Role of microRNAs in sight-threatening diabetic retinopathy in Chinese patients: abridged secondary publication

YY Cheung *, CH Lee, DTW Lui, CHY Fong, VSY Cheung, JHC Mak, RLC Wong, WS Chow, YC Woo, A Xu, PC Sham, KSL Lam †

KEY MESSAGES

1. MicroRNA-related single nucleotide polymorphisms showed suggestive associations with sight-threatening diabetic retinopathy (STDR) in Chinese patients with type 2 diabetes.
2. MicroRNA profiling analysis identified several microRNAs that were differentially expressed between incident STDR cases and non-STDR controls.
3. Circulating microRNAs demonstrated the potential to serve as non-invasive biomarkers for prediction of STDR development.
4. Independent validation studies are required to confirm the findings of this study.

Hong Kong Med J 2024;30(Suppl 5):S17-20
HMRF project number: 06172266

[1,2] YY Cheung, [1,2] CH Lee, [1] DTW Lui, [1] CHY Fong, [1] VSY Cheung, [1] JHC Mak, [1] RLC Wong, [1] WS Chow, [1] YC Woo, [1,2] A Xu, [3] PC Sham, [1,2] KSL Lam

[1] *Department of Medicine, School of Clinical Medicine, The University of Hong Kong, Hong Kong SAR, China*
[2] *State Key Laboratory of Pharmaceutical Biotechnology, The University of Hong Kong, Hong Kong SAR, China*
[3] *Department of Psychiatry, School of Clinical Medicine, The University of Hong Kong, Hong Kong SAR, China*

* Principal applicant: cyy0219@hku.hk
† Corresponding author: ksllam@hku.hk

Introduction

Diabetic retinopathy (DR) is the most common microvascular complication of diabetes and can lead to vision loss. MicroRNAs (miRNAs) are a group of short, highly conserved, single-stranded, small, non-coding RNAs approximately 22 nucleotides in length. miRNAs post-transcriptionally modulate gene expression through mRNA degradation and the inhibition of protein translation via binding between the 3'-untranslated region of target mRNAs and the miRNA seed region. Dysregulated miRNA expression may play a role in the development of DR. Single nucleotide polymorphisms (SNPs) can alter miRNA function; SNPs involved in miRNA regulatory networks, such as those located in specific miRNA-encoding sequences or in miRNA-binding sites within the 3'-untranslated region of target genes, may have diverse functional consequences. These SNPs can alter miRNA expression, increase or decrease miRNA-target interactions, and create or disrupt miRNA-target interactions. This study aimed to (1) identify the miRNAs involved in sight-threatening (ST) DR by detecting STDR-associated miRNA-related genetic variants from a cross-sectional case-control genome-wide association study (GWAS) of STDR and a multiphase prospective nested case-control genome-wide circulating microRNA expression profiling analysis, and (2) evaluate the potential of the identified circulating miRNAs to serve as biomarkers for prediction of STDR development.

Methods

A cross-sectional case-control GWAS of STDR was conducted; 1000 STDR cases and 2195 non-STDR controls (mostly from the Hong Kong West Diabetes Registry cohort[1]) were included to identify miRNA-related variants that are associated with STDR. The grading of STDR was determined by ophthalmologists, based on the United Kingdom National Screening Committee classification.[2]

A multiphase prospective nested case-control genome-wide circulating miRNA expression profiling study, involving 124 incident STDR cases and 124 controls recruited from the same cohort,[1] was performed to identify novel circulating miRNAs that are related to STDR development. The potential for the identified circulating miRNAs to serve as non-invasive biomarkers for STDR prediction was then evaluated. The discovery phase included 24 incident STDR cases and 24 controls who remained free of STDR; they were matched in terms of age, sex, diabetes duration, haemoglobin A1c level, and hypertension status. Candidate miRNAs were then carried forward to the training phase for validation of the findings from next-generation sequencing. Significant miRNAs were then quantified by reverse transcription (RT)–quantitative polymerase chain reaction (qPCR) for the remaining 100 incident STDR cases and 100 controls for final validation.

STDR cases were defined as patients with type 2 diabetes mellitus who had either pre-proliferative DR (grade R2) or proliferative DR (grade R3). Non-

STDR controls were defined as patients without retinopathy or with background retinopathy (grade R1). Incident STDR cases were defined as patients who developed STDR between baseline assessment and 31 December 2017. Individuals who remained free of STDR through 31 December 2017 were regarded as controls in the miRNA profiling analysis.

For the detection of STDR-associated miRNA-related genetic variants, all participants were genotyped. Imputation was performed to maximise genetic coverage. miRNAs were extracted and used to generate the sequencing libraries, which were subsequently sequenced as paired-end reads of 151 base pairs. Candidate miRNAs were measured by RT-qPCR in the training and validation phases. A previously reported reference miRNA for DR, hsa-miR-328-3p, was used to normalise between-sample variation in RNA isolation.

