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Hypermethylation of miRNA-17~92 cluster in peripheral blood mononuclear cells in diabetic retinopathy

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Abstract

Background and Aims: Diabetic retinopathy (DR) is the most common complication of diabetes. The inflammatory milieu of diabetes results in changes throughout the body. This study asked whether epigenetic changes in peripheral blood mononuclear cells (PBMCs) reflect DR severity.

Methods: PBMCs were separated from the whole blood of DR individuals using density gradient centrifugation. DNA was isolated, and methylation of micro-RNA (miR)-17~92 cluster was evaluated.

Results: We observed that the miR-17~92 cluster was hypermethylated in DR individuals; specifically, this change was most remarkable with proliferative-DR (PDR).

Conclusions: miR-17~92 methylation in PBMCs could help understand DR's pathogenesis and identify individuals at the risk of severe DR for early intervention.

Keywords

Diabetic retinopathy; miRNA; epigenetic regulation

1. Introduction

Diabetic retinopathy (DR) is the most common and progressive complication of diabetes. While there is an alarming increase in diabetics worldwide, treatment options are minimal.

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6. Author Contribution

QL performed experiments; SB conducted statistical analysis; GE helped with statistical analysis design and supervision; AB designed the research, supervised the overall project and wrote the manuscript.

Conflict of interest

AB is an ad hoc Staff Pharmacist at CVS Health/Aetna, this work does not reflect any views or opinions of CVS Health/Aetna. and the authors report no other conflicts of interest with this research.

A more specific problem is the multifactorial nature of the disease pathogenesis. miRNA's are small ~22 nucleotide non-coding RNA's known to play an important role in gene regulation; miRNA bind to a specific sequence at the three prime untranslated region (3'UTR) of their target mRNA to induce translational repression[1]. A miRNA cluster is a set of more than two miRNAs transcribed from physically adjacent miRNA genes; additionally, miRNAs in a cluster are transcribed in a similar orientation, and they are not separated by a transcription unit or by a miRNA in the opposite orientation[2]. Usually, miRNA clusters are two or three miRNAs; however, larger clusters also exist; for example, a miR-17~92 cluster consists of 6 miRNAs miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1[3]. miRNAs could be regulated by epigenetic changes such as DNA methylation. Previous studies demonstrate hypermethylation of miR-17~92 and downregulation of miR-17~92 cluster in fibrosis of tissues obtained from individuals with idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disorder (COPD)[4]. We previously reported that miR-92a levels of peripheral blood circulating angiogenic cells (CACs) reflect changes in the retinopathy status of diabetic individuals[5]. In this study using relatively a simple assay for methylation of miR-17~92 and a small volume of peripheral blood mononuclear cells (PBMCs), we show the miR-17~92 is hypermethylated in DR, of note miR-17~92 hypermethylation changes were highest after a change in DR status from mild non-proliferative DR (NPDR) to proliferative diabetic retinopathy (PDR).

2. Methods

The study was approved by Institutional Review Board (IRB) and conducted after obtaining informed consent and abiding by the declaration of Helsinki. Peripheral blood from the following groups (i) Control, (ii) Diabetes with no DR, (iii) mild NPDR, (iv) moderate NPDR, (v) PDR, and (vi) severe PDR were obtained from the Indiana Biobank (Indianapolis, IN, USA). Study participants reported the race, and the race categories were defined based on the US Office of Management and Budget's Revisions to the Standards for the Classification of Federal Data on Race and Ethnicity[6]. To isolate PMBCs, whole blood was diluted 1:1 in PBS and gently layered on top of the Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). The sample was centrifuged for 30 minutes at 400 x g with the brakes off. The PMBCs at the interface were collected and washed twice in PBS buffer. The DNA was extracted using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Germantown, MD, USA #80224). Next, 250 ng of DNA was digested using the EpiTect Methyl II DNA Restriction Kit (Qiagen, #335452) according to the manufacturer's protocol. After digestion, EpiTect Methyl II qPCR Arrays (Qiagen) were performed; the enzyme reactions were mixed with RT^2 SYBR Green ROX qPCR Mastermix (Qiagen, # 330520) and primers (EPHS1190055–1A, EPHS115450–1A, and EPHS115451–1A) to quantify gene promoter methylation.

