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ORIGINAL ARTICLE

Metformin Alters the MicroRNA Profile of High Glucose-Induced Human Umbilical Vein Endothelial Cells

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ABSTRAK

MikroRNA (miRNA) menyumbang kepada perkembangan komplikasi vaskular akibat diabetes. Metformin telah terbukti dapat melindungi daripada disfungsi endotelium dalam kalangan pesakit diabetes; namun, ia masih belum jelas sama ada kesan ini diperantarai melalui modulasi miRNA. Untuk memahami kesan metformin terhadap profil miRNA dalam sel endotelium yang dirawat dengan glukosa tinggi, kami telah menggunakan penjujukan generasi hadapan (NGS) dan seterusnya menjalankan analisis bioinformatik untuk meneroka gen sasaran serta laluan yang terlibat. Hasil NGS mengenal pasti sebanyak 19 miRNA yang diekspresi secara berbeza, termasuk sembilan miRNA berekspresi rendah dan 10 miRNA berekspresi tinggi. Analisis ontologi gen menunjukkan fungsi molekul dan proses biologi seperti pengikatan reseptor kelas ErbB-2 dan pengawalaturan aktiviti reseptor yang penting dalam memperantara penglibatan metformin terhadap tekanan oksidatif dan apoptosis yang disebabkan oleh diabetes. Analisis tapak jalan Kyoto Encyclopedia of Genes and Genomes (KEGG) pula menghubungkan miRNA dengan tapak jalan berkaitan diabetes, seperti diabetes mellitus jenis II dan sistem isyarat fosfatidilinositol. Tambahan lagi, analisis rangkaian interaksi protein mengenalpasti beberapa gen utama, seperti dehidrogenase gliseraldehid-3-fosfat, sirtuin 1, dan Jun proto-onkogen sebagai gen hub dalam memperantarai tekanan oksidatif dan apoptosis. Kesimpulannya, kajian ini menunjukkan metformin memberi kesan perlindungan

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terhadap disfungsi endotelium vaskular diabetes melalui modulasi miRNA. Penemuan ini boleh membantu untuk membangunkan intervensi terapeutik yang lebih bersasar bagi penyakit diabetes.
Kata kunci: *Diabetes; glukosa tinggi; metformin; mikroRNA; sel endotelium vena umbilikus manusia*

ABSTRACT

MicroRNAs (miRNAs) contribute to the progression of vascular complications in diabetes. Metformin has been shown to protect against endothelial dysfunction in diabetes; however, it remained unclear whether this effect was mediated through the modulation of miRNAs. To gain insight into the effects of metformin on miRNA profile in endothelial cells treated with high glucose, we utilised next gene sequencing (NGS) and further conducted the bioinformatic analyses to explore the associated target genes and pathways. NGS revealed 19 miRNAs with differential expression, including nine downregulated and 10 upregulated miRNAs. Gene ontology highlighted molecular functions and biological processes such as ErbB-2 class receptor binding and receptor activity regulation, which are crucial in mediating the involvement of metformin in diabetes-induced oxidative stress and apoptosis. Kyoto Encyclopedia of Genes and Genomes pathway analysis connected these miRNAs to diabetes-related pathways, such as type II diabetes mellitus and phosphatidylinositol signaling system. Furthermore, network analysis of protein interaction identified important genes, such as glyceraldehyde-3-phosphate dehydrogenase, sirtuin 1 and Jun proto-oncogene as hub genes in modulating oxidative stress and apoptosis. In conclusion, this study elucidates how metformin exerts its protective effects on diabetic vascular endothelial dysfunction by modulating miRNAs. These findings could help develop more targeted therapeutic interventions for diabetes.
Keywords: Diabetes; high glucose; human umbilical vein endothelial cells; metformin; microRNA

INTRODUCTION

Cardiovascular disease remains a top cause of mortality and morbidity among individuals with diabetes. It is projected that by 2045, the global diabetic population will surge from the current 425 million to an estimated 629 million (Syukri et al. 2023). One of the defining characteristics of diabetes is the presence of hyperglycaemia (Tamel Selvan et al. 2023). Hyperglycaemia enhances the reactive oxygen species (ROS) generation, leading to oxidative stress and apoptosis, which eventually, result in the development of diabetic vascular endothelial dysfunction.

The mechanisms contributing to oxidative stress in diabetes are multifactorial. They include autooxidative glycation, nonenzymatic glycation, activation of the sorbitol pathway, increased inflammation and tissue injury resulting from changes in the antioxidant defense system (Mogole et al. 2020; Yusof et al. 2020). These interconnected processes promote the production of ROS, amplifying overall oxidative burden in diabetic patients. Among the cells affected by diabetes, endothelial cells are notably vulnerable to the impact of diabetes-induced oxidative stress (Alvarez et al. 2024).

Hyperglycaemia in diabetes also leads to apoptosis of endothelial cells. Hyperglycaemia disrupts the equilibrium of proteins that promote or inhibit apoptosis in human umbilical vein endothelial cells (HUVECs), leading to cytochrome c release from

mitochondria (Sapian et al. 2023). Cytochrome c initiates a cascade of events that ultimately activate caspase-3 and c-Jun N-terminal protein kinase (JNK). This cascade is driven by elevated levels of ROS, which play a pivotal role in mediating hyperglycaemia-induced apoptosis (Tang et al. 2020). Additionally, hyperglycaemia induces apoptosis through the calcium/calmodulin-dependent protein kinase II (CaMKII) activation (Ni et al. 2022). Hyperglycaemia activates CaMKII, which in turn, triggers multiple downstream pathways that promote apoptosis. CaMKII activation leads to mitochondrial dysfunction, enhanced ROS production and cytochrome c release, all of which reinforce the apoptotic signaling network (Benchoula et al. 2023). Furthermore, CaMKII increases the activity of pro-apoptotic factors, including caspases, further promoting endothelial cell death (Yang et al. 2021).

