximum gene list size allowed by IPA, the miRNAs were analyzed using the microRNA Target Filter limited to experimentally validated miRNA-mRNA interactions. Target genes were further analyzed for over-represented biological functions and canonical pathways using the IPA database.

Statistical analysis. Statistical analyses were performed using R 3.1.2 software (The R Project for Statistical Computing; https://www.R-project.org). The Shapiro-Wilk test was used to test for normal distribution of continuous variables, and subsequently, continuous variables were expressed as the mean ± standard deviation for normally distributed ones and the median (first quartile - third quartile) for the variables that deviated from a normal distribution. Categorical variables were presented as frequencies and percentages. Student's t-test (for normally distributed variables) and the Mann-Whitney test (for the variables deviating from a normal distribution) were used to compare continuous variables. Fisher's exact test

Table II. Differential miRNAs between the first day of AMI and the stable phase following myocardial infarction in the study group.

miRNA	Fold change	P-value
hsa-miR-133b	45.764	9.0E-06
hsa-miR-133a	30.127	1.6E-04
hsa-miR-208a	27.074	3.0E-06
hsa-miR-1	12.139	2.0E-03
hsa-miR-30a-5p	3.643	7.0E-03
hsa-miR-629-5p	2.573	4.5E-02
hsa-miR-20b-5p	2.570	2.4E-02
hsa-miR-22-5p	2.360	1.1E-02
hsa-miR-145-5p	1.776	9.0E-03
hsa-miR-22-3p	1.507	2.0E-02
hsa-miR-486-5p	1.402	3.8E-02
hsa-miR-451a	1.349	3.3E-02
hsa-miR-92a-3p	1.327	1.0E-02
hsa-miR-93-5p	1.133	3.1E-02
hsa-let-7i-5p	-1.198	1.8E-02
hsa-miR-148b-3p	-1.226	3.4E-02
hsa-miR-103a-3p	-1.237	1.3E-02
hsa-miR-223-3p	-1.237	1.7E-02
hsa-miR-652-3p	-1.251	3.6E-02
hsa-miR-26b-5p	-1.257	4.2E-02
hsa-miR-107	-1.272	7.0E-03
hsa-miR-199a-3p	-1.278	3.0E-02
hsa-miR-151a-5p	-1.326	9.0E-03
hsa-miR-30b-5p	-1.414	3.0E-03
hsa-miR-181a-5p	-1.436	4.6E-02
hsa-miR-142-3p	-1.693	3.6E-04
hsa-miR-374b-5p	-1.829	3.6E-04
hsa-miR-335-5p	-2.384	1.5E-02
hsa-miR-505-3p	-2.873	5.0E-03
hsa-miR-885-5p	-2.898	4.0E-02
hsa-miR-326	-3.535	7.0E-03
hsa-miR-301a-3p	-4.147	7.0E-03

Data are presented as the fold change relative to the expression of patients six months following AMI. P-values were calculated using a paired two-tailed Student's t-test. Only miRNA species with $P < 0.05$ are shown. AMI, acute myocardial infarction; miRNA, microRNA.

was used to compare categorical variables. Statistical significance between miRNAs that were differentially expressed in study and validation groups was determined using either a paired, two-tailed Student's t-test (admission compared with six months following AMI) or an unpaired, two-tailed Student's t-test (admission compared with the control group). $P < 0.05$ was taken to indicate a statistically significant value. Principal component analysis (PCA) was performed using GenEx software (version 6.0; MultiD Analyses AB). Receiver operating characteristic (ROC) curve analysis and the area under the curve (AUC) were used to estimate the ability of biomarkers to distinguish the AMI group from the control

