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ORIGINAL ARTICLE

Hsa-miR-584-5p as a novel candidate biomarker in Turkish men with severe coronary artery disease

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Abstract

Coronary artery disease (CAD) is still the preliminary cause of mortality and morbidity in the developed world. Identification of novel predictive and therapeutic biomarkers is crucial for accurate diagnosis, prognosis and treatment of the CAD. The aim of this study was to detect novel candidate miRNA biomarker that may be used in the management of CAD. We performed miRNA profiling in whole blood samples of angiographically confirmed Turkish men with CAD and non-CAD controls with insignificant coronary stenosis. Validation of microarray results was performed by qRT-PCR in a larger cohort of 62 samples. We subsequently assessed the diagnostic value of the miRNA and correlations of miRNA with clinical parameters. miRNA-target identification and network analyses were conducted by Ingenuity Pathway Analysis (IPA) software. Hsa-miR-584-5p was one of the top significantly dysregulated miRNA observed in miRNA microarray. Men-specific down-regulation ($p = 0.040$) of hsa-miR-584-5p was confirmed by qRT-PCR. ROC curve analysis highlighted the potential diagnostic value of hsa-miR-584-5p with a power area under the curve (AUC) of 0.714 and 0.643 in men and in total sample, respectively. The expression levels of hsa-miR-584-5p showed inverse correlation with stenosis and Gensini scores. IPA revealed *CDH13* as the only CAD related predicted target for the miRNA with biological evidence of its involvement in CAD. This study suggests that hsa-miR-584-5p, known to be tumor suppressor miRNA, as a candidate biomarker for CAD and highlighted its putative role in the CAD pathogenesis. The validation of results in larger samples incorporating functional studies warrant further research.

Keywords Atherosclerosis · miRNA · Microarray · IPA · T-Cadherin · Stenosis

Introduction

Coronary artery disease (CAD) is still the leading cause of mortality and disability in the developed world. It is imperative to identify novel biomarkers of diagnostic and prognostic value to alleviate the burden of CAD worldwide. Recent genome-wide association studies (GWASs) have paved the way for unravelling genetic risk factors involved in the etiopathogenesis of CAD [1–3] However, common variants

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identified by GWASs explain only 6% of the heritability of CAD so a large proportion of the heritability of CAD is still yet to be determined [4, 5]. In the past few years, researchers have been investigating the yet undiscovered heritability by exploring the roles of microRNA (miRNA) in cardiovascular disease and there is now accumulating evidence that miRNAs contribute to the development of atherosclerosis, plaque development and pathogenesis of CAD [6–9].

MiRNAs are short (20–23 nucleotides in length) non-coding RNAs that bind to the 3′untranslated region (3′UTR) of messenger RNA and regulate gene expression at the post-transcriptional level by inhibiting or degrading the translation of mRNAs [10]. MiRNAs can easily be measured and detected in tissues and different types of body fluids (e.g. blood, plasma, serum, urine, saliva, tears) in highly stable form and different expression patterns [11]. Tissue and cell-specific expressions of miRNAs have drawn much attention by researchers making them the potential target of cutting edge medical therapies for human diseases [12–15]. However, the rapid evolution of bioinformatic tools has enabled the identification of miRNA targets and their putative roles in disease pathogenesis and related pathways. In humans, around 2654 miRNAs have been identified and are estimated to regulate at least 60% of the protein-coding genes [last accessed on 03 March 2019 (miRBase 20- https://www.miRbase.org/)] [16].

In recent years, growing evidence emphasized that altered expressions of extracellular and intracellular miRNA associate with pathophysiological conditions and suggest their potential usage as risk assessment and therapeutic interventions for various human diseases including CAD [17–19]. Thus, assessing the expression profiles of miRNAs in CAD patients and non-CAD controls has a tremendous clinical impact by leading to biomarker discoveries for CAD.

In this study, we aimed to investigate the distinct signatures of miRNA expressions in whole bloods of Turkish patients with angiographically confirmed CAD and non-CAD controls with angiographically insignificant coronary lesions. Bioinformatic and statistical analyses were performed to comprehensively identify target genes, construct the miRNA-target gene-disease network and correlate validated miRNA with lipoprotein lipid-profiles.

