Integrated bioinformatics analysis reveals novel key biomarkers and potential candidate small molecule drugs in diabetic nephropathy

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Research Article

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Abstract

The underlying molecular mechanisms of diabetic nephropathy (DN) have yet not been investigated clearly. In this investigation, we aimed to identify key genes involved in the pathogenesis and prognosis of DN. We selected expression profiling by high throughput sequencing dataset GSE142025 from Gene Expression Omnibus (GEO) database. The differentially expressed genes (DEGs) between DN and normal control samples were analyzed with limma package. Gene ontology (GO) and REACTOME enrichment analysis were performed using ToppGene. Then we established the protein-protein interaction (PPI) network, miRNA-DEG regulatory network and TF-DEG regulatory network. The diagnostic values of hub genes were performed through receiver operating characteristic (ROC) curve analysis. Finally, the candidate small molecules as potential drugs to treat DM were predicted using molecular docking studies. Through expression profiling by high throughput sequencing dataset, a total of 549 DEGs were detected including 275 up regulated and 274 down regulated genes. Biological process analysis of functional enrichment showed these DEGs were mainly enriched in cell activation, response to hormone, cell surface, integral component of plasma membrane, signaling receptor binding, lipid binding, immunoregulatory interactions between a lymphoid and a non-lymphoid cell and biological oxidations. DEGs with high degree of connectivity (MDFI, LCK, BTK, IRF4, PRKCB, EGR1, JUN, FOS, ALB and NR4A1) were selected as hub genes from protein-protein interaction (PPI) network, miRNA-DEG regulatory network and TF-DEG regulatory network. The ROC curve analysis confirmed that hub genes were high diagnostic values. Finally, the significant small molecules were obtained based on molecular docking studies. Our results indicated that MDFI, LCK, BTK, IRF4, PRKCB, EGR1, JUN, FOS, ALB and NR4A1 could be the potential novel biomarkers for GC diagnosis prognosis and the promising therapeutic targets. The present study may be crucial to understanding the molecular mechanism of DN initiation and progression.

Introduction

Diabetic nephropathy (DN) is a common and devastating microvascular complication of the kidneys induced by diabetes mellitus [1]. The incidence of DN is reported to be 30% to 40% patients with diabetes [2] and is the main cause of end-stage renal disease throughout the world in both developed and developing countries [3]. Numerous risk factors may affect DN progression [4]; however, how these factors affect the development of DN requires further study and no effective method has been developed. Despite important developments toward an understanding of the pathophysiology of DN, early diagnosis, therapeutic interference, and underlying molecular pathogenesis hover a require [5]. Therefore, enlighten the rare nature belonging to DN is predominant in expand therapies to improve patient outcome.

The exact mechanisms of DN are still unknown. A number of investigation have reported possible roles of some genes and pathways such as UCP1-3 [6] and JAK/STAT3 signaling pathway [7] in the development of DN. However, these reports only concentrated on any certain molecule, gene or pathway, ignoring that the development process involves aberrant expression of a variety of genes and pathways,
among which some proteins might interact with other proteins and thus play a essential role in the DN [8]. Hub genes may act as prognostic or diagnostic biomarkers or treatment targets for DN [9]. Therefore, it is urgent to search new biomarkers for DN with a powerful genome-wide technology.

The high-throughput platforms for analysis of gene expression, such as high throughput RNA sequencing, are increasingly valued as promising tools in medical field with great clinical applications: molecular diagnosis, prognosis prediction and new drug targets discovery [10]. The Gene Expression Omnibus (GEO) is a database and online resource for the gene expression of any species. In the current investigation, expression profiling by high throughput sequencing dataset (GSE142025) was downloaded. In total, there are 28 DN samples and 8 normal control samples datasets available. A data processing standard was used to filter the DEGs on the limma package of R language, followed by Gene Ontology (GO) and pathway enrichment analyses using ToppGene software. The DEGs protein-protein interaction (PPI) network and modular analysis were integrated using InnateDB interactome software to identify hub genes in DN. The regulatory network of miRNA and TF was constructed and the target genes with high degree of connectivity were selected. Receiver operating characteristic (ROC) curve analysis was used to predicting power of the gene signature. Molecular docking experiment was implemented for selected hub genes. This study will enhance our understanding of the molecular mechanisms of DN.

