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# Circulating miRNA-106b-5p As a Potential Biomarker for Coronary Artery Disease

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**Article type:** **ABSTRACT**

**Original Article**

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Coronary artery diseases (CAD) represent a significant global health concern and are recognized as a primary contributor to mortality on a worldwide scale. Early diagnosis of CAD is one of promising goal to manage this disorder. Recent investigations have highlighted the pivotal involvement of microRNAs (miRNAs) in diverse health conditions, notably CAD. The principal objective of this investigation was to identify appropriate miRNAs that could be employed for the early detection of CAD**.** In the present study, we analyzed dataset of CAD (GSE113079) and 100 differentially expressed mRNAs (DEmRNAs) were detected. The miRNAs that have a significant interaction with DEmRNAs were chosen. By computational prediction method, 5 miRNAs (miR-106b-5p, miR-20a-3p, miR-17-3p, miR-146a-5p, and miR-155-3p) were selected. Finally, we assessed the anticipated expression levels of microRNAs in CAD patients and healthy control groups. Our findings revealed a statistically significant elevation solely in the expression level of miR-106b-5p within the CAD group when compared to the control group ($p>0.001$). Our study demonstrated an elevation in the expression of miR-106b-5p in individuals diagnosed with CAD. This microRNA may be used as a diagnostic biomarker in patients with CAD. However, further investigations are needed to confirm these results.

**Keywords:** MicroRNA, coronary artery disease, miRNA-mRNA intraction, qRT-PCR

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## Introduction

Coronary artery diseases (CAD) are a common cause of death worldwide. (1, 2). They also cause high costs for the healthcare system (3). Early detection of cardiovascular disease is therefore one of the most important challenges that has attracted the attention of researchers (4). Although several studies have been carried out in this area and various biomarkers have been introduced, the results have not been very promising (5-7).

miRNAs function as epigenetic regulators, altering gene expression patterns and signaling pathways by targeting specific cellular messenger RNA (mRNA)(8, 9). Numerous studies have shown the considerable influence of these molecules on the control of vital biological processes such as cell division, apoptosis, angiogenesis, cell migration and cell growth (10-13). Circulating miRNAs represent a promising advance in the field of diagnostic biomarkers for CAD. The expression profiles of these microRNAs change in response to various pathological conditions, including CAD. Circulating miRNAs offer high sensitivity and specificity as they allow simultaneous analysis of multiple microRNAs, providing a more comprehensive molecular signature of the disease. This versatile approach does not only improve the early detection of CAD, but also enables the identification of disease subtypes and the assessment of disease progression, which represents a significant advantage over conventional diagnostic methods (14-16).

The aim of the present study was to predict and validate miRNA, which are significantly upregulated in CAD patients, and to evaluate their potential as diagnostic biomarkers. To this end, we selected an expression dataset of CAD by searching in the Gene Expression Omnibus (GEO) database (GSE113079). Gene expression analysis of this dataset revealed 50 up-regulated and 50 down-regulated mRNAs that were statistically significantly differentially expressed between CAD and controls. Subsequently, the miRNAs that showed significant interaction with the downregulated mRNAs were selected. Finally, the predicted expression of miRNA in whole blood of CAD patients was analyzed.

## Materials and methods

### Research design

We conducted a case-control study. Initially, the Limma package in Bioconductor was utilized to identify differentially expressed mRNAs from the GSE113079 dataset (GPL20115 Agilent-067406 Human CBC lncRNA + mRNA microarray V4.0, Probe name version). The selection process was focused on mining statistically significant differentially expressed mRNAs (DEmRNAs), discerned by their expression value disparities between CAD and control samples, with a log2 fold change of $\geq |1|$ and an adjusted p-value threshold of 0.05. These criteria were employed to identify mRNAs that exhibited significant differential expression (16), the dataset contains 141 peripheral blood samples (93 CAD and 48 control).

In the next step, to determine these DEmRNAs interaction with miRNA, miRNet 2.0 database (https://www.miRnet.ca/) was used to select miRNAs as candidate biomarkers, and 5 miRNAs (miR-106b-5p, miR-146a-5p, miR-17-3p, miR-20a-3p and miR-155-3p) were selected.