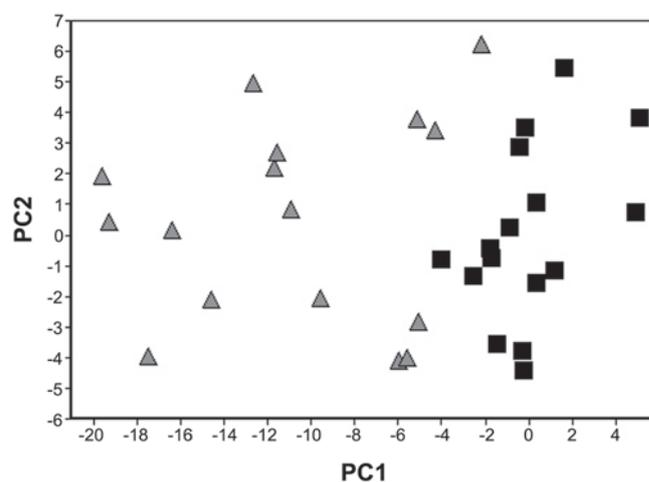


Figure 2. Principal component analysis of plasma miRNA profiles. Principal component analysis of the miRNA expression data clusters plasma collected on admission (grey triangles) and six months following acute myocardial infarction (black squares) into their respective groups. Normalized values were used for principal component analysis. miRNA, microRNA.

group. The optimal cut-off points for each miRNA were determined using the highest sum of sensitivity and specificity.

Results

Patient characteristics. In the present study, patients with STEMI who were treated with primary percutaneous revascularization were included. The mean age of participants was 54.9 ± 11.3 years for the study group ($n=16$) and 58.2 ± 11.1 years for the validation group ($n=14$). Clinical characteristics of patients from the two groups are shown in Table I.

Identification of differentially expressed miRNAs in the plasma of patients with AMI. miRNA profiling was performed on plasma samples derived from patients on the first day of AMI ($n=16$), and on samples from the identical patients collected six months following AMI ($n=16$, stable phase), which reduced the impact of inter-individual variability. Following data analysis, miRNA candidates were selected on the basis of fulfillment of the criterion of significance ($P < 0.05$) in the comparison between admission and six months following AMI. A total of 32 miRNAs (14 up- and 18 down-regulated) were differentially quantified in the acute phase of MI compared with the stable phase following MI (Table II).

PCA was performed on the miRNA results from the analyzed samples to determine how the 32 differentially expressed miRNAs were distributed among the samples from the first day of AMI, and those collected six months afterwards. As shown in Fig. 2, the PCA clearly separated the plasma samples on admission from those collected six months following AMI. This suggests that the observed miRNA differences are associated with the pathophysiology of MI, and these miRNAs might constitute an early biomarker signature for AMI.

Validation of selected miRNAs in an independent group of patients with AMI. miRNA candidates for validation were selected following an extensive review of the literature on the basis of their inferred relevance to cardiovascular

Table III. RT-qPCR quantification of selected miRNAs on admission vs. six months following AMI in study and validation groups.

miRNA	Admission vs. six months			
	Study group		Validation group	
	Fold change	P-value	Fold change	P-value
hsa-miR-133b	45.764	<0.001	5.449	<0.05
hsa-miR-22-5p	2.360	<0.05	4.872	<0.01
hsa-miR-374b-5p	-1.829	<0.001	1.642	NS

Results were normalized to miR-19b-3p and UniSp2. Data for the study group are taken from Table II. miRNA, microRNA; NS, non-significant; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Table IV. RT-qPCR quantification of the two selected miRNAs on admission vs. the control group in the validation group.

miRNA	Admission vs. control group	
	Validation group	
	Fold change	P-value
hsa-miR-133b	7.273	<0.05
hsa-miR-22-5p	4.505	<0.01

