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Research Article

Network Pharmacology and Molecular Docking Study on the Pharmacological Mechanism of Verbascoside for the Treatment of High-Altitude Renal Disease

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Abstract

Background and Objective: Low air pressure and hypoxia conditions at high altitudes adversely affect the respiratory, circulatory and nervous systems, which in turn accelerates the onset and progression of chronic kidney disease. Recent studies have shown the protective effect of verbascoside (VB) against kidney injury due to its antioxidant properties. However, the pharmacologic mechanism of VB on high-altitude renal disease remains unclear. Hence, the objective of this study was to explore the key targets and possible molecular mechanisms of VB against high-altitude renal disease through network pharmacology, molecular docking and *in vitro* experiments.

Materials and Methods: Targets of VB, high-altitude renal disease and hypoxia-induced pathways were obtained. Venn diagram identified key targets. The GO and KEGG analyses revealed of VB involvement in biological processes and pathways in high-altitude renal disease. Molecular docking studied of VB binding interactions with hub genes. The CCK-8 and qRT-PCR assessed cell viability and mRNA expression of EGLN1, HMOX1 and BCL-2 in hypoxia-induced HK-2 cells. **Results:** The EGLN1, HMOX1 and BCL-2 were identified as VB target genes against high-altitude renal disease. The VB directly interacted with them in the HIF-1 signaling pathway. The miR-409-3p and miR-495-3p might regulate these genes. The VB significantly increased HK-2 cell viability and decreased mRNA levels of EGLN1, HMOX1 and BCL-2.

Conclusion: Under hypoxic conditions, VB potentially treats high-altitude renal disease by downregulating EGLN1, HMOX1 and BCL-2 mRNA expression, contributing to improved renal function and disease progression delay in high-altitude renal disease.

Key words: High-altitude renal disease, verbascoside, molecular docking, network pharmacology, hypoxia induction

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

High-altitude renal disease, also known as high-altitude nephropathy, is a condition characterized by renal dysfunction that occurs in individuals living at high altitudes¹. The characteristic of high-altitude areas is the thin atmosphere, with atmospheric pressure and oxygen partial pressure decreasing with increasing altitude². High-altitude renal disease is particularly prevalent in populations residing in mountainous regions, such as the Andean and Himalayan populations³. It is characterized by renal hypoxia, oxidative stress, inflammation and impaired renal function. Renal hypoxia, specifically within the tubulointerstitium, presents as a potential prognostic indicator concerning the advancement of Chronic Kidney Disease (CKD)⁴. Analysis of the transcriptome across various organs of yaks residing at altitudes of 3400, 4200 and 5000 m revealed that in the differentially expressed genes of the kidney, 6.25% were associated with hypoxia. This ranked second in prevalence after the heart at 2%². Given the geographical context, high-altitude renal disease shares a formation mechanism akin to hypoxia-induced CKD. High-altitude renal disease is additionally characterized by high-altitude polycythemia, hyperuricemia, systemic hypertension and microalbuminuria². Despite being a significant health concern in high-altitude areas, effective treatment strategies for high-altitude renal disease are limited.

In recent years, natural compounds have gained attention for their potential therapeutic properties in various diseases, including kidney disorders. Verbascoside (VB, also known as acteoside), a bioactive compound derived from many medicinal plant families such as Verbenaceae and *Plantago* species, has shown promising effects in ameliorating renal injuries⁵. The VB has been shown to alleviate diabetic kidney disease by modulating podocyte damage associated with NR4A1-LKB1-AMPK signaling⁶. It exhibits antioxidant, anti-inflammatory and cytoprotective properties, allowing it to ameliorate memory damage caused by hypoxic at altitude by modulating oxidative stress and mTOR signaling pathways⁷, suggesting its potential role in mitigating high-altitude renal disease progression. However, the precise pharmacological mechanisms underlying the therapeutic effects of VB in delaying the progression of high-altitude renal disease remain largely unknown.

Network pharmacology is a powerful tool that integrates network analysis, drug-target prediction and pathway enrichment analysis to elucidate the complex interactions between drugs, targets and diseases⁸. It has been extensively utilized to unravel the intricate mechanisms of drug therapy,

such as chongcaoyishen decoction against CKD⁹, astragaloside IV alleviating renal fibrosis¹⁰, Bushen Huashi decoction against kidney stones¹¹, etc. Molecular docking, on the other hand, provides insights into the binding affinity and potential interactions between small molecules and target proteins. Currently, many studies have utilized network pharmacology and molecular docking approaches to reveal the mechanism of drug action on diseases^{12,13}. Meanwhile, this approach has emerged as a promising way to accelerate drug discovery¹⁴. However, there are no systematic network pharmacology studies on VB against high-altitude renal disease.

Therefore, this study aimed to reveal the mechanism of VB in high-altitude renal disease treatment and predict its key targets as well as signaling pathways via network pharmacology, molecular docking and *in vitro* validation. These findings will provide a scientific basis for studying the complex mechanisms of drug action.

MATERIALS AND METHODS

Study area: This study was conducted in the Department of Nephrology, The 940th Hospital of Joint Logistics Support Force of Chinese People's Liberation Army from January, 2023 to June, 2023.

Data download and pre-processing: The CKD-related microarray expression dataset GSE66494 (8 healthy control samples, 53 CKD patient samples) was downloaded in the Gene Expression Omnibus (GEO) database¹⁵. Probe annotation of the dataset was performed based on the GPL6480 platform. The keyword "Acteoside" was imported to retrieve known relevant drug target genes from the GeneCards¹⁶, the Comparative Toxicogenomics Database¹⁷, HIT2¹⁸ and SymMap¹⁹. Moreover, the drug target genes were also predicted based on the Simplified Molecular-Input Line-Entry System (SMILES) structure of VB in SuperPred²⁰ and SwissTargetPrediction²¹ databases.

Enrichment analysis of Gene Set Variation Analysis (GSVA) based on hypoxia-induced related pathways: Hypoxia-induced gene sets were screened using MSigDB²². The activity scores of the GSE66494 dataset with the hypoxia-induced genes were predicted using the GSVA package in R 4.2.

Weighted Gene Co-Expression Network Analysis (WGCNA): The WGCNA provides a holistic view of the structure of gene co-expression networks and the correlation between specific biological processes (BP), which is often applied to screen biomarkers, discover potential therapeutic targets and explain

mechanisms of disease progression²³. The WGCNA package in R4.2.0 software was used to analyze GSE66494 gene expression data. The expressions of all genes were grouped to eliminate outliers. The correlation coefficients between gene pairs were calculated using the Pearson method and transformed into similarity measures. Based on the dynamic pruning algorithm (merge cut height = 0.35), the gene co-expression network was divided into different gene modules. The optimal matrix transformation threshold (R squared cut) was selected as 0.85 and the minimum module size (min module size) was set to 30 by adjusting the WGCNA parameters. The core genes of every module were identified by selecting the genes associated with both CKD and hypoxia-induced factor pathway scores²⁴.