Meanwhile, a miRNA-mRNA interaction network was built using blood tissue data and, the network was created using the program Cytoscape 3.9.0. Moreover, for evaluating a gene list based on functional

annotation, Metascape (https://metascape.org/gp/index.html#/main/step1) was utilized to perform GSEA (Gene Set Enrichment Analysis) (18).

Then, in the verification phase, we randomly selected 44 serum samples from men who were candidates for coronary artery bypass graft (CABG) and 48 serum samples from normal healthy persons of the same age as control group, to examine the miRNAs' expression patterns and their diagnostic capacities.

In the concluding phase, the investigation employed Receiver-operating characteristic (ROC) curves and the area under the ROC curve (AUC) to evaluate the diagnostic efficacy, specificity, and sensitivity of the identified miRNAs that exhibited statistically significant expression differences between healthy individuals and CAD patients.

**Study population**

The study enrolled forty-four male candidates for coronary artery bypass graft (CABG) at Shafa Hospital in Kerman, Iran. A questionnaire was employed to gather demographic information, including details about smoking, family history of diseases, and drug history. Exclusion criteria comprised contagious and chronic inflammatory diseases, oral contraception, hormone replacement therapy, history of cancer, and prior coronary angioplasty experience (17). Additionally, a normal healthy control group consisting of 48 individuals of similar age was selected. Ethical approval for the study was obtained from the Ethics Committee of Kerman University of Medical Sciences (#IR.KMU.AH.REC.1403.033), and all participants provided an informed consent.

**Anthropometric and clinical assessment**

Blood pressure, TG, Cholesterol, height, weight, BMI, HDL-c and LDL-c were measured from all participants as anthropometric and clinical parameters in standard condition.

**RNA isolation and cDNA synthesis**

For RNA extraction, an additional 500 microliters of Trizol were added to the samples. These samples were then incubated on ice for 10 minutes. Next, 350 microliters of chloroform (Merck, Germany) were added to the mixture, and the solution was incubated on ice for 5 minutes. Following this, the mixture underwent centrifugation at 12,000×g for 15 minutes at 4 ℃ for separation of the supernatant. An equivalent amount of isopropanol (Merck, Germany) was then mixed with the supernatant and incubated at -20 ℃ for 1 hour. The sample was then centrifuged again at 12,000×g at 4 ℃ for 20 minutes. After centrifugation, the liquid portion was discarded. The pellet was rinsed twice, first with 99% ethanol and then with 70% ethanol. The final step involved dissolving the RNA in 30 microliters of nuclease-free DEPC-treated water and preserving it at -80 ℃ for subsequent procedures. The assessment of RNA quality, integrity, and purity was accomplished by employing 1.5% agarose gel electrophoresis and an absorption ratio of 260/280 and 260/230.

To measure the levels of the miRNAs, cDNA was synthesized through a reverse transcription process. This process utilized specialized multi-scribe reverse transcriptase (MLV) and stem-loop primer designed for each gene. A reaction mix of 500 ng RNA, 1.5 µL stem-loop (100 nM), and distilled water to achieve a total volume of 10 µL was prepared. After incubation at 65 °C for 20 minutes, the mixture was cooled down on ice. Next, 4 µL of 5X first-strand Buffer, 1 µL of dNTPs (10 mM), 1 µL of MLV (200 U/µL), and 0.5 µL of RNase inhibitor (40 U/µL) were added to the reaction. The resulting 16.5 µL reactions were processed in

a Biometra TAdvanced thermal cycler at 16°C for 30 minutes, then at 42 °C for 60 minutes, and finally at 85 °C for 5 minutes. Following this, the reactions were maintained at 4 °C. All reverse transcriptase reactions, including control samples without template and reverse transcriptase, were conducted in duplicate.