The PLINK software was used for data management and manipulation. Stringent quality control was applied. In total, 7 645 048 polymorphic SNPs were examined to determine their associations with STDR in 943 STDR cases and 2072 non-STDR controls. A score-based test was used to examine the associations of SNPs with STDR; adjustment was made for age, sex, diabetes duration, haemoglobin A1c, hypertension status, and the first five principal components. Genome-wide significance was defined as $P<5 \times 10^{-8}$. The 'EBSeq' package in R software was used to identify differentially expressed miRNAs. To analyse the RT-qPCR results, the expression levels of miRNAs were normalised to the level of hsa-miR-328-3p. The Mann-Whitney *U* test was used to compare the expression levels of miRNAs in the matched case and control groups. Receiver operating characteristic curves and areas under the curve (AUCs) were used to evaluate the diagnostic utility of serum miRNAs.

Results

In total, 132 index SNPs showed suggestive associations with STDR after adjustment for covariates ($P<5\times10^{-5}$), although none achieved genome-wide significance. miRNA-related SNPs showing associations with STDR included *MIR2054-INTU* rs1344262 (odds ratio [OR]=1.36, 95% confidence interval [CI]=1.22-1.53, $P=9.13 \times10^{-8}$), CCBE1 rs1048008 (OR=0.77, 95% CI=0.68-0.87, $P=5.29 \times10^{-6}$), which was predicted to influence the binding of hsa-miR-183-5p, *AKAP13* rs117010213 (OR=2.63, 95% CI=1.70-4.07, $P=1.11 \times10^{-5}$), which may influence the binding of hsa-miR-1972; and *NMNAT1* rs10779735 (OR=0.75, 95% CI=0.65-0.86, $P=4.24 \times10^{-5}$), which was predicted to disrupt the binding site for hsa-miR-130b-5p and may create a binding site for hsa-miR-129-5p (Table 1).

In total, 2588 mature miRNAs were expressed in 48 serum samples. miRNAs were considered differentially expressed if their $\log_2$ fold change was ≥2 or ≤2 with false discovery rate–adjusted P<0.001. Eighteen miRNAs were upregulated in the incident STDR cases, including miR-183-5p. The expression levels of these 18 miRNAs were determined by RT-qPCR; 10 of them were significantly upregulated in the incident STDR case group (all P<0.05, false discovery rate–adjusted P<0.1, log2 fold change ≥2). These 10 miRNAs were then carried forward to the validation phase. All except hsa-miR-26b-5p were validated and displayed significant differences between the incident STDR cases and controls (all P<0.05, false discovery rate–adjusted P<0.05, Table 2).

Individual miRNAs only showed moderate AUCs ranging from 0.641 to 0.692 (Fig). The combination of all nine miRNAs showed a markedly increased AUC of 0.847 (95% CI=0.796-0.898). The AUC of a clinical model comprising risk factors

TABLE 1. Association results of microRNA-related genetic variants ($P<5 \times10^{-5}$)

Single nucleotide polymorphism	Nearest genes	Related microRNA	Odd ratios (95% confidence interval)	Adjusted P value	Functional relevance
rs1344262	*MIR2054-INTU*	hsa-miR-2054	1.36 (1.22-1.53)	9.13×10^{-8}	AKT3 was predicted to be a target of miR-2054. AKT regulates the expression of vascular endothelial growth factor, which plays a role in vascular dysfunction during diabetic retinopathy.
rs1048008	*CCBE1*	hsa-miR-183-5p	0.77 (0.68-0.87)	5.29×10^{-6}	Silencing miR-183 in mice with diabetic retinopathy inhibits tube formation and cell growth in the vascular endothelial cells by inhibiting the PI3K/Akt/vascular endothelial growth factor signalling pathway.
rs117010213	*AKAP13*	hsa-miR-1972	2.63 (1.70-4.07)	1.11×10^{-5}	miR-1972 promotes angiogenesis by targeting the p53/mechanistic target of rapamycin pathway.
rs10779735	*NMNAT1*	hsa-miR-130b-5p; hsa-miR-129-5p	0.75 (0.65-0.86)	4.24×10^{-5}	The expression of miR-130b is significantly upregulated in the vitreous humour of patients with proliferative vitreoretinopathy. Upregulation of hsa-miR-129-5p inhibits angiogenesis, cell migration, and invasion.

