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Correlation of circulatory VEGF and miRNA 126 in angiographically proven coronary artery disease and its clinical importance

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ABSTRACT

Background: Atherosclerotic ischemic coronary artery disease (CAD) is a major cause of morbidity and mortality in both developed and developing countries, indicating the need for the discovery of deeper molecular insights of CAD mechanisms, biomarkers, and innovative therapeutic targets. Vascular endothelial growth factor (VEGF) may have some role, in the progression of human coronary atherosclerosis. Circulating microRNAs (miR) have a potential as a diagnostic as well as a prognostic biomarker of cardiovascular disease and dysfunctions. **Aims and Objectives:** The aim of the study was to correlate the levels of miRNA-126 and VEGF in the serum of CAD patients. **Materials and Methods:** The study was conducted on 100 cases of freshly diagnosed CAD patients (angiographically proven) and 80 normal healthy patients. Various serum biomarkers were estimated. The level of serum VEGF and Mi-RNA was correlated for CAD patients. **Results:** Highly significantly difference was found in levels of hemoglobin A1C, low density lipoprotein, total cholesterol, and total homocysteine while high-density lipoproteins and triglycerides were less significantly different for controls and cases. VEGF levels of CAD cases were found to be very significantly higher as compared to controls. The miRNA-126 expression was found to be lower in the CAD subjects compared to non-CAD subjects. **Conclusion:** The study was suggested the role of VEGF in atherogenesis and plaque instability. No significant correlation was found among between miR-126 levels and VEGF levels among CAD cases. The study is limited by a smaller sample size and was single-centered; therefore, multicentric study with a larger patient cohort needs to be done and to validate our findings.

Key words: Circulating biomarker; Coronary artery disease; Vascular endothelial growth factor; Mi-RNA

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INTRODUCTION

Globally coronary artery disease (CAD) is a major public health problem. Cardiovascular disease was the most common underlying cause of death in the world in 2013, accounting for an estimated 17.3 million of 54 million total deaths, or 31.5% of all global deaths.[1] In Asian adults over 18 years of age, CAD prevalence is 4.3% which is likely to increase by approximately 18% by 2030.[2] In developing countries, mortality from CAD is increasing while in developed countries, it was declining.[3]

In CAD, coronary arteries are obliterated or obstructed due to atherosclerosis causing restriction in blood flow and lack of oxygen supply to myocardium.[4,5] In a severe case, total obstruction of the artery causes myocardial infarction. Vascular endothelial growth factor (VEGF) is an important angiogenic factor related with endothelial function. Pathological findings demonstrate distinct expression of VEGF and its receptors in atherosclerotic lesions in human coronary arteries and VEGF may have some role, in the progression of human coronary atherosclerosis.[6] It is still debatable whether VEGF is proatherosclerotic or antiatherosclerotic factor. In

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addition, as an endothelial-enriched miRNA, miR-126 has been reported to play important roles in modulating vascular development and angiogenesis[7,8] miR-126 can be detected and precisely quantified in the circulation. In cardiovascular pathology, circulating miR-126 concentrations is markedly reduced and negatively correlated with pathological determinants.

In human coronaries, VEGF and its receptors are not detected in normal coronary segments but are expressed in atherosclerotic areas, more specifically in endothelial cells of microcapillaries, in macrophages, and in partially differentiated SMC.[9]

It is well established that miRNAs play critical roles in physiological and pathological processes in the cardiovascular system, such as endothelial dysfunction, inflammation, apoptosis, angiogenesis, atherosclerosis, and neointimal hyperplasia or restenosis.[10]

In clinical practice, high sensitivity cardiac troponin test is widely used due to high accuracy in diagnosing acute myocardial infarction (AMI); therefore, it is difficult for new biomarkers to demonstrate the significant added value on top of cardiac troponins.[11] The unspecific elevation of troponin levels can be present in case of non-ischemic heart failure, renal failure, myocarditis, arrhythmias, and pulmonary embolism due to myocardial injury.[11,12] One of the major limits of cTnT is that multiple dosage at different time is needed and patients are ordered to stay in the emergency room for 3–6 h after arrival. *De facto* measurement of circulating miRNAs requires quantitative real-time polymerase chain reaction (qRT-PCR), which is a time-consuming technique, in comparison with detection of hs-cTnT (approximately 30 min) and the 2015 ESC guidelines recommend the use of a rapid rule-out protocol (0 h and 1 h or 0 and 3 h) when hscTnT is available.[13] The use of qRT-PCR is currently the limiting factor in terms of rapid detection of circulating miRNAs.