**Quantitative reverse transcription (qRT-) PCR**

The RT-qPCR method was performed using a standardized protocol from YektaTajhiz, within the Mic qPCR Cycler instrument. The PCR mix, with a total volume of 10 µL, comprised 3.6 µL of RT product, 6.25 µL of YektaTajhiz 2× qPCR Probe Master Mix, 0.2 µL of probe (100 nM), 1 µL of forward primer (100 nM), and 1 µL of reverse primer (100 nM). The reactions proceeded through a 10-minute preheating phase at 95°C, followed by 40 rounds of denaturation (15 sec at 95°C), annealing (15 sec at 60°C), and extension (15 sec at 72 °C). Each reaction was replicated to ensure accuracy. The results, quantifying the PCR's performance, were analyzed using the $2^{-\Delta\Delta CT}$ method. U6 was used as internal control to normalized the miRNAs expression levels. The stem-loop sequences and forward primers and universal reverse primer sequences are detailed in Table 1.

**Table 1.** Stem loops sequence, forward oligonucleotide and universal reverse sequence

| **Stem loop** | **5'→3' Sequence** | |
|---|---|---|
| hsa-miR-17-3p | GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCTGCCATC GAGCAGCATAC CTACAA | |
| hsa-miR-20a-3p | GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCTGCCATC GAGCAGCATAC CTACCT | |
| hsa-miR-155-3p | GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCTGCCATC GAGCAGCATAC TGTTAA | |
| hsa-miR-146a-5p | GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCTGCCATC GAGCAGCATAC AACCCA | |
| hsa-miR-106b-5p | GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCTGCCATC GAGCAGCATAC ATCTGC | |
| U6 | GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCTGCCATC GAGCAGCATAC CGAATT | |
| **Forward Primers** | **5'→3' Sequence** | **product Size(bp)** |
| hsa-miR-17-3p | ACTGCAGTGAAGGCACTTGTAG | 100 |
| hsa-miR-20a-3p | TAAAGTGCTTATAGTGCAGGTAG | 100 |
| hsa-miR-155-3p | CTCCTACATATTAGCATTAACA | 100 |
| hsa-miR-146a-5p | TGAGAACTGAATTCCATGGGTT | 100 |
| hsa-miR-106b-5p | TAAAGTGCTGACAGTGCAGAT | 100 |
| U6 | GCAAGGATGACACGCAAATTCG | 100 |
| Universal Reverse Primer | GCTGCTACCTCGGACCCT | |

**Statistical analysis**

Utilizing SPSS software, version 22, a comprehensive statistical analysis of the dataset was conducted. Data were represented either as mean ± standard error of the mean (SEM) or as frequency and percentages. In comparing the quantitative and qualitative aspects of two distinct groups, the independent samples Student's t-test/ Mann-Whitney U test and chi-square tests were applied. To measure the correlation between continuous variables, Pearson and Spearman's rho correlation coefficients were calculated. The Receiver

Operating Characteristic (ROC) curve and the Area Under the Curve (AUC) were applied to assess the diagnostic potential of studied miRNAs and lncRNAs. Statistical significance was determined by setting the threshold for p-values<0.05. Each experimental setup included at least two replicates for every condition.

## Results

### Data collection and identification of DEGs

This investigation employed the GSE113079 dataset (GPL20115 Agilent-067406 Human CBC lncRNA + mRNA microarray V4.0, Probe name version). The dataset encompasses 141 peripheral blood samples, comprising 93 from individuals with CAD and 48 from controls. Utilizing the Limma package in the R programming language, a comprehensive analysis of differential expression was conducted. A total of 852 differentially expressed genes were identified, with 445 exhibiting up-regulation and 407 displaying down-regulation (Supplementary File 1). For detection miRNAs-mRNAs interaction, we get 50 top significant DEmRNAs, 25 Up DEmRNAs and 25 down DEmRNAs (Table 2).