Materials And Methods

Data source

The DN expression profiling by high throughput sequencing dataset GSE142025 was downloaded from the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo) [11]. The dataset GSE142025 was based on the GPL20301 platform (Illumina HiSeq 4000 (Homo sapiens)), including 28 DN samples and 8 normal control samples.

Identification of DEGs

Identify a gene that are differentially expressed across experimental conditions, was used to identify DEGs in the GSE142025 dataset with the limma package of R language, which had been processed, normalized and transformed. An adjusted P-value was retrieved by implement the Benjamini-Hochberg false discovery rate (FDR) correction on the original P-value, and a fold change threshold was preferred based on our plan to target on statistically significant DEGs [12]. Only genes with a fold change > 1.35 for up regulated genes and fold change < -1.24 for down regulated genes, and adjusted P-value <0.05 were considered as statistically significant DEGs. A volcano plot and heat map of the identified DEGs was also constructed, using an R package.

Gene ontology and pathway enrichment analysis of DEGs

In the current investigation, the significant enrichment analysis of DEGs was assessed based on the Gene Ontology (GO) and REACTOME using the ToppGene (ToppFun)
Construction of protein-protein interaction (PPI) network

The online database InnateDB interactome [16] was used to construct a PPI network of the proteins encoded by DEGs. Then, Cytoscape software (Version 3.8.1, National Institute of General Medical Sciences) [17] was utilized to perform protein interaction association network analysis and analyze the interaction correlation of the candidate proteins encoded by the DEGs in DN. Next, the Network Analyzer plugin for Cytoscape was applied to calculate node degree [18], betweenness centrality [19], stress centrality [20] and closeness centrality [21]. Finally, the PEWCC1 [22] for Cytoscape was used to collect the significant modules in the PPI network complex.

Integrated regulatory network construction

The integrated regulatory network of miRNAs (microRNAs) and TFs (transcription factors) was constructed based on standardized integration of numerous high-throughput datasets. It granted a plan including a set of hub genes, miRNAs and TFs for analyzing multi-level regulation in DN. The miRNAs associated with DEGs were selected from miRNet [23] Database (The integration database of TarBase, miRTarBase, miRecords, miRanda (S mansoni only), miR2Disease, HMDD, PhenomiR, SM2miR, PharmacomiR, EpimiR, starBase, TransmiR, ADmiRE, and TAM 2.0), and TFs associated with DEGs were selected from NetworkAnalyst database [24] Database (The integration database of JASPAR). The miRNA-DEG regulatory network and TF-DEG regulatory network were constructed by using Cytoscape software, which is open source software for visualizing complex networks.

Validation of the hub genes

The diagnostic value of validated hub genes was assessed using receiver operating characteristic (ROC) curve analysis using the pROC in R with GLM prediction model [25] and area under the curve (AUC) was calculated.

Molecular docking studies
The module SYBYL-X 2.0 perpetual software were used for Surflex-Docking of the designed molecules. The molecules were sketched by using ChemDraw Software and imported and saved in sdf. format using openbabel free software. The protein structures of CyclinB1 (CCNB1) its co-crystallised protein of PDB code 4Y72, 5H0V and Four and half LIM domains 2 (FHL2) its NMR structure of proteins 2D8Z and 2EHE was retrieved from Protein Data Bank [26-27]. Together with the TRIPOS force field, GasteigerHuckel (GH) charges were added to all designed derivatives for the structure optimization process. In addition, energy minimization was carried out using MMFF94s and MMFF94 algorithm process. Protein processing was carried out after the incorporation of protein. The co-crystallized ligand and all water molecules were removed from the crystal structure; more hydrogens were added and the side chain was set. TRIPOS force field was used for the minimization of structure. The compounds’ interaction efficiency with the receptor was represented by the Surflex-Dock score in kcal / mol units. The interaction between the protein and the ligand, the best pose was incorporated into the molecular area. The visualisation of ligand interaction with receptor is done by using discovery studio visualizer.