Drugs such as metformin are frequently prescribed to lower blood glucose levels and minimise the diabetes complications. Metformin has antioxidative and anti-apoptotic activities that protect against endothelial dysfunction (Manica et al. 2023). Metformin also enhances vascular function and decreases mortality and cardiovascular complication in diabetic patients (Triggle et al. 2022). The effectiveness of metformin in attenuating endothelial dysfunction has been demonstrated not only in clinical trials, but also in *in vivo* and *in vitro* studies (Han et al. 2018).

Recent studies have discovered a strong connection between miRNAs and the pathogenesis of diabetic vascular complications (Ismail et al. 2023; Szostak et al. 2023). MiRNAs are small RNA molecules, typically 20-24 nucleotides in size, that function in the regulation of gene expression at the post-transcriptional level (Mohd Isa et al. 2023). Notably, changes in miRNA expression are linked to oxidative stress and apoptosis under diabetic conditions (Qadir et al. 2019; Vezza et al. 2021; Wei et al. 2020). In particular, the miR-21, miR-25, miR-145, miR-106b, miR-233, miR-214, miR-30e-5p, miR-455-5p, miR-203 and miR-200 family are implicated in diabetic oxidative stress (Qadir et al. 2019), whereas miR-770-5p, miR-195 and miR-218 are linked to apoptosis in diabetes (Szostak et al. 2023; Zhang & Chen 2020).

In earlier study, we showed that high glucose exposure in HUVECs resulted in the differential expression of 17 miRNAs (Othman et al. 2023). Interestingly, metformin has been demonstrated to influence the expression of circulating miRNAs in type 2 diabetic patients (Demirsoy et al. 2018). Nonetheless, it remained unclear whether the protective effect of metformin on endothelial dysfunction is mediated through the modulation of miRNAs, and there is a scarcity of studies thoroughly investigating the miRNA profiling in high glucose-induced endothelial cells treated with metformin.

HUVECs are an ideal model for studying oxidative stress due to their susceptibility to oxidative damage and their relevance to cardiovascular diseases. These cells possess a variety of antioxidant systems that work together to mitigate ROS-induced damage (Medina-Leyte et al. 2020). Additionally, HUVECs can be easily isolated and are readily available from discarded umbilical cords upon delivery, making them an accessible and plentiful supply of endothelial cells (Kocherova et al. 2019).

Therefore, in this research, we utilised next generation sequencing (NGS) to explore the role of metformin in the miRNA profile of high glucose-induced HUVECs, with a particular focus on oxidative stress and apoptosis. Subsequent bioinformatic analyses utilised the GOSeq tool to elucidate the gene

ontology (GO) terms associated with enriched genes, emphasising notable differences in gene expression and its enrichment in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Computational prediction of miRNA-mRNA interactions employed the miRanda algorithm, with the STRING database facilitating the construction of a protein-protein interaction (PPI) signaling network. Subsequently, the network was thoroughly explored through Cytoscape to identify hub genes within this network. The results obtained from this study could offer a more comprehensive view of the mechanisms underlying the protective effects of metformin on diabetic vascular endothelial dysfunction. Identification of the specific miRNA and its target molecular pathway may help in the early detection of diabetic vascular complications, thus allowing timely intervention.

MATERIALS AND METHODS

HUVECs Isolation, Culture and Treatment

Ethical approval was obtained from the Universiti Kebangsaan Malaysia (UKM) Research Ethics Committee (project approval code: UKM PPI/111/8/JEP-2023-246) and all participants signed informed written consent prior to their involvement. Freshly harvested umbilical veins were used to isolate HUVECs through an enzyme digestion procedure as in a previous study (Hamid et al. 2022). In brief, umbilical cords donated by healthy mothers were rinsed with Dulbecco's phosphate-buffered saline (Gibco, Grand Island, New York) and treated with 0.1% (w/v) collagenase type I (MedChemExpress, Monmouth Junction, NJ, USA). The freshly isolated cells were grown in a medium supplemented with 10% fetal bovine serum (ScienCell Research Laboratories, Carlsbad, CA, USA). HUVECs were confirmed by their characteristic cobblestone appearance and the detection of endothelial markers (CD31 and von Willebrand factor) through immunocytochemistry (Hamid et al. 2022). HUVECs from passage 3-4 at 80% confluency were utilised for all experiments. HUVECs were then divided into a high glucose group (HG; treated with 33.3 mM d-glucose), and a high glucose + metformin group (HG+Met; subjected to concomitant treatment with 33.3 mM d-glucose and 10 µM metformin). All treatments were administered for a duration of 24 hours. The selection of glucose and metformin doses, as well as the duration of treatment, was based on established models from prior research (Han et al. 2018; Wang et al. 2016).

Total RNA Extraction

HUVECs underwent total RNA isolation using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol. The purity, integrity and concentration of the isolated total RNA were measured with an Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) to confirm its suitability for NGS sequencing.

Small RNA Library Construction and Sequencing

Complementary DNA (cDNA) libraries from the RNA samples were created and analysed using Illumina Hiseq, with a paired-end chemistry of 2 x 150 bp. The raw reads were preprocessed with Trimmomatic (V0.30) program to eliminate adaptors, improve quality and filter by specific size. Then, FastQC (V0.10.1) was used to perform quality control checks on the trimmed data. The sequences were mapped to a complete human reference genome (GRCh38.104) with Bowtie2 (V2.1.0).

Known miRNAs were identified by aligning clean reads to the miRbase database, while novel miRNAs were detected using miRDeep2 software. Following this, transcripts per million (TPM) normalisation was applied to the raw reads. DESeq2 was utilised to identify differentially expressed miRNAs based on a negative binomial distribution model. To control the false discovery rate, the Benjamini-Hochberg method was applied, setting a p-value threshold of <0.05. Additionally, a minimum two-fold change in gene expression was required to identify differentially expressed miRNAs. The data from this study have been submitted to GEO, listed under accession code PRJNA1091147.