Material and methods

Study design and subjects

This study was designed in three steps; (1) miRNA profiling by microarray in discovery sample (n = 7), (2) Validation of dysregulated miRNA by qRT-PCR (Quantitative Real-time PCR) in the extended sample (n = 62), (3) Bioinformatic analyses to identify miRNA-target and miRNA-CAD related target interactions by Ingenuity Pathway Analysis (IPA) Software. The study workflow is shown in Fig. 1.

The study samples used in this study were recruited from T.C Ufuk University Medical Center Cardiology Department between 2014 and 2016; all samples were aged 18 years or over and underwent invasive coronary angiography due to stable angina pectoris, ischemia and acute coronary syndrome. Selected seven men [four patients > 90% luminal stenosis narrowing and three non-CAD controls (< 20% stenosis)] were used for miRNA microarray analysis. MiRNA validation by qRT-PCR was performed in 62 samples [26 CAD patients who had at least 70% luminal stenosis narrowing in their epicardial coronary artery and 36 non-CAD controls (< 20% stenosis)] (Supplemental Table 1). Two independent operators were evaluated coronary luminal narrowing following the guidelines of ACC/AHA for classification of coronary lesions. SYNTAX (Synergy between percutaneous coronary intervention with Taxus and Cardiac Surgery) Scores (SYNTAX score) and Gensini Scores (GS) were calculated for each sample following angiography to evaluate the complexity and severity of CAD. Whole sample collection and analysis processes were conducted in compliance with the ethical guidelines of the Declaration of Helsinki and approved by Institutional Review Board at Istanbul

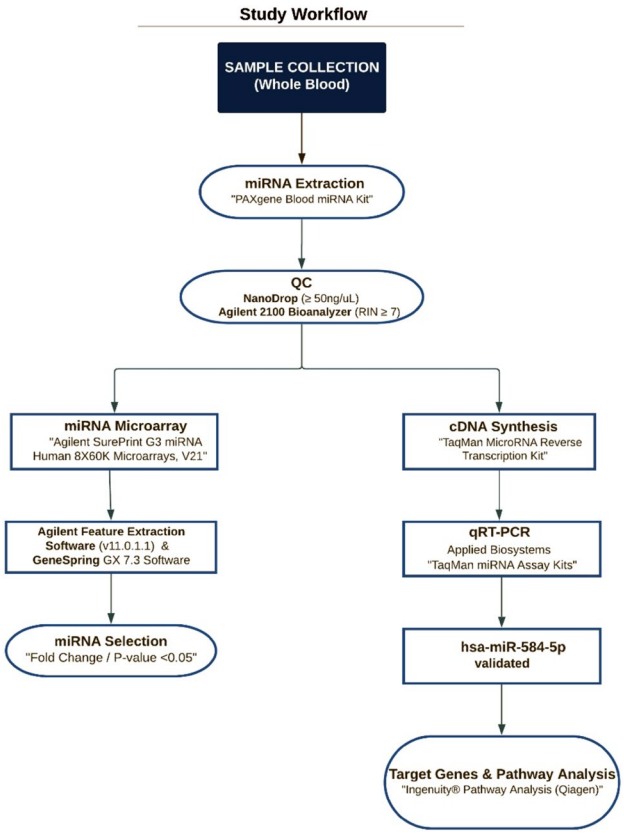

Fig. 1 The workflow of the study

University, Istanbul Medical School, Sehremini, Istanbul, Turkey. The written informed consents were obtained from all participants and all experiments were performed in accordance with the approved guidelines and regulations.

Laboratory test

Blood serum samples were collected before coronary angiography and stored at − 80 °C until analyzed. Any lipemic, icterus and hemolysis specimens were excluded. Analyses of biochemical parameters were determined in two central laboratories. Concentrations of total cholesterol (TC), fasting triglycerides, glucose, High-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured by UniCel DxC 800 (Beckman Coulter, USA). Glycosylated Hemoglobin (HbA1c) levels were analyzed using the High-Performance Liquid Chromatography (HPLC) system which is considered as the gold standard. The results were given as the ratio of HbA1c to total hemoglobin in percentages (HbA1c %).