Results

Identification of DEGs

DN and normal control samples (28 and 9, respectively) were first analyzed. Limma was used to analyze the series of each chip and to identify the DEGs. Following analysis of GSE142025 dataset, 549 DEGs (275 up regulated and 274 down regulated) genes were identified (Fig. 1. and Table 1). The results of the cluster analysis of DEGs revealed significant differences between the DN and normal control samples (Fig. 2).

Gene ontology and pathway enrichment analysis of DEGs

The identified DEGs were uploaded to the online software ToppGene for GO and REACTOME pathway enrichment analyses and results are listed in Table 2 and Table 3. The results of the GO analysis revealed that up regulated genes were significantly enriched in BP, including cell activation and regulation of immune system process, whereas down regulated genes were significantly enriched in response to hormone and ion transport. In terms of CC, the up regulated genes were enriched in cell surface and intrinsic component of plasma membrane, whereas down regulated genes were enriched in integral component of plasma membrane and nuclear chromatin. In terms of MF, the up regulated genes were enriched in signaling receptor binding and identical protein binding, whereas down regulated genes were enriched in lipid binding and transporter activity. REACTOME pathway analysis revealed that the up regulated genes were highly associated with pathways including immunoregulatory interactions between a lymphoid and a non-lymphoid cell, and innate immune system, whereas down regulated genes were significantly enriched in biological oxidations and GPCR ligand binding.

Construction of protein-protein interaction (PPI) network
The DEG expression profiles in DN were constructed according to the information in the InnateDB interactome database. The PPI network of DEGs is consisted of 2718 nodes and 4477 edges (Fig. 3A). There are 10 genes selected as hub genes, such as MDFI, LCK, BTK, IRF4, PRKCB, EGR1, JUN, FOS, ALB and NR4A1 are listed in Table 4. A two significant modules were obtained from PPI network of DEGs using PEWCC1, including 15 nodes and 36 edges (Fig. 3B) and 7 nodes and 12 edges (Fig. 3C). Gene ontology and pathway enrichment analysis revealed that genes in these modules were mainly involved in innate immune system, immunoregulatory interactions between a lymphoid and a non-lymphoid cell, cell activation, regulation of immune system process, cell surface, response to hormone, cytokine signaling in immune system, metabolism of proteins and nuclear chromatin.

**Integrated regulatory network construction**

The miRNA-DEG regulatory network had 8997 interactions (involving 1973 miRNAs and 248 DEGs) (Fig. 4A). Moreover, COL1A1 was targeted by 178 miRNAs (ex, hsa-mir-4492), IRF4 was targeted by 140 miRNAs (ex, hsa-mir-4319), MYBL2 was targeted by 83 miRNAs (ex, hsa-mir-637), PRKCB was targeted by 81 miRNAs (ex, hsa-mir-1261), IL2RB was targeted by 54 miRNAs (ex, hsa-mir-4300), JUN was targeted by 144 miRNAs (ex, hsa-mir-3943), EGR1 was targeted by 132 miRNAs (ex, hsa-mir-548e-3p), ZFP36 was targeted by 130 miRNAs (ex, hsa-mir-6077), FOS was targeted by 105 miRNAs (ex, hsa-mir-5586-5p) and DUSP1 was targeted by 97 miRNAs (ex, hsa-mir-4458) are listed in Table 5. The TF-DEG regulatory network had 1954 interactions (involving 81 TFs and 250 DEGs) (Fig. 4B). Moreover, IRF4 was targeted by 10 TFs (ex, NFATC2), LCK was targeted by 10 TFs (ex, YY1), RET was targeted by 10 TFs (ex, NR2C2), MAP1LC3C was targeted by 10 TFs (ex, MAX), IL2RB was targeted by 8 TFs (ex, PDX1), ATF3 was targeted by 19 TFs (ex, TP53), EGR1 was targeted by 16 TFs (ex, ARID3A), JUNB was targeted by 15 TFs (ex, SRF), FOS was targeted by 13 TFs (ex, CREB1) and PTPRO was targeted by 13 TFs (ex, NR3C1) are listed in Table 5.

