• Basic Research •

Key genes and regulatory networks for diabetic retinopathy based on hypoxia-related genes: a bioinformatics analysis

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Received: 2023-11-16 Accepted: 2024-04-01

Abstract

● **AIM:** To prevent neovascularization in diabetic retinopathy (DR) patients and partially control disease progression.

● **METHODS:** Hypoxia-related differentially expressed genes (DEGs) were identified from the GSE60436 and GSE102485 datasets, followed by gene ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Potential candidate drugs were screened using the CMap database. Subsequently, a protein-protein interaction (PPI) network was constructed to identify hypoxia-related hub genes. A nomogram was generated using the rms R package, and the correlation of hub genes was analyzed using the Hmisc R package. The clinical significance of hub genes was validated by comparing their expression levels between disease and normal groups and constructing receiver operating characteristic curve (ROC) curves. Finally, a hypoxia-related miRNA-transcription factor (TF)-Hub gene network was constructed using the NetworkAnalyst online tool.

● **RESULTS:** Totally 48 hypoxia-related DEGs and screened 10 potential candidate drugs with interaction relationships to upregulated hypoxia-related genes were identified, such as ruxolitinib, meprylcaine, and deferiprone. In addition, 8 hub genes were also identified: glycogen phosphorylase muscle associated (*PYGM*), glyceraldehyde-3-phosphate dehydrogenase spermatogenic (*GAPDHS*), enolase 3 (*ENO3*), aldolase fructose-bisphosphate C (*ALDOC*), phosphoglucomutase 2 (*PGM2*), enolase 2 (*ENO2*), phosphoglycerate mutase 2 (*PGAM2*), and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (*PFKFB3*). Based on hub gene predictions, the miRNA-TF-Hub gene network revealed complex interactions between 163 miRNAs, 77 TFs, and hub genes. The results of ROC showed that the except for *GAPDHS*, the area under curve (AUC) values of the other 7 hub genes were greater than 0.758, indicating their favorable diagnostic performance.

● **CONCLUSION:** *PYGM*, *GAPDHS*, *ENO3*, *ALDOC*, *PGM2*, *ENO2*, *PGAM2*, and *PFKFB3* are hub genes in DR, and hypoxia-related hub genes exhibited favorable diagnostic performance.

● **KEYWORDS:** diabetic retinopathy; hypoxia-related genes; hub genes; miRNA-TF-Hub gene; drug prediction
DOI:10.18240/ijo.2024.08.04

Citation: Yu CH, Wu CX, Li D, Gong LL, Lyu XD, Yang J. Key genes and regulatory networks for diabetic retinopathy based on hypoxia-related genes: a bioinformatics analysis. *Int J Ophthalmol* 2024;17(8):1411-1417

INTRODUCTION

Diabetic retinopathy (DR) is a type of retinal damage induced by diabetes that is classified as a complication. In severe cases, it can cause blindness[1]. DR patients typically present with symptoms such as hemorrhage, exudation, cotton wool spots, microaneurysms, and neovascularization in the ocular fundus[2-3]. When retinal capillary perfusion is totally interrupted, vascular endothelial growth factor (VEGF) stimulates the formation of new blood vessels at the margin of the non-perfused area, moving DR from the non-proliferative stage to the proliferative stage. In this stage, previously clogged retinal capillaries cannot be restored, resulting in permanent retinal ischemia in the afflicted area[4-6]. Therefore, early discovery, treatment, and control are critical for diabetic

patients, particularly those with DR[7]. Currently, despite the availability of surgical, laser, and anti-VEGF treatments to prevent neovascularization in DR patients and partially control disease progression, the cure rate for DR remains low due to the complexity of the condition and the influence of factors such as hypertension, hyperglycemia, and a high body mass index[8-11].