Stem-loop Reverse Transcription-Quantitative Polymerase Chain Reaction Verification of Selected MiRNA

Stem-loop quantitative polymerase chain reaction (qPCR) was used to validate the expression of the significantly downregulated novel miR-1133, confirming the results obtained from NGS sequencing. As part of this process, cDNA was synthesised from 5 ng of total RNA enriched with small RNA using the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Biosearch Technologies, Hoddesdon, UK), incorporating 0.1 µM of a specific stem-loop primer. A 10 µL reaction mix was used for the qPCR of novel miR-1133, consisting of 3 µL of 60 diluted cDNA, 0.5 µM each of forward and reverse primers and 5 µL of 2 miRCURY SYBR Green Master Mix. Table 1 provided the detailed primer sequences. The reactions were performed on a CFX96™ Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) with an initial denaturation step at 95°C for 10 minutes. This was followed by 40 cycles comprising 10 seconds at 95°C for denaturation, 30 seconds at 60°C for annealing, and 10 seconds at 72°C for elongation; ending with a final elongation at 40°C for 1 second. To evaluate the specificity of the amplification, the reaction kinetics of each primer set were confirmed through analysis of the melting profiles. Bio-Rad CFX Manager software version 3.1 was used to detect and evaluate amplification signals during the elongation step. The $2^{-\Delta\Delta CT}$ method was then applied to quantify miRNA expressions levels and data were normalised against the U6 gene, serving as the internal control.

Differential Gene Expression and Pathway Analysis

To determine GO terms annotating with a set of enriched genes, the GOSeq tool (version 1.34.1) was used, with significance set at $p < 0.05$. Additionally, enriched KEGG pathways were identified by mapping significant differential gene expression.

TABLE 1: Primer sequence for qPCR

Target miRNA		Primer sequence (5'-3')	PCR product size (bp)
1133	Forward	GCTGGGCGGCTTGCTGG	72
	Reverse	GTAGGATGCCGCTCTCAG	
U6	Forward	CTCGCTTCGGCAGCACA	94
	Reverse	AACGCTTCACGAATTTGCGT	
Stem-loop miR-1133	GTTGGCTCTGGTAGGATGCCGCTCTCAGGGCATCCTACCAGAGCCAAACCGAGCC		

Computational Analysis of miRNA-mRNA Interactions and Gene Networks

We employed the miRanda algorithm, a target prediction tool to predict interaction between miRNAs and their target genes. This computational method was designed to identify potential binding sites of miRNAs on target mRNA sequences, based on sequence complementarity and energy stability of the miRNA-mRNA duplex. To generate a network illustrating interactions among proteins encoded by the target genes, the STRING database (http://string-db.org/, retrieved on February 28, 2024) was utilised. Subsequently, hub genes, which were important genes within this network, were determined by assessing their connectivity degree through Cytoscape software (version 3.6.1).

Statistical Analysis

The qPCR data analysis was conducted using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA). The Shapiro-Wilk test was applied to assess whether the dataset was normally distributed. The results were reported as mean ± SEM. Group comparisons of the qPCR data were performed using the unpaired t-test, a suitable statistical method for evaluating differences between two independent groups with normally distributed data. Statistical significance was defined as $p < 0.05$.

RESULTS

NGS Sequencing Analyses

To delineate the miRNA expression profile, we conducted comprehensive NGS analyses involving three samples of high glucose-induced HUVECs with or without metformin treatment. A total of 1,737 miRNAs were detected across all samples, with 800 miRNAs detected in both the HG and HG+Met groups, as depicted in Figure 1.

MiRNAs were deemed differentially expressed if their expression exhibited a fold change more than 2 and a *p*-value below 0.05, as determined by DESeq2 analysis. A total of 19 miRNAs (novel miRNA-610, hsa-

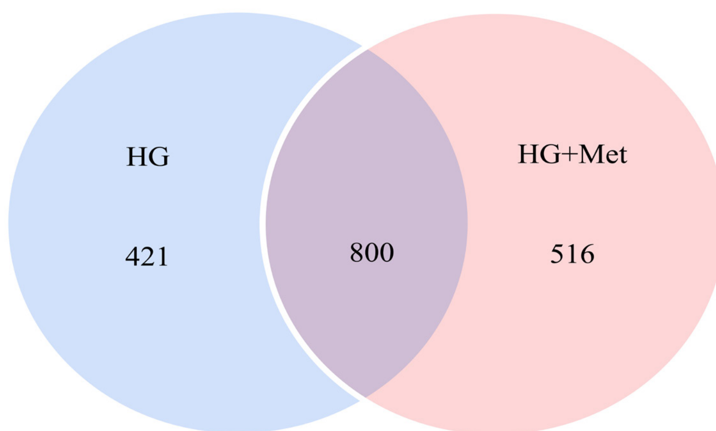

FIGURE 1: Venn diagram illustrated the number of miRNAs identified in high glucose-induced HUVECs treated with or without metformin (n = 3) (HG: high glucose; Met: metformin)

miR-10397-5p, hsa-miR-4750-5p, hsa-miR-6869-5p, hsa-miR-6741-5p, hsa-miR-5100, novel miRNA-1103, novel miRNA-233, novel miRNA-243, hsa-miR-3691-5p, novel miRNA-194, novel miRNA-90, hsa-miR-3620-3p, novel miRNA-710, novel miRNA-81, novel miRNA-310, novel miRNA-1133, novel miRNA-615, novel miRNA-1023) were differentially expressed in the HG+Met group compared to the HG group (fold change ≥ 2.0, p < 0.05). Among these, 10 miRNAs were upregulated by metformin treatment (novel miRNA-610, hsa-miR-10397-5p, hsa-miR-4750-5p, hsa-miR-6869-5p, hsa-miR-6741-5p, hsa-miR-5100, novel miRNA-1103, novel miRNA-233, novel miRNA-243, hsa-miR-3691-5p), while nine miRNAs were downregulated by metformin treatment (novel miRNA-194, novel miRNA-90, hsa-miR-3620-3p, novel miRNA-710, novel miRNA-81, novel miRNA-310, novel miRNA-1133, novel miRNA-615, novel miRNA-1023) (Figure 2, Table 2).

