Identification the Hub Genes of Hirudin Intervention of Diabetic Nephropathy Based On Bioinformatic Analysis and Molecular Docking

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Research Article

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Abstract

Background: Diabetic nephropathy (DN) is one of the common complications of diabetes, it can cause a disproportionate burden. Leeches are widely used to treat DN in China, and hirudin is the main component of leeches. However, its pharmacological mechanisms and molecular targets are unclear. This work aimed to explore new biomarkers of DN and reveal the mechanism of hirudin in DN.

Methods: Expression microarray datas between kidney tissues of DN and control individuals were obtained from the GEO database, and differentially expressed genes were identified as DN-related targets using the robust rank aggregation analysis. The SEA, GeneCards and SwissTargetPrediction databases were used to predict targets of hirudin. A protein-protein interaction network of DN-hirudin was conducted by Cytoscape software. GO and KEGG pathway enrichment analyses were carried out to explore the involved pharmacological mechanism of hirudin in treatment of DN. And molecular docking was adopted to predict the hub targets of hirudin.

Results: A total of 30 significant DEGs (16 up- and 14 down-regulated) were identified. ATF3, SLC22A8 and TGF-B1 are likely as new biomarkers of DN. The 42 candidate targets were identified in PPI network. GO analysis revealed that these targets were enriched with ubiquitin protein ligase, transcription factor binding and nuclear transport. The KEGG pathways were enriched, including AGE-RAGE signaling pathway in diabetic complications, PI3K-Akt signaling pathway, Focal adhesion. The docking results showed that hirudin could easily dock with ITGA4, EGFR and ESR1. **Conclusion:** Our study demonstrates that hirudin is involved in DN therapy through a multi-targeted, multi-pathway approach. It provides a basis for further research on its mechanism of action.

Introduction

Diabetic nephropathy (DN) is one of the most common and serious chronic complications of diabetes, its incidence has nearly doubled in the past decade, and accounts for 30%-40% of all diabetic patients [1]. A study revealed a increasing trend of chronic kidney disease (CKD) in China from 2010 to 2015 [2], the proportion of CKD hospitalized population associated with diabetes is 1.10%, which has surpassed glomerulonephritis (0.75%) as the leading cause of end-stage renal disease (ESRD) in China.

The most significant clinical manifestations of DN are varying degrees of proteinuria and progressive loss of renal function. The pathogenesis of DN is the disorder of glucose metabolism, the changes of renal hemodynamics, oxidative stress, and inflammatory reaction activation. The early pathological changes are glomerular hypertrophy, basement membrane thickening; after the progression of DN, it is characterized by the accumulation of extracellular matrix(ECM), glomerular sclerosis and tubulointerstitial fibrosis, which eventually lead to renal fibrosis and renal failure [3,4]. However, there is still a lack of effective therapeutic treatment for DN. It is urgent to discover the treatment options to help in better patient prognosis.

Hirudin is the most potent natural anticoagulant substance derived from the salivary glands of medicinal leeches by Haycraft in 1884, and later named by Markwardt [5]. Hirudin, a 65 amino acid polypeptide with a molecular weight of approximately 7000 Da, has multiple pharmacological effects including improving circulation, relieving inflammation reaction and inhibiting the production of fibrosis. Many studies have revealed that hirudin could protect the kidneys against inflammation [6], inhibit the clot formation [7,8], alleviation organ damage and decrease fibrosis [8,9]. It has been reported that hirudin is used in hemodialysis as an anticoagulant, and activated partial thromboplastin times were lower compared to heparin [10]. Hirudin renoprotective effect on Renal Interstitial Fibrosis (RIF) by inhibiting of TGF- β 1/Smad and NF- κ B signaling and decreasing the apoptosis of renal tubular epithelial cells were verified [8,9]. And hirudin can inhibit thrombin-induced autophagy to reduce brain damage [11,12].