During hypoxia, the body's oxygen supply and demand are interrupted, resulting in disruptions in numerous life processes that promote cellular survival. For instance, to meet the rapid proliferation of tumor cells, these cells undergo metabolic reprogramming to adapt to the hypoxic environment by relying on glycolysis for energy production[12-13]. Furthermore, hypoxia is present in many disorders and plays a crucial role in their progression, including Alzheimer's disease, Parkinson's disease, and DR[14-15]. Shiba *et al*[16] found that intermittent hypoxia and reoxygenation are prevalent in proliferative DR patients. Similarly, Ramsey and Arden[17] found that during the dark period, diabetic retinas cannot meet the extra metabolic load imposed by rod photoreceptors for dark adaptation, exacerbating retinal hypoxia and driving excessive VEGF synthesis. Conversely, limiting dark adaptation can increase the prevalence of DR[17]. Therefore, controlling oxygen balance in DR holds promise for improving disease management. However, the relationship between hypoxia-related genes and the progression of DR is uncertain.

In this study, we conducted a comprehensive analysis of hypoxia-related genes in DR for the first time. We collected a hypoxia-related gene set from public databases and selected hub genes for disease diagnosis. Additionally, we constructed a miRNA-transcription factor (TF)-Hub gene interaction network and predicted potential therapeutic drugs for DR. Through these investigations, we aimed to further elucidate the relationship between hypoxia-related genes and DR and provide insights for mechanistic and drug studies in DR.

MATERIALS AND METHODS

Acquisition of Public Data The publicly available DR datasets, GSE60436 and GSE102485, were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/). The GSE60436 dataset consisted of 3 normal samples and 6 DR samples, while the GSE102485 dataset included 3 normal samples and 22 DR samples. A collection of 200 hypoxia-related genes was gathered from the MSigDB (https://www.gsea-msigdb.org/gsea/msigdb)[18].

Identification and Enrichment Analysis of Hypoxia-Related Differentially Expressed Genes in DR Differential analysis was performed using the limma R package in R software on the normal and DR sample data from the GSE60436 dataset. Differentially expressed genes (DEGs) in DR were identified with $P<0.05$ and $|\log(FC)| >1$ as criteria. Venn analysis was then conducted to identify the overlapping genes between the DEGs and the hypoxia-related genes, representing the hypoxia-related DEGs in DR. Subsequently, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of the hypoxia-related DEGs in DR were performed using the clusterProfiler package in R software, with a significance threshold of $P<0.05$.

Identification of Potential Drugs for DR The top 30 upregulated genes among the hypoxia-related DEGs in DR were selected and input into the CMap database (https://portals.broadinstitute.org/cmap/) to obtain information on potential drugs. Negative scores indicated that the drugs might have therapeutic benefits for the disease[19].

Selection of Hub Genes The protein-protein interaction (PPI) network of the hypoxia-related DEGs in DR was constructed by inputting the genes into the STRING database (https://string-db.org/) with a confidence score>0.4. Subsequently, hub genes were selected using the MCODE plugin and the MCC algorithm of the cytoHubba plugin in Cytoscape software.

Correlation Analysis and Diagnostic Performance Evaluation of Hub Genes Using the hub genes screened from the Cytoscape, a nomogram was constructed in the GSE60436 dataset using the rms R package. Additionally, the correlation between hub genes was analyzed using the Hmisc R package. Furthermore, the expression levels of hub genes were compared between the disease group and the normal group in the GSE102485 dataset, and the clinical significance of hub genes was validated using Receiver operating characteristic curve (ROC) analysis.

Prediction of miRNA-TF-Hub Gene Interaction Network The hub genes were input into the NetworkAnalyst online tool (https://www.networkanalyst.ca/) to obtain the miRNA-TF-Hub gene interaction network. The following parameters were set: Designated organism: Homo sapiens (human); Collection ID type: official gene symbol; Gene-miRNA interaction database: miRTarBase v8.0 database; Gene-TF interaction database: ENCODE database.