Verification of Novel miR-1133 by Stem-loop qPCR

We utilised stem-loop qPCR to confirm the reliability of our NGS results. In our previous study, we demonstrated that novel miRNA-1133 was the most upregulated miRNA in high glucose-induced HUVECs (Othman et al. 2023). Interestingly, NGS results in this study showed that novel miRNA-1133 was

TABLE 2: The microRNAs (miRNAs) that exhibited differential expression following metformin treatment in hyperglycemia-induced HUVECs (fold change ≥ 2, p<0.05, n = 3)

miRNA	Fold Change	Type of Regulation	p-value
Novel miRNA-610	5.743223	Up	0.002915
hsa-miR-10397-5p	5.652655	Up	0.003591
hsa-miR-4750-5p	4.953312	Up	0.012767
hsa-miR-6869-5p	4.87057	Up	0.016081
hsa-miR-6741-5p	4.552475	Up	0.025295
hsa-miR-5100	4.417298	Up	0.039046
Novel miRNA-1103	4.385443	Up	0.034022
Novel miRNA-233	4.248727	Up	0.041399
Novel miRNA-243	4.141174	Up	0.047912
hsa-miR-3691-5p	3.160931	Up	0.026608
Novel miRNA-194	-4.32653	Down	0.037016
Novel miRNA-90	-4.58318	Down	0.025278
hsa-miR-3620-3p	-4.71078	Down	0.020656
Novel miRNA-710	-4.84702	Down	0.016444
Novel miRNA-81	-5.25252	Down	0.010327
Novel miRNA-310	-5.379	Down	0.006156
Novel miRNA-1133	-5.39236	Down	0.005345
Novel miRNA-615	-6.0859	Down	0.001307
Novel miRNA-1023	-6.0859	Down	0.001307

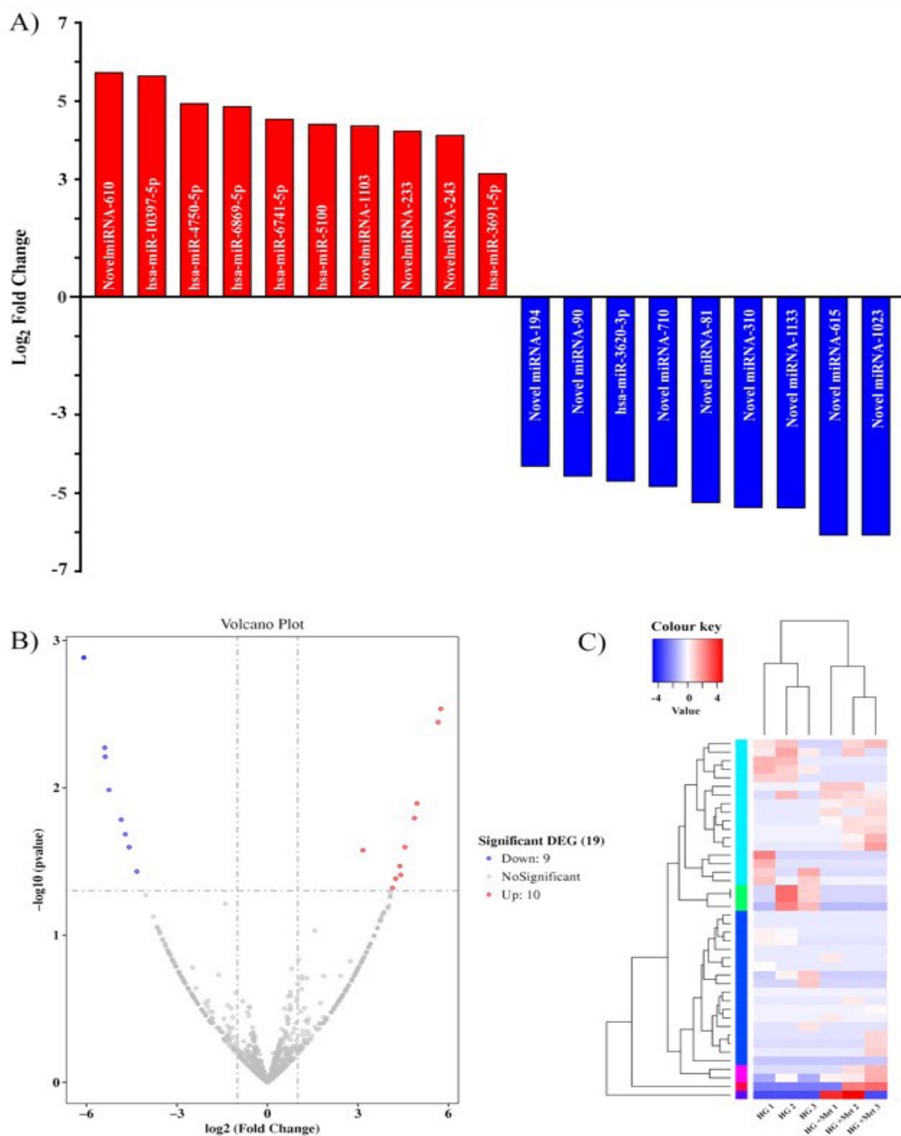

FIGURE 2: Analysis of miRNA expression profile with metformin treatment in high glucose-induced HUVECs (n = 3) (A) The differential expression of miRNAs in high glucose-induced HUVECs treated with metformin, showing both up-regulated (red) and down-regulated (blue) miRNAs; (B) a volcano plot displaying the fold change of miRNA expression in the samples (log2 fold change) with their statistical significance (-log10 (p-value)), color-coded to distinguish the significantly upregulated (red), downregulated (blue), and non-significant (grey) miRNAs; (C) a heatmap accompanied by dendrograms, demonstrating the expression levels of significantly altered miRNAs, with the color key indicating expression levels, ranging from low (blue) to high (red)

among the downregulated miRNAs following metformin treatment in high glucose-induced HUVECs (Table 2). Therefore, we validated the expression of novel miRNA-1133 using stem-loop qPCR. The findings proved that metformin treatment caused a 0.34-fold downregulation of novel miR-1133 expression compared to untreated high glucose-induced HUVECs ($p < 0.01$) (Figure 3).