Identification of potential target genes for the effects of VB on CKD: The Venn diagram²⁵ was utilized to show the intersection of VB-target genes and key genes in WGCNA. The Venn diagram was constructed using software R4.2.0.

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis: Utilizing R4.2.0 software, GO and KEGG enrichment analysis was conducted on the common genes identified in the Venn diagram²⁶. The top 15 GO terms and pathways were graphically represented in column charts based on a significance threshold of $p < 0.05$. The enrichment assessment employed the hypergeometric distribution model to determine the statistical significance of the association between the target gene set and specific GO terms and biological pathways:

$$P = 1 - \sum_{i=0}^{k-1} \frac{\binom{M}{i} \binom{N-M}{n-i}}{\binom{N}{n}}$$

In this context, "N" represents the total number of genes, "M" denotes the count of annotated genes in the GO database, "n" indicates the input target genes and "k" signifies the number of genes shared between the overall gene pool and those annotated in GO. The resulting p-value, obtained through statistical testing, quantifies the strength of association between the input target genes and the respective GO terms or pathways. Associations are considered significant when the p-value is less than 0.05.

Mapping of disease-associated metabolites and gene synergistic regulatory pathways: To identify metabolites associated with the development of CKD, a comprehensive literature search was conducted²⁷. Additionally, the Human

Metabolome Database (HMDB) was queried to retrieve relevant metabolite information. The retrieved metabolites were screened and filtered based on their relevance to CKD. Metabolites meeting the predefined criteria were selected for further analysis. Gene expression data from the GSE66494 dataset were utilized to validate the expression levels of genes associated with the screened metabolites. Differential expression analysis was performed using a t-test to identify differentially expressed genes. The disease-related metabolites and differentially expressed genes were subjected to a co-pathway enrichment analysis using KEGG Mapper. The KEGG Mapper tool facilitated the identification of coordinated pathways involving the screened metabolites and genes.

Prediction of mRNA-miRNA: The TargetScanHuman database was utilized to predict potential miRNAs of target genes. Separate analyses were performed for each gene of interest. The predicted miRNAs of target genes were used to construct a miRNA-mRNA regulatory network. Cytoscape, a network visualization and analysis tool, was employed for network construction.

Molecular docking verification: The protein structures of genes involved in the Hypoxia-Inducing Factor-1 (HIF-1) signaling pathway in pdb format were downloaded from the RCSB Protein Data Bank²⁸. The sdf structure of VB was downloaded from PubChem and converted to a uniform PDB format using Pymol software. The receptor and ligand were preprocessed accordingly to ensure effective docking of the results. Water molecules of ligands were removed in Pymol software and automatic charge adjustment calculations were performed in AutoDock 1.5.6 software to determine the root of the ligand and select the twistable bond. The file was saved in pdbqt format. Water molecules of the receptor and the original ligand were removed in Pymol software, keeping only the single chain. Hydrogen, amino acids and charge calculations were optimized in AutoDock 1.5.6 software and the format was converted to pdbqt. The active docking pocket was determined in AutoDock 1.5.6 software based on the spatial position of the original ligand in the receptor. Finally, molecular docking was performed based on the command line version of AutoDock Vina 1.1.2.

Cell culture: The human renal proximal tubular cell line (HK-2), sourced from Procell Life Science and Technologies Co., Ltd. (#CL-0109, Wuhan, China), was employed in the study. The HK-2 cells were cultivated in Dulbecco's Modified Eagle's Medium/Nutrient Ham's Mixture F-12 medium supplemented with 10% fetal bovine serum, 100 U/mL

penicillin and 100 µg/mL streptomycin. The cells were maintained at a temperature of 37°C within a humidified incubator featuring 5% CO₂. To experiment, the cells were categorized into three distinct groups: Normoxia, hypoxia and hypoxia+VB. The normoxia group cells were cultured for 24 hrs in an incubator with an oxygen concentration of 21%. On the other hand, cells designated for the hypoxia group were subjected to an environment containing 1% oxygen for 24 hrs. In the hypoxia+VB group, cells were pretreated with 100 µM VB²⁹ for a duration of 3 hrs before being exposed to hypoxia conditions.

Cell Counting Kit-8 (CCK-8) assay: For assessing cell viability following 24 hrs hypoxia/normoxia exposure, the CCK-8 assay was employed. In this procedure, a cell suspension (100 µL/well) was introduced into 96-well plates, which were subsequently placed in a 5% CO₂ incubator at 37°C for pre-culturing. Following this initial incubation period, 10 µL of CCK-8 reaction solution (Beyotime, Shanghai, China) was introduced into each well. The plates were then subjected to an additional 2 hrs incubation within the incubator. The subsequent quantification was performed by measuring the absorbance at 450 nm using a microplate reader (Wuxi HIWELL DIATEK Instruments, Jiangsu, China, #: DR-3518G).

Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR) analysis: The mRNA expression levels of Egl-9 Family Hypoxia Inducible Factor 1 (EGLN1), Heme Oxygenase-1 (HMOX1) and B-Cell Lymphoma 2 (BCL-2) in each group of cells were detected by qPCR. Total RNA was extracted from cells using Trizol reagent (Invitrogen, California, USA) according to the manufacturer's instructions. FastKing One-Step Genome cDNA First-Strand Synthesis Kit (TIANGEN, Beijing, China) was used to transcribe RNA into cDNA, providing the template for PCR. The SYBR Green PCR Master Mix (Lifeint, Xiamen, China) was employed for qRT-PCR analysis. Thermal cycling was performed as follows: 95°C for 3 min (denaturation), followed by 40 cycles of 95 for 12 sec and 62°C for 40 sec. Sequences of primers were as follows: EGLN1 (human) forward primer: 5'-TGT GCG GGA AGA TGG AGA AC-3', reverse primer: 5'-GTG CTC CTT GCA GCA GTA GA-3'; HMOX1 (human) forward primer: 5'-ACT CCC TGG AGA TGA CTC CC-3', reverse primer: 5'-TCT TGC ACT TTG TTG CTG GC-3'; BCL-2 (human) forward primer: 5'-GAA CTG GGG GAG GAT TGT GG-3', reverse primer: 5'-GCC GGT TCA GGT ACT CAG TC-3'; GAPDH forward primer: 5'-GAG AAG GCT GGG GCT CAT TT-3', reverse primer: 5'-AGT GAT GGC ATG GAC TGT GG-3'. The GAPDH was used as an internal control and the relative expression of these genes was calculated using the 2^{-ΔΔCT} method.