Results were normalized to miR-19b-3p and UniSp2. miRNA, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

disease. Additionally, the potential candidates were filtered for highly expressed miRNAs according to inspection of their raw Cp values in individual samples. The validation was performed on serum samples of an independent patient group on admission (n=14) and samples from the identical patients collected six months following AMI (n=14), and a control group (n=7). The levels of these miRNAs were quantified using RT-qPCR for individual miRNAs. Three miRNAs were further investigated: miR-133b, which is known to be associated with MI; miR-374b-5p, which limited literature has suggested has an involvement in MI; and miR-22-5p, which has not yet been reported to be associated with MI, and therefore may be a possible novel biomarker. Two of the miRNAs, miR-133b and miR-22-5p, demonstrated significant differences in the comparison between admission and six months following AMI, the direction and magnitude of the changes reflecting reasonably well those found in the profiling stage (Table III). These two miRNAs were further investigated in the patients with AMI and the control group. As shown in Table IV, the expression levels of miR-133b and miR-22-5p were significantly increased in patients with AMI compared with the control group.

Diagnostic accuracy of selected circulating miRNAs. To evaluate the diagnostic value of miR-133b and miR-22-5p as potential biomarkers of AMI, ROC curve analysis, together

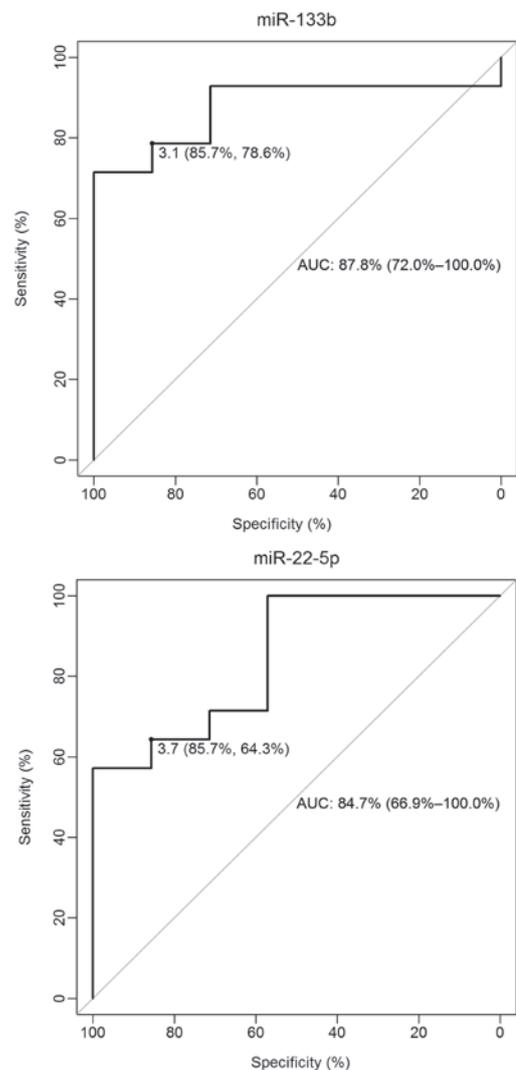


Figure 3. ROC curves for miR-133b and miR-22-5p. AUC, area under the curve; ROC, receiver operating characteristic.

with calculation of the AUC, was performed. As shown in Fig. 3, the ROC curves of miR-133b and miR-22-5p reflected a good separation between the patients with AMI and the control group, with AUC measurements of 87.8% [95% confidence