GO Analysis

To investigate the effects of the 19 differentially expressed miRNAs, we conducted GO term analysis to annotate their 6289 target genes (Figure 4). This analysis described the pathways and processes influenced by metformin in the context of oxidative stress and apoptosis in HUVECs. The y-axis displayed GO terms associated with molecular functions, biological processes and cellular components while the x-axis indicated the significance of these terms represented by the negative logarithm to the base 10 of the *p*-value (-log10(*p*-value)).

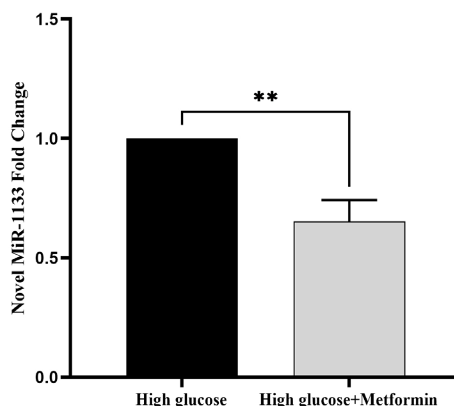

FIGURE 3: Metformin downregulated the expression of novel miR-1133 in high glucose-induced HUVECs, as validated by stem-loop quantitative PCR. Values are shown as mean ± SEM, n = 6 (** p<0.01 vs. high glucose)

In the molecular function category, there was an enrichment in 11 GO terms. The most significant term was extracellular matrix constituent, lubricant activity (GO:0030197), followed by ErbB-2 class receptor binding (GO:0005176). Additional terms of significance

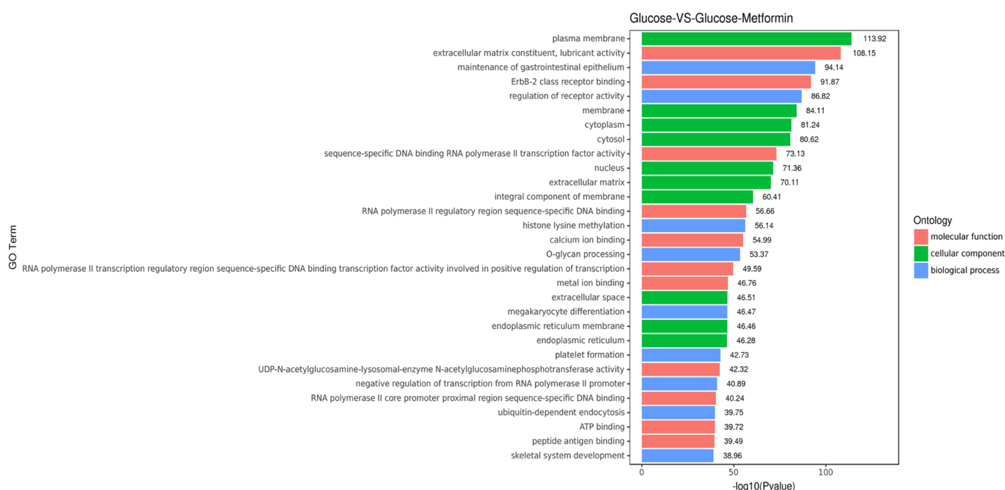

FIGURE 4: The GO term enrichment analysis for high glucose versus high glucose + metformin groups, categorising terms by molecular function (green), cellular component (red), and biological process (blue), with the significance of each term represented by -log10(p-value)

included those related to the sequence-specific DNA binding RNA polymerase II transcription factor activity (GO:0000981), RNA polymerase II regulatory region sequence-specific DNA binding (GO:0000977), calcium ion binding (GO:0005509), RNA polymerase II transcription regulatory region sequence-specific DNA binding transcription factor activity involved in positive regulation of transcription (GO:0001228), metal ion binding (GO:0046872), UDP-N-acetylglucoseamine-lysosomal-enzyme N-acetylglucoseaminephosphotransferase activity (GO:0003976), RNA polymerase II core promoter proximal region sequence-specific DNA binding (GO:0000978), ATP binding (GO:0005524) and peptide antigen binding (GO:0042605).

In the biological process category, the analysis revealed enrichment across nine GO terms. The most prominent of these was maintenance of gastrointestinal epithelium (GO:0030277). Additionally, regulation of receptor activity (GO:0010469), histone lysine methylation (GO:0034968), O-glycan processing (GO:0016266), megakaryocyte differentiation (GO:0030219), platelet formation (GO:0030220), negative regulation of transcription from RNA polymerase II promoter (GO:0000122), ubiquitin-dependent endocytosis (GO:0070086), and skeletal system development (GO:0001501) were enriched.

The analysis further emphasised the enrichment of cellular component, which included the endoplasmic reticulum (GO:0005783), endoplasmic reticulum membrane (GO:0005789), extracellular space (GO:0005615), integral component of membrane (GO:0016021), extracellular matrix (GO:0031012), nucleus (GO:0005634), cytosol (GO:0005829), cytoplasm (GO:0005737), membrane (GO:0016020) and plasma membrane (GO:0005886).

KEGG Pathway Analysis

The KEGG pathway analysis highlighted the enrichment of multiple pathways in HUVECs treated with metformin under hyperglycaemic conditions (Figure 5). Pathways exhibiting notable gene involvement and low *q*-values, ranging from 7.63×10^{-21} to 2.35×10^{-6}, included those related to viral myocarditis, KO05416; various types of N-glycan biosynthesis, KO00513; type II diabetes mellitus, KO04930; type I diabetes mellitus, KO04940; transcriptional misregulation in cancer, KO05202; synaptic vesicle cycle, KO04721; RNA degradation, KO03018; plant-pathogen interaction, KO04626; phosphatidylinositol signaling system, KO04070; other types of O-glycan biosynthesis, KO00514; notch signaling pathway, KO04330; insulin secretion, KO04911; inositol phosphate metabolism, KO00562; inflammatory bowel disease (IBD), KO05321; HTLV-I infection, KO05166; graft-versus-host disease, KO05332; glycosylphosphatidylinositol(GPI)-anchor biosynthesis, KO00563; glycosphingolipid biosynthesis - lacto and neolacto series, KO00601; glycosaminoglycan biosynthesis - keratan sulfate, KO00533; glycerophospholipid metabolism, KO00564; fructose and mannose metabolism, KO00051; flavone and flavonol biosynthesis, KO00944; dorso-ventral axis formation, KO04320; circadian entrainment, KO04713; cell adhesion molecules (CAMs), KO04514; calcium signaling pathway, KO04020; autoimmune thyroid disease, KO05320; asthma, KO05310; allograft rejection, KO05330 and ABC transporters, KO02010.