Statistical analysis: All data are presented as Mean ± Standard Deviation of three independent experiments. One-way analysis of variance followed by Tukey's test was used to compare differences between groups. All statistical analysis were done on GraphPad 7.0 software and p<0.05 was considered statistically significant.

RESULTS

Identification of high-altitude renal disease-related genes:

A total of 19582 genes associated with CKD were obtained after annotation of the dataset GSE66494 downloaded at GEO on the GPL6480 platform. To reveal the correlation between CKD-related genes in GSE66494 and a hypoxia environment in high-altitude, three gene sets (regulatory gene expression induced by hypoxia, cellular response to hypoxia and P53 hypoxia pathway) related to hypoxia induction were screened from MSigDB. As shown in Fig. 1a, the CKD group had higher scores of hypoxia-induced related pathway activity compared to the control group. This finding implied that there might be physiological and molecular changes associated with the hypoxic environment in patients with CKD. When the soft threshold was selected as 9 (Fig. 1b), the constructed network conformed to the scale-free distribution. Nine clustering modules (Fig. 1c) were obtained by WGCNA analysis. Among these modules, five modules (yellow, blue, black, magenta, turquoise) included 7137 genes that were correlated with CKD and hypoxia-induced pathway scores (Fig. 1d). Altogether, 6279 genes showed significant relevance with CKD and hypoxia-induced pathway scores, which were considered high-altitude renal disease-related target genes and were further analyzed.

Identification of potential target genes for the effects of VB

on CKD: After combining search results, 418 genes related to VB were obtained. To find out the target genes of VB against high-altitude renal disease, 6,279 genes were identified by WGCNA and 418 VB target genes were intersected and 97 overlapping genes were identified (Fig. 2a).

GO and KEGG enrichment analysis: To delve deeper into the roles of the 97 shared genes, a comprehensive biological function enrichment analysis was undertaken. The outcomes unveiled a total of 524 enriched GO terms and 81 KEGG pathways, all having a significance level of p<0.05. The GO enrichment analysis outcomes were categorized into three key aspects: BP, cellular component (CC) and molecular function (MF). From each category, the top 15 functions with the most significant p-values were selected for further

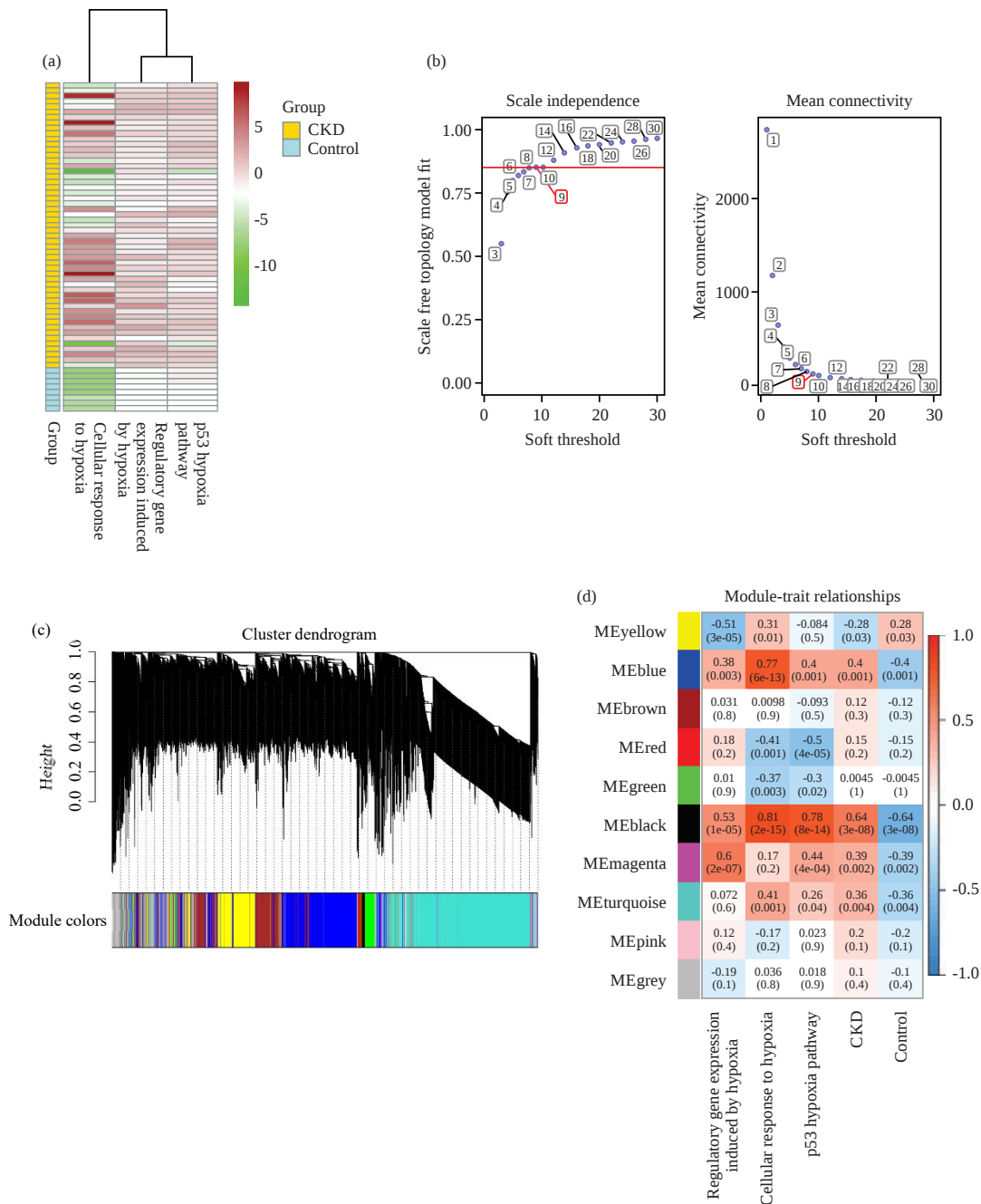


Fig. 1(a-d): GSVA and WGCNA analysis, (a) Heat map of GSVA pathway scores, (b) Distribution of soft threshold and connectivity in WGCNA, (c) Heat map of module clustering in WGCNA and (d) Heat map of correlation between clustering module and sample phenotype data