The role of miRNAs as novel biomarkers in the early diagnosis of AMI is debated. Therefore, the present study is aimed to evaluate circulating miRNA 126 in patients with coronary artery disease and its use as biomarker for diagnosis of CAD.

Aims and objectives

To find out the levels of miRNA-126 and VEGF in the serum of CAD patients and assess the possible correlation between elevation in the levels of specific miRNA and levels of different lipids and lipoproteins including angiogenesis factors, that is, VEGF.

MATERIALS AND METHODS

The case–control study was done at the Department of Pathology, King George's Medical University, Lucknow, from August 2017 to August 2018. One hundred and eighty patients attending the general outpatient department (OPD) or admitted to indoor facility of the department of Cardiology, KGMU, Lucknow, were recruited for the study. The study was approved by the Institutional Ethical Committee before subject recruitment. A written informed consent was also taken from the patients.

The patients between 40 and 81 years of either sex with clinical evidence of hyperlipidemia and freshly diagnosed CAD by angiographically were included in the study. Normal healthy patient attending OPD for other than CAD complaint was selected for controls.

Patients with a history of significant concomitant diseases including hepatic failure, renal failure, abnormal liver function, hepatitis, cardiomyopathy, congenital heart disease, bleeding disorders, previous thoracic irradiation therapy, and malignant diseases were excluded from the study.

CAD was defined as at least one major epicardial vessel with >50% stenosis, assessed by quantitative coronary angiography. Hyperlipidemia was defined as total cholesterol (TC) level of ≥250 mg/dL and/or low-density lipoproteins (LDL) level of ≥160 mg/dL, or if the patient was being treated with lipid-lowering medication. Hypertension was defined as resting systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg or in the presence of active antihypertensive treatment. Individuals who formerly or currently smoked ≥10 cigarettes per day for at least 2 years were defined as smokers.

Detailed history including age, sex, educational status, nature of work, body mass index, smoking habit, alcoholic, hypertension, diet, diabetes mellitus, medical history, and lifestyle habits was recorded. Risk factors were determined by questionnaire, physician diagnosis, and/or treatment for hypertension, hyperlipidemia, and diabetes.

Samples collection and serum isolation

3.5 mL of peripheral blood was collected in plain and EDTA vials (NOVAC, POLYMED, POLY MEDICURE LTD, India) from cases and controls. Serum was separated by centrifugation at 1900 g for 10 min, followed by a 10 min high-speed centrifugation at 16,000 g and stored at −80°C until further processing. To avoid potential contamination of leukocytes, all samples were processed within 1 h of the collection.

Biochemical examination

Biochemical parameters including Plasma Glucose (mg/dL), hemoglobin A1C (HbA1c) (%) (D-10 Bio-Rad, USA), TC, triglyceride (TG), high-density lipoproteins (HDL)-cholesterol, LDL- cholesterol, very LDL-cholesterol, Folate II, Vitamin B12, Vitamin D 25-OH, and total homocysteine (HCY2) were recorded. All biochemical parameters were measured by a fully automated biochemical analyzer (ARCHITECT i2000SR, Abbott Diagnostic and Selectra ProXL, ELITech Group).

Serum VEGF level estimation

Serum VEGF was determined manually by high sensitivity indirect sandwich enzyme-linked immunosorbent assay (ELISA) procedure using RayBio Human VEGF ELISA kit and the reading was recorded by iMark™ Microplate Absorbance Reader (BIOS) at 450 nanometer according to the instruction by the manufacturer.