With the development of the high-throughput sequencing technique and bio-informatics, much progress has been made in screening potential biomarkers and therapeutic targets. However, there are some problems in the common gene expression analysis, such as sample sizes, different microarray platforms and the high rate of false-positive results. As a remedy, the robust rank aggregation (RRA) method has been used to identify differentially expressed genes (DEGs) based on multiple datasets in various diseases [13], which could arrange each gene in each dataset randomly. To the best of our knowledge, previous studies have not used the RRA method to identify DEGs of DN, which facilitated us to carry out a new study. Moreover, network-target and multi-component therapies are increasingly recommended in pharmaceutical research [14]. At the molecular level, network-target and multi-component therapies have been increasingly recommended in research. From the molecular level, multiple-target and multi-pathway ingredients are effective therapeutics for diseases with complex pathology and physiology. Network pharmacology, as a novel method, breaks through the traditional concept of one drug, one target, one disease. By using computer simulations and databases, it can display networks of compound-target, and target-disease to explore mechanisms of medicines [15].

The purpose of this study was to analysis differential expressed genes of DN using the RRA approach. In addition, a network pharmacology was constructed to explore the potential mechanism of hirudin on DN, and drug-target-biological function/pathway interactions analyzed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway [16,17].

Results

Identification of DEGs in DN

Based on the inclusion and exclusion criteria, three GEO databases were included, series:GSE30528 [18], GSE47183 [19] and GSE99339 [20]. Both GSE47183 and GSE99339 have two datasets for different platforms, GSE47183 was divided into GSE47183_GPL11670 and GSE47183_GPL14663. GSE99339 only selected the dataset of GPL19184 according to the inclusion and exclusion criteria. The information of these datasets was shown in Table 1. All four datasets were normalized and standardized, and the

DEGs were identified by limma package in R software. The DEGs were represented by red and blue dots, which were shown in the four volcano plots (Fig 1).

Table 1

Characteristics of the included microarray datasets

GSE ID	Platform	Samples	Tissues	Country	Year
GSE30528	GPL571	9D and 13C	Glomeruli	USA	2011
GSE47183	GPL11670	7D and 3C	Glomeruli	USA	2013
GSE47183	GPL14663	7D and 13C	Glomeruli	USA	2013
GSE99339	GPL19184	7D and 8C	Glomeruli	USA	2017
GSE: Gene Expression Omnibus Series; GPL: Gene Expression Omnibus Platform;D: Diabetic nephropathy; C: Control.					

Then, the gene lists in four datasets were integrated using the RRA analysis according to the threshold of P value < 0.05. The smaller the P value, the higher the credibility of differential gene expressions. A total of 30 significant DEGs (16 up- and 14 down-regulated) were identified (Additional file 1). And the top 30 DEGs were shown in a heatmap (Fig 2). ATF3, SLC22A8 and TGF-B1 were the top 3 exceptionally expressed genes, they may have a critical role in the DN progression.

Identification of candidate targets for hirudin

Based on the structure prediction of hirudin from the SEA-SwissTargetPrediction and GeneCards databases, we got 522 targets totally. A Venn diagram summarized 12 common targets after excluding the duplicate targets (Fig.S1). The data of the three databases of all genes were shown in Additional file 2. And the shared targets ACE, ANPEP, CTSD, F2, ITGA4, ITGB1, ITGB3, ITGB7, MMP10, MMP9, REN, and STAT3 may be identified as potential targets for hirudin.

PPI Networks analysis

The PPI networks of hirudin putative targets and DN-related targets were visualized with Cytoscape software, respectively. We predicted two PPI networks contained 886 nodes and 1028 nodes, and merged the two results into a new PPI. Then, we analyzed the topological properties of the PPI networks to identify the targets for hirudin against DN. The PPI network contained 195 nodes and 2522 edges(Fig.3A), and the median degree of all nodes was 24. According to the median degree, we further extracted a network of 99 nodes and 1638 edges(Fig.3B). Finally, the 42 candidate targets were identified with degree=38, betweenness=55.16, closeness=0.587 (Fig.3C).

GO and KEGG enrichment analyses

We imported the 42 candidate genes to perform GO and KEGG analyses. The data of GO analysis and KEGG analysis were shown in Supplementary information. The top 10 significant terms of GO (including biological process, molecular function and cellular component) and 10 KEGG pathways were analyzed.