(a) Horizontal axis is pathway name and vertical axis is sample grouping. Brown indicates high pathway activity scores, and green indicates low pathway activity scores, (c) Different colors represent different modules and each module represents a class of genes with high similarity of expression patterns and (d) Top number in the color block is the correlation and the bottom number is the p-value of the correlation

examination, as depicted in Fig. 2(b-d). In the BP domain, enrichments encompassed xenobiotic stimulus, response to alcohol, regulation of membrane potential, regulation of cytosolic calcium ion concentration and calcium ion

homeostasis, among others (Fig. 2b). Within the CC domain, notable GO terms included transporter complex, transmembrane transporter complex, synaptic membrane, membrane raft and membrane microdomain (Fig. 2c). In

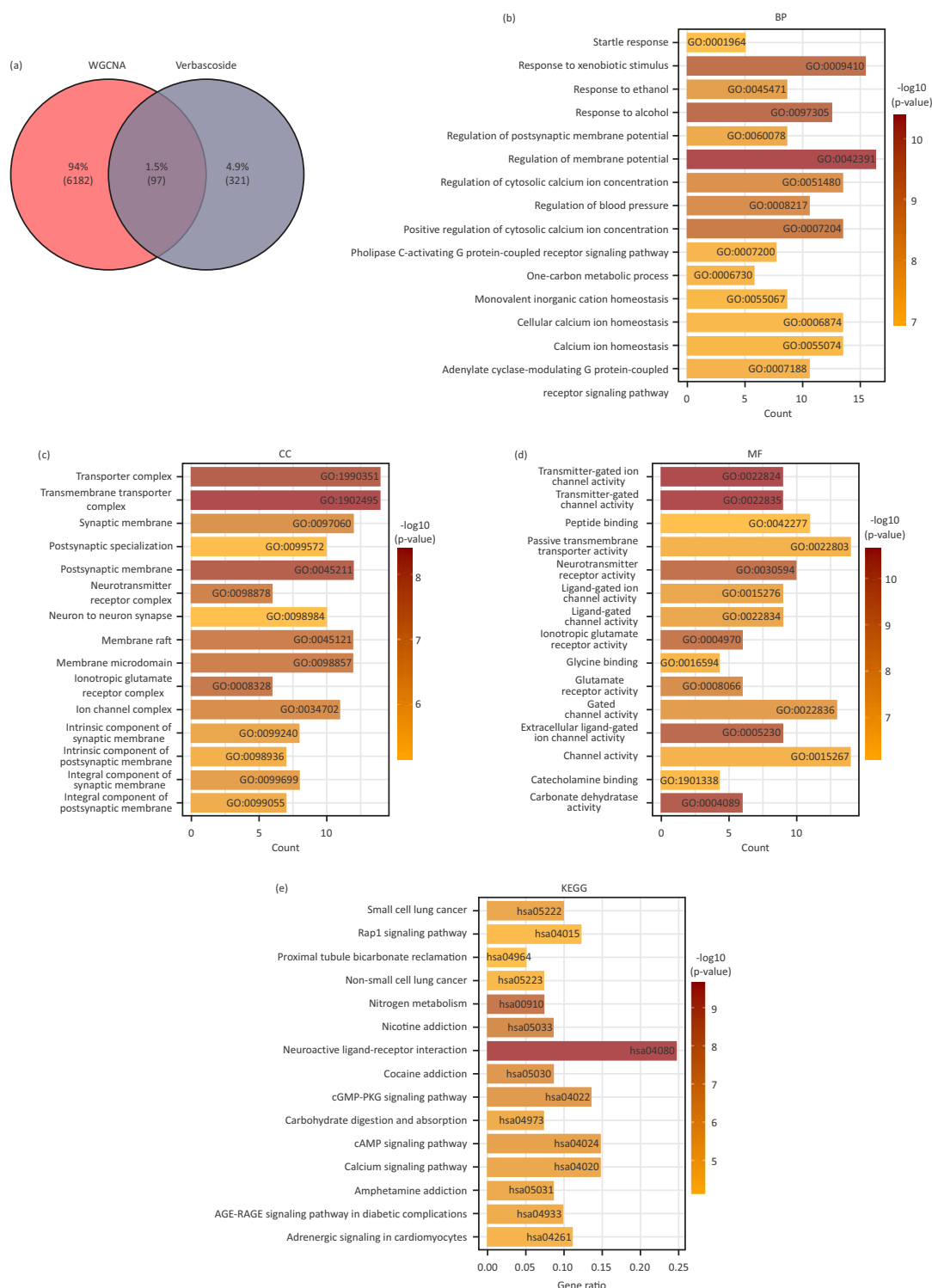


Fig. 2(a-e): Venn Diagram and enrichment analysis, (a) Intersection of VB target genes and key genes in WGCNA, (b) Histogram chart of the biological process from GO enrichment analysis, (c) Histogram chart of the cellular component from GO enrichment analysis, (d) Histogram chart of the molecular function from GO enrichment analysis and (e) Histogram chart from KEGG enrichment analysis

X-axis and Y-axis show the enrichment factor and pathways, respectively and the darker the color, the smaller the p-value