miRNA extraction

Venous blood samples were collected into PAXgene Blood RNA Tubes (PreAnalytiX, Hombrechtikon, Switzerland) and miRNA was isolated from whole blood using the PAXgene miRNA isolation kit (PreAnalytiX) by following the manufacturer's protocol. The samples were stored at − 20 °C for 24 h until miRNA extraction. The quantity and quality of the RNA were evaluated using NanoDrop spectrophotometer 2000 (Thermo Scientific, Waltham, MA, USA) and RNA Integrity Number (RIN value) was assessed by using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano kit (Agilent Technologies, Palo Alto, CA). Samples with a RIN ≥ 7 were used for further downstream analyses.

miRNA microarray analysis

MiRNA expression profiles were analyzed by using the Agilent miRNA microarray system in selected four patients with proven severe atherosclerotic CAD (SYNTAX score ≥ 33) and three non-CAD controls. MiRNA profiling (Agilent SurePrint G3 miRNA Human 8X60K Microarrays, v21) were assessed by miRNA Complete Labeling and Hybridization Kit (Agilent Technologies) using 1.5 µg of total RNA according to the manufacturer's instructions. Agilent Technologies G4900DA SureScann was used for detecting signals and data was analyzed by using Agilent Feature Extraction Software (v11.0.1.1) and GeneSpring GX 7.3 software (Agilent). All statistical analyses were performed by using R statistical package v3.1.2. Differentially expressed miRNAs were determined with a fold change and *p*-value threshold of > 1.5 and < 0.05, respectively.

Validation of miRNA by quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was carried out using the "TaqMan MicroRNA Assay Kit" (Applied Biosystems Life Technologies, Foster City, CA, USA) and with hydrolysis probe (Assay ID: hsa-miR-584-5p) in 26 CAD patients [SYNTAX score ≥ 33 and severe coronary lesion (≥ 70%)] and 36 non-CAD controls. "TaqMan MiRNA Reverse Transcription (RT)" kit was used for cDNA synthesis from 10 ng total RNA samples according to manufacturer's instructions. The qRT-PCR was performed with 2.5 ul product on Roche LightCycler 480 Real-Time PCR System. All qRT-PCR reactions were run in triplicates. We used Comparative threshold cycle (Ct) method (2^{-DDCt} method) was used for the analysis of the data and the results were expressed as the relative quantification (RQ) values. Small nucleolar RNA (snoRNA), SNORD44 (RNU44) (Assay ID: 001,094, Assay name: RNU44) was used as an endogenous control for normalization of hsa-miR-584-5p expression.

Statistical analyses

The prevalence of hypertension, diabetes mellitus, lipid lowering drug usage and smoking history between CAD cases and non-CAD were compared using the chi-square test. Quantitative variables were presented as mean values ± standard deviations (SD) and categorical variables were presented as percentages. Correlations between miRNAs and biochemical parameters were examined with non-parametric Spearman correlation analysis. Mann–Whitney U test was performed to analyze the difference in whole blood miRNA expression between CAD and non-CAD controls. *P*-value, confidence interval (CI), and SD were calculated using Ct values obtained from qRT-PCR results. Receiver operating characteristic (ROC) curves were also generated, and areas under the ROC curves (AUC) were calculated to obtain sensitivity and specificity. The AUC value ≥ 0.5 was set as cut off for inferring the diagnostic value for the distinction between control and patient outcomes. SPSS (v21.0, SPSS Inc, Chicago, IL, USA) and GraphPad Prism (v.8) were used to perform statistical analyses. Statistical significance was defined by $p < 0.05$ for all tests.

Bioinformatic analysis

Target molecules of validated miRNA and miRNA-target interactions were examined by using the MicroRNA Target Filter feature implemented in Ingenuity Pathway Analysis (IPA) software (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). The MicroRNA Target Filter retrieve information from TargetScan, TarBase, miRecords database incorporating Ingenuity Knowledge Base and provide results for experimentally

validated and predicted target genes supported with literature-curated Ingenuity Knowledge Base. Identified target genes can be filtered based on confidence level, function/presence in species, role in diseases, tissues and cell lines, pathways, molecules and location of the molecule. Specific filters were applied to analyze the miRNA-CAD related target genes relationships and the network was constructed by using the Pathway Build tool in IPA.