RESULTS

Identification and Enrichment Analysis of DEGs The differential expression analysis of the GSE60436 dataset revealed a total of 2244 downregulated genes and 775 upregulated genes (Figure 1A). Taking the intersection of these DEGs with hypoxia-related genes, we obtained 48 hypoxia-related DEGs (Figure 1B). The enrichment analysis results showed that these 48 DEGs were mainly enriched in KEGG signaling pathways such as the hypoxia inducible factor 1 (HIF-1) signaling pathway, glycolysis/gluconeogenesis, biosynthesis of amino acids, Glucagon signaling pathway, carbon metabolism, and p53 signaling pathway (Figure 1C). Additionally, they were enriched in GO terms including

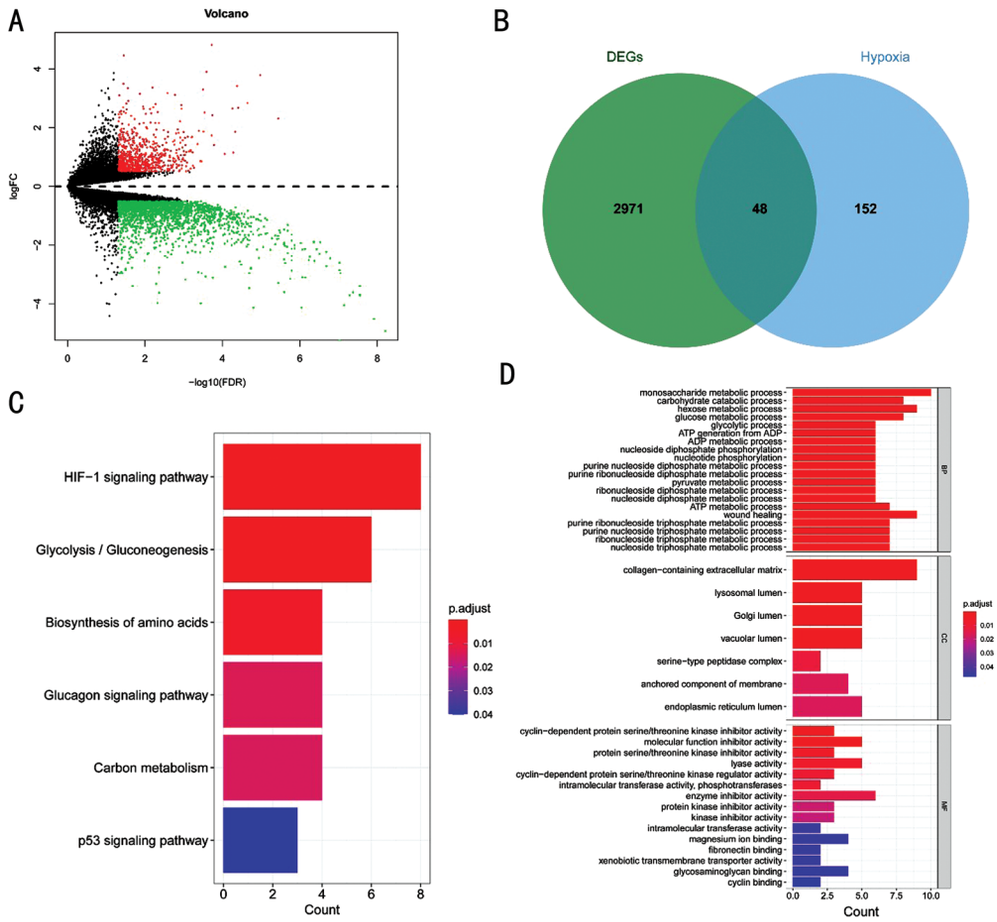

Figure 1 Identification of DEGs and enrichment analysis of function and pathway A: Selection of DEGs in the GSE60436 dataset; B: Selection of hypoxia-related DEGs; C: KEGG analysis of hypoxia-related DEGs; D: GO analysis of hypoxia-related DEGs. FC: Fold change; FDR: False discovery rate; DEGs: Differentially expressed genes.

monosaccharide metabolic process, glycolytic process, cyclin-dependent protein serine/threonine kinase inhibitor activity, molecular function inhibitor activity, protein serine/threonine kinase inhibitor activity, lyase activity, and cyclin-dependent protein serine/threonine kinase regulator activity (Figure 1D).