Statistics:

Associations of continuous variables with methylation were evaluated using correlation coefficients, and associations with categorical variables were tested using Kruskal-Wallis tests. A two-sided 5% significance level was used for all tests. Analyses were performed using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA).

3. Results

DNA methylation for a miR-17~92 promoter in PBMCs was studied in 40 subjects (56.1 % males and 43.9 % females). The study participants had the following distribution for the race, White (64.9%), Black or African American (29.8%), Asian (1.8%), more than one race (1.8%), and other (1.8%) (Table 1A). The average age of study participants was 58.11 yrs (Table 1B); there was no effect of sex, race, or age on miR-17~92 methylation. Among metabolic parameters, glycated hemoglobin, the duration of diabetes, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides that were studied, the duration of diabetes exhibited a significant difference (p=0.01) in miR-17~92 methylation (Table 1B). Methylation of miR-17~92 was upregulated in both diabetes and DR; however, this increase was significant (p=0.01) for the DR group (Table 1C). The methylation of miR-17~92 was increased upon an increase in severity of DR from mild NPDR to severe PDR. The difference in methylation of miR-17~92 was statistically significant between PDR & mild NPDR groups (p=0.02) and severe PDR & mild NPDR groups (p=0.04; Table 1D).

4. Discussion

A series of studies demonstrate a critical role of miR-17~92 cluster in immune diseases, cardiovascular disorders, normal development [3], and retinoblastoma. Adding to these diseases, this study, for the first time, highlighted the potential role of the miR-17~92 cluster in DR by showing an increase in its hypermethylation. In particular, there was a significant increase in miR-17~92 methylation between NPDR and PDR groups, thus identifying a critical point in DR progression when the subjects are likely at the risk of progressing to a more severe form. Furthermore, as the miR-17~92 measurements were performed on a small volume of PBMCs and using a simple commercially available system, hypermethylation of miR-17~92 could be conveniently measured in clinical samples.

It is intriguing to note an increase in miR-17~92 in PDR than NPDR subjects. Previous studies have shown a similar increase in methylation of miR-17~92 in lung fibroblasts of individuals with IPF and COPD[4]. The miR-17~92 cluster targets fibrotic genes such as transforming growth factor-beta-1 (TGF-β1)[7] and connective tissue growth factor (CTGF)[8], and both are known to be implicated in the pathogenesis of DR. Thus, we speculate that changes in miR-17~92 in PBMCs may reflect fibrotic changes in the retina which may lead to the development of fibrovascular membrane (FVM) formation in PDR, further studies are warranted to shed some light in this direction. In conclusion, our study identified the potential role of miR-17~92 in DR by showing hypermethylation of its promoter, which may help determine its pathogenic mechanism.

Limitations

While this study highlighted hypermethylation of miR-17~92 in PBMCs of DR individuals, there are some limitations; for example, the current study design could not allow assessing changes in fibrotic genes such as TGF-β1 or CTGF in FVMs and miR-17~92 methylation in PBMCs simultaneously. Furthermore, based on the previous studies we focused on miR-17~92 methylation, it would be interesting to assess epigenetic changes

in additional miRNAs or other targets of epigenetic modifications such as histone modification. Additionally, it would be interesting to study the correlation in the severity of retinopathy and miR-17~92, methylation using retinal assessments such as optical coherence tomography angiography (OCT-A); however, at present, this information is not available for this study.