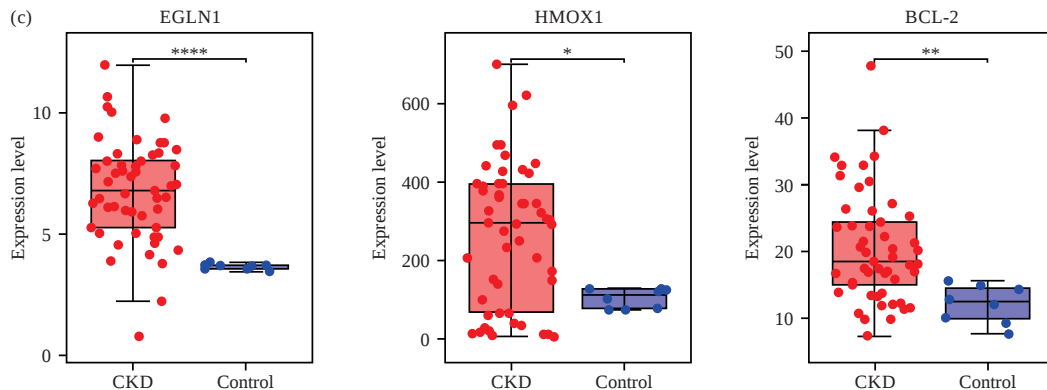
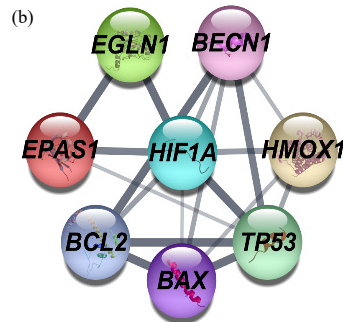
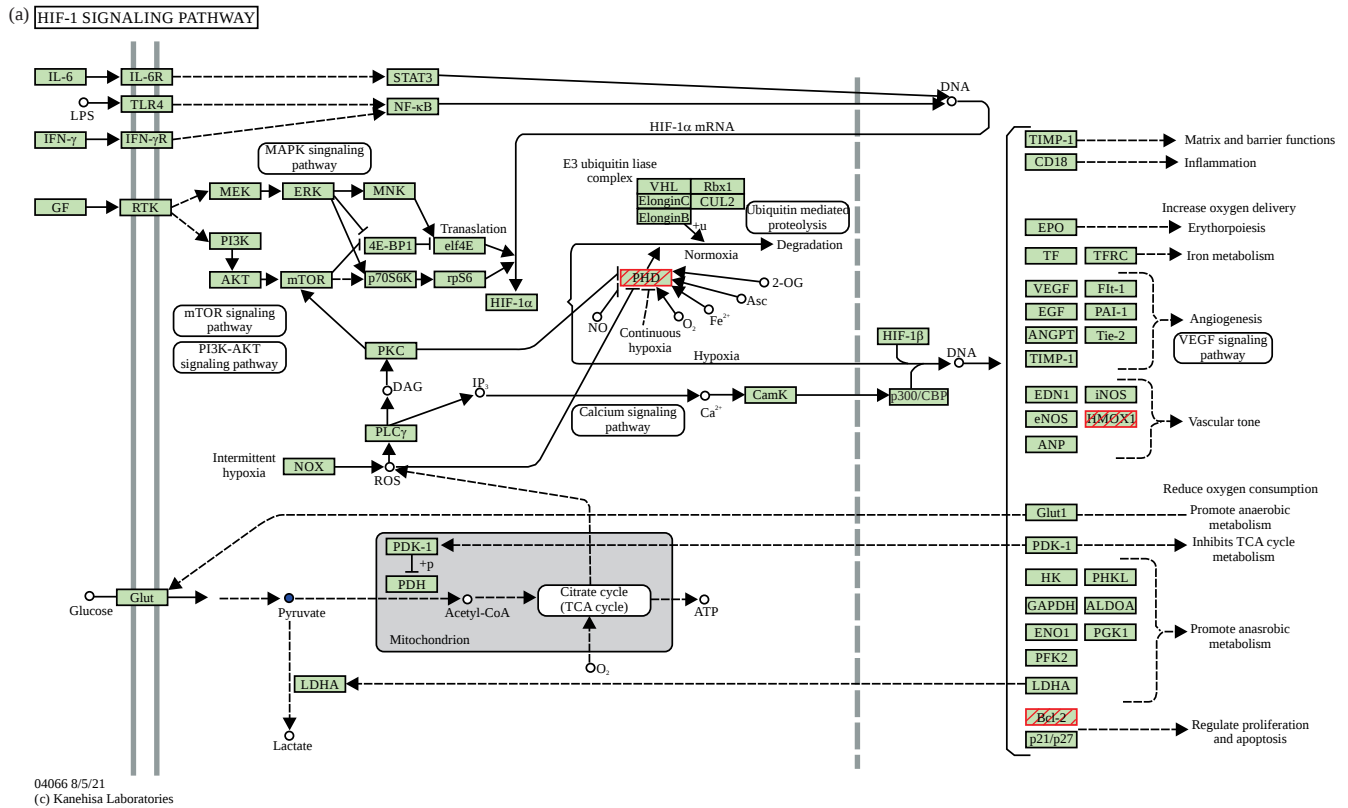


Fig.3(a-c): Mapping of CKD-associated metabolites and gene synergistic regulatory pathways, (a) Mechanism of the HIF-1 signaling pathway involving the synergistic participation of metabolites and genes associated with CKD, (b) Interaction network of EGLN1, HMOX1 and BCL-2 and (C) Boxplot of differential expression of EGLN1, HMOX1 and BCL-2 in GSE66494

(a) Metabolite Pyruvate is marked in blue dot and genes are marked in red shaded boxes: EGLN1, HMOX1, BCL-2, * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$

Table 1: Metabolites and genes related to chronic kidney disease

Name	KEGG_ID
3,5-Diiodo-L-tyrosine	C01060
3-Methylhistidine	C01152
Isethionate	C05123
Glucaric acid	C00818
Trimethylamine N-oxide	C01104
Cytidine	C00475
Gluconate	C00257
D-Glucuronic acid	C00191
Guanidinosuccinic acid	C03139
Pimelic acid	C02656
Citramalic acid	C00815
Trigonelline	C01004
Quinolinic acid	C03722
Choline	C00114
Pyruvate	C00022
Isocitrate	C00451
ATP1A1	476
PARP1	142
TDP1	55775
HMOX1	3162
PRKCD	5580
ATP1A4	480
AGTR1	185
BCL2	596
TGFBR2	7048
NR3C1	2908
ATP1A3	478
EGLN1	54583
REN	5972
S1PR1	1901
RXRB	6257
SCD	6319
CHD1	1105
CDK2	1017
ALKBH2	121642

terms of MF, enriched terms comprised peptide binding, passive transmembrane transporter activity, neurotransmitter receptor activity, gated channel activity and channel activity (Fig. 2d). Visualizing the relationship between the target and pathways, the KEGG pathway enrichment network diagram (Fig. 2e) indicated that VB might exert its influence on high-altitude renal disease treatment through these pathways. The KEGG functional enrichment analysis results indicated that VB potentially impacts high-altitude renal disease triggered by hypoxia mainly through pathways like neuroactive ligand-receptor interaction, CGMP-PKG signaling pathway, CAMP signaling pathway and calcium signaling pathway, among others.