Prediction of Candidate Drugs using CMap Through the CMap database, we obtained 10 candidate drugs (LY-364947, PJ-34, ruxolitinib, CG-930, prima-1-met, tyrphostin-AG-112, SB-202190, meprylcaine, and deferiprone) that may interact with upregulated genes related to hypoxia (Table 1). These drugs were found to have various effects, including transforming growth factor β (TGF-β) receptor inhibitor, poly (ADP-ribose) polymerase (PARP) inhibitor, Janus kinase (JAK) inhibitors, thioredoxin inhibitor, protein tyrosine kinase inhibitor, p38 mitogen-activated protein kinase (MAPK) inhibitor, local anesthetic, and chelating agent (Table 1).

Selection of Hypoxia-Related Hub Genes Analysis using the STRING database revealed complex interactions among the hypoxia-related DEGs (Figure 2A). By intersecting the results of the MCC algorithm and the MCODE algorithm, we identified 8 hub genes: glycogen phosphorylase muscle associated (*PYGM*), glyceraldehyde-3-phosphate dehydrogenase spermatogenic (*GAPDHS*), enolase 3 (*ENO3*), aldolase fructose-bisphosphate C (*ALDOC*), phosphoglucomutase 2 (*PGM2*), enolase 2 (*ENO2*), phosphoglycerate mutase 2 (*PGAM2*), and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (*PFKFB3*; Figure 2B).

Table 1 Ten drug candidates obtained from the CMAP database that may have interactions with hypoxia-related upregulated genes

Name	ID	Description	Score
LY-364947	BRD-K06234293	TGF beta receptor inhibitor	-99.93
PJ-34	BRD-K11853856	PARP inhibitor	-99.93
Ruxolitinib	BRD-K53972329	JAK inhibitor	-99.89
CG-930	BRD-K84085265	JAK inhibitor	-99.89
Prima-1-met	BRD-K49456190	thioredoxin inhibitor	-99.89
Tyrphostin-AG-112	BRD-K01192156	Protein tyrosine kinase inhibitor	-99.89
SB-202190	BRD-K54330070	p38 MAPK inhibitor	-99.89
Meprylcaine	BRD-K65417056	Local anesthetic	-99.89
Deferiprone	BRD-K06878038	Chelating agent	-99.82

TGF-β: Transforming growth factor β; PARP: Poly (ADP-ribose) polymerase; JAK: Janus kinase; MAPK: Mitogen-activated protein kinase.

Analysis of Hypoxia-Related Hub Genes Correlation analysis of the hub genes revealed significant relationships among the 8 hub genes (P<0.05). *PGM2* showed significant negative correlations with *PYGM*, *GAPDHS*, and *PGAM2*,