**Table 2**. Top DEmRNAs based on log2 fold change.

| | Symbols |
|---|---|
| **50 to Up DEmRNAs** | CFAP74(KIAA1751),ACTBL2,BIRC7,KIF17,NMNAT2,NEURL1B,SAXO1(FAM154A),FTMT,OPN4,TRPM5,ARPIN(C15orf38),NUPR1,AVPR1B,PYDC2,PLA2R1,SHANK1,CPEB1,TREH,OR4C3,TM4SF5,MYL2,EYS,MYF5,NOG,FXYD3,MB,OTC,PIH1D2,WFDC2,ADAM21,MED18,FERMT2,ATP1A4,COL23A1,SLC19A3,ANTXR1,PAX8,KRTAP43,ADRA1A,MUC3A,KRT32,LY6H,KITLG,CDRT15L2,PKD1L2,OR5A2,KRTAP4-7,CRYAA,OR2J2,ZNF492 |
| **Down 50 top DEmRNAs** | ARHGEF33,PDE4D,RBPJL,PAK2,MTRNR2L9,C22orf31,CCL4,IL1A,CSNK1A1,AREG,KIR2DS4,AKAP5,FAM169A,NELFA(WHSC2),BNC2,FAM47A,SH2D4A,LONRF3,KRT33B,CD300A,PDGFD,TP53AIP1,IL18R1,F2R,IL1B,LOC400682,PDGFRB,IKZF5,FBXW2,RCN2,HOPX,DLEU7,ANKRD54,ANGEL2,CKLF,ADSS,B3GNT7,OSM,CCL20,ANP32C,SH2D2A,ZNF548,BTN2A1,SYT11,RECQL,NAMPT,SMURF1,IL18RAP,SERPINB2,PDLIM2 |

### Finding mRNAs for each DEmiRNAs

Thirty-one microRNAs were discovered to interact with or target 37 top important mRNAs (among 50 top DEmRNAs) (Supplementary File 2) after evaluating the list of significant mRNAs in the miRNet database based on blood tissue. Among the 31 microRNAs that targeted the DEmRNAs, miR-106b-5p, miR-146a-5p and miR-155-3p, miR-17-3p, miR-20a-3p had the most interaction with their targets. The interaction network is shown in Figure 1.

### Enrichment analysis of DEmiRNAs targets

The results of enrichment analysis of top DEmRNAs targets (Figure 2A) revealed that the genes involved in thrombin pathway, muscle tissue development, apoptsis signaling pathway in response to DNA damage, cAMP signaling pathway, regulation of blood circulation, glutamatergic synapse were significantly enriched.

### Anthropometric and clinical parameters

The anthropometric and clinical parameters are shown in Table 3. According to the findings presented in Table 3, observed a significant difference in weight, BMI, systolic blood pressure, LDL-c and smoking

between control group and CAD group. And, data did not show any significant difference in the age and height of the two groups.