TABLE 2. Results of the validation phase of the microRNA profiling analysis

microRNA	Fold change	P value	False discovery rate–adjusted P value
hsa-miR-744-5p	1.28	4.38×10^{-6}	2.33×10^{-5}
hsa-miR-16-5p	1.77	4.65×10^{-6}	2.33×10^{-5}
hsa-miR-143-3p	1.65	8.56×10^{-6}	2.85×10^{-5}
hsa-miR-24-3p	1.31	1.15×10^{-5}	2.88×10^{-5}
hsa-let-7g-5p	1.31	3.29×10^{-5}	6.58×10^{-5}
hsa-miR-103a-3p	1.27	3.76×10^{-4}	6.27×10^{-4}
hsa-miR-126-5p	1.29	6.00×10^{-4}	8.57×10^{-4}
hsa-let-7d-5p	1.11	0.0187	0.023
hsa-miR-26a-5p	1.12	0.0434	0.048
hsa-miR-26b-5p	1.10	0.116	0.116

of haemoglobin A1c level, estimated glomerular filtration rate, body mass index, and hypertension was 0.560 (95% CI=0.480-0.640). When the nine miRNAs were included, the AUC significantly increased to 0.868 (95% CI=0.820-0.915, Fig).

Discussion

In the GWAS, the strongest association with STDR was exhibited by the intergenic variant rs1344262, located approximately 535 kb downstream of *MIR2054* and approximately 1.6 Mb distant from *INTU*. The 3'-untranslated region of AKT serine/threonine kinase 3 (AKT3) was predicted to be a target of miR-2054. AKT regulates the expression of vascular endothelial growth factor (VEGF), which plays an important role in vascular dysfunction during DR.[3] We detected suggestive associations of three genetic variants, which may affect various miRNA binding sites. However, none of these SNPs reached genome-wide significance.

Despite the relatively small sample size, we were able to identify substantially differentially expressed miRNAs. Some of these have demonstrated potential functional relevance to DR development. Several members of the let-7 miRNA family, such as hsa-let-7a-5p, reportedly are associated with DR. Due to their identical seed regions, all let-7 family members are suspected to possess similar functions. We demonstrated that the circulating levels of hsa-let-7d-5p and hsa-let-7g-5p were significantly elevated in patients with incident STDR. Hypoxia-inducible factor-1α upregulates let-7 and let-7-targeted argonaute 1, leading to reduced VEGF mRNA expression and angiogenesis.[4] Furthermore, let-7 family miRNAs were highly expressed in vascular cells, such as endothelial cells and vascular smooth muscle cells, suggesting