RNA isolation from serum

Total RNA was extracted using a Trizol-based miRNA isolation protocol (Invitrogen, Carlsbad, CA, USA). Finally, the RNA concentrations were measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc. Wilmington, USA), and the RNA samples were stored at −80°C for future use.

cDNA synthesis and quantitative real-time PCR

Total RNA extracted from serum was initially reverse transcribed using (Multiscribe) MuLV reverse transcriptase kit (Cat. no. K1622, Thermo Fisher Scientific, USA). cDNA was amplified with miR126 (hsa-miR-126-5p, Cat no. 4427975) and RNU6 (Cat no. 4427975). Then, qRT PCR was carried out on the 7500 real-time PCR system (Applied Biosystems, USA) using TaqMan® Universal Master Mix II No UNG (Applied Biosystem, USA) according to the manufacturer's instructions. The relative expression levels of miRNAs were calculated using the 2ΔΔCt method and fold-changes were calculated for each miRNA.[14,15]

Statistical analysis

The statistical analysis was done using Statistical Package for the Social Sciences Version 21.0 statistical Analysis Software. Discrete variables were represented as numbers, percentages, and mean±SD. Categorical variables were analyzed by the Chi-square tests and Student's "t" test. Analysis of variance was used to compare within group and between group variances among the study groups. The Pearson correlation coefficient is an estimate for correlation. In all analyses, statistical significance was accepted at $P<0.05$.

RESULTS

The study was conducted on 180 subjects out of which, 100 cases of angiographically proven CAD cases and 80 controls matched with age and gender. Most of the parameters are similar for cases and controls except age which was significantly higher for cases (53±10) as compared to controls (42±10) (Table 1).

Highly significantly difference was found in levels of HbA1c, LDL, TC, and total HCY2 while HDL and triglycerides were less significantly different for controls and cases. Both Vitamin B12 and total HCY2 levels were found to be higher for cases compared to control but the difference is not significant for Vitamin B12. All other parameters have higher average levels for controls compared to cases (Table 2).

VEGF levels of cases (259.68±222.33 units) were found to be significantly higher as compared to Controls (120.76±104.80 units). Although miR126 levels of controls (0.721±0.362 units) were found to be higher as compared to that of Cases (0.649±0.274 units), this difference was not found to be statistically significant (Table 3).

Association of HbA1c and miR-126 levels was found to be statistically significant. CAD cases with lower HbA1c levels, that is, <6 and 6–8 had significantly raised miR-126 levels as compared to those with high HbA1c levels, that is, >8 (0.702±0.283 and 0.653±0.266 units vs. 0.427±0.153 units). No significant association of other laboratory parameters of cases was found with miR-126 and VEGF levels (Table 4).

Correlation of miR-126 levels and VEGF levels among CAD cases was found to be non-significant, unidirectional and level of correlation was weak ($r=0.007$; $P=0.948$) (Figure 1).

DISCUSSION

CAD is caused by atherosclerotic lesion which ultimately narrows the vessel lumen, causing the ischemic symptoms and cardiac dysfunction. CAD has been one of the

Table 1: Comparison of average values of physical attributes of cases and controls

Variables	Cases (n=100)		Controls (n=80)		Significance of differences
	Mean	SD	Mean	SD	"P"
Height (in cm)	161.7	9.3	162.6	11.2	0.56
Weight (in kg)	61.7	10.9	63.8	10.6	0.19
Age (in years)	53	10	42	10	<0.001
BMI	24	4.2	24.6	3.4	0.3
Hypertensive	23	23.0	12	15.0	0.178

BMI: Body mass index

Table 2: Comparison of biochemical parameters of coronary artery disease cases and controls

Variables	Cases (n=100)		Controls (n=80)		Significance of differences	
	Mean	SD	Mean	SD	"t"	"P"
BMI	24.00	4.14	24.66	3.31	−1.158	0.248
HbA1c	6.53	1.59	5.50	0.71	5.383	<0.001
HDL	42.18	13.41	48.33	12.72	−3.129	0.002
LDL	57.54	31.14	82.58	45.09	−4.397	<0.001
VLDL	30.23	14.91	34.74	34.15	−1.187	0.237
TG	144.28	64.87	176.92	124.29	−2.269	0.024
TC	129.22	40.30	169.10	46.15	−6.184	<0.001
Folate II	13.10	10.83	13.35	10.57	−0.153	0.878
Vitamin B12	332.43	334.22	250.64	239.33	1.843	0.067
Vitamin D	21.34	13.78	23.14	25.56	−0.602	0.548
Total HCY2	22.58	11.67	2.48	2.39	15.141	<0.001