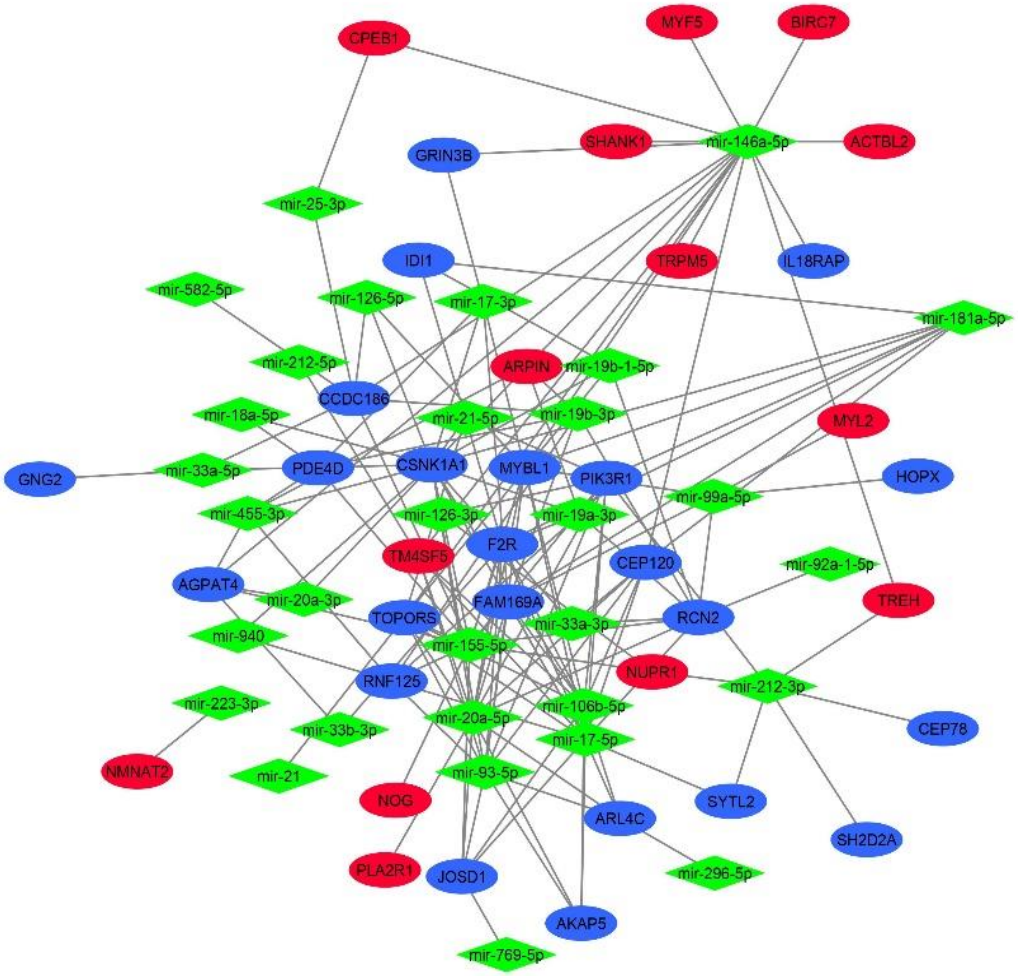


**Fig. 1**. miRNA-mRNA interaction network, green diamonds represent miRNAs and red circles represent Up-DEmRNAs in this study.

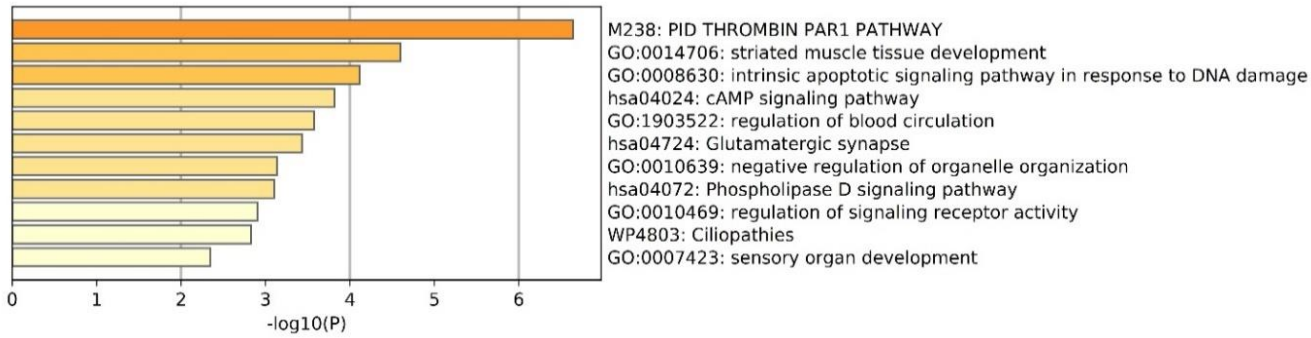


**Fig. 2**. GSEA analysis for targets of top DEmRNAs.

**Table 3**. Anthropometric and clinical characteristics of control and CAD groups.

| | Control | CAD |
|---|---|---|
| N | 48 | 44 |
| Age | 58.92 ± 11.25 | 59.54 ± 9.48 |
| weight | 65.69 ± 15.52 | 84.25 ± 13.17** |
| Height | 169.05 ± 15.22 | 171.02 ± 14.11 |
| Body Mass Index (BMI) | 25.11 ± 3.25 | 28.65 ± 5.51** |
| Systolic blood pressure | 128.36 ± 23.05 | 147.12 ± 38.18*** |
| Smoking% | 21.69 | 48.15* |
| TG (mmol/L) | 1.62 (1.01–1.89) | 1.56 (1.12–1.92) |
| LDL-c (mmol/L) | 2.03 + 0.31 | 2.58±1.02* |
| HDL-c (mmol/L) | 1.27 + 0.14 | 1.21 + 0.23 |

Data are shown as mean ± SD. Compared with the control group: * p < 0.05, ** p < 0.01, *** p < 0.001.