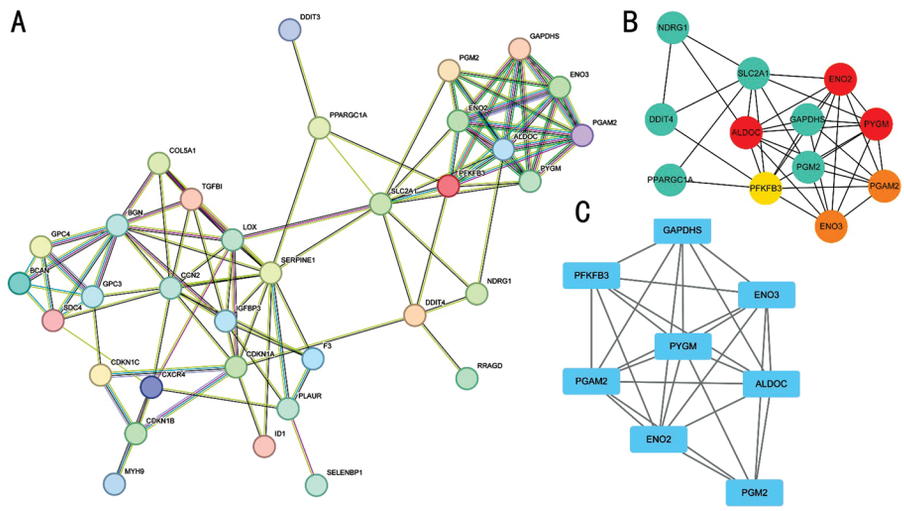

Figure 2 A PPI network was constructed using STRING database to screen hub genes A: Interaction network of hypoxia-related DEGs constructed based on the STRING database; B: Identification of hub genes using the MCC algorithm; C: Identification of hub genes using the MCODE algorithm. PPI: Protein-protein interaction; DEGs: Differentially expressed genes.