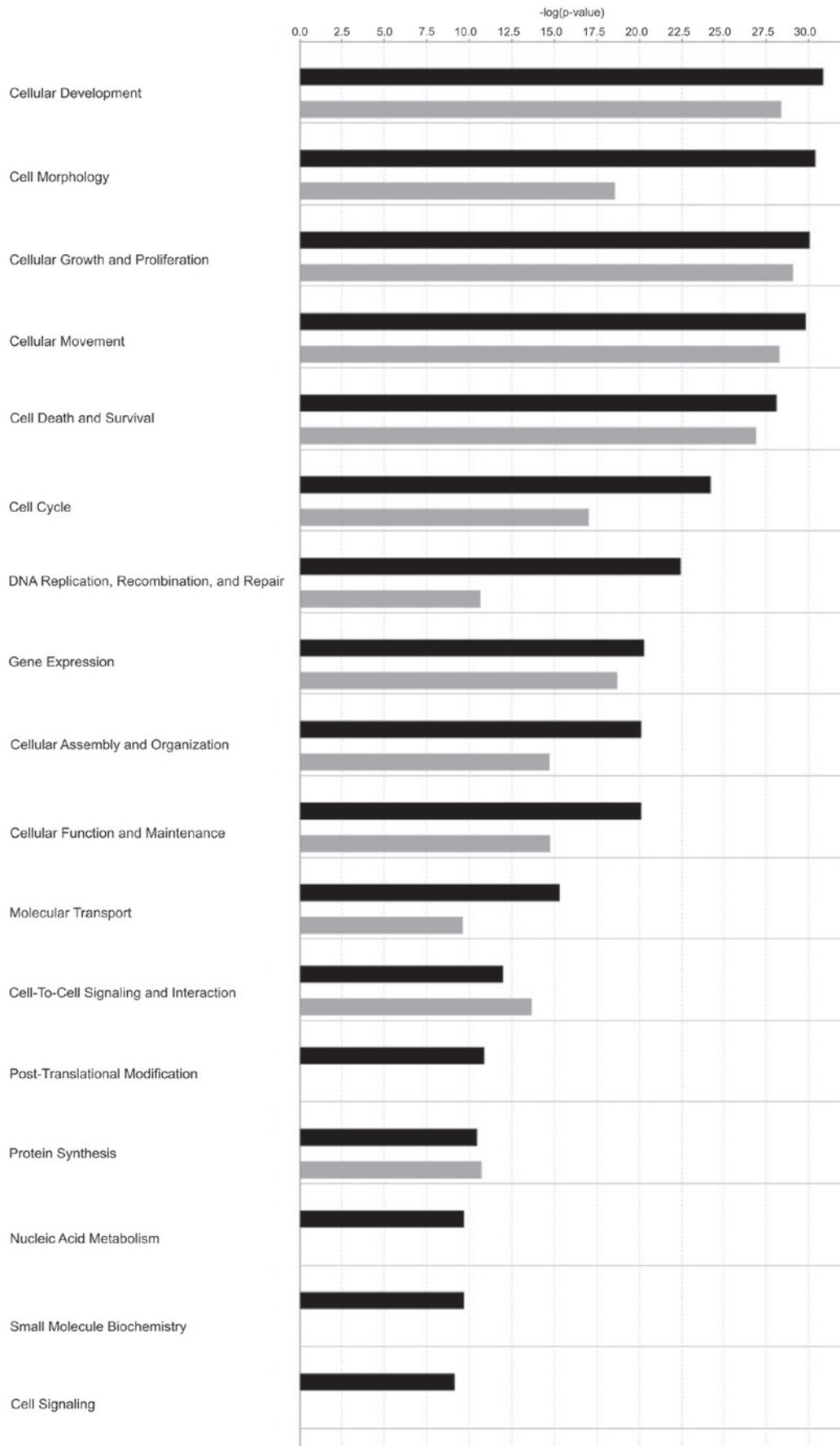


Figure 4. Molecular and cellular functions associated with differentially expressed miRNAs. Ingenuity pathway analysis of miRNA sets differentially expressed on admission compared with six months after acute myocardial infarction was performed. The x-axis of the diagram displays the significance, which was calculated using the Fisher exact test and expressed as the negative exponent of the P-value. Pathways associated with up-regulated miRNAs are shown in black, whereas those pathways associated with down-regulated miRNAs are shown in grey. miRNA, microRNA.