HbA1c: Hemoglobin A1C, BMI: Body mass index, HDL: High-density lipoproteins, LDL: Low-density lipoproteins, VLDL: Very low-density lipoproteins, TG: Triglyceride, TC: Total cholesterol, HCY2: Homocysteine

Table 3: Fold change in circulating MiR126 and VEGF levels in coronary artery disease (CAD) cases and controls

Variables	Cases (n=100)		Controls (n=80)		Significance of differences	
	Mean	SD	Mean	SD	"t"	"P"
VEGF	259.68	222.33	120.76	104.80	5.148	<0.001
MiR126	0.649	0.274	0.721	0.362	−1.528	0.128

VEGF: Vascular endothelial growth factor

Table 4: Association of circulating miR-126 and VEGF laboratory parameters (cases)

Variables	miR-126				VEGF			
	Mean	SD	"t"/F	"P"	Mean	SD	"t"/F	"P"
HbA1c								
>8 (n=11)	0.427	0.153	4.733	0.011	202.09	154.17	1.265	0.287
6–8 (n=47)	0.653	0.266			237.87	200.06		
<6 (n=42)	0.702	0.283			299.18	256.16		
Total cholesterol								
<130 (n=59)	0.653	0.285	0.092	0.912	271.23	233.70	0.633	0.533
130–200 (n=35)	0.635	0.254			256.61	217.08		
>200 (n=6)	0.683	0.324			164.06	110.16		
Total triglyceride								
<30 (n=52)	0.650	0.282	0.016	0.984	271.32	237.99	0.849	0.431
30–200 (n=34)	0.652	0.289			271.49	207.43		
>200 (n=13)	0.637	0.219			187.79	196.30		
HDL								
<40 (n=49)	0.658	0.266	0.097	0.907	289.67	259.41	1.054	0.352
40–60 (n=44)	0.644	0.267			238.30	187.64		
>60 (n=7)	0.611	0.403			184.18	86.94		
LDL								
≤130 (n=95)	0.650	0.272	0.148	0.882	257.52	218.61	-0.423	0.673
>130 (n=5)	0.631	0.354			300.88	313.16		
Folate II								
0–44 (n=94)	0.657	0.278	1.155	0.251	264.27	225.47	0.815	0.417
>44 (n=6)	0.524	0.187			187.85	162.97		
Vitamin D								
≤25 (n=72)	0.655	0.271	0.387	0.699	271.10	239.55	0.822	0.413
>25 (n=28)	0.632	0.286			230.32	170.60		
Vitamin B12								
0–300 (n=70)	0.649	0.273	-0.007	0.994	279.58	243.38	1.373	0.173
>300 (n=30)	0.649	0.283			213.26	156.92		
Total HCY2								
0–20 (n=48)	0.663	0.275	0.502	0.617	278.61	211.96	0.816	0.416
>20 (n=52)	0.635	0.276			242.21	232.16		

VEGF: Vascular endothelial growth factor, HbA1c: Hemoglobin A1C, HDL: High-density lipoproteins, LDL: Low-density lipoproteins, HCY2: Homocysteine