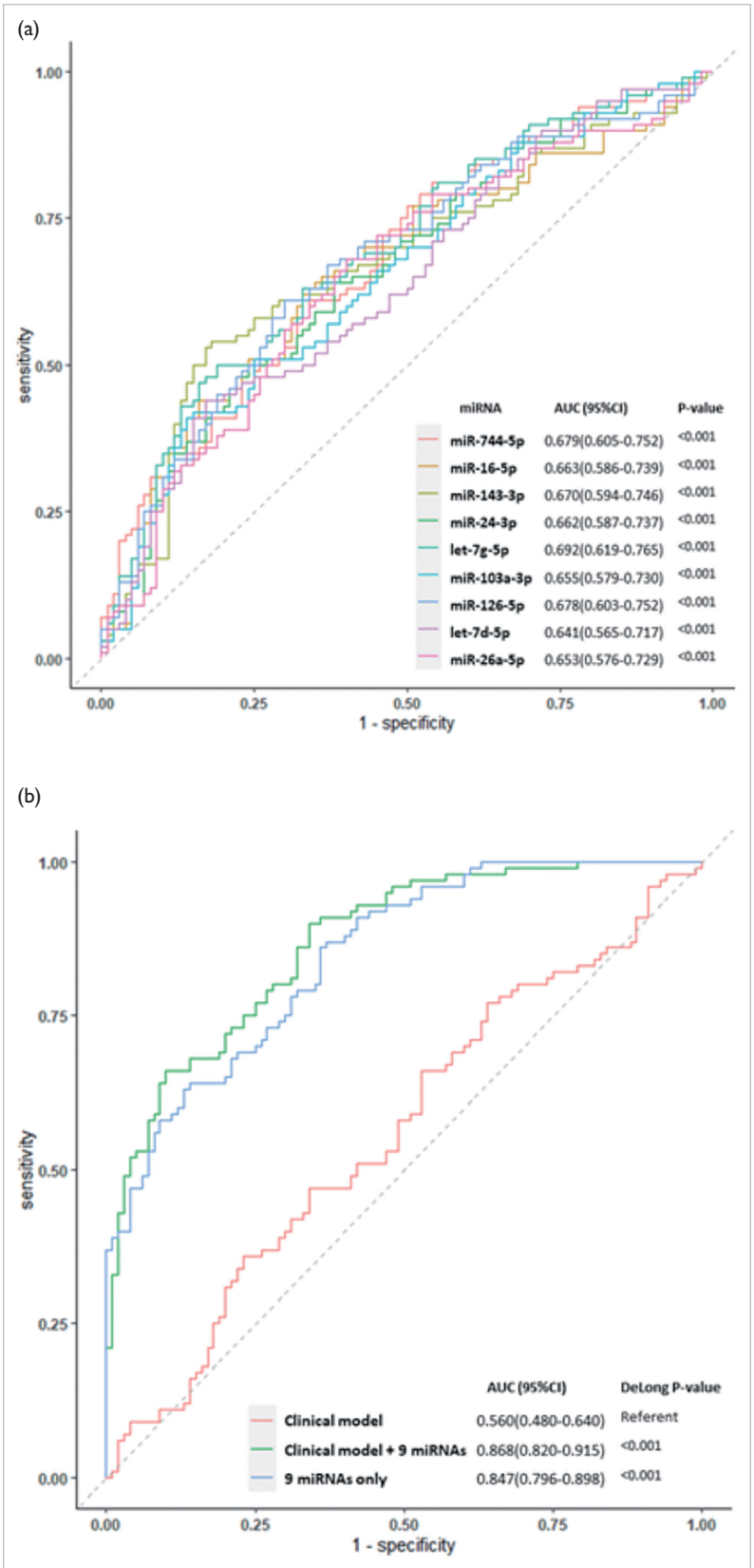

FIG. Receiver operating characteristic curve analysis for discrimination of incident sight-threatening diabetic retinopathy (STDR) cases and non-STDR controls: (a) the areas under the curve (AUCs) of all nine individual miRNAs range from 0.641 to 0.692, and (b) the AUCs of a clinical model (including risk factors of haemoglobin A1c level, estimated glomerular filtration rate, body mass index, and hypertension status), a model of nine miRNAs, and a combination of both; the AUC increases from 0.560 in the clinical model to 0.868 when the nine miRNAs are added.

that these miRNAs contribute to the regulation of vascular cell phenotypes. Patients with proliferative DR reportedly have elevated vitreous levels of hsa-miR-24-3p.[5] In the present study, serum hsa-miR-24-3p levels were increased in patients with incident STDR. VEGF and transforming growth factor β have roles in DR development. The predicted target genes for hsa-miR-24-3p were enriched in both VEGF and transforming growth factor β signalling pathways. Angiogenesis, a hallmark of DR development, was identified as an enriched subcategory in the GO enrichment analysis of predicted target genes of the identified miRNAs. Moreover, KEGG pathway enrichment analysis revealed that the predicted targets of these miRNAs were involved in the AGE-RAGE and MAPK signalling pathways, both closely related to DR development. These findings suggest that the identified miRNAs participate in STDR by regulating these pathways.

Although each individual miRNA only has moderate predictive value for STDR development (AUC=0.641-0.692), the combination of the nine miRNAs yielded a high AUC of 0.847. Furthermore, addition of the nine miRNAs to a clinical model significantly improved the AUC from 0.561 to 0.868, suggesting the potential for using these miRNAs as non-invasive biomarkers to predict STDR development.

Conclusion

Several potential miRNA-related SNPs are associated with STDR. We successfully validated a panel of miRNAs that are associated with incident STDR; they can serve as non-invasive biomarkers for predicting STDR development. Independent validation studies with larger sample sizes are warranted to validate our findings. Further functional analyses are required to elucidate the functional roles of the identified miRNAs in DR.

Funding

This study was supported by the Health and Medical Research Fund, Health Bureau, Hong Kong SAR Government (#06172266). The full report is available from the Health and Medical Research Fund website (https://rfs2.healthbureau.gov.hk).

References

1. Lee CH, Cheung CYY, Woo YC, et al. Prospective associations of circulating adipocyte fatty acid-binding protein levels with risks of renal outcomes and mortality in type 2 diabetes. Diabetologia 2019;62:169-77.
2. Harding S, Greenwood R, Aldington S, et al. Grading and disease management in national screening for diabetic retinopathy in England and Wales. Diabet Med 2003;20:965-71.
3. Qin B, Liu J, Liu S, Li B, Ren J. MiR-20b targets AKT3 and modulates vascular endothelial growth factor-mediated changes in diabetic retinopathy. Acta Biochim Biophys Sin (Shanghai) 2016;48:732-40.
4. Chen Z, Lai TC, Jan YH, et al. Hypoxia-responsive miRNAs target argonaute 1 to promote angiogenesis. J Clin Invest 2013;123:1057-67.
5. Guo J, Zhou P, Pan M, et al. Relationship between elevated microRNAs and growth factors levels in the vitreous of patients with proliferative diabetic retinopathy. J Diabetes Complications 2021;35:108021.