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(Research Article)

Epigenetic Effect of MicroRNA in Coronary Heart Disease with and without Diabetes

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Abstract: Coronary heart disease (CHD), as well as diabetes illness, remain the cause of mortality worldwide; hence, founding new biomarkers measurable in the initial stage of illness is necessary to accelerate treatment efficiency. Recently evidence has demonstrated alterations in miRNA levels associated with deregulated gene expression in diabetes and CHD. This case-control study investigates the probable effect of miR-146a rs2910164 C /G polymorphism in diabetes and atherosclerotic cardiovascular disease in Egyptian patients. The study comprised 210 individuals, including 70 CHD patients without diabetes, 70 CHD patients with diabetes, and 70 healthy controls, of Egyptian origin. Anthropometric and blood biochemical parameters were measured as well genetic analysis for rs2910164 C/G polymorphism was performed for all subjects using TaqMan real-time PCR assay. Our results revealed that; the biomedical parameters have a significant correlation between CHD with and without diabetes patients and healthy controls with a p-value <0.05. Analyses of genotype distribution for (rs2910164 C/G) revealed a significant association in both CHD with and without diabetes and control (odd ratio= 1.56, confidence interval (CI 95 %) = (0.06-0.63)) and (odd ratio= 0.88, (CI 95 %) = (0.83-0.92)) respectively. The current study's findings indicated that the selected polymorphism, miR-146a rs2910164 could represent a useful biomarker for susceptibility to CHD and diabetes in the Egyptian people.

Keywords: coronary heart disease; diabetes; Polymorphisms; microRNAs; TaqMan

1. INTRODUCTION

Despite powerful new improvements in identification, prevention, diagnosis, treatment, and disease management; cardiovascular diseases (CVDs) remain an extensive challenge forcing a bulky responsibility on healthcare systems[1]. Atherosclerosis is a complicated process that establishes endothelial cell dysfunction in the coronary artery and causes luminal narrowing, blocking blood flow to the heart and leading to an acute heart attack[2,3]. Diabetes Mellitus (DM) is a significant causative point for cardiovascular diseases[4]. Even recently diagnosed diabetes patients had one or more vascular complications, and CHD is the prominent cause of death among diabetics[4, 5]. Moreover, Patients with DM die from CHD two to six times than those who don't[6]. However, there are few biomarkers for diagnosing the early stage of CVD from diabetes[4]. MicroRNAs, discovered in 1933 by Ambros and Ruvkun group, are not protein-coding RNA[7]

These small non-coding molecules have critical roles in several pathophysiological actions, including cellular mechanisms such as the cell cycle[8,9]. After ten years of the discovery, scientists start to discover it as a biomarker for cancer, followed by many other diseases. Micro RNA proved its efficiency in disease detection due to its quick synthesis, specificity, and it's easily detection[10]. Currently, MiRNA has been used in many diseases (epilepsy, cancer...) as a diagnostic and prognostic biomarker due to its mutated expression[11]. MiRNA- 146a was regulated by nuclear factor-κB-dependent (NF-κB) through Toll-like receptors such as TLR4 which is activated by lipopolysaccharide, and other cytokines such as interleukin-1β or tumor necrosis factor TNF-a, also increased the levels of mature miR-146a. MiR-146a SNP rs2910146 is located in the pre-miR-146a and

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associated with various diseases where inflammation is an important issue[12,13]. Human miR-146 exists in two forms, miR-146a and miR-146b, which are highly expressed in macrophages, mononuclear cells, and T lymphocytes[14].

Consequently, this study determines the correlation of the polymorphism of MicroRNA146a rs2910164 and its involvement strengthens the risk of developing these diseases in the Egyptian populations.

2. METHODS

1.1 Study subjects

The current study is a case-control based on the Helsinki Declaration. All participants wrote an informed consent for genetic and biochemical analyses. A total of 70 CHD patients without diabetes, 70 CHD patients with diabetes, and 70 healthy volunteers (control group) were recruited for the current study from September 2019 to November 2021.