Results

Baseline characteristics of study subjects

A total of 62 subjects with angiographically documented stenosis were enrolled in the current study. Of 62 subjects, 26 had severe coronary lesions (≥ 70%) and 36 samples had angiographically insignificant coronary lesions (< 20% stenosis). Patients with obstructive CAD had higher incidence of family history of cardiovascular diseases. In addition, Type 2 diabetes was more prevalent among CAD patients compared to the control group. The mean age of participants with angiographically significant and insignificant CAD are 60.46 and 57.33 years, respectively. The baseline characteristics of the study participants are shown in Supplemental Table 1.

MiRNA microarray analysis

In total, we identified ten miRNAs, which were differentially expressed (DEMs) in whole blood of CAD patients (n = 4) compared to non-CAD controls (n = 3) (Supplemental Table 2). Hsa-miR-3960 and hsa-miR-584-5p were the top miRNAs that were significantly down-regulated in CAD group compared to non-CAD with a *p*-value of 0.004 (− 1.571 fold change) and 0.003 (− 1.858 fold change), respectively. Volcano plot and hierarchical clustering analysis plots showing the DEMs can be found in Fig. 2. Hsa-miR-584-5p with the highest fold change among all DEMs were chosen for quantification with qRT-PCR and further bioinformatic analyses.

Quantitative RT-PCR results of hsa-miR-584-5p

Expression profiles of hsa-miR-584-5p were analyzed by qRT-PCR in the total sample of 26 CAD with CAD and of 36 non-CAD controls (Supplemental Table 3 and Fig. 3). Hsa-miR-584-5p was found to be down-regulated in CAD patients when comparing with non-CAD with a marginally significant *p*-value ($p = 0.056$) in total sample and in men ($p = 0.041$). Quantitative RT-PCR result also showed a trend of down-regulation ($p > 0.05$) of hsa-miR-584-5p in women with CAD.

Specificity and sensitivity of hsa-miR-584-5p for CAD patients

The diagnostic accuracy of hsa-miR-584-5p as a candidate biomarker for distinguishing CAD patients from non-CAD controls was evaluated by ROC curve with the area under the curve (AUC) value. In whole group, the AUC value of hsa-miR-584-5p for the CAD patients was 0.643 (95% CI 0.502–0.784, $p = 0.055$). As depicted in Fig. 4a, b, hsa-miR-584-5p has higher specific and sensitive diagnostic value for men (AUC = 0.714, 95% CI 0.531–0.897, $p = 0.041$) when compared to women (AUC = 0.562, 95% CI 0.361–0.764, $p = 0.606$). Results of ROC curve analysis were shown in Supplemental Table 3 and ROC graphs can be found in Fig. 4.

IPA

Following validation of hsa-miR-584-5p by qRT-PCR, we explored its molecular targets and relationships with CAD by using IPA software (QIAGEN Inc., Germany). MiRNA target filter tool in IPA enabled us to define target genes by filtering disease and biological functions using the data gathered from Ingenuity Knowledge Base. Mining target genes of hsa-miR-584-5p by filtering disease categories as 'Cardiovascular Disease' identified 29 unique predicted target genes categorized based on the evidence for their role in cardiovascular conditions including CAD. Particularly, the majority of the targets were linked to anemia which is an independent risk factor for CAD (Supplemental Table 4). When we select the disease function as 'Coronary Artery Disease', *CDH13 (Cadherin 13, T-Cadherin)* was the only predicted target that had strong scientific evidence for associate with CAD Fig. 5 illustrates the network analysis generated from IPA and canonical pathways associated with *CDH13*. The network representations of the CDH13 involvement in the four canonical pathways can be found in Supplemental Figs 1–4.

Correlation of hsa-miR-584-5p with clinical parameters

We further assessed the correlation of hsa-miR-584-5p with available clinical parameters and found the expression level of