FIGURE 5: Scatter plot of KEGG pathway enrichment of differentially expressed genes. Pathways with higher gene numbers and lower q-values were more significantly enriched, indicating their potential regulatory role modulated by metformin in hyperglycemic conditions

Target Gene Prediction and Protein-Protein Interaction Network Analysis of Differentially Expressed miRNAs

The predicted targets included 1,534 genes for the upregulated miRNAs and 4,755 genes for the downregulated miRNAs (Supplementary Materials, Table 3). Among the upregulated miRNAs, hsa-miR-4750-5p was identified as having the highest number of target genes, with a total of 582 genes. In contrast, novel miRNA-1133 showed the largest number of target genes found in the downregulated miRNAs, totaling 3387 genes.

CytoHubba plugin in Cytoscape was utilised in our PPI network analysis to identify hub genes that play a critical role in influencing oxidative stress and apoptosis in diabetes. Hub genes are characterised by their extensive interactions with other genes throughout the network (Jiao et al. 2023). The network structure was assessed individually for genes targeted by

TABLE 3: Number of predictive target genes of differentially expressed miRNAs

Upregulated miRNA	Number	Downregulated miRNA	Number
Novel miRNA-610	25	Novel miRNA-194	29
hsa-miR-10397-5p	29	Novel miRNA-90	20
hsa-miR-4750-5p	582	hsa-miR-3620-3p	877
hsa-miR-6869-5p	49	Novel miRNA-710	10
hsa-miR-6741-5p	126	Novel miRNA-81	366
hsa-miR-5100	39	Novel miRNA-310	58
Novel miRNA-1103	546	Novel miRNA-1133	3387
Novel miRNA-233	38	Novel miRNA-615	4
Novel miRNA-243	0	Novel miRNA-1023	4
hsa-miR-3691-5p	100		
Total	1534	Total	4755

both upregulated and downregulated miRNAs (Figure 6, Table 4). Figure 6A showed the gene interaction network influenced by upregulated miRNAs, displaying a densely interconnected network. Nodes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin beta (ACTB), epidermal growth factor receptor (EGFR), jun proto-oncogene, AP-1 transcription factor subunit (JUN), calmodulin 3 (CALM3), estrogen receptor 1 (ESR1), mitogen-activated protein kinase 3 (MAPK3), CD4 molecule (CD4), notch 1 (NOTCH1), erb-B2 receptor tyrosine kinase 2 (ERBB2), sirtuin 1 (SIRT1), SMAD family member 4 (SMAD4), nucleophosmin 1 (NPM1), CD8A molecule (CD8A), discs large MAGUK scaffold protein 4 (DLG4), eukaryotic translation elongation factor 2 (EEF2), SMAD family member 2 (SMAD2), F-box and WD repeat domain containing 7 (FBXW7), fibroblast growth factor receptor 1 (FGFR1) and protein tyrosine phosphatase, non-receptor type 11 (PTPN11) exhibited a high degree centrality, highlighting their potential role as pivotal regulatory hubs in the underlying biological processes affected by upregulated miRNAs.

Conversely, Figure 6B visualised the interaction network for genes targeted by downregulated miRNAs. Here, nodes like RAC-alpha serine/threonine-protein kinase (AKT1), ACTB, MYC proto-oncogene, bHLH transcription factor (MYC), EGFR, ubiquitin A-52 residue ribosomal protein fusion product 1 (UBA52), ribosomal protein S27a (RPS27A), signal transducer and activator of transcription 3 (STAT3), ESR1, cell division control protein 42 homolog (CDC42), ras homolog family member A (RHOA), CALM3, protein kinase, cAMP-dependent, catalytic, alpha (PRKACA), H3 histone, family 2 (H3-2), bromodomain-containing protein 4 (BRD4), CD44 molecule (Indian blood group) (CD44), peroxidasin homolog (PXDN), CREB binding protein (CREBBP), DLG4, MDM2 proto-oncogene (MDM2) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) demonstrated a substantial degree of connectivity.

DISCUSSION

This study reveals significant changes in the miRNA expression profile following treatment with metformin in high glucose-induced HUVECs. Through NGS, we detected