The GO terms of significant biological process (BP) revealed that protein localization to nucleus, cellular response to heat, regulation of protein localization to nucleus, nucleocytoplasmic transport, nuclear transport, and positive regulation of establishment of protein localization were enriched (Fig.4A).

The top 10 remarkably enriched in molecular function (MF) terms were ubiquitin-like protein ligase binding, DNA-binding transcription factor binding, RNA polymerase II-specific DNA-binding transcription factor binding, histone deacetylase binding, phosphoprotein binding, steroid hormone receptor binding, cadherin binding, nuclear hormone receptor binding and nuclear receptor binding (Fig.4B).

In the GO analysis of cellular component (CC), focal adhesion, cell-substrate junction, vesicle lumen, secretory granule lumen, cytoplasmic vesicle lumen, complex of collagen trimers, melanosome, pigment granule, transcription regulator complex, and chromosomal region were mainly involved (Fig.4C).

Furthermore, KEGG pathway enrichment analysis including AGE-RAGE signaling pathway in diabetic complications, PI3K-Akt signaling pathway, Focal adhesion, Viral carcinogenesis, HIF-1 signaling pathway, Relaxin signaling pathway, MAPK signaling pathway, Kaposi sarcoma-associated herpesvirus infection, and were significantly identified (Fig.5). The chord diagram was visualized based on the significantly enriched pathways and related genes (Fig.S2).

Molecular docking of hub genes

The hub genes in PPI network were identified using a Cytoscape plugin, CytoHubba.

The PPI suggested that ITGA4 was the core target gene. Other several genes, such as NTRK1, FN1, ESR1, STAT3, EGFR and VCAM1 (Fig.6). They might be the key target genes for hirudin against DN. The 3D structures of the above hub genes were searched from RCSB PDB database and molecular simulations were performed using AutoDock Tools to dock with hirudin. The docking results showed that hirudin could easily dock and bind the active pockets of ITGA4, EGFR, ESR1, evaluated binding energies -6.94, -4.32, -2.48.kcal/mol (Fig.7).

Discussion

DN is kidney damage caused by persistent hyperglycemia, and present with massive proteinuria and progressive deterioration of kidney function [21]. As a progressive vascular complications of diabetes, DN is strongly associated with cardiovascular and cerebrovascular disease, as well as increased mortality of diabetes patients [1]. Thus, it is especially critical to identify the pathogenesis of DN and find novel

therapeutic targets. To my knowledge, no study applying the RRA method to determine differential genes in DN was conducted. In this study, we included four microarray studies, analyzed DEGs of DN and normal groups, and the integration results of co-differential genes were obtained by RRA analysis. In total, 30 DEGs were obtained from four datasets comprising 14 down-regulated and 16 up-regulated genes. The 10 most important genes are ATF3, SLC22A8, TGF β 1, SRPX2, ZNF415, NLGN4Y, PBK, C1QA, AFP and CYP27B1, which may be a novel biomarker for the prediction and diagnosis of DN. Transforming growth factor- β 1 (TGF β 1), demonstrated as a central pathogenic mediator, has been identified to play a detrimental role in the development of progressive renal fibrosis [22,23]. Our study identified the hub genes in DN, transcriptional analysis has recently become a promising tool to investigate the specific molecular mechanisms for disease progression.

In this study, a hirudin-targets-DN network was constructed to elucidate the potential mechanisms on DN treatment with hirudin. PPI network were merged to obtain the candidate targets, and parameters (Degree, Betweenness and Closeness) were set for screening more accurate targets. The topology analysis of the network showed that 42 targets were identified and conducted the functional enrichment analysis. Then, GO enrichment terms included classification of 3782 BP terms, 405 CC terms and 428 MF terms. The results revealed that hirudin regulated some biological processes, such as protein localization to nucleus, nuclear transport and regulation of protein localization to nucleus; the enriched CC terms were cell-substrate junction, vesicle lumen and transcription regulator complex; by applying MF terms analysis, most of the differential genes were enriched in ubiquitin protein ligase, transcription factor binding, histone deacetylase binding and steroid hormone receptor binding. These results are similar to several published studies, the development of DN is closely associated with transcription factors and protein modifications [23-25]. Therefore, hirudin may help to regulate these enrichment function in the treatment of DN.