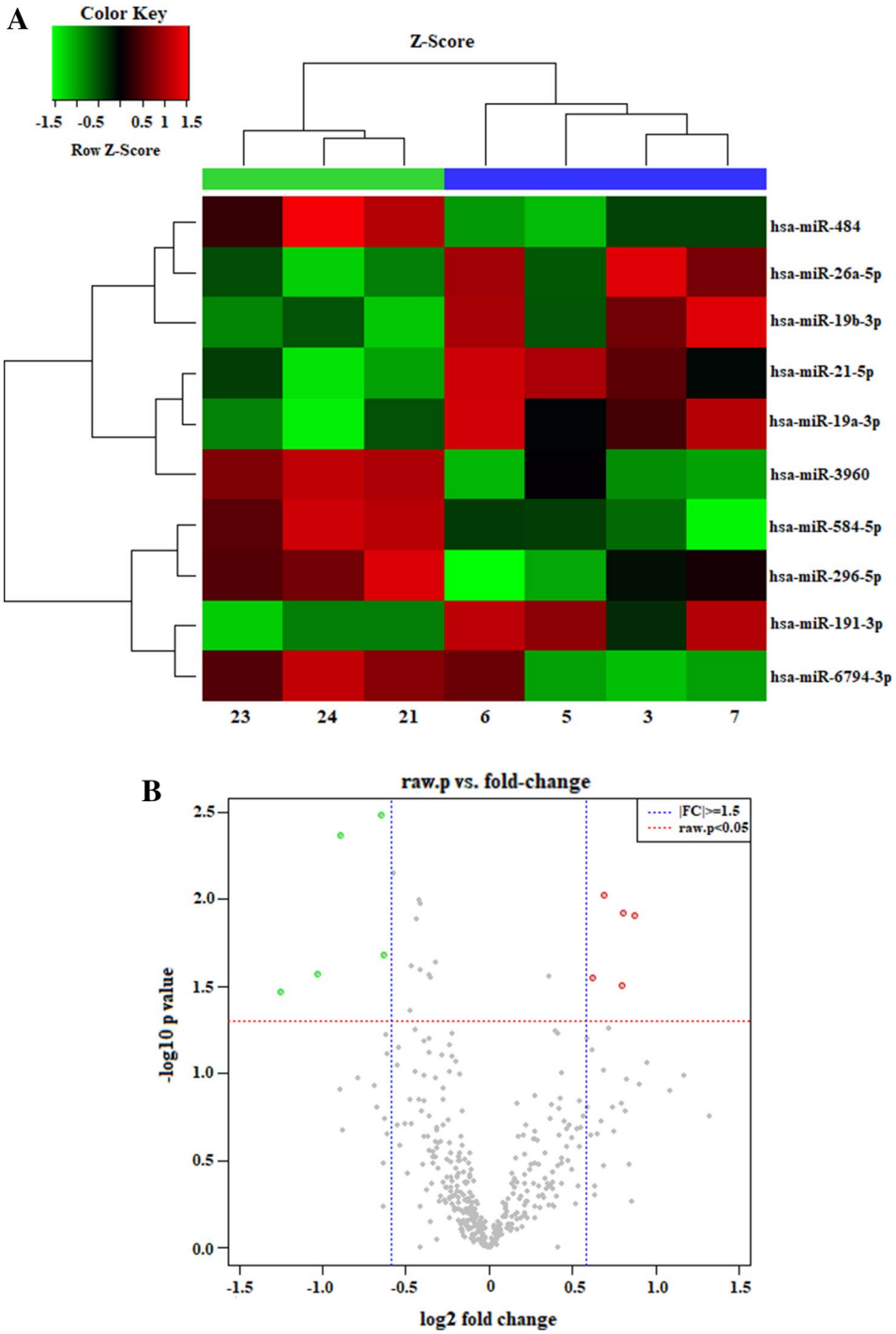

Fig. 2 Expression profiles of whole blood miRNAs in CAD patients (n = 4) and non-CAD controls (n = 3). **a** Hierarchical clustering analysis for the selected differentially expressed miRNAs. The horizontal axis represents the samples, and the left vertical axis represents the miRNA (blue bar: CAD; green: non-CAD controls). Red and green represent up-regulation and down-regulation separately. **b** The volcano plot illustrates differentially expressed miRNAs: dots in grey indicted the miRNAs that did not reach significant changes of expression; dots in green on the down indicated the miRNAs that had significant down-regulation of expression, and dots in red on the up indicated the miRNAs that had significant up-regulation of expression. (Color figure online)

hsa-miR-584-5p was significantly negatively correlated with stenosis (r = −0.27, $p = 0.037$) and Gensini score (r = −0.26, $p = 0.039$) in total sample (n = 62) (Fig. 6). However, no significant correlations were observed when analyzing CAD and non-CAD controls separately. The inverse association of hsa-miR-584-5p with stenosis ($p = 0.015$) and Gensini scores ($p = 0.005$) also remained significant in multiple linear association test after controlling for covariates such as sex, age, Type 2 Diabetes, lipid lowering drug, and family history (Supplemental Table 5).