Hub genes of VB against high-altitude renal disease:

Following, the CKD-related metabolites and genes synergistic regulatory pathways were also identified. A total of 16 metabolites were identified to be related to CKD by searching the literature and the HMDB database (Table 1). Expression levels of the 97 intersecting genes in the

Venn diagram were validated in the GSE66494 dataset. As shown in Table 1, 19 genes were found to be differentially expressed according to a t-test statistical analysis. The screened CKD-related metabolites and the 19 differentially expressed genes were subjected to a co-pathway enrichment analysis using KEGG Mapper. This analysis identified the involvement of EGLN1, HMOX1, BCL-2 and pyruvate in the HIF-1 signaling pathway (Fig. 3a). The STRING database was utilized to predict regulatory relationships among EGLN1, HMOX1 and BCL-2. It revealed that these three genes may indirectly regulate each other through HIF1A (Fig. 3b). Additionally, the expression levels of EGLN1, HMOX1 and BCL-2 were further validated in the GSE66494 dataset, where all three genes exhibited higher expression in the CKD group compared to the control group ($p < 0.05$, Fig. 3c). According to these results, EGLN1, HMOX1 and BCL-2 were identified as hub genes in VB for the treatment of high-altitude renal disease.

Prediction of mRNA-miRNA: Using TargetScanHuman, EGLN1, HMOX1 and BCL-2 were predicted to be potentially regulated by 56, 79 and 144 miRNAs, respectively. As shown in Fig. 4, a network was used to visualize the interactions between the three genes (EGLN1, HMOX1 and BCL-2) and their target miRNAs. Within the predicted miRNAs, two miRNAs, miR-409-3p and miR-495-3p, were found to potentially co-regulate EGLN1, HMOX1 and BCL-2. These miRNAs may play a role in the coordinated regulation of the three genes.

Molecular docking verification: Molecular docking was performed between EGLN1, HMOX1, BCL-2 and VB in Fig. 5(a-c). The docking results were evaluated based on the binding energy and the formation of hydrogen bonds between the receptor and ligand, using a screening criterion of binding energy less than -5.0 kcal/mol and the ability to form hydrogen bonds³⁰. The docking results meeting these criteria are summarized in Table 2. By molecular docking simulation, the binding energies of BCL-2, EGLN1 and HMOX1 with VB were -10.2, -6.7 and -6.3 kcal/mol, respectively, demonstrating good interactions. Additionally, hydrogen bonds were formed between the receptor and ligand, further supporting the stability of the complex.

VB downregulated EGLN1, HMOX1 and BCL-2 in hypoxia-induced HK-2 cells:

To validate the outcomes of network pharmacology and molecular docking analyses, hypoxia-induced HK-2 cells were treated with VB. Results showed that cell viability of HK-2 cells in the hypoxia group was reduced

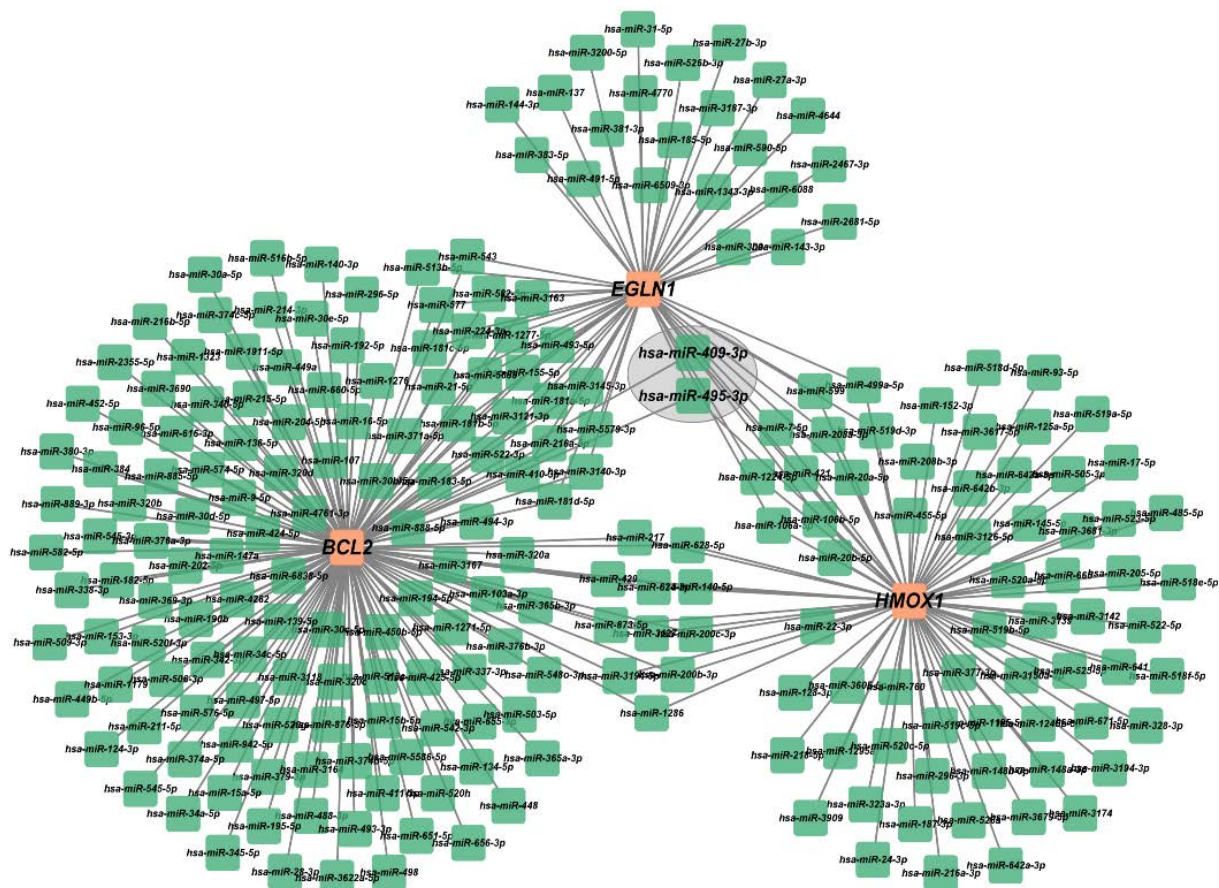


Fig. 4: Network diagram of mRNA-miRNA relationships

Orange rectangles represent genes and the green rectangles represent miRNAs. The miRNAs in the gray background are the common miRNAs that regulate EGLN1, HMOX1 and BCL-2