Biochemical parameters such as lipid profile, fasting blood sugar, liver enzymes, total creatine kinase activity, and kidney functions were determined using the standard protocols at the clinical laboratory of The National Heart Institute (NHI). We measured anthropometric variables, Body mass index (BMI), and Blood pressure. Furthermore, the questionnaire recorded information on smoking and a family history of CHD and diabetes. Biochemical characteristics of patients and controls were given in Table 1 and Table 2, respectively.

1.2 Sampling

10 ml of venous blood was collected from each participant (patients and control groups) for genotype analysis and measurements of blood biochemical parameters.

1.3 Genetic analysis (Genomic DNA extraction and genotyping)

Two mL of whole venous blood was drowned in EDTA tubes. The DNA was extracted from the nucleated blood cells by salting protocol [15]. The integrity of the recovered DNA was assessed by gel electrophoresis. NanoDrop spectrophotometer was used for measuring DNA concentration and purity. DNA was stored at -80° C until used.

The polymorphism of miRNA146a rs2910164 C/G was genotyped by using the TaqMan real-time PCR technique using the pre-designed assay (SNP ID: rs2910164, Catalog No, 4351379) for allelic discrimination, containing specific TaqMan probes with fluorescent dyes for each allele (VIC specific for mutant allele and FAM specific for wild type allele). The total volume of PCR was 20µL, with 5µl DNA, 10µl TaqMan Universal PCR Master Mix, 0.05µl (40X) Assay Mix, and 4.5µl of RNase-free water. The polymerase chain reaction conditions were a pre-denaturation cycle at 95°C for 10 minutes, then 45 denaturation cycles at 95°C for 10 seconds and annealing at 60°C for 30 seconds. For genotyping quality control, deionized water was used to replace template DNA as a negative control, and 10% of samples were randomly selected and duplicated, with a 100% match rate. TaqMan Genotyper Software analyzed data. (Version 1.3.1).

1.4 Statistical analyses

This study conducted statistical analysis for the data using SPSS V20.0 software. Normally distributed data were compared by using student's t-test. Data are presented as mean ± SD from three different groups. A P-value less than 0.05 was deemed statistically significant. ANOVA compared demographic characteristics for continuous data and Pearson's χ^2 test to compare categorical data frequencies between participants. Allele frequencies for each participant were stratified and calculated based on the gene counting method.

2. Results

2.1 Clinical and biochemical characteristics of the study subjects

Table 1 illustrates the general characteristic data of enrolled subjects. This study included 140 CHD with and without diabetes patients, including 91 men and 49 women with mean age (of 57.7 ± 6.2) years, and 70 volunteers were involved in a healthy control group, comprising 19 men and 51 women with mean age (47.57± 6.41) years. Statistically significant differences in sex and age were found between CHD with and without diabetes patients compared to the controls group (P=0.001 and P=0.02, respectively). Other clinical data, including body weight, BMI, SBP, and DBP, were significantly higher in CHD with and without diabetes patients than in controls (P<0.05). In addition, we observed significantly higher levels of the biomedical parameters such as (TC, TG, LDL-C, fasting blood sugar, liver enzymes, and kidney functions) and lower HDL-C levels in CHD with and without diabetes patients compared to the controls (P<0.05).

Table 2 illustrates the general characteristic data of enrolled subjects. This study included 70 CHD with diabetes patients, including 50 Men and 20 women with mean age (of 58.34± 5.8), and 70 CHD without diabetes patients, comprising 41 men and 29 women with mean age (57.04 ± 6.58) years. No statistically considerable differences in sex, age, body weight, and BMI were found between CHD with diabetes patients compared to CHD without diabetes (P>0.05). Other clinical data including, SBP and DBP were significantly higher in CHD with diabetes patients than in CHD ithout diabetes patients (P=0.025 and 0.005, respectively).