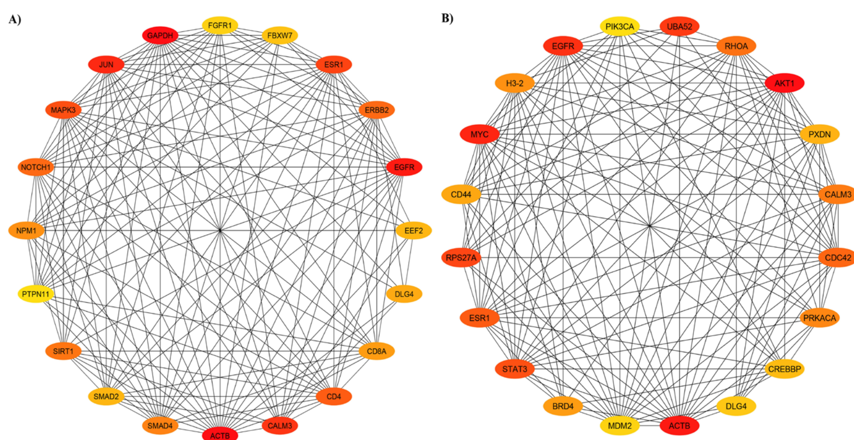

FIGURE 6: Top 20 hub genes within the PPI network of target genes regulated by the (A) upregulated and (B) downregulated miRNAs were determined by STRING-based analysis and visualised using the Cytoscape plugin, CytoHubba, to ascertain their interaction degrees. Nodes were color-coded from red to yellow, indicating a gradient from the highest- to lowest-ranked nodes based on their degree of connectivity. ACTB: actin beta; AKT1: RAC-alpha serine/threonine-protein kinase; BRD4: Bromodomain-containing protein 4; CALM3: Calmodulin 3; CDC42: Cell division control protein 42 homolog; CD4: CD4 molecule; CD44: CD44 molecule (Indian blood group); CD8A: CD8a molecule; CREBBP: CREB binding protein; DLG4: Discs large MAGUK scaffold protein 4; EEF2: Eukaryotic Translation Elongation Factor 2; EGFR: Epidermal growth factor receptor; ERBB2: Erb-B2 receptor tyrosine kinase 2; ESR1: Estrogen receptor 1; FBXW7: F-Box and WD repeat domain containing 7; FGFR1: Fibroblast growth factor receptor 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; H3-2: H3 histone, family 2; JUN: Jun proto-oncogene, AP-1 transcription factor subunit; MAPK3: Mitogen-Activated Protein Kinase 3; MDM2: MDM2 proto-oncogene; MYC: MYC proto-oncogene, bHLH transcription factor; NOTCH1: Notch 1; NPM1: Nucleophosmin 1; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PRKACA: Protein kinase, cAMP-dependent, catalytic, alpha; PXDN: Peroxidasin homolog; RHOA: Ras homolog family member A; RPS27A: Ribosomal protein S27a; SIRT1: Sirtuin 1; SMAD2: SMAD family member 2; SMAD4: SMAD family member 4; STAT3: Signal transducer and activator of transcription 3; UBA52: Ubiquitin A-52 residue ribosomal protein fusion product 1; PTPN11: Protein tyrosine phosphatase, non-receptor type 11

19 differentially expressed miRNAs upon treatment with metformin. Among these, 10 miRNAs exhibited upregulation, while nine miRNAs showed downregulation. This dual modulatory effect on miRNA expression indicates that metformin may contribute to the prevention of vascular endothelial dysfunction associated with diabetes by regulating miRNA expression. Subsequent analysis was conducted on the 19 differentially expressed miRNAs, along with their 6289 target genes using GO term and KEGG pathways. This analysis yielded crucial understanding of the molecular mechanisms that drive the therapeutic effects of metformin, revealing a total of 3930 GO terms delineating molecular functions, biological processes and cellular components.

Within the enriched molecular functions associated with metformin's role in high glucose-induced oxidative stress, significant GO terms include ErbB-2 class receptor binding (GO:0005176), calcium ion binding (GO:0005509), metal ion binding (GO:0046872), ATP binding (GO:0005524), sequence-specific DNA binding RNA polymerase II transcription factor activity (GO:0000981), and RNA polymerase II

TABLE 4: Hub genes with the highest degree of connectivity in the protein-protein interactions network

Upregulated miRNAs		Downregulated miRNAs	
Gene symbol	**Degree**	**Gene symbol**	**Degree**
GAPDH	161	AKT1	472
ACTB	161	ACTB	451
EGFR	140	MYC	392
JUN	113	EGFR	361
CALM3	112	UBA52	340
ESR1	109	RPS27A	331
MAPK3	86	STAT3	292
CD4	80	ESR1	268
NOTCH1	76	CDC42	254
ERBB2	76	RHOA	250
SIRT1	75	CALM3	235
SMAD4	70	PRKACA	216
NPM1	69	H3-2	200
CD8A	67	BRD4	198
DLG4	63	CD44	196
EEF2	57	PXDN	193
SMAD2	57	CREBBP	182
FBXW7	56	DLG4	177
FGFR1	55	MDM2	172
PTPN11	54	PIK3CA	167

transcription regulatory region sequence-specific DNA binding transcription factor activity involved in positive regulation of transcription (GO:0001228). Inhibition of ErbB-2 receptor and disruption of calcium signaling have been shown to increase the generation of ROS and trigger apoptosis (Cao et al. 2023; Chen et al. 2023). Metformin has been shown to reverse ethanol-induced oxidative stress and apoptosis in cardiomyocytes in an ErbB2-dependent manner (Chen et al. 2023). Metformin also modulates intracellular calcium levels, particularly in response to oxidative challenges such as exposure to hydrogen peroxide (Cao et al. 2023).

Moreover, metal ions such as copper have a role in causing oxidative stress via the Fenton reaction (Rossmann et al. 2023). The enrichment of the GO term metal ion binding (GO:0046872) by metformin suggests that its effectiveness extends to neutralising copper-induced oxidative mechanisms. Meanwhile, the GO term ATP-binding (GO:0005524) connects to metformin's role in high glucose-induced oxidative stress. Metformin boosts mitochondrial membrane potential and ATP production from ADP. This demonstrates metformin's effect on ATP-binding proteins and mitochondrial function, which is crucial for cellular energy balance and oxidative stress management (Szymczak-Pajor et al. 2022). Furthermore, transcription factors such

as Nrf2 regulate genes crucial for antioxidant defense and apoptosis control. Metformin has been shown to activate the AKT/Nrf2 pathway, leading to nuclear translocation of Nrf2 and activation of antioxidant genes, including catalase (CAT), heme oxygenase-1 (HO-1) and superoxide dismutase 2 (SOD2) (Chen et al. 2023).

The GO terms associated with biological processes enriched by metformin's effects in addressing high glucose-induced oxidative stress include regulation of receptor activity (GO:0010469, histone lysine methylation (GO:0034968), O-glycan processing (GO:0016266), and negative regulation of transcription from RNA polymerase II promoter (GO:0000122). Regulating receptor activity, notably through AMPK activation, is key under hyperglycaemic conditions. Metformin activates AMPK, which in turn increases the expression of antioxidant enzymes and reduces ROS level. This mechanism protects cells from oxidative damage, highlighting metformin's protective role against the adverse effects of hyperglycaemia (Ren et al. 2020). Similarly, histone lysine methylation is essential for the regulation of genes associated with oxidative stress and apoptosis. Metformin alters histone marks and targets demethylases such as lysine demethylase 6A (KDM6A/UTX), thereby modifying gene expression related to antioxidant enzymes and apoptotic pathway (Yang et al. 2022).