**Validation of the hub genes**

A ROC curve was plotted to evaluate the diagnostic value of MDFI, LCK, BTK, IRF4, PRKCB, EGR1, JUN, FOS, ALB and NR4A1 (Fig. 5). The AUCs for the 10 genes were 0.946, 0.853, 0.835, 0.871, 0.804, 0.991, 0.964, 0.964, 0.839 and 0.982, respectively.

**Molecular docking studies**

In the current research, the docking simulation was conducted to recognize the active site conformation and major interactions responsible for complex stability with the binding sites receptor. Novel molecules containing thiazolidindione heterocyclic ring were designed and performed docking studies using Sybyl X 2.1 drug design software. Molecules containing thiazolidindione heterocyclic ring is designed based on the structure of the pioglitazone, is most commonly used alone or in combination with other antidiabetic drug. In diabetic nephropathy pregablin is used to relieve the pain and is taken as a standard. The proteins which are over expressed in diabetic nephropathy are selected for docking studies. The X- RAY crystallographic structure of one proteins of each over expressed PRKCB its co-crystallised protein of PDB...
code 5T5T and IRF4 its co-crystallised protein of PDB code 5UTZ were selected for docking. The investigations of designed molecules were performed to identify the potential molecule. The most of the designed molecules obtained C-score greater than 5 and are active having the c-score greater than 5 are said to be an active, among total of 56 designed molecules few molecules have excellent good binding energy (C-score) greater than 8 respectively. Few of the designed molecules SALPYR 12, SALISO 11 and SLPYR 13 (Fig. 6) shown excellent binding score of 8.518, 8.437 and 8.083 with 5T5T and no molecules with excellent binding score with 5UTZ respectively. Few of the molecules BENZPYR 7 and BENZPYR 1 obtained with moderate binding score of 4.5897 and 5.5881 with 5T5T and molecules of SALISO 2, SALISO 8 & SALPYR 4 with binding score of 5.8485, 5.5892 & 5.5222 with PDB code 5UTZ respectively. Molecules of BENZPYR 01, BENZPYR 5, BENZPYR 2 and SALPYR 7 shown weak binding score of 4.581, 4.4581, 4.4092 and 4.3984 and the molecules SLPYR 11, BENZISO 5, BENZISO 7 and BENZISO 13 with binding score 3.604, 3.5198, 3.5005 and 3.4955 with 5T5T and 5UTZ, the values are depicted in Table 6. The binding score of the predicted molecules are compared with that of the standard Pregablin used in diabetic nephropathy, the standard obtained moderate binding score with 5T5T and weak binding score with 5UTZ respectively. The molecule 26 has highest binding score its interaction with protein 5T5T and hydrogen bonding and other bonding interactions with amino acids are depicted by 3D and 2D figures (Fig. 7 and Fig. 8).

**Discussion**

DN remains end-stage renal disease worldwide because of its complicated molecular mechanisms and cellular heterogeneity, and its prevalence rise every year [28]. Therefore, recognition of DN may offer clinicians novel tools that can be used to treat the disease. Extensive genomic investigations showing the effects of genes have accepted noticeable attention. Many potential and valuable genes must be identified to develop the clinical outcome for DN patients. However, the number of specific molecular biomarkers that can be used to show therapeutic effects is still limited, and prognostic factors are essential for the treatment of DN patients. Therefore, to diminish mortality and develop DN prognosis, there is a critical demand for the screening of molecular biomarkers of DN.