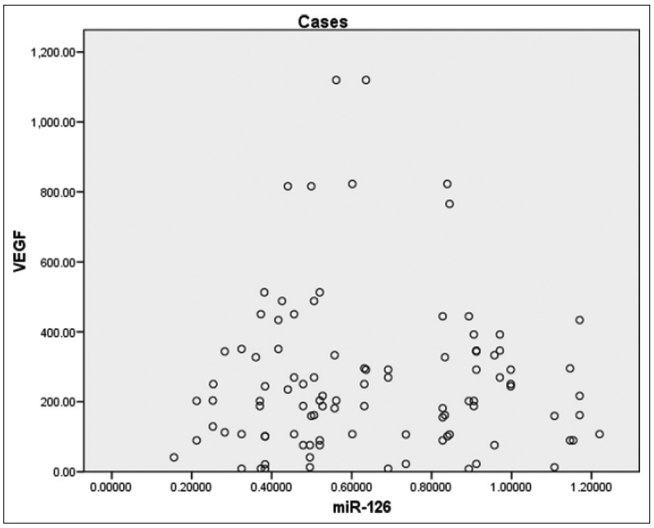

Figure 1: Correlation of miR-126 and VEGF among cases

major health problems worldwide despite the advances in treatments. Thus, there is a crucial need to find new biomarkers and therapeutic targets for CAD to further improve the prognosis of these patients.[16]

In the present case–control study of 180 subjects, 100 were angiographically proven CAD patients and 80 were age-, gender-matched healthy controls. Mean age was 53 and 42 years in case and control group, respectively. Majority of the subjects enrolled in the study were aged >45 years, that is, 74.0% cases and 61.3% controls, respectively.

The prevalence of hypertension was less in control (15%) versus patients (23%) with CAD. Similarly, results were found in the study done by Long et al.,[8] and Sun et al.,[17] Sun et al., found the prevalence of hypertension (50% in patients without CAD versus 60% in with CAD) and diabetes (30% vs. 25%) were not different between two groups.

The glycemic control was poor in CAD patients compared to control with mean value of 6.53 and 5.5 in cases and control, respectively (P<0.001). This is in concordance with Shanoli et al.,[18] study, in which diabetes was found to be dominant in CAD-positive group but hypertension was equally distributed in both CAD and non-CAD group.

The mean value of HDL of cases and control is 42.18 and 48.33 in mg/dL, respectively, showing a significant difference of P<0.002 supporting that a higher value of HDL has a protective role in heart disease. However, the mean value of LDL, TG, and TC were significantly lower in CAD cases compared to control with P<0.001, P=0.024, and P<0.001, respectively. Similar results shown by Sun et al.,[17] Long et al.,[8] and Shanoli et al.,[18] Shanoli et al., in their study found no significant differences in TG, TC, and non-HDL level among cases and controls suggesting that these levels have less predictive power on the incidence of CAD in this Eastern Indian population which differs from the studies done in the past in this country. In favor of this finding, some recent research has provided more evidence suggesting that smaller LDL particles are more atherogenic and hence more closely associated with CHD, regardless of total or LDL cholesterol levels.[18]

In the study of Sun Xiao (2012)[17] in which they analyzed micro RNA 126 between 31 patients with CAD and 36 patients without CAD along with the biochemical parameters and found the lipid profiles were similar between two groups due to medications. This can also be the reason of lower mean values of LDL, TG, and TC in our study as most of the CAD patients are follow-up patients with undergoing statins or standard treatment.

The mean level of Vitamin D was slightly lower in CAD cases compared to control but the difference was not significant. Similarly, no significant difference was noted between Vitamin B12 and Folate which definitely indicates a need to extend the study on a larger population to determine the normal range in CAD group and detailed questionnaire regarding multivitamin supplements.

The total HCY2 levels were found to be higher for cases compared to control P<0.001. This is in concordance with the studies done by Shenoy et al.,[19] and Sadeghian et al.,[20] Similarly in the study of Shenoy et al.,[19] fasting serum HCY2 levels in CAD patients were significantly higher than patients without CAD (P<0.001). Furthermore, homocyseine levels correlated significantly with increasing severity of CAD (P<0.001). In the study of Sadeghian et al., the mean plasma level of HCY2 in patients (19.3±1.7 μmol/lit) was significantly higher than the control group (13.9±0.9 μmol/lit) (P<0.005) and other factors such as cigarette smoking, increasing age, diabetes mellitus, and hypertension did not have any statistically significant role in hyperhomocysteinemia.