Numerous studies have shown that multiple signaling pathways play a key role in the pathogenesis of DN. In our study, KEGG results suggested that these DEGs were mainly enriched in the AGE-RAGE signaling pathway in diabetic complications, PI3K-Akt signaling pathway, Focal adhesion, Viral carcinogenesis and HIF-1 signaling pathway. AGE-RAGE signaling pathway plays a central role in diabetic complications, AGE receptors (RAGE) accumulation can cause oxidative stress and chronic inflammation, and activate different intracellular signaling pathways such as PI3K/AKT, JAK/STAT and NF- κ B [26-27]. And some studies reported that AGE could inhibit autophagy, and the reduced AGE can promote autophagy to be involved in the treatment of DN [28,29].

Previous evidence suggested that PI3K-AKT signaling pathway is involved in regulating cellular metabolism, cell proliferation and differentiation, and cell autophagy. High glucose induced can accelerate tubular cell apoptosis and renal fibrosis by increasing AKT expression and transdifferentiation of tubular epithelial cells [26-27].

And further studies confirmed that inhibition of the PI3K/AKT pathway can improve lysosome function and autophagy activity in podocytes and delays DN [30-32]. Our study revealed that hirudin might

regulate PI3K/AKT pathway in the treatment of DN.

It has been found that Focal adhesion is closely correlated with cell proliferation and differentiation, as well as maintenance of cell morphology. The ECM-integrin-cytoskeleton axis is involved in the pathological changes of many diseases.

Integrin is a key molecule which mediates the connection between the cell and extracellular environment. Recently, the relationship between renal damage and Integrin signalling has attracted more and more attentions from the researchers. Previous studies have shown that DN is correlated with podocytes detachment from the glomerular basement membrane (GBM), and Integrin family members are the major podocyte adhesion molecules. Integrin Alpha 4 (ITGA4), Integrin Beta 3 (ITGB3) Integrin Beta 4 (ITGB4) have been found to be differentially expressed in the glomeruli and tubules of DN. Previous findings confirmed that proteinuria and renal damage can accompanied by a decrease in Integrin proteins [33].

Pedchenko reported that interactions of integrin mediated cell-ECM might contribute to the development of diabetic nephropathy [34]. EGFR is reported to act with integrins in regulating cell-ECM interactions [35]. It is well known that EGFR is widely expressed in the renal disease, and EGFR inhibition or knockdown can reduces podocyte injury. ECM is a driver factor of fibrosis in DN, ECM deposition can lead to tubulointerstitial fibrosis. Some studies confirmed that Hirudin has anticoagulant and anti-inflammatory effects and antagonize the deposition of ECM [8-11]. In order to predict the hub targets of hirudin in the treatment of DN, top 10 hub genes for DN treatment with hirudin were screened by CytoHubba plugin. As we can see from molecular docking analysis, ITGA4, EGFR and ESR1 revealed good binding forces with hirudin. The findings reflect the multi-target and multi-pathway actions of hirudin against DN.

Therefore, these results also confirm that hirudin can play a therapeutic role in DN by regulating these targets and enrichment pathways. However, There are still some problems in our study. We just predicted the potential functional mechanism of hirudin on DN, future work should focus on further molecular mechanism researches and additional experimental validations.

Conclusions

Overall, hirudin has obvious advantages in the treatment of DN, and we found the mechanism and possible targets of hirudin by network pharmacological analysis and molecular docking simulation. ITGA4, EGFR and ESR1 were promising to be the hub genes of hirudin for treatment of DN. However, further experimental validation is needed in the future.

Materials And Methods

Microarray datasets of DN

We used the keywords “DN”, “Diabetic nephropathy”, “DKD” and “Diabetic kidney disease” to retrieve the gene expression datasets through The Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/>) [36]. The inclusion and exclusion criteria were as follows: (1) the gene expression profile was from micro-dissected glomeruli; (2) datasets was the transcriptome expression data; (3) datasets contained at least 10 samples. The flow diagram of this study was shown in Figure 8.