5. Acknowledgments

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Highlights

- Diabetic retinopathy (DR) is the most common complication of diabetes
- Epigenetic changes play an important role in DR's pathogenesis
- miRNA 17~92 cluster in mononuclear cells is hypermethylated in DR
- miRNA 17~92 methylation may help with DR pathogenesis and early intervention

Table 1.

miR-17~92 methylation in PBMC's of individuals with DR.

A. Association between miR-17~92 methylation and study demographics

Variable		Number (%) of partcipants	P-value[e]
Sex			0.56[a]
	Male	32 (56.1%)	
	Female	25 (43.9%)	
	Missing	2	
Race	Asian	1 (1.8%)	0.46[a]
	Black or African American	17 (29.8%)	
	More than one race	1 (1.8%)	
	Other	1 (1.8%)	
	White	37 (64.9%)	
	Missing	2	

B. Correlation coefficients between miR-17~92 methylation and age, duration of diabetes, metabolic parameters[b]

Variable	Mean (SD)	Spearman's Correlation	P-value[e]
Age (years)	58.11 (14.2)	0.04	0.83
Diabetes duration (Days)	5101.5 (2750.5)	0.50	**0.01**
HbA1c(%)	9.0 (2.5)	0.33	0.16
Cholesterol (Total)	157.9 (40.2)	−0.27	0.23
HDL (mg/dL)	47.8 (11.6)	−0.12	0.61
LDL (mg/dL)	81.9 (27.5)	−0.39	0.07
Triglycerides (mg/dL)	134.7 (105.7)	0.07	0.78

C. miR-17~92 methylation in control, diabetes and DR[c]

Diagnosis	N	Mean	Std Dev	Median	Lower Quartile	Upper Quartile	Min	Max	Statistical Significance[e] P-value (comparison)
Control	7	0.03	0.03	0.02	0.01	0.06	0.00	0.09	0.14 (Control vs Diabetes)
Diabetes	7	0.10	0.14	0.04	0.02	0.09	0.02	0.41	0.07 (Diabetes vs DR)
DR	26	0.39	0.75	0.13	0.09	0.24	0.00	3.64	**0.01** (DR vs Control)

D. miR-17~92 methylation in different subpopulations of DR[d]

Diagnosis	N	Mean	Std Dev	Median	Lower Quartile	Upper Quartile	Min	Max	Statistical Significance[e] P-value (comparison)
Mild NPDR	10	0.18	0.31	0.05	0.01	0.10	0.00	0.90	0.09 (Mild NPDR vs Mod NPDR)
Mod NPDR	4	0.15	0.05	0.13	0.12	0.18	0.12	0.22	0.09 (Mod NPDR vs PDR)
PDR	7	0.51	0.53	0.24	0.16	1.08	0.12	1.44	**0.02** (PDR vs Mild NPDR) 0.09 (PDR vs Mod NPDR)

D. miR-17~92 methylation in different subpopulations of DR[d]

Diagnosis	N	Mean	Std Dev	Median	Lower Quartile	Upper Quartile	Min	Max	Statistical Significance[e] P-value (comparison)
Severe PDR	5	0.85	1.56	0.16	0.11	0.23	0.11	3.64	**0.04** (Severe PDR vs Mild NPDR) 0.29 (Severe PDR vs PDR) 0.81 (Severe PDR vs Mod NPDR)

Abbreviations: HbA1c, hemoglobin A1c; LDL, low-density lipoprotein; HDL, high-density lipoprotein; DR, diabetic retinopathy,NPDR,non-proliferative DR;Mod NPDR,moderate non-proliferative;PDR,proliferative DR; std dev, standard deviation.

[a] P-value tests for the association of the variable with methylation% using a Kruskal-Wallis test.

[b] P-value tests for the association of the variable with methylation% using a Spearman's correlation coefficient.

[C] Kruskal Wallis test value to know whether methylation is different between groups: 0.01

[d] Kruskal Wallis test value to know whether methylation is different between groups: 0.02

[e] statistical significance: p-value (in bold) less than 0.05 was considered to be statistically significant