To better understand the genetic modifications occurring during DN advancement, bioinformatics methods were used to extract data from the GSE7803 and GSE142025 expression profiling by high throughput sequencing. In this investigation, we identified 549 DEGs (275 up regulated and 274 downregulated) between DN and normal control. Xie et al [29] and Zhou et al [30] demonstrate that increased activity of polymorphic CFHR1 and RGS1 genes play a key role in nephropathy progression. Previous investigations report that polymorphic GREM1 gene plays an essential role in progression of DN [31]. Sun et al [32] find that CCL19 is responsible for renal inflammation and fibrosis in DN. Martinelli-Boneschi et al [33] revealed that polymorphic COL6A5 gene is involved in neuropathic chronic itch, but this gene might be liable for progression of DN. Hall et al [34] observed that the expression of CIDEC (cell death inducing DFFA like effector c) play key role in obesity, but this gene might be involved in DN progression. NR4A1 drives DN growth through mitochondrial fission and mitophagy [35]. Recent study has reported that low expression of NR4A2 is associated with myocardial infarction [36], but this gene might be linked
with progression of DN. EGR1 is required for fibrosis and inflammatory response in DN [37]. ATF3 expression has been implicated in DN [38]. Polymorphic NR4A3 gene may contribute to type 2 diabetes progression [39], but this gene might be associated with development of DN. KLK1 functions in DN progression can be used for predicting the progression and prognosis of the disease [40].

A series of DEGs were discovered to be enriched in the GO functions and pathways. SERPINA3 [41], IKZF1 [42], BTK (Bruton tyrosine kinase) [43], C1QA [44], CD1C [45] and CCL13 [46] have a key role in lupus nephritis, but these genes might be liable for advancement of DN. TNFSF14 [47], ITGAL (integrin subunit alpha L) [48], PLAC8 [49], ADRA2A [50], CCL21 [51], ALOX5 [52], CNR2 [53], COL1A1 [54], WNT7A [55], SLAMF1 [56], CD3D [57], LTF (lactotransferrin) [58], MIR27B [59], PDK4 [60], UCN3 [61], PCK1 [62], CEL (carboxyl ester lipase) [63], TRPM6 [64], MTTP (microsomal triglyceride transfer protein) [65], CYP2C8 [66] and CYP3A4 [67] have important role in the progression of type 2 diabetes via inflammation, but these genes might be crucial role in DN progression. Zhang el al [68], Ellenbroek et al [69], Guo et al [70] and Tillmanns et al [71] have shown that MZB1, LAIR1, MIR142 and FAP (fibroblast activation protein alpha) modulating mitochondrial function and alleviating inflammation in myocardial infarction, but these genes might be involved in progression of DN. IRF4 plays a key role in the obesity-induced insulin resistance [72], but this gene might be responsible for development of DN. MDK (midkine) [73], CCR2 [74], SAA1 [75], C3 [76], CD19 [77], CCR5 [78], CXCR3 [79], FABP4 [80], GDF15 [81], IGF2 [82], IGFBP1 [83] and IL6 [84] are important in the progression of DN through inflammation. A previous study has shown that UBASH3A [85], SIRPG (signal regulatory protein gamma) [86], IKZF3 [87], CD1D [88], CD2 [89], CD48 [90], CD247 [91] and CYP27B1 [92] are liable for progression of type 1 diabetes through inflammation, but these genes might be key for progression of DN. SIT1 [93], JAML (junction adhesion molecule like) [94], TIMP1 [95], PRKCB (protein kinase C beta) [96], MMP7 [97], WNT7B [98], WNT10A [99], DUSP1 [100], WT1 [101], APOC3 [102], ERRFI1 [103], HCN2 [104], MME (membrane metalloendopeptidase) [105], STRA6 [106], SLC12A3 [107] and GC (GC vitamin D binding protein) [108] expedites epithelial to mesenchymal transition and renal fibrosis in DN. Previous studies have found CFD (complement factor D) [109], DOCK2 [110], LYZ (lysozyme) [111], CD5L [112], SCARA5 [113], VCAN (versican) [114], GDF5 [115], SFRP2 [116], BTG2 [117], ZFP36 [118], GPR3 [119], OLR1 [120], PM20D1 [121] and UGT2B7 [122] to be expressed in obesity, but these genes might be liable for advancement of DN. Polymorphic FCRL3 [123], FCGR2B [124], COMP (cartilage oligomeric matrix protein) [125], ERFE (erythroferrone) [126] and NPHS1 [127] expression can be altered by inflammation, which might involved in nephropathy. The expression of COL1A2 [128], LCK (LCK proto-oncogene, Src family tyrosine kinase) [129], LCN2 [130] and APOB (apolipoprotein B) [131] are key for progression of diabetic retinopathy, but these genes might be essential for DN development. COL3A1 [132], PER1 [133], JUN (Jun proto-oncogene, AP-1 transcription factor subunit) [134], SLC26A4 [135], F2RL3 [136], CYP4A11 [137] and CYP4F2 [138] play an important role in the hypertension, but these genes might be involved in progression of DN.