Differentially expressed genes analysis

First, the gene expression matrixes and related annotation documents were downloaded from GEO, and normalization and log2 transformation were performed. Then, annotation documents were mapped the microarray probes to gene symbols individually. If a single gene mapped to multiple probes, the data was merged and calculated the average value. And the “limma” package (the linear models for microarray data) in R platform (v 4.0.2) was utilized to determine the DEGs. The DEGs with P value < 0.05 and $|\log_2 \text{fold change(FC)}| > 0.5$ were considered as potential hub genes, and the integrated up-regulated and down-regulated genes were saved for subsequent analysis.

RRA analysis

The RRA method could integrate the DEGs from several microarrays [13]. The lists of up-regulated and down-regulated genes were screened using RRA package with P value < 0.05 and $|\log_2 \text{FC}| > 0.5$. The top 30 of DEGs were mapped on a heatmap, which were regarded as DN-related targets.

Active compound screening

Based on Pubchem database (<https://pubchem.ncbi.nlm.nih.gov/>), the structure of hirudin in SDF format was downloaded (Fig S3). To predict the most putative protein targets of active ingredients, the SEA (Similarity Ensemble Approach, <http://sea.bkslab.org/>), SwissTargetPrediction (<http://www.swisstargetprediction.ch/index.php>) and Gene Cards (<https://www.genecards.org/>) were used to forecast the compound-protein interactions based on the structure of hirudin, individually [37,38]. Eventually, the candidate targets of the three databases were obtained as the actionable targets of hirudin after the duplications were removed.

Construction of compound-disease target network

The Protein-protein interaction (PPI) network of hirudin targets and DN-related targets was merged with Cytoscape (version 3.7.2, <https://www.cytoscape.org/>) software. The nodes represent the targets, while the edges indicate the compound-protein interactions. The nodes with topological interaction in the PPI network were screened by calculating Degree Centrality (DC), Betweenness Centrality (BC), Closeness Centrality (CC). And the Parameters of the node greater than the median of all nodes was selected as a

hub gene. These parameters represent the topological importance, and these definitions and computational formulas have been applied in network pharmacology and systems pharmacology [39].

GO and KEGG pathway enrichment analyses

The GO includes biological process (BP), molecular function (MF) and cell component (CC). To predict the potential function of gene targets, the GO and KEGG pathway enrichment analysis were carried out by the clusterProfiler package in R, and the results were visualized using the ggplot2 package in R [40,41]. The gene-pathway network was constructed to screen the hub targets that hirudin treated DN [42].

Hub genes and molecular docking

CytoHubba is a plugin for exploring important nodes in biological networks based on network topology analysis, which provides 12 methods of topology analysis based on shortest paths. Cytohubba is used to identify hub genes. Molecular docking is a tool to predict the binding mode and binding affinities of a ligand with a protein of known 3D structure. PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) was used to download the structures of hirudin, and the molecule structure was saved to mol2 format files. The 3D structure of the target proteins (hub genes from cytoHubba) were downloaded from the RCSB PDB database (<https://www.rcsb.org/>). The molecular docking simulation was conducted with AutoDock Tools (version 1.5.6, <http://mgltools.scripps.edu/>). The docking operation process followed the software instruction. The target model was prepared by removing ligands and water molecules. Polar hydrogen atoms and charges were added to the target using Autodock tool (ADT), and it was saved as a PDBQT file. To visualize the 3D structure of the docking result, PyMOL software (version 2.3, <https://pymol.org/2/>) was used [43].