Table 1. Clinical and biochemical characteristics of CHD with and without diabetes patients and healthy controls

Variables	CHD with and without diabetes cases	Healthy Control	P-Value Sig(2-tailed)
No. (%)	140 (100%)	70 (100%)	--------
Gender (%) Men Women	 91(65%) 49(35%)	 19(27%) 51(73%)	0.001*
Age	57.7 ± 6.2	47.7± 6.41	0.02*
Wt. (Kg)	87.96±9.83	79.6 ±7.9	0.04*
Body mass index (BMI) (kg/m^2)	29.67± 3.9	27.97 ±3.03	0.001*
fasting blood glucose (FBG)	158.9 ±71.4	105.9 ±14.6	0.002*
Total cholesterol (mg/dl)	212.00±40.4	134.00±21.1	0.002*
HDL-Cholesterol (mg/dl)	38.56 ±8.6	49.53 ±9.07	0.012*
LDL-Cholesterol (mg/dl)	141.9±39.3	96.06 ±19.2	0.001*
Triglyceride (mg/dl)	182.9± 48.08	88.7 ±57.92	0.000*
Systolic Blood Pressure (mmHg)	127 ±12.7	114 ± 4.9	0.031*
Diastolic Blood Pressure (mmHg)	85.8±9.3	74± 4.9	0.039*
Smoking Yes No	 37 103	 42 28	0.000*
Serum Urea (mg/dl)	68.85±49.6	24.25±7.92	0.000*
Serum Creatinine (mg/dl)	1.41±0.81	0.70±0.24	0.000*
Alanine aminotransferase(ALT) (U/L)	89.92±29.5	21.64± 8.43	0.014*
Aspartate aminotransferase (AST) (U/L)	84.5±128.6	24.6±11.19	0.000*
Creatine kinase(CK) (U/L)	231.5±25.5	65.87±32.9	0.000*

- The results are expressed as n (%) and Mean ±SD, *p< 0.005 is deemed statistically significant.

Table 2. Correlation of clinical and biochemical characteristics of CHD with diabetes patients and CHD without diabetes

Variables	CHD with diabetes cases	CHD without diabetes cases	P-Value Sig (2-tailed)
No. (%)	70 (100%)	70 (100%)	--------
Gender (%) Men Women	 50(71%) 20(23%)	 41(59%) 29(41%)	0.156
Age	58.34±5.8	57.04±6.58	0.812
Wt. (Kg)	79.6±7.9	88.96±9.83	0.906
BMI (kg/m^2)	29.47±4.1	29.87±3.6	0.664
Blood Sugar (FBG)	212.81±64.48	105.09±15.4	0.000*
TC (mg/dl)	204.01±37.26	220.31±42.02	0.001*
HDL-C (mg/dl)	37.9±8.48	39.23 ±8.7	0.125
LDL-C (mg/dl)	154.14±51.93	145.47±47.86	0.082
TG (mg/dl)	176.86±39.47	186.97± 55.21	0.061
Systolic BP (mmHg)	129.57 ± 12.56	126 ±12.67	0.025*
DiastolicBP (mmHg)	87.36± 8.67	84.14±9.7	0.005*
Smoking Yes No.	 19 51	 18 52	0.850
Serum Urea (mg/dl)	71.37±52.87	66.33±46.47	0.429
Serum creatinine (mg/dl)	1.37±0.74	1.45±0.88	0.380
ALT (U/L)	102.27±9.84	193.31±7.12	0.059
AST (U/L)	151.89±13.68	67.1±15.3	0.056
CK (U/L)	483.5±4.49	378.67±4.86	0.329

The results are expressed as n (%) and Mean ±SD, *p< 0.005 is deemed statistically significant.

In addition, we observed significantly higher levels of fasting blood sugar in CHD with diabetes patients than in CHD without diabetes patients (P<0.001) and significantly lower the total cholesterol levels in CHD with diabetes patients than in CHD without diabetes patients (P<0.001). At the same time, there was no statistically significant difference with other biomedical parameters such as (TG, LDL-C, liver enzymes, and kidney functions) and lower HDL-C levels in CHD with diabetes patients compared to CHD without diabetes patients (P>0.05).

2.2 Frequencies of SNP genotype between patients and controls

Polymorphism in miRNA sequence (rs2910164 C/G in miRNA146a) was successfully genotyped in all CHD with and without diabetes patients and healthy controls using TaqMan SNP Genotyping Assay. Regarding TaqMan Genotype Software, the findings of the allelic discrimination data were shown as a plot of Allele 1(G) (VIC dye) and Allele 2 (C) (FAM dye) respectively as given in Figures (1).