## Analysis of the expression level of the predicted miRNAs

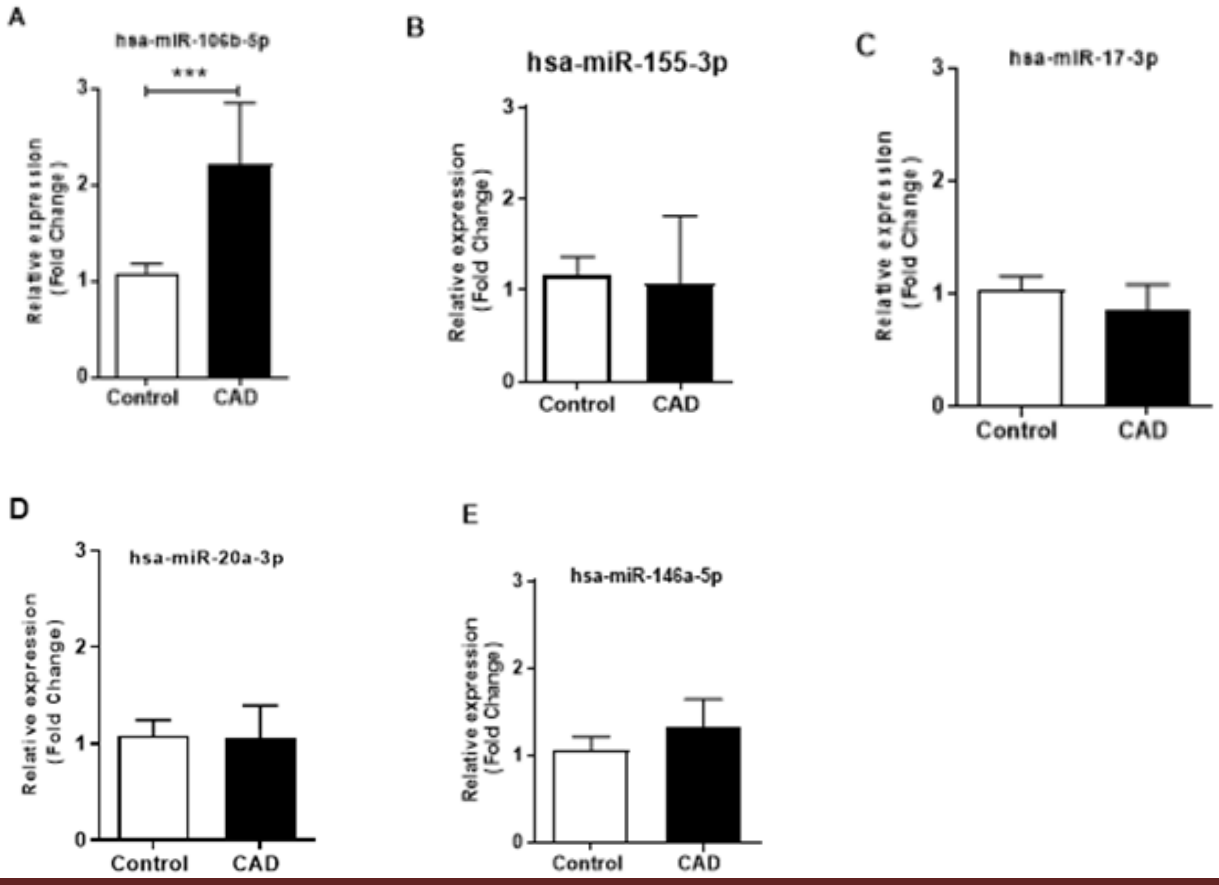


**Fig. 3**. Comparison of the expression levels of serum (A) miR-106b-5p, (B) miR-155-3p, (C) miR-17-3p, (D) miR-20a-3p and (F) miR-146a-5p in CAD patients and healthy groups. CAD: Coronary artery disease. (*> 0.05, **> 0.01, ***> 0.001)

To evaluate the expression levels of miR-106b-5p, miR-155-3p, miR-17-3p, miR-20a-3p and miR-146a-5p, we used the stem loop qRT-PCR. As depicted in Figure 3, the expression level of miR-106b-5p was the only one that exhibited a statistically significant increase in the CAD group compared to the control group (p-value > 0.001) (Figure 3A).