Abbreviations

ACE: Angiotensin Converting Enzyme; AFP: Alpha Fetoprotein; ANPEP: Alanyl Aminopeptidase; ATF3: Activating transcription factor 3; CKD: Chronic kidney disease; CTSD: Cathepsin D; CYP27B1: Cytochrome P450 Family 27 Subfamily B Member 1; C1QA: Complement C1q A Chain; DEG: Different expression genes; DKD: Diabetic kidney disease; DN: Diabetic nephropathy; ECM: Extracellular matrix; EGFR: Epidermal growth factor receptor; ESRD: End-stage renal disease; ESR1: Estrogen Receptor 1; FN1: Fibronectin 1; F2: Coagulation Factor II; GEO: Gene Expression Omnibus; ITGA4: Integrin Alpha 4; ITGB1: Integrin beta 1; ITGB3: Integrin beta 3; ITGB7: Integrin beta 7; MMP9: Matrix Metalloproteinase 9; MMP10: Matrix Metalloproteinase 10; NLGN4Y: Neuroligin 4 Y; NTRK1: Neurotrophic Receptor Tyrosine Kinase 1; PBK: PDZ Binding Kinase; PPI: Protein-protein interaction; REN: Renin; RIF: Renal Interstitial Fibrosis; RRA: Robust rank aggregation; SEA: Similarity Ensemble Approach; SLC22A8: Solute carrier family 22 member 8; SRPX2: Sushi Repeat Containing Protein X-Linked 2; STAT3: Signal Transducer And Activator Of Transcription 3; TCM: Traditional Chinese Medicine; TGF- β 1: Transforming Growth Factor Beta-1; VCAM1: Vascular Cell Adhesion Molecule 1; ZNF415: Zinc Finger Protein 415.

Declarations

Acknowledgments

All the authors have read and approved the manuscript in all respects for publication.

Authors' Contributions

HT Y and C J designed the project idea; W L and HX Y performed the study; W L and HX Y analyzed the data and wrote the manuscript; JQ L and XY L assisted in the preparation of the manuscript; Ke Zhong, GR F helped with data collection; . All authors agree to be accountable for all aspects of work ensuring integrity and accuracy. All the authors read and approved the final manuscript.

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Availability of data and materials

This study was a network pharmacology combined bioinformatics analysis based on data from an open database, which involved no ethic problem. And the raw data of this study are derived from Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>), Pubchem database (<https://pubchem.ncbi.nlm.nih.gov/>), SwissTargetPrediction (<http://www.Swisstargetprediction.ch/index.php>), the SEA (<http://sea.bkslab.org/>), and Gene Cards (<https://www.genecards.org/>), which are publicly available databases.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Figures

Figure 1

The volcano plots of differentially expressed genes. Red points indicate up-regulated genes, blue points indicate down-regulated genes, and gray points indicate genes with no significant difference. (A) GSE30528. (B) GSE47183 based on GPL11670 platform. (C) GSE47183 based on GPL14663 platform. (D) GSE99339 based on GPL19184 platform.

Figure 2

Heatmap of the top 30 differentially expressed genes by robust rank aggregation analysis. Red represents high expression of genes in DN patients, while blue represents down expression of genes.

Figure 3

Identification of key subnetwork. (A) The first PPI network of hirudin against DN, and the red genes were identified with degree higher than median (degree > 24). (B) The second PPI network from A, and yellow genes were screened with some scores higher than median (degree > 38, Betweenness > 55.16, Closeness > 0.587). (C) The key subnetwork of hirudin against DN after two filtrations.

Figure 4

GO and KEGG enrichment analysis. The color of the nodes is shown in a gradient from red to blue according to the descending order of the P value. The size of nodes is arranged according to the ascending order of the number of gene counts.(A) The enriched biological process (BP) terms. (B) The enriched molecular function (MF) terms. (C) The enriched cellular component (CC) terms.

Figure 5

KEGG enrichment analysis of target genes of the top 10 pathways. The color of the item is shown in a gradient from red to blue according to the descending order of the P value. The length of item is arranged according to the ascending order of the number of gene counts.

Figure 6

The top 10 hub genes of hirudin against DN using Cytoscape, cytohubba plugin. The key level of nodes is arranged according to the color from red to yellow.

Figure 7

Molecular docking of the key genes in the subnetwork. (A) Molecular docking between hirudin and protein ITGA4. (B) Molecular docking between hirudin and protein EFGR. (C) Molecular docking between hirudin and protein ESR1.

Figure 8

Flow chart of the whole study.

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