The statistical analysis revealed that genotype frequency and allele distribution of polymorphism (rs2910164 C/G) showed significant differences between CHD patients and healthy controls groups ($p < 0.05$), while there were no significant differences between CHD with and without diabetes patients groups as given in Tables (3 and 4) and Figures (2). The genotype distributions of the miRNA-146a rs2910164 (GG) were significantly shown in the patient's group with and without diabetes compared to the control group ($\chi^2 = 20.97$, P-value= 0.01 and $\chi^2 = 13.04$, P-value = 0.000, respectively).

Regards to allele frequency distribution, there was a statically significant higher proportion of G allele of rs2910164 carriers in CHD with and without diabetes patients groups than in the controls group (P-value =0.04), with OR value of 1.97 (95% CI: 1.37-2.8), and (P-value =0.002), with OR value of 1.97 (95% CI: 1.717-2.18) respectively.

4. DISCUSSION

Coronary Heart disease and diabetes mellitus are remarkable public health problems[16,17]. Atherosclerosis, along with diabetes mellitus, suggests having a connection through different pathophysiological pathways[18]. SNPs in miRNAs are powerful biomarkers having indicative or a prognostic value and also a guide to investigating the disease causative factors[19]. Microribonucleic acids (miRNAs) are of particular interest in this field, including miR-146a. It has been widely studied because of its important regulatory role in inflammatory and immune processes[20,21]. Recently, the association between miRNAs polymorphisms in CHD and diabetes risks are drawing more attention[22,23]. In this study, we have validated the association between polymorphism of miRNA146a rs2910164 with the risk of the development of CHD and diabetes by comparing CHD with and without diabetes patients with controls groups from unrelated Egyptian populations.

According to previous studies, many risk factors were associated with coronary artery diseases and diabetes development, such as age and men's gender, hypertension, and obesity[24]. In the current study, the analysis revealed a significant (age and sex) difference found among CHD and controls which may signify that increase in age and gender could lead to a higher risk of developing CHD. Furthermore, both the CHD patients and controls groups revealed differences in the biochemical markers which are higher in the patients' group than in the controls, in contrast, a significantly lower level of HDLC was observed in CHD patients. However, no considerable differences were obtained between both CHD with and without diabetes patients groups regarding liver and kidney functions and lipid profile except total cholesterol; this could be explained by the drugs taken by patients to control biochemical markers. These results came following Sung et al., who reported that the people with a history of CHD, hypertension, diabetes, BMI, plasma TG, and TC concentrations were higher in CHD patients compared with controls (P < 0.05) however, plasma HDL-C concentrations were significantly lower in CHD patients[25,26].

We also found that there is an association between (rs2910164 in miR-146a) genotype with coronary heart disease and diabetes risks. Concerning miR-146a rs2910164 C/G, we observed statistically significant differences in its genotype and allele frequencies among both groups and the control group. Our data indicated that subjects carrying the homozygote genotype (GG) are more susceptible to the risk of CHD and diabetes than those carrying GC and CC genotypes. In agreement with our results, several reports revealed an association between rs2910164 C/G and CHD and diabetes risks with increased genotype distribution and allele distributions of rs2910164 in CHD patients compared with healthy controls[27,13,28]. In addition, Xie et al. conducted a meta-analysis of more than eight thousand participants; his results showed a clear association between CHD and MiR-146a through alleles and genotype distribution comparison. [29].