Based on the PPI network and module analysis, we obtained top hub genes in the whole network. Onions et al. [139] showed that ALB (albumin) was potential biomarkers of DN and disease progression. Novel biomarkers such as MDFI (MyoD family inhibitor), FOS (Fos proto-oncogene, AP-1 transcription factor
subunit), SH2D1A, SLA2, TRAT1, CD3E, JUNB and FOSB might play an important role in the development of DN.

Based on the miRNA-DEG regulatory network and TF-DEG regulatory network, we obtained target in the whole network. Recent investigation reported that the dysregulated activity of MYBL2 was associated with myocardial infarction progression [140], but this gene might be linked with progression of DN. Many investigation have reported the hsa-mir-637 [141] and NR3C1 [142] were linked with progression of hypertension, but these genes might be liable for advancement of DN. Wang et al. [143] noted that hsa-mir-1261 was associated with development of DN. Li et al [144] reported that hsa-mir-4458 expression was an independent marker of prognosis in myocardial infarction, but this gene might be involved in DN. Keller et al [145], Fujimoto et al [146] and Xu et al [147] demonstrated that expression of NFATC2, PDX1 and CREB1 were involved in type 2 diabetes, but these genes might be key for progression of DN. Wang et al. [148], Zhang et al. [149] and Zhao et al. [150] found that YY1, TP53 and SRF (serum-response factor) played a key role in DN through the epithelial–mesenchymal transition. Novel targets such as IL2RB, hsa-mir-4492, hsa-mir-4319, hsa-mir-4300, hsa-mir-3943, hsa-mir-548e-3p, hsa-mir-6077, hsa-mir-5586-5p, RET (ret proto-oncogene), MAP1LC3C, PTPRO (protein tyrosine phosphatase receptor type O), NR2C2, MAX (myc-associated factor X) and ARID3A might be responsible for progression of DN.

In the present investigation, the DEGs of DN and normal control samples were analyzed to achieve a better understanding of DN. GO and pathway enrichment analyses of DEGs were applied, and the protein–protein interaction (PPI) network, module and miRNA-DEG regulatory network and TF-DEG regulatory network of these DEGs were also constructed. ROC analysis and molecular docking experiments conducted. The aim of this investigation was to identify essential genes and pathways in DN using bioinformatics analysis, and then to explore the intrinsic mechanisms of DN and distinguish new potential diagnostic and therapeutic biomarkers of DN. We anticipated that these investigations will provide further insight of DN pathogenesis and advancement at the molecular level.

**Declarations**

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**Conflict of interest**

The authors declare that they have no conflict of interest.

**Ethical approval**

This article does not contain any studies with human participants or animals performed by any of the authors.
Informed consent

No informed consent because this study does not contain human or animals participants.

Availability of data and materials

The datasets supporting the conclusions of this article are available in the GEO (Gene Expression Omnibus) (https://www.ncbi.nlm.nih.gov/geo/) repository. [(GSE142025) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE142025]

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author Contributions

H.J - Methodology and validation
B.V - Writing original draft, and review and editing
N.J - Software and resources
A.T - Formal analysis and validation
C.V- Investigation and resources
I.K - Supervision and resources

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**Tables**

Due to technical limitations, Tables 1-6 are only available as a download in the supplemental files section.