Furthermore, disruptions in O-glycan processing under hyperglycaemic state highlight the significance of post-translational modifications in endothelial cell function (Loaeza-Reyes et al. 2021). Such findings complement evidence suggesting that metformin intervenes by potentially correcting these glycosylation patterns. Besides, the negative regulation of transcription from the RNA polymerase II promoter draws attention to genetic regulation mechanisms essential for cell survival under hyperglycaemic stress. Metformin has been shown to activate AMPK, leading to the inactivation of transcription factors or coactivators involved in the transcription of genes that exacerbate oxidative stress and endothelial dysfunction (Madsen et al. 2015).

The subsequent KEGG pathway analysis revealed that metformin influenced pathways associated with high glucose-induced oxidative stress. Uncovering 77 KEGG pathways, the analysis highlights metformin's capacity to target metabolic disruptions in type II diabetes mellitus, enhancing insulin sensitivity and glucose uptake, and potentially attenuating oxidative stress (Angelika et al. 2024). The drug also influences the phosphatidylinositol signaling system, essential for cell growth, apoptosis and metabolic regulation, potentially optimising insulin action and glucose metabolism (Kaneto et al. 2021). Additionally, metformin's role extends to calcium signaling and glycerophospholipid metabolism, reducing oxidative stress and the generation of pro-oxidant molecules within endothelial cells (Cao et al. 2023; Chen et al. 2022). Lastly, by modulating ABC transporter function, metformin aids the protective cellular response against oxidative damage, illustrating its comprehensive approach to mitigating the sequelae of oxidative stress in hyperglycaemic environments (Ye et al. 2020).

The predictive target gene analysis, as detailed in Table 3, highlights the extensive regulatory capacity of both the upregulated and downregulated miRNAs. Notably, hsa-miR-4750-5p and novel miRNA-1133 emerged as the miRNAs having the most targeted genes among upregulated and downregulated categories, respectively. The wide target spectrum suggests that a single miRNA can influence multiple pathways, particularly in

oxidative stress and apoptosis mechanisms. We also identified a subset of genes within the top 30 of the PPI networks that exhibit direct connections to metformin's therapeutic effects in mitigating oxidative stress induced by high glucose. Among these, GAPDH, EGFR, JUN and SIRT1 were influenced by the upregulated miRNAs, while AKT1, MYC, STAT3, PIK3CA, ESR1, CDC42 and RHOA were modulated by the downregulated miRNAs.

GAPDH is primarily recognised not only for its involvement in glycolysis, but also for its role in managing oxidative stress. This suggests that metformin lowers oxidative stress by enhancing GAPDH activity and cellular metabolism (Haydinger et al. 2023). Meanwhile, EGFR is involved in cell growth and survival pathways (Sheng et al. 2021). Metformin's influence on EGFR signaling could be pivotal in strengthening cellular defenses against oxidative stress, in line with the drug's antioxidative properties (Cao et al. 2023). Additionally, metformin could inhibit JUN activation caused by oxidative stress, thus protecting cells from apoptosis (Jung et al. 2012). Looking at SIRT1, which regulates stress responses and mitochondrial function, it is clear that metformin's activation of pathways that stimulate SIRT1 expression could strengthen the cellular antioxidative defenses against oxidative stress (Ren et al. 2020).

Furthermore, the gene AKT1, which is central for glucose metabolism and cell survival, is one of the identified top hub genes. Metformin's activation of AMPK might refine AKT1 activity, promoting a more favourable energy balance and diminishing oxidative stress (Ren et al. 2020). MYC and STAT3 further exemplify metformin's ability to downregulate pathways that exacerbate metabolic stress and oxidative damage, while also maintaining anti-inflammatory and antioxidative defenses (An et al. 2023; Xiang et al. 2015). The role of PIK3CA in metabolic processes and survival (Guan et al. 2024; Maffei et al. 2018; Yang et al. 2019), alongside ESR1's regulation of oxidative stress and metabolic balance (Baghel & Srivastava 2020), emphasise metformin's interaction with the PI3K/AKT pathway and estrogen signaling, respectively. This interaction enhances cellular responses to oxidative challenges. Finally, CDC42 and RHOA, which regulate the cytoskeleton and cell cycle (Huang et al. 2019; Kumar et al. 2014), represent the adaptive ability metformin confers upon cells to withstand oxidative stress.

However, despite the comprehensive analysis of the effect of metformin on the miRNA profile of high glucose-induced HUVECs, this study has certain limitations. Conducted with a limited sample size (n = 3), the findings' generalisability may be constrained, highlighting the need for further validation with a larger sample size. The exclusive use of HUVECs, although insightful for high glucose-induced endothelial dysfunction, limits the applicability of results across different endothelial cell types and in vivo scenarios. Moreover, further functional validation is needed to elucidate the precise roles of the differentially expressed miRNAs in mediating metformin's impact on endothelial oxidative stress and apoptosis. Additionally, the mechanisms through which these miRNAs influence endothelial responses to metformin in hyperglycaemic conditions remain partially explored. Lastly, the translation of these findings into clinical strategies for managing diabetes-related vascular complications remains speculative, necessitating additional research to assess the therapeutic potential of metformin in targeting specific miRNAs.

CONCLUSION

This study provides an in-depth analysis of metformin's effect on the miRNA profile in high glucose-induced endothelial cells treated with metformin. It identifies 19 differentially expressed miRNAs, including nine downregulated and 10 upregulated miRNAs. Through bioinformatic analyses, this study elucidates the role, signaling pathways, target genes, and protein interactions associated with oxidative stress and apoptosis, potentially contributing to metformin's protective effects on the vasculature. These results enhance our understanding of the mechanisms underlying metformin's protection against diabetic vascular endothelial dysfunction, suggesting avenues for future research into miRNA-based therapies for diabetes and its vascular complications.

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