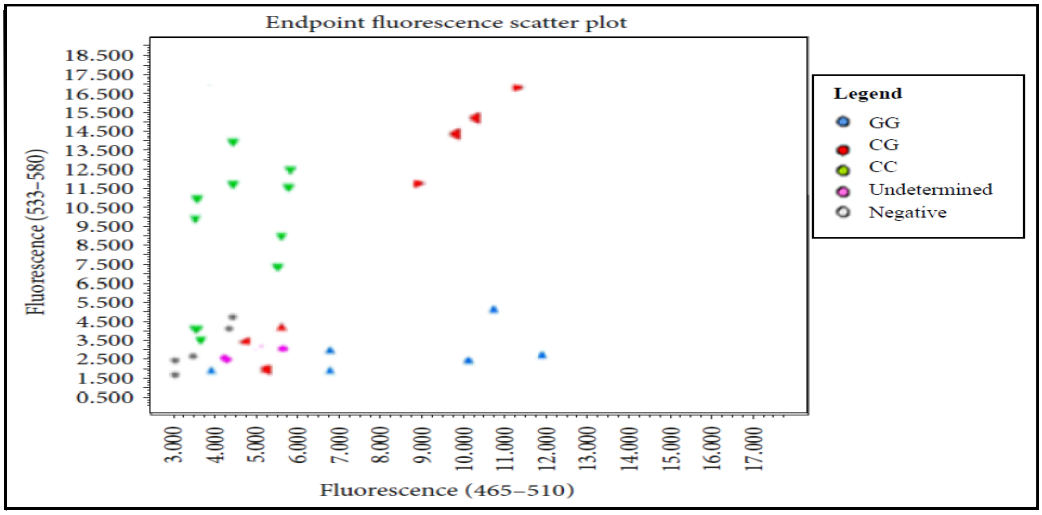

Figure 1. Allelic discrimination plot showing the various genotypes of miRNA-146a (rs2910164 C/G)

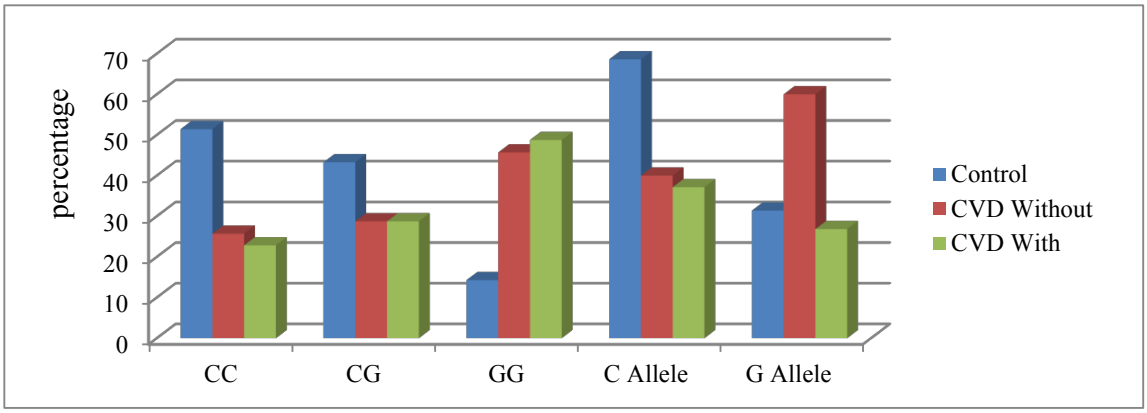

Figure 2. The percentage of *miRNA146a* (rs2910164 C/G) genotype in different studied groups.

Table 3. Association of miRNA-146a polymorphism in CHD with diabetes and control groups

MIR146a SNP	Genotypes Allele	CHD with diabetes n(%)	Healthy Control n (%)	Adjusted OR (95% CI)	Pearson X^2 value	P-value Sig. (2-tailed)
rs2910164 G/C Variant	**Genotype n (%)**	**n=70**	**n=70**	-	-	-
	CC	16 (22.85%)	36 (51.42%)	3.57(1.72-7.03)	**Ref.**	
	CG	20(28.75%)	24(43.28%)	1.30(0.63-2.66)	5.30	**0.466**
	GG	34 (48.75%)	10 (14.28%)	1.56(0.06-0.63)	20.97	**0.01****
	Allelen (%)	**n=140**	**n=140**	-	-	-
	C (Normal)	52(37.14%)	96(68.6 %)	0.34(0.39-0.76)	**Ref.**	
	G (Mutant)	88 (26.85%)	44 (31.4%)	1.97(1.37-2.8)	21.12	**0.04****

Comparisons were carried out by chi-square X^2 test, The results are expressed as n (%). N: number, %: percentage, OR: odds ratio, CI: confidence interval, *p< 0.005 is deemed statistically significant.