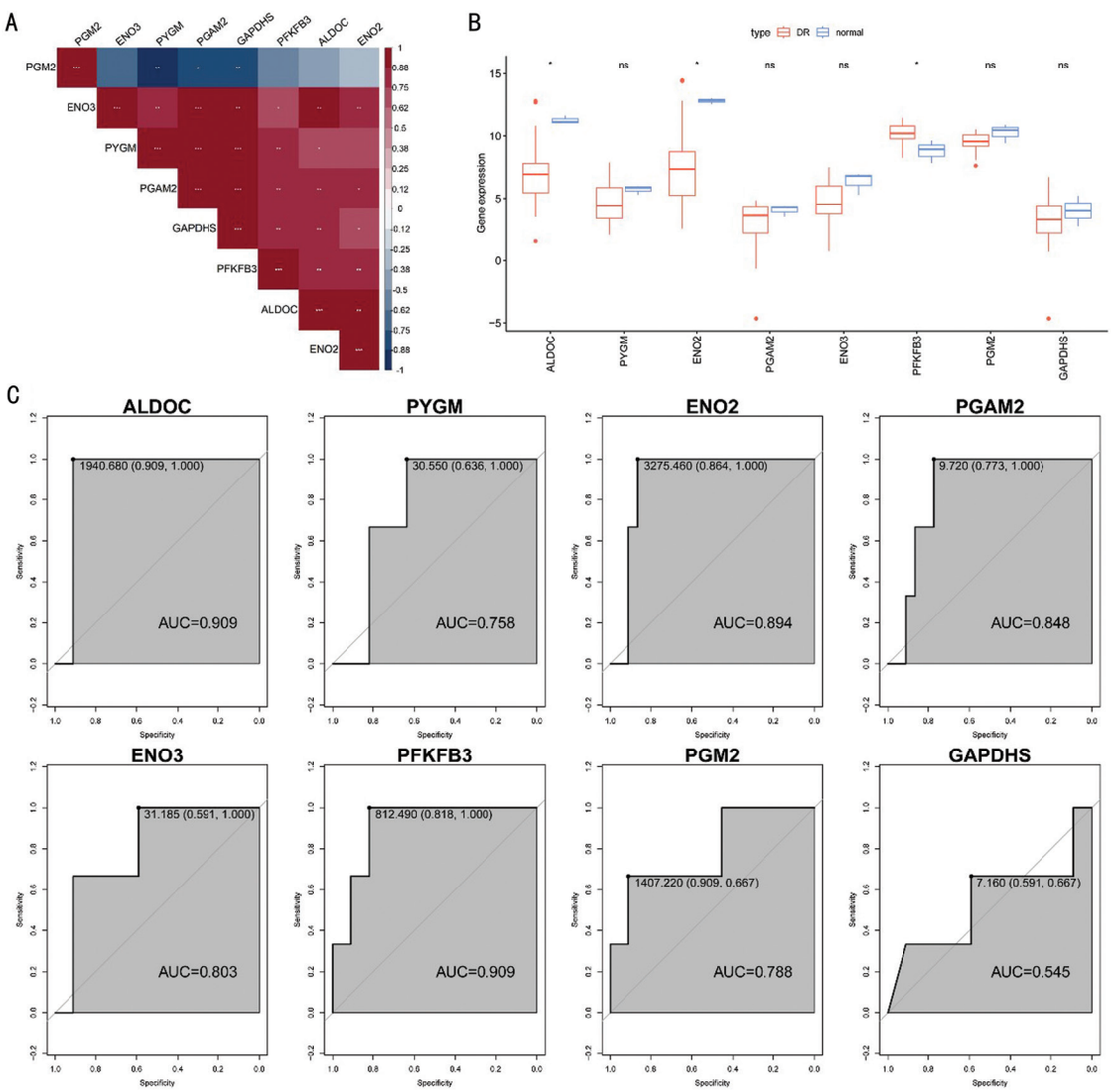

Figure 3 Analysis of correlation between hub genes and diagnostic performance A: Correlation analysis among hub genes; B: Differential expression analysis of hub genes in the GSE102485 dataset; C: ROC curves of hub genes in the GSE102485 dataset. ROC: Receiver operating characteristic.

while *PYGM* showed significant positive correlations with *GAPDHS* and *PGAM2* (Figure 3A). Further analysis of the expression levels of hub genes in the GSE102485 dataset showed that *ALDOC* and *ENO3* were significantly under

expressed in the DR group (P<0.05), while *PFKFB3* was significantly overexpressed in the DR group (P<0.05). The expression differences of the other hub genes (*PYGM*, *GAPDHS*, *PGM2*, *ENO2*, and *PGAM2*) between the normal and DR groups were not significant (P>0.05; Figure 3B). Analysis of ROC curves revealed that all hub genes, except for *GAPDHS*, had area under curve (AUC) values greater than 0.758, indicating their favorable diagnostic performance (Figure 3C). Additionally, the nomogram constructed based on the GSE60436 dataset indicated that these 8 hub genes could predict disease risk (Figure 4).

miRNA-TF-Hub Gene Interaction Network Using the NetworkAnalyst online tool, we screened for miRNAs and TFs that may target the hub genes and constructed the corresponding network. The results showed that we predicted a total of 163 miRNAs and 77 TFs, which may have complex interactions with the hub genes. Among them, hsa-mir-335-5p as a miRNA targeted *ALDOC*, *GAPDHS*, and *PFKFB3* simultaneously, while hsa-mir-423-5p and hsa-mir-3184-5p as miRNAs targeted *PFKFB3* and *ENO2* simultaneously. Metastasis-associated protein 2 (MTA2) as a TF targeted *ENO3*, *ENO2*, and *PFKFB3* simultaneously (Figure 5).

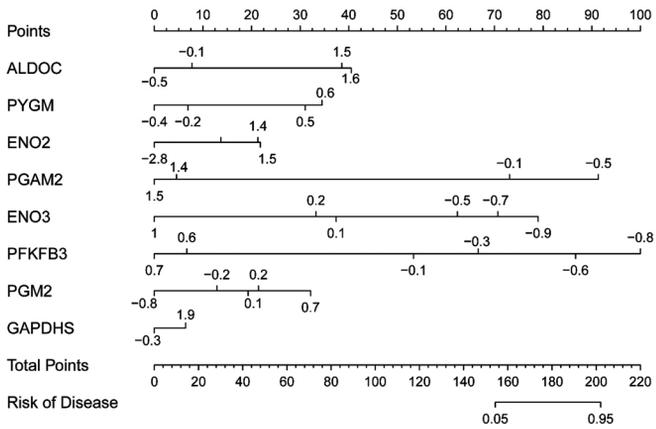

Figure 4 Nomogram of hub genes constructed using the GSE60436 dataset.