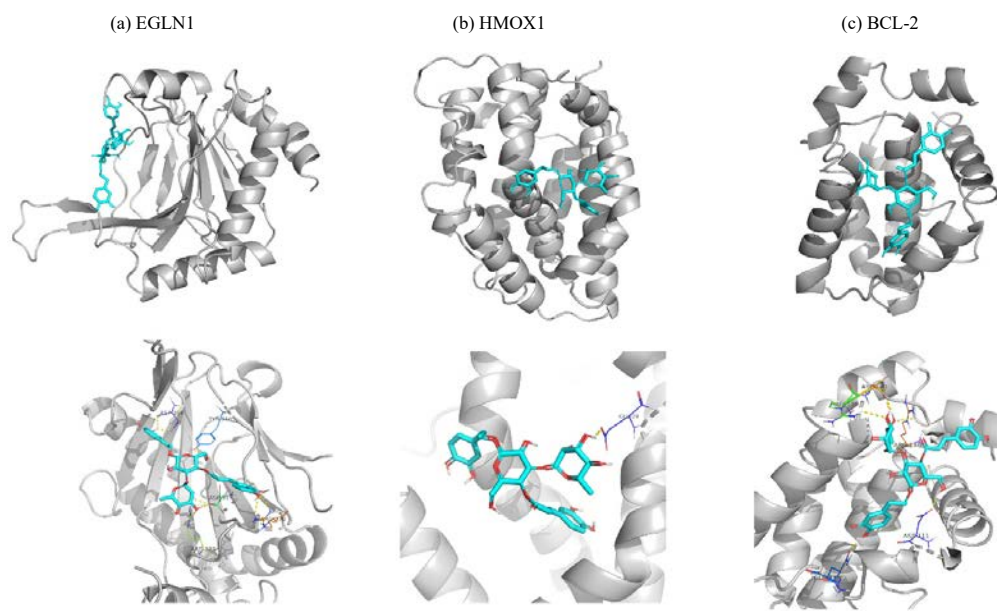


Fig.5(a-c): Molecular docking results (above) and specific docking sites (below) of VB with (a) EGLN1, (b) HMOX1 and (c) BCL-2

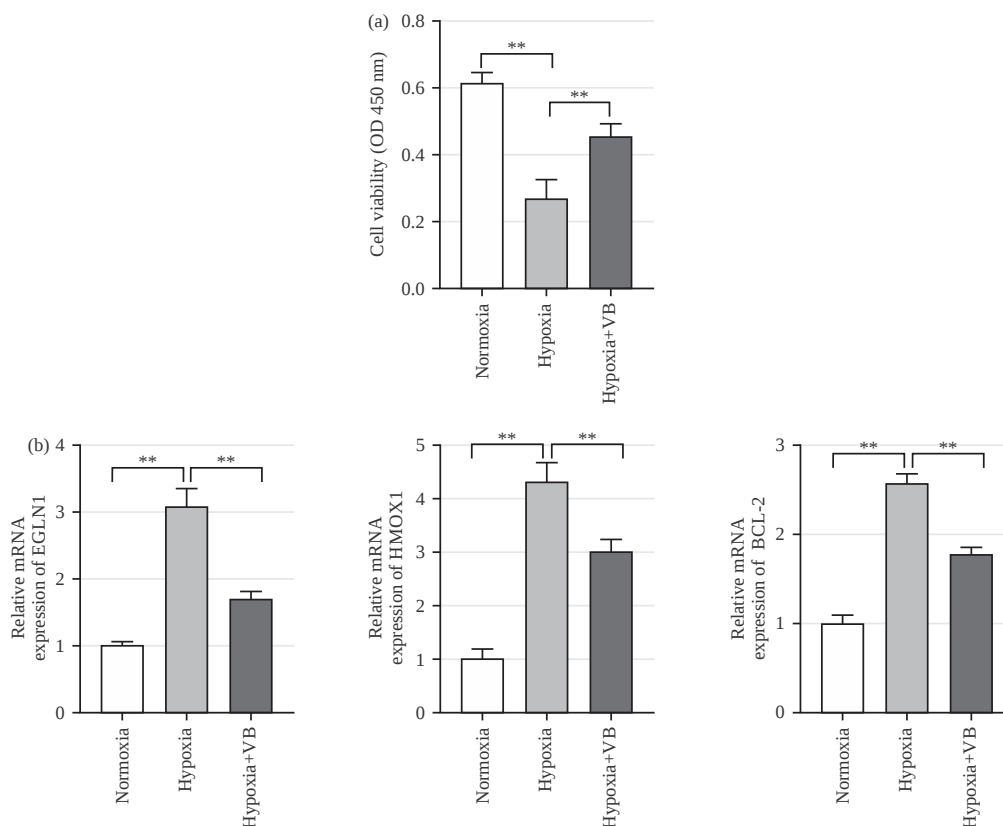


Fig. 6(a-b): VB downregulated EGLN1, HMOX1 and BCL-2 in hypoxia-induced HK-2 cells, (a) CCK-8 detected the cell viability of HK-2 cells and (b) qRT-PCR detected the relative mRNA expression of EGLN1, HMOX1 and BCL-2 in HK-2 cells

** $p < 0.01$ and human renal proximal tubular cell line HK-2 cells were pre-treated with 100 μM VB for 3 hrs and further cultured in the hypoxia atmosphere for 24 hrs to induce a high-altitude renal disease cell model

Table 2: Free binding energy of verbascoside and 3 target genes

Target	PDB	PubChem_ID	Compound	Free binding energy (kcal/mol)
BCL-2	6o0k	5281800	Verbascoside	-10.2
EGLN1	2g19			-6.7
HMOX1	1n45			-6.3

compared to that in the normoxia group ($p < 0.01$), but VB elevated cell viability in the hypoxia group ($p < 0.01$, Fig. 6a). In addition, expression levels of EGLN1, HMOX1 and BCL-2 were all upregulated in the hypoxia group compared to the normoxia group ($p < 0.01$, Fig. 6b). The VB significantly downregulated expression of these three genes in hypoxia-induced cells ($p < 0.01$, Fig. 6b). These results indicated that VB might treat high-altitude renal disease by downregulating EGLN1, HMOX1 and BCL-2.

DISCUSSION

In this study, 97 potential therapeutic targets were obtained after screening for common targets of CKD, hypoxia and VB. These genes were involved in the regulation of HIF-1

signaling pathway. The STRING was further utilized to identify the key targets of VB against high-altitude renal disease, including EGLN1, HMOX1 and BCL-2. Meanwhile, these key genes were targeted by multiple miRNAs, especially miR-409-3p and miR-495-3p. Molecular docking proved that the three genes had satisfactory binding abilities with VB. Subsequently, *in vitro* experiments were applied to confirm these findings. The results showed that hypoxia could significantly inhibited the viability of HK-2 cells and greatly promote the expression levels of EGLN1, HMOX1 and BCL-2. As expected, the addition of VB effectively reversed the inhibition of cell activity and the abnormal expression of key genes caused by hypoxia. These findings provide an important scientific basis for further research on the mechanism of VB in the treatment of high-altitude renal disease.