Table 4. Association of miRNA-146a polymorphism in CHD without diabetes and control groups

MIR146a SNP	Genotypes / Allele	CHD without diabetes n (%)	Healthy Control n (%)	Adjusted OR (95% CI)	Pearson X^2 value	P-value Sig. (2-tailed)
rs2910164 G/C Variant	**Genotype n (%)**	**n=70**	**n=70**	-	-	-
	CC	18 (25.7 %)	36 (51.42%)	3.57(1.72-7.03)	**Ref.**	
	CG	20 (28.57%)	24(43.28%)	0.90 (0.55-1.47)	0.176	**0.706**
	GG	32 (45.7%)	10 (14.28%)	0.88(0.83-0.92)	13.04	**0.000****
	Allele n (%)	**n=140**	**n=140**	-	-	-
	C (Normal)	56(40%)	96(68.6 %)	0.631(0.45-0.84)	**Ref.**	
	G (Mutant)	84(60.0%)	44 (31.4%)	1.97(1.717-2.18)	13.725	**0.002****

Comparisons were carried out by chi-square X^2 test. The results are expressed as n (%), N: number, %: percentage, OR: odds ratio, CI: confidence interval, *p< 0.005 is deemed statistically significant.

More ever Alipoor et al showed that circulating miR-146a was significantly higher in controls compared to diabetic patients. Furthermore, the rs2910164- G allele is associated with a reduction in expression levels of miR-146a[27]. MiR-146a is one of the most important miRNAs that shown to regulate inflammatory response and promote cell proliferation[19,30]. SNP rs2910164 includes alteration of C into G nucleotide substitution, leading to a mismatch in the stem or secondary structure of miRNA146a precursor; subsequently lowering the expression of pre- miR-146a[31]. Moreover, different studies have shown that miR-146a can reduce pro-inflammatory cytokine production through down-regulating interleukin-1 receptor-associated kinase 1 (IRAK) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) in macrophages[32]. Thus, the downregulation of miR-146a may increase the vascular damage response as well inflammatory process-related to atherosclerosis[33], also activation of NF-kappa B has been noted as a primary factor in the pathophysiology and complications of diabetes[11].

5. CONCLUSIONS

Our approach demonstrates a significant correlation between miR-146a rs2910164 C/G genetic variant and an increasing risk of coronary heart disease with diabetes patients. rs2910164 is also assumed as a potential prognostic biomarker for CHD patients with diabetes in Egyptian population. These specific genetic traits, environmental factors and personal habits could affect the prevalence of CAD with diabetes. More studies could be done in more considerable sample size to evaluate the ability of miR-146a in CAD cases prognostic.

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Conflicts of Interest: The authors declare no conflict of interest.

Ethical Statement: Study protocol was approved by the National Heart Institute Ethical Committee (NHI), Egypt (EC number: 5/2019) and Faculty of Pharmacy, Girls, Al-Azhar University (REC number: 261).

Author Contribution: This work was carried out in collaboration between all authors.**Heba Mohamed Ahmed**: methodology, formal analysis, and writing the manuscript. **Doha E. Ellakwa**: supervision, data curation, editing manuscript, and gaining ethical approval. **Khalda Sayed Amr**: the practical part, data analysis, validation, and editing of the manuscript.**Amr Mohamed Zaher**: the sample collection, editing the manuscript, and gaining ethical approval.

List of Abbreviations:
CHD: Coronary heart disease.
miRNA: MicroRNAs (Microribonucleic acids).
CVDs: cardiovascular diseases.
DM: Diabetes Mellitus.
NF-?B: nuclear factor-?B-dependent.
TNF: tumor necrosis factor.
NHI: National Heart Institute.
BMI: Body mass index.
EDTA: Ethylenediaminetetraacetic acid.
DNA: Deoxyribonucleic acid.
PCR: Polymerase chain reaction.
SNP: Single nucleotide polymorphism.
ANOVA: Analysis of variance.
SBP: systolic blood pressure.
DBP: diastolic blood pressure.
IRAK: interleukin-1 receptor-associated kinase 1.
TRAF6: TNF Receptor Associated Factor 6.

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