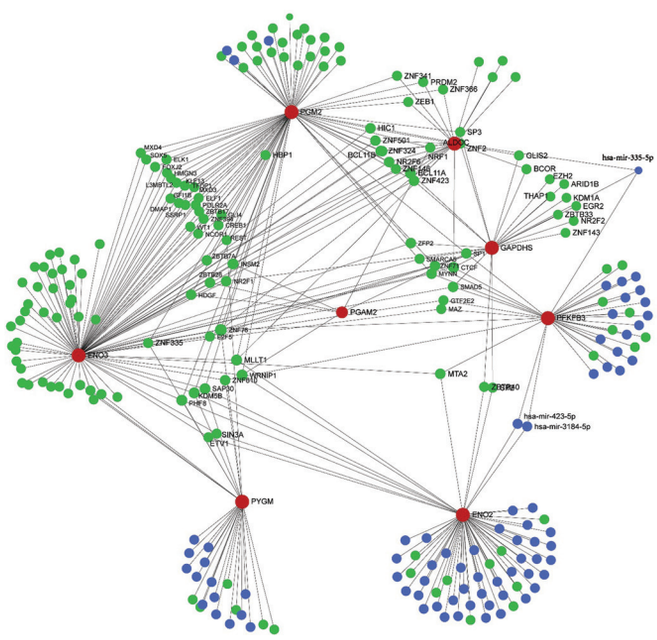

Figure 5 Construction of the miRNA-TF-Hub gene network Red: Hub genes; Green: TFs; Blue: miRNAs. TF: Transcription factor.

DISCUSSION

Diabetes has long been acknowledged as a global public health issue. DR, a primary microvascular complication of diabetes, has a global prevalence of 34.6% and has contributed significantly to the global disease burden[20]. The etiology and pathogenesis of DR are complex. The proliferative stage of DR is defined by excessive angiogenesis, and pathological angiogenesis can promote inflammation and immunological dysfunction by exacerbating ischemia and disrupting oxygen and nutrient supply, thus creating a vicious cycle of angiogenesis and disease progression[21]. However, current research on the pathologic mechanisms of DR focuses mostly on VEGF-related issues, and progress in this field is limited[22]. As a result, pursuing more DR research avenues is critical to understanding the pathogenesis of DR. Research indicates that hypoxia increases the expression of hypoxia inducible factor 1 subunit alpha (HIF-1α) in human retinal endothelial cells and promotes retinal angiogenesis in DR by upregulating proangiogenic growth factors[23]. Therefore, these findings collectively suggest that hypoxia is commonly implicated in DR and may influence the disease development. The goal of this study was to identify hypoxia-related genes with potential diagnostic and therapeutic value in DR and explore possible regulatory networks.

We identified 48 hypoxia-related DEGs through differential expression analysis and Venn analysis. Enrichment analysis revealed that these genes were primarily enriched in KEGG signaling pathways such as the HIF-1 signaling pathway, biosynthesis of amino acids, and the p53 signaling pathway. Hypoxia-inducible factors are key regulatory factors in the oxygen response during hypoxia. Wang *et al*[24] demonstrated through network analysis and experimental validation that modulating the expression levels of key genes in the HIF-1 signaling pathway [upregulating nitric oxide synthase 3 (NOS3) and heme oxygenase 1 (HMOX1) and downregulating vascular endothelial growth factor A (VEGFA), serpin family E member 1 (SERPINE1), and nitric oxide synthase 2 (NOS2)] substantially prevented the development of DR. Amino acids are the fundamental building blocks of proteins, and previous research has linked amino acid synthesis and metabolism to retinal diseases such as age-related macular degeneration, DR, and retinopathy of prematurity. Intraocular samples from DR patients show enrichment in alanine, aspartate, and glutamate metabolism[25]. According to Li *et al*[26], activation of the p53 signaling pathway induces apoptosis of retinal

endothelial cells. Taken together, these findings suggest that hypoxia-related genes may influence the progression of DR by modulating these signaling pathways.