VEGF levels of cases (259.68±222.33 units) were found to be significantly higher as compared to Controls (120.76±104.80 units) with P<0.001. In addition, similar results have been shown by study of Blann et al.,[21] Lin et al.,[6] Blann et al., reported increased VEGF concentrations in CAD patients compared to healthy controls.

The miR126 levels of controls (0.721±0.362 units) were found to be higher as compared to that of Cases (0.649±0.274 units) but this difference was not found to be statistically significant. This is in concordance with the study of Fichtlscherer et al.,[22] Sun et al.,[17] Li et al.,[7] Sun et al., in their study found a mean value of miR-126 was

lower in CAD cases than non-CAD cases but that was not significantly downregulated in the blood of patients with CAD as compared to patients without CAD, suggesting that this miRNA is not a marker specific for significant CAD. However, Li et al.,[7] in which they categorized the 110 CAD patients based on the diseased vessel according to their coronary angiography results as follows: 26 patients with an isolated left main CAD (LMCA) (24%), 22 patients with LMCA plus single-vessel disease (20%), 20 patients with LMCA plus double-vessel disease (18%), 15 patients with LMCA plus triple-vessel disease (14%), and 27 patients with three-vessel disease (25%). The expression level of miR-126-5p in patients with multi-vessel disease was lower than the control group. However, the expression of circulating miR-126-5p was not dramatically down-regulated in patients with an isolated LMCA compared with the control subjects.

In this study, the expression of miR-126 was not significantly down-regulated in CAD patients compared to the controls, so that the down-regulation of circulating miR-126 must reach a threshold plaque burden of diseased vessel and there is a need of extensive study of miR-126 expression on the different groups on the ground of severity of CAD.

Association of HbA1c and miR-126 levels was found to be statistically significant. CAD cases with lower HbA1c levels, that is, <6 and 6–8 had significantly raised miR-126 levels as compared to those with poor glycemic control of high HbA1c levels, that is, >8 (0.702±0.283 and 0.653±0.266 units vs. 0.427±0.153 units).

No significant association of other laboratory parameters such a LDL, HDL, TC, TG, Vitamin B12, Folate, Vitamin D, and total HCY of cases was found with miR-126 and VEGF levels. This is in accordance with the study of Blann et al.,[21] found no other significant correlations/associations between any risk factor (lipids, smoking, and blood pressure) with VEGF. However, interestingly, the level of miR-126 was significantly decreased in patients with CAD and high LDL cholesterol level. In contrast, the level of miR-126 was significantly increased when LDL cholesterol was high in patients who had risk factors for CAD but did not have angiographically significant CAD. To the best of our knowledge, no study has been done so far to establish the association between miR 126 and VEGF with Vitamin B12, Folate, Vitamin D, and total HCY.

Correlation of miR-126 levels and VEGF levels among CAD cases was found to be non-significant, unidirectional and level of correlation was weak (r=0.007; P=0.948). Although very few studies had found the inverse correlation of mi126 with VEGF. Downregulation of miR-126 increases VEGF activity has been reported by Amr et al.,[23] and Sasahira et al.,[24] In this context, we wish to further conduct multicentric studies with larger patient's cohort to validate the change in the miRNA levels with VEGF levels.

Limitations of the study

This study is limited by smaller sample size and was single-centered; therefore, multicentric study with larger patient's cohort needs to be done and to validate our findings. Multicentric studies with a larger patient's cohort will help to validate the change in microRNA-126 levels to confirm its importance as diagnostic biomarker for the CAD-suffering population.

CONCLUSION

VEGF levels of CAD cases were found to be very significantly higher as compared to controls which suggest the role of VEGF may in atherogenesis and plaque instability. The miRNA-126 expression was found to be lower in the CAD group compared to non-CAD subjects but the difference was not significant. Probably because the down-regulation of circulating miR-126 must reach a threshold plaque burden of diseased vessel. Hence, there is a need of extensive study of miR-126 expression on the different groups on the ground of severity of CAD. No significant correlation was found among between miR-126 levels and VEGF levels among CAD cases. No significant association of laboratory parameters such as LDL, HDL, TC, TG, Vitamin B12, Folate, Vitamin D, and total HCY of cases was found either with miR-126 or VEGF levels.

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