Furthermore, the data revealed no noteworthy distinctions in the expression levels of miR-155-3p, miR-17-3p, miR-20a-3p, and miR-146a-5p between the CAD group and the control group (Fig 3B, 3C, 3D and 3E).

**The potential diagnostic efficacy of miR-106b-5p in the context of CAD**

The diagnostic performance of miR-106b-5p was assessed using ROC curve analysis, as depicted in Figure 4, the AUC value for miR-106b-5p was determined to be 0.8975 (95% CI: 0.8265-0.9685, P <0.001). With an optimal cutoff value of 1.75, the sensitivity and specificity were found to be 70% and 95%, respectively.

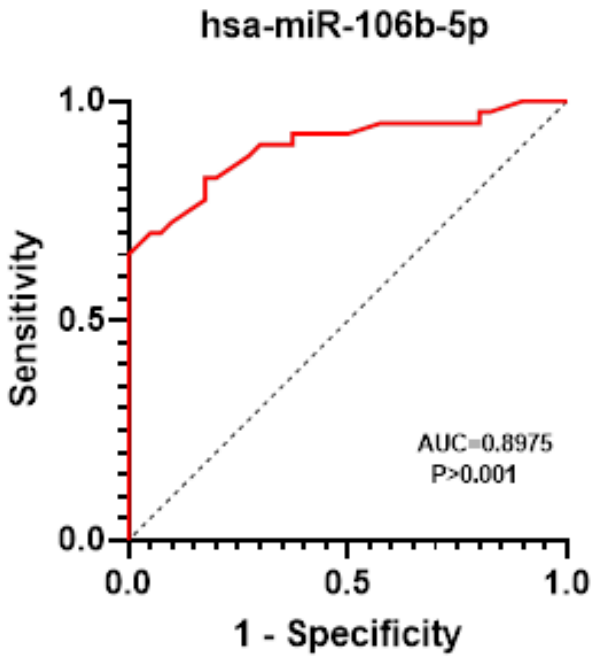


**Fig. 4**. Receiver operating characteristic (ROC) curve analysis of has-miR-106b-5p to discriminate CAD patients from healthy controls.

**Correlation between miR-106b-5p expression levels and CAD risk factors**

Regarding risk factors for CAD, it was observed in the control group that LDL-c exhibited a correlation with miR-106b-5p expression levels (r=−0.532, P=0.023). Additionally, in the patient group, miR-106b-5p expression levels were linked with weight (r=−0.358, P=0.041) and BMI (r=−0.463, P=0.015) (Table 4).

**Association between miR-106b-5p serum levels and CAD risk factors**

In stepwise multiple linear regression analysis, the concentration of LDL-c (β, 0.038; p<0.001) and BMI (β, 0.058; p<0.01) were found to have a significant independent association with miR-106b-5p serum level.

**Table 4.** Correlations between miR-106b-5p serum levels and CAD risk factors.

| | Control | | CAD | |
|---|---|---|---|---|
| | **R Values** | **P Values** | **R Values** | P Values |
| Age | 0.086 | 0.235 | -0.189 | 0.45 |
| weight | 0.261 | 0.131 | 0.358 | 0.041* |
| Height | -0.325 | 0.26 | -0.241 | 0.089 |
| Body Mass Index (BMI) | -0.321 | 0.065 | 0.463 | 0.015* |
| Systolic blood pressure | 0.420 | 0.201 | -0.236 | 0.162 |
| TG | 0.025 | 0.125 | -0.097 | 0.101 |
| LDL-c | -0.532 | 0.023* | 0.823 | 0.341 |
| HDL-c | 0.287 | 0.149 | -0.574 | 0.097 |

Correlations were assessed using Spearman's correlation coefficients. The significant correlations are marked with an asterisk

## Discussion

CVD, notably coronary heart disease, stand as significant contributors to mortality in diverse societies. The timely identification of these diseases holds paramount importance. Researchers face the challenge of identifying specific biomarkers that can facilitate the early diagnosis of such conditions (18, 19).