Through screening, we identified eight hub genes related to hypoxia, including *PYGM*, *GAPDHS*, *ENO3*, *ALDOC*, *PGM2*, *ENO2*, *PGAM2*, and *PFKFB3*. The relationships between *PYGM*, *ENO3*, *ALDOC*, *PGM2*, *ENO2*, and DR have yet to be deciphered. However, there have been reports regarding *GAPDHS*, *PGAM2*, and *PFKFB3*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is a glycolytic enzyme that catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. Kanwar and Kowluru's[27] study found that inhibiting GAPDH can activate its upstream and downstream signaling pathways, thereby promoting retinal lesions in DR rats. PGAM2, namely phosphoglycerate mutase 2, is a key enzyme in the glycolysis process. Previous studies have reported abnormal expression of PGAM2 in DR rats and confirmed it as a key hub gene in laser treatment for DR rats[28-29]. PFKFB3 is one of the isoforms of the bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB1-4) and is widely expressed in human tissues, including the retina[30-31]. Min *et al*[32] found that activating the HIF1α-PFKFB3 pathway promotes angiogenesis and neurodegeneration in DR, leading to disease progression. These findings collectively suggest that these hub genes may play a significant role in the progression of DR.

Furthermore, this study employed the NetworkAnalyst online platform to screen for miRNAs and TFs that could target hub genes and create associated network diagrams. Among these molecules, hsa-mir-335-5p targeted *ALDOC*, *GAPDHS*, and *PFKFB3* simultaneously, hsa-mir-423-5p targeted *PFKFB3* and *ENO2* simultaneously, and MTA2, as a TF, targeted *ENO3*, *ENO2*, and PFKFB3 simultaneously. Hirota *et al*[33] discovered that eyes with proliferative DR have higher expression levels of hsa-mir-423-5p in the vitreous than eyes with macular holes, implying that abnormal expression of hsa-mir-423-5p may be associated with the onset of DR. Tokarski *et al*[34] found that under hypoxic conditions, hsa-mir-335-5p may affect the proliferation and migration of key progenitor and mature endothelial cells in angiogenesis by regulating the HGFA-HGF-c-Met signaling pathway. MTA2 is a metastasis-associated protein. Zhu *et al*[35] found that MTA2 interacts with HIF-1α, inhibits the transcription of E-cadherin, and promotes the progression of pancreatic cancer, albeit no link to DR has been established. In summary, understanding the significance of hypoxia-related hub genes and their potential miRNAs and TFs may help to understand the relationship between hypoxia-related genes and the progression of DR, as well as providing a basis for future mechanistic investigations.

In conclusion, the study used bioinformatics tools to extensively examine the involvement of hypoxia-related genes in DR and proposed key hub genes. However, this study does have some drawbacks. Although numerous datasets were included in this investigation, the sample sizes of each dataset were limited. Additionally, the proposed hub genes and candidate drugs require empirical validations. Furthermore, it should be highlighted that, while we created a probable miRNA-TF-Hub gene network, the accuracy of the results needs further research validation as they were predicted using online databases.

ACKNOWLEDGEMENTS

Authors' contributions: Conceptualization: Yu CH; Data curation: Yang J; Formal analysis: Wu CX; Funding acquisition: Yu CH; Investigation: Wu CX; Methodology: Yu CH; Project administration: Yu CH; Resources: Lyu XD; Software: Wu CX; Supervision: Lyu XD; Validation: Li D; Visualization: Lyu XD; Writing – original draft: Gong LL; Writing – review & editing: Yang J.

Foundations: Supported by Scientific Research Project of Xianning Central Hospital in 2022 (No.2022XYB020); Science and Technology Plan Project of Xianning Municipal in 2022 (No.2022SFYF014).

Conflicts of Interest: Yu CH, None; **Wu CX,** None; **Li D,** None; **Gong LL,** None; **Lyu XD,** None; **Yang J,** None.

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