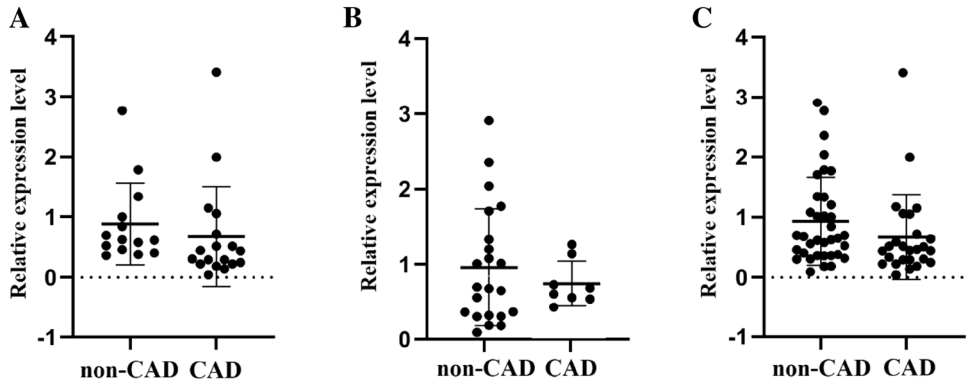

Fig. 3 Relative expression levels of hsa-miR-584-5p. The figure depicts relative expression levels of hsa-miR-584-5p in **a** men ($p=0.04$), **b** women ($p=0.629$) and **c** in all samples ($p=0.056$). Y-axis indicates the relative expression level

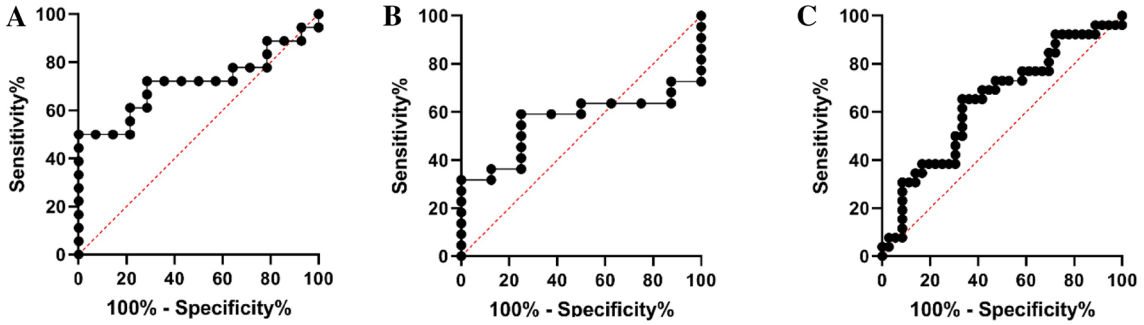

Fig. 4 Specificity and Sensitivity of hsa-miR-584-5p for CAD patients. Results of ROC analysis for hsa-miR-584-5p in whole blood of non-CAD and CAD groups were shown in A, B and C. The area under the ROC curve (AUC) for hsa-miR-584-5p are 0.714, 0.562, 0.643 in men with CAD (**a**), women with CAD (**b**) and in all samples (**c**), respectively