The ameliorative effect of VB on renal function has been demonstrated and it can attenuate renal fibrosis in rats with unilateral ureteral obstruction by improving macrophage infiltration⁵. Furthermore, VB may improve renal function by reducing serum creatinine, blood urea nitrogen, urinary protein and the expression of related proteins and it may improve renal inflammation and fibrosis induced by unilateral ureteral obstruction in rats by triggering the HMGN1/TLR4/TREM-1 pathway³¹. It has been confirmed that VB alleviated podocyte injury associated with diabetic kidney disease by modulating the NR4A1-LKB1-AMPK signaling pathway⁶. Based on this, the positive effect of VB on high-altitude renal disease can be speculated. Through the enrichment analysis of shared pathways among 16 metabolites associated with the progression of CKD and 97 key target genes involved in hypoxia regulation by VB, EGLN1, HMOX1 and BCL-2 were identified as targets genes for VB against high-altitude renal disease.

The EGLN1 locus is characterized by positive selection in Tibetan and Andean populations living at altitudes greater than 3500 m³². The EGLN1 acts as an oxygen sensor to regulate the cellular response to hypoxia by degrading HIF proteins in an oxygen-dependent manner³³. Zeng *et al.*³⁴ found that the knockdown of prolyl hydroxylase 2 (PHD2) encoded by EGLN1 in endothelial cells significantly inhibited angiotensin-induced renal fibrosis, reactive oxygen species formation and iron accumulation. The HMOX1 is responsible for converting the heme produced by the decomposition of hemoglobin into carbon monoxide, iron ions (Fe²⁺) and bilirubin³⁵. A study conducted by Eddie-Amadi *et al.*³⁶ revealed that banana peel exerts anti-inflammatory and anti-apoptotic effects by activating the Nrf2/Hmx-1 pathway and inhibiting the Nfκb pathway, thereby ameliorating metal mixture-induced liver and kidney damage. The BCL-2, as an anti-apoptotic protein, inhibits the occurrence of cell apoptosis³⁷. An existing study suggested that the regulation of BCL-2 could alleviate mitochondrial damage and apoptosis in cells experiencing acute renal injury³⁸. The VB has been found to play a chemoprotective role by modulating the processes of oxidative stress and apoptosis (involving BCL-2 gene), which exerts a therapeutic effect on hepatocellular carcinoma³⁹. The VB significantly reduced the mRNA expression levels of EGLN1, HMOX1 and BCL-2. These results revealed that VB might exert its therapeutic effects by downregulating EGLN1, HMOX1 and BCL-2.

Moreover, EGLN1, HMOX1 and BCL-2 were found to collectively regulate the HIF-1 signaling pathway and they may be indirectly regulated by HIF1A. A study by

Viswanatha *et al.*⁴⁰ has shown that VB attenuates ischemia-reperfusion brain injury in Wistar rats via modulation of HIF-1α, NF-κB and VEGF pathways. The HIF pathway is one of the most important signaling pathways under low-oxygen conditions⁴¹. In a low-oxygen environment, the stability of HIF proteins increases, leading to the activation of the HIF pathway. Activation of the hypoxia and HIF pathway alters the gene expression involved in renal and immune cell metabolism, thereby disrupting their functions⁴². Multiple lines of evidence suggest that HIF-1α serves as a profibrotic effector in renal diseases⁴². As an example, the stimulation of HIF-1α due to hypoxia within renal tubular epithelial cells triggers the promotion of epithelial-mesenchymal transition *in vitro*. This process is linked to tubulointerstitial fibrosis in mice with unilateral ureteral obstruction and individuals afflicted by CKD⁴³. Moreover, HIF activation could also play an important role in providing renal protection during acute kidney injury⁴¹. The EGLN1 encodes an enzyme that regulates the stability of HIF-1α⁴⁴, while HMOX1 encodes an enzyme that plays a role in cellular antioxidant defenses and protection against oxidative stress^{37,45}. In general, the HIF-1 signaling pathway played an important regulatory and protective role in the development of high-altitude renal disease.

In addition, by constructing the miRNA-mRNA relationship network, miR-409-3p and miR-495-3p might play a role in the coordinated regulation of three target genes (EGLN1, HMOX1 and BCL-2), which could be used as potential drug targets. In the study of Wang *et al.*⁴⁶, phosphatidylinositol-dependent kinase 1 in clear cell renal cell carcinoma was regulated by miR-409-3p under hypoxic conditions to promote its cellular glycolysis. The miR-495-3p has been investigated as a signal for the development of clear cell renal cell carcinoma⁴⁷ and possibly a novel biomarker for sepsis-associated acute kidney injury⁴⁸. This could contribute to the development of new therapeutic strategies, including miRNA targeting or drug development.

It is worth noting that current study has certain limitations. First, molecular docking simulations provide insights into the potential binding interactions. Still, *in vivo*, studies are needed to confirm the physiological relevance of these interactions and the overall therapeutic effects of VB in high-altitude renal disease. Additionally, the specific mechanism of the HIF-1 signaling pathway in the treatment of high-altitude renal disease by VB still requires further studies. Although miR-409-3p and miR-495-3p show promising potential as drug targets for high-altitude renal disease, there remains a gap in their study, warranting further investigation.

CONCLUSION

In summary, this study highlights the potential of VB as a therapeutic agent for high-altitude renal disease. Through network pharmacology and molecular docking techniques, the interaction between VB and target genes (EGLN1, HMOX1 and BCL-2) was revealed. The EGLN1, HMOX1 and BCL-2 were found to collectively regulate the HIF-1 signaling pathway and they may be indirectly regulated by HIF1A. Moreover, VB might alleviate high-altitude renal disease by downregulating the mRNA expression of specific genes (EGLN1, HMOX1 and BCL-2). These findings contribute to the development of targeted therapies for high-altitude renal disease.

SIGNIFICANCE STATEMENT

In this study, network pharmacology and molecular docking techniques were employed to explore the potential mechanism of verbascoside (VB) against high-altitude renal disease and the key therapeutic targets were identified as EGLN1, HMOX1 and BCL-2. Moreover, the therapeutic effect of VB on high-altitude renal disease may derive from its modulation of HIF-1 signaling pathway and involve the precise regulation of several upstream miRNAs, such as miR-409-3p and miR-495-3p. This study confirmed the therapeutic potential of VB for high-altitude renal disease and provided scientific basis for its clinical application. Based on the above findings, further revelation of the active compounds and exact mechanism of VB in the treatment of high-altitude renal disease is essential.

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