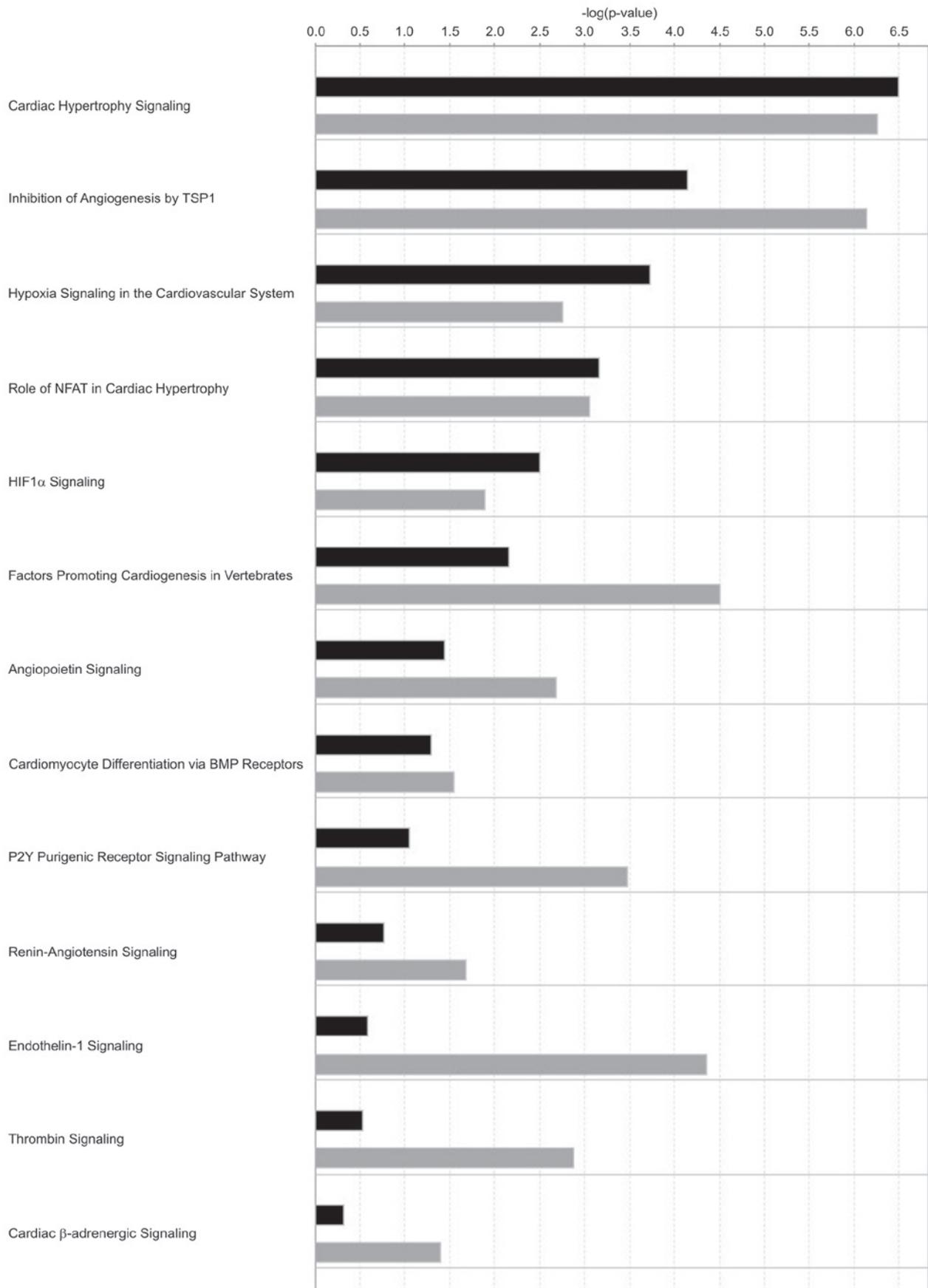


Figure 5. Predicted cardiovascular signaling pathways regulated by miRNAs differentially expressed in AMI. Ingenuity pathway analysis of miRNA sets differentially expressed on admission compared with six months following AMI was performed. The x-axis of the diagram displays the significance, which was calculated using the Fisher exact test and expressed as the negative exponent of the P-value. Pathways associated with up-regulated miRNAs are shown in black, whereas those pathways associated with down-regulated miRNAs are shown in grey. AMI, acute myocardial infarction; miRNA, microRNA; TSP-1, thrombospondin 1; NFAT, nuclear factor of activated T-cells; HIF-1 α , hypoxia-inducible factor 1 α ; BMP, bone morphogenetic protein.