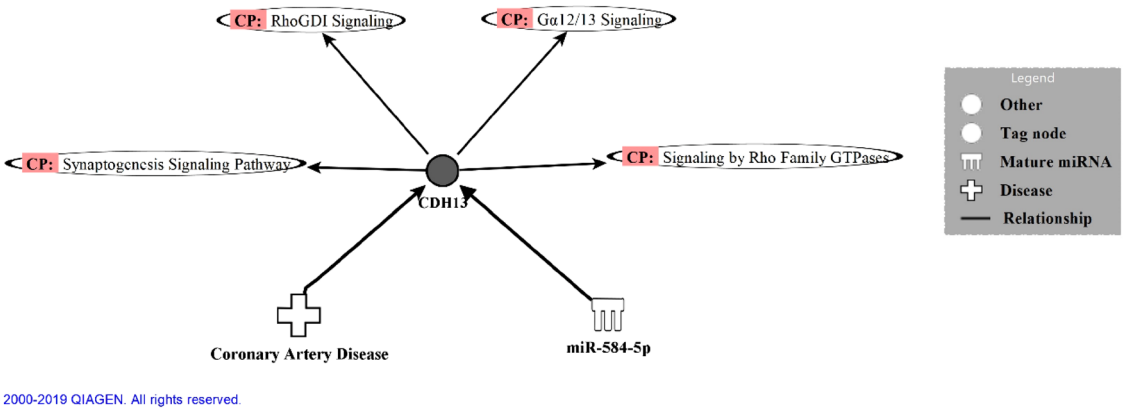

Fig. 5 Network plot generated from Ingenuity Pathway Analysis software (IPA, Qiagen Inc., Germany) showing the relationship of hsa-miR-584-5p with CAD-associated predicted target and related Canonical Pathways (CP). Target gene prediction is based on the TargetScan. TargetScan CS (Context++ scores) for *CDH13* is -0.295

Discussion

Reducing the global burden of CAD is still one of the major research areas for public health researchers. Easily detectable, sensitive and specific biomarker discoveries are of great importance for accurate diagnosis, risk prediction, prognosis and effective management of CAD. miRNAs have been proposed to be ideal biomarkers for several pathologic conditions including CAD due to their superior properties such as their easy detectability, tissue-specific expressions, presence of different body fluids and conservations among species [20–22].

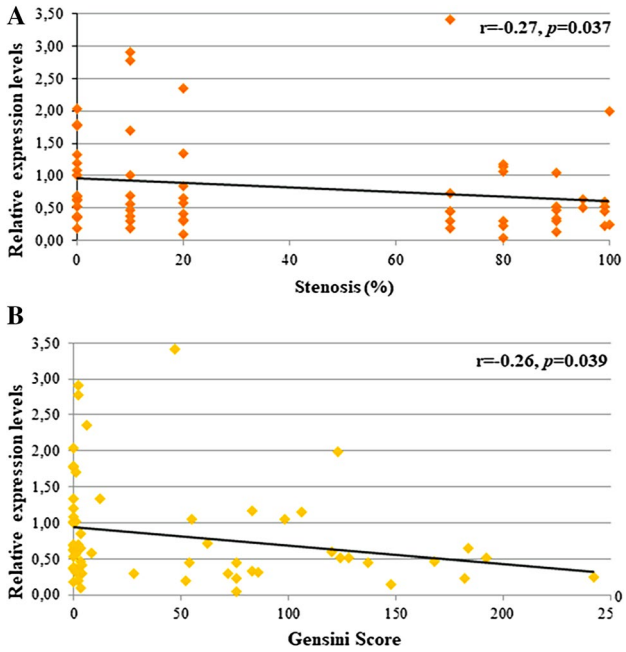

Fig. 6 Correlation analysis of hsa-miR-584-5p with stenosis and Gensini Scores (GS) (n = 62). a Correlation between stenosis and hsa-miR-584-5p. **b** Correlation between GS and hsa-miR-584-5p

In this study, we assessed the difference in whole blood miRNA expression profiles of angiographically confirmed CAD patients and non-CAD controls to identify a novel candidate miRNA biomarker for CAD. Significantly different expressions of ten miRNA were identified in miRNA microarray analyses and we selected hsa-miR-584-5p for validation by qRT-PCR. Our qRT-PCR results were consistent with miRNA array and hsa-miR-584-5p was down-regulated in men with CAD compared to non-CAD controls. We also observed the inverse correlation of expression levels of hsa-miR-584-5p with the severity of CAD and stenosis implicating hsa-miR-584-5p down-regulation may subsequently activates distinct pathways involving in the CAD pathogenesis. Remarkably, our ROC analysis shed light on the potential diagnostic biomarker property of hsa-miR-584-5p to distinguish CAD patients from non-CAD controls.