The miRNAs special characteristics, along with the simplicity and specificity of identification methods, have attracted the attention of researchers (20, 21). To this end, the GSE113079 dataset was analyzed to detect differential expression mRNAs (DEmRNAs) in CAD patients and healthy people. The 100 top significant DEmRNAs were used to determine the interaction with miRNAs. Enrichment analysis of the top DEmRNAs was performed to detect signaling pathways in which the DEmRNAs are involved. Moreover, the data showed miR-106b-5p, miR-146a-5p, miR-17-3p, miR-20a-3p and miR-155-3p had the most affinity to the 50 top DEmRNAs. To validate the outcomes of the bioinformatics analysis and to identify miRNAs suitable as biomarkers for diagnosing CAD, the predicted miRNAs were assessed in both CAD patients and healthy individuals.

Data showed that out of the five predicted miRNAs, only miR-106b-5p expression levels in CAD patients were significantly higher than in healthy participants. Moreover, the expression levels of the other predicted miRs (miR-146a-5p, miR-17-3p, miR-20a-3p and miR-155-3p) did not show any significant differences between CAD patients and healthy individuals. It is worth noting that CAD patients had a non-significant increase in miRNA-146a-5p expression in comparison to healthy controls.

Given this result, this miRNA-106b-5p could be considered a candidate for a diagnostic biomarker for CAD. This result is protected by studies showing that miRNA-106b-5p is altered in heart patients (22, 23), Elgebaly et al. reported that miR-106b-5p along with miR-137 can be used for the early detection of suspected CAD.

In this context, Lui *et al*. showed that the concentration of serum miR-106 in individuals with heart disease exhibits a significant increase compared to that in healthy individuals. They concluded that the abnormal levels of serum miR-106 may serve as predictive indicators for heart disease risk (24).

In an investigation of miRNA co-expression in atherosclerosis patients, miR-106b-5p showed the most significant difference compared to healthy individuals. This microRNA targets various signaling pathways in vascular endothelial cells, highlighting its potential key role in the regulatory network of gene expression related to atherosclerosis (25).

While there is some evidence to suggest that miR-106b may play a role in the development of CAD, the exact nature of this role is still not fully understood. Moreover, considering the conventional role of miRNAs in binding to target genes to influence biological functions (26), this study suggests that the elevated miR-106 levels in the serum of CAD patients may potentially change the expression of the downstream target genes. This, in turn, could contribute to the development of CAD.

Moreover, miRNA-mRNA interaction and gene enrichment analysis showed the link between the predicted 5 miRNAs and Thrombin activation PAR1 pathway. It has been reported that thrombin activates PAR1 pathway on endothelial cells, leukocytes and smooth muscle cells in the vessels. This triggers inflammatory reactions, promotes the formation of thrombi and increases the proliferation and migration of smooth muscle cells in the arterial walls (27). Together, these processes contribute to plaque instability, thrombotic events and the progression of atherosclerosis, which lead to negative cardiovascular consequences such as myocardial infarction. Targeting this pathway with antithrombotic therapies or inhibitors of PAR1 signaling represents a potential strategy to reduce cardiovascular risk and improve outcomes in patients with CAD (27).

In summary, based on the comprehensive analyses and results presented in this study, miR-106b-5p emerges as a promising biomarker for the detection of coronary artery disease (CAD). Through rigorous bioinformatics analysis and experimental validation using qRT-PCR, we found that miR-106b-5p is significantly upregulated in CAD patients compared to healthy controls. This differential expression suggests its potential utility as a diagnostic marker for CAD. The study employed robust methods such as differential gene expression analysis and miRNA-mRNA interaction network construction, which confirmed the association of miR-106b-5p with CAD-related signaling pathways. Importantly, ROC curve analysis showed high sensitivity and specificity of miR-106b-5p in discriminating CAD patients from healthy individuals, with an AUC value of 0.8975. These results emphasize the potential of miR-106b-5p as a non-invasive biomarker for the early detection of CAD and offer advantages over current diagnostic methods. However, further research is needed to validate these results in different populations and to elucidate the underlying molecular mechanisms driving the association of miR-106b-5p with CAD.

In conclusion, miR-106b-5p is a promising, valuable diagnostic tool for CAD and paves the way for future studies aimed at improving cardiovascular disease management and patient outcomes. However, future research efforts should focus on conducting studies with larger sample sizes and long-term follow-up of patients to confirm and extend these initial results.

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