interval (CI): 72.0-100.0] and 84.7% (95% CI: 66.9-100.0), respectively. ROC curves yielded an optimal cut-off value of 3.1 for miR-133b, with a sensitivity of 78.6% and a specificity of 85.7%, and an optimal cut-off value of 3.7 for miR-22-5p, with a sensitivity of 64.3% and a specificity of 85.7%. These results suggest that miR-133b and miR-22-5p are of good diagnostic value for patients with AMI.

Discussion

Previous studies have revealed that heart-specific miRNAs are released into the circulation during AMI, and therefore may be used to detect and monitor myocardial injury (13,14). In the present study it has been confirmed that the well-known 'cardiac miRNAs' of ongoing early myocardial damage, miR-1, miR-133a, miR-133b and miR-208a, are significantly up-regulated in AMI. An additional 28 differentially expressed miRNAs that were apparently associated with AMI were also identified. To determine the biological significance of miRNAs dysregulated in AMI, *in silico* target prediction was performed using the IPA software (Qiagen, Inc.). Targeting information was available for 22 out of the 32 miRNAs in the database, resulting in a total of 412 experimentally validated target mRNAs for up-regulated miRNAs, and a total of 304 experimentally validated mRNAs for down-regulated miRNAs. Functional analysis revealed that targets for both up- and down-regulated miRNAs were generally involved in identical molecular and cellular functions (Fig. 4). Only post-translational modification, nucleic acid metabolism, small molecule biochemistry and cell signaling were predicted to be associated with up-regulated miRNAs. In addition, several pathways involved in cardiovascular signaling were revealed to be associated with the canonical pathway analysis, the most important being cardiac hypertrophy signaling, inhibition of angiogenesis by thrombospondin 1 and hypoxia signaling in the cardiovascular system (Fig. 5). These findings reveal that the identified miRNAs could have a role in the pathogenesis of MI through their ability to negatively regulate the expression of genes that govern processes important for myocardial function.

Numerous biochemical biomarkers of MI are commonly used in clinical practice [e.g. cardiac troponins (Tn) I and T, creatinine kinase isoenzyme MB, N-terminal pro B-type natriuretic peptide and B-type natriuretic peptide] (15). However, it should be noted that an increase in the levels of these biochemical biomarkers above reference values may also occur in various other disease states not necessarily associated with MI (16). Therefore, selected miRNAs or miRNA sets, particularly when combined with clinical parameters, are likely to be more specific biomarkers of MI. Additionally, studying their mechanism of action should provide an improved understanding of the changes that occur in the myocardium, and determine the potential role of extracellular miRNAs as paracrine signaling molecules.

The up-regulation of miR-133b and miR-22-5p in two independent patient groups using serum or plasma confirmed the high diagnostic value of these miRNAs. The ROC curve analysis revealed that the AUCs of miR-133b and miR-22-5p were 87.8 and 84.7%, indicating that they may be clinically practicable biomarkers for AMI diagnosis. The major novel finding reported in the present study is the up-regulation of miR-22-5p

in the acute phase of STEMI. To date, miR-22-3p originating from the same hairpin has been studied in depth to elucidate its role in cardiovascular remodeling (17) and heart failure (18). To the best of our knowledge, no previous data regarding a role for miR-22-5p in cardiovascular diseases is available, albeit a recent study has demonstrated that up-regulation of mmu-miR-22-5p may prevent myocardium regeneration in 7-day-old mice (19).

In conclusion, the present study has reported an altered miRNA expression profile associated with AMI. A group of 32 circulating miRNAs that are significantly up- or down-regulated in AMI compared with the stable phase of the disease has been described. The circulating miRNA, miR-22-5p, has been identified as a novel diagnostic biomarker of AMI.

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