Hsa-miR-584-5p, located on chromosome 5q32, is known to be tumor-suppressor miRNA [23] and have been recently shown to down-regulated in certain cancers including human neuroblastoma [24], thyroid carcinoma [25], glioma [26] and human clear cell renal cell carcinoma [27]. Meanwhile, consistent with our results, Weber et al. also reported the down-regulation of hsa-miR-584-5p in whole bloods of CAD patients who underwent ACE/ARB therapy [28]. This study highlights the gender-specific expression patterns of hsa-miR-584-5p and points out that its postulated role in CAD pathogenesis differentiates in male and females. Gender-associated expressions of miRNAs and their possible gender-associate functions in human diseases have been also affirmed by prior research [29–32]. Thus, the role of hsa-miR-584-5p as a sex-specific biomarker for CAD needs to be further investigated.

Hsa-miR-584-5p validation was followed by bioinformatics analysis using IPA and provided 29 experimental and predicted targets involving in the pathways associated with cardiovascular disease. Yet, *CDH13 (Cadherin 13, T-Cadherin)* was the only target gene identified when we filtered the disease categories as CAD. The *CDH13* was a predicted target based on TargetScan algorithms and has no experimental validation. Thus, further studies are warranted to investigate the hsa-miR-584-5p mediated regulation of *CDH13* and their role in CAD pathogenesis.

CDH13 gene encodes T-Cadherin (T-CAD, H-Cadherin, Cadherin-13), which is an unclassical member of the cadherin superfamily, is known to be adiponectin receptor and anchored to the plasma membrane through glycosylphosphatidylinositol (GPI) [33, 34]. Experimental studies indicated the intriguing role of T-Cadherin in the pathobiology of human diseases such as cancer, lung disease, neurological disorders, metabolic disease and cardiovascular disease [34, 35]. Moreover, its amino-acid sequence is highly conserved in all vertebrates and this support the notion that T-cadherin has biological significance for human biology [34]. T-Cadherin expressed by smooth muscle cells (SMC) and endothelial cells have been shown to participate in cell migration, proliferation and survival (Supplemental Figs. 1–4) [36–38]. High levels of T-Cadherin has been also observed in diseased vascular endothelial and smooth muscle cells during atherosclerosis [39, 40]. Genetic association studies also reveal *CDH13* sequence variants associated with CAD and cardiometabolic phenotypes and the results imply that *CDH13* sequence variations contribute to the fluctuation of plasma T-Cadherin levels [41–43]. Furthermore, *CDH13* was found as one of the 15 novel risk loci for CAD in a recent meta-analysis and it was suggested to be a novel candidate gene for vessel disease due to its involvement in blood vessel development [3]. Yet, the knowledge of the function of T-Cadherin on the cardiovascular system is still limited and require to be clarified by further investigations.

Several miRNA biomarker candidates for CAD using different sources (i.e. whole blood, platelets, plasma, serum, microvehicles) have already been postulated by many studies [18, 44–48]. Suggested miRNA biomarkers may have lack of therapeutic and diagnostic efficacy due to the discrepancy in study design so it is highly noteworthy to validate the results in distinct populations for the usage of the miRNA in clinical applications. Our study design is remarkable in terms of using samples from a not well-studied population, Turks, and reveal population specific expressions of miRNAs in Turkish samples. However, to the best of our knowledge, this is the first study that evaluates the expression profiles of

whole blood miRNAs in Turkish samples. Notably, we did not obtain consistent results for previously DEMs in CAD that were detected in plasma or serum. In agreement with our findings, the striking patterns of variation in miRNA expressions in different body fluids (whole blood, serum and plasma) were observed in previous studies as well [49–51].

However, our current research has some limitations. Our research only includes the validation of hsa-miR-584 yet other differently expressed miRNAs determined in miRNA array still need to be quantitatively validated and further assessed for their diagnostic and prognostic implications in CAD. Besides the relatively small size of sample used in the study, identifying miRNA target by only bioinformatic tools without doing experiments were also drawbacks of the study.

In conclusion, we found different expression patterns of hsa-miR-584-5p between Turkish patients with CAD and non-CAD while postulated its diagnostic value for CAD by further analyses. Our findings require to be validated in larger samples and future comprehensive research may clarify the functional relevance of hsa-miR-584-5p with *CDH13* and CAD.

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Author contributions NC, DP designed and conducted the experiments. AFE. and BE contributed materials. NC, DP, BD contributed to data analysis and wrote the manuscript. NC, DP, BD, AFE and BE interpreted the results. All authors reviewed the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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