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ABOUT COVER

Editorial Board Member of *World Journal of Diabetes*, Dr. She-Yu Li, MD, is an Associate Professor and Specialist of Endocrinology and Diabetology at West China Hospital Sichuan University. He is the co-chief and co-founder of MAGIC China Center and board member of the Diabetes and Obesity Study Group of the Chinese Diabetes Society. He received his MD degree from the West China School of Medicine, Sichuan University in 2012, after which he worked as a visiting researcher at Ninewells Hospital, University of Dundee (Scotland; 2017 to 2019). His research involves electronic medical record-based real-world studies and evidence translation in the fields of diabetes, obesity, and related diseases. These efforts have led to the publication of over 100 papers in peer-reviewed journals. (L-Editor: Filipodia)

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EDITORIAL

Is there a role for glucagon-like peptide-1 receptor agonists in the management of diabetic nephropathy?

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Abstract

Chronic kidney disease constitutes a major microvascular complication of diabetes mellitus. Accumulating data suggest that glucagon-like peptide-1 receptor agonists (GLP-1 RAs) might have a role in the management of diabetic kidney disease (DKD). GLP-1 RAs appear to reduce the incidence of persistent macro-albuminuria in patients with type 2 diabetes mellitus. This beneficial effect appears to be mediated not only by the glucose-lowering action of these agents but also on their blood pressure lowering, anti-inflammatory and antioxidant effects. On the other hand, GLP-1 RAs do not appear to affect the rate of decline of glomerular filtration rate. However, this might be due to the relatively short duration of the trials that evaluated their effects on DKD. Moreover, these trials were not designed nor powered to assess renal outcomes. Given than macrolbuminuria is a strong risk factor for the progression of DKD, it might be expected that GLP-1 RAs will prevent the deterioration in renal function in the long term. Nevertheless, this remains to be shown in appropriately designed randomized controlled trials in patients with DKD.

Key Words: Diabetic nephropathy; Type 2 diabetes mellitus; Glucagon-like peptide-1 receptor agonists; Liraglutide; Dulaglutide; Semaglutide

Core Tip: Glucagon-like peptide-1 receptor agonists prevent the development of persistent macroalbuminuria in patients with type 2 diabetes mellitus. However, it is unclear whether they delay the decline in glomerular filtration rate in this population. Long-term trials are needed to clarify the role of these agents in the management of diabetic nephropathy.

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INTRODUCTION

Chronic kidney disease (CKD) constitutes a major microvascular complication of diabetes mellitus (DM) and occurs both in type 1 and type 2 DM (T2DM)[1]. The prevalence of diabetic nephropathy is 50% in patients with type 1 DM and 30%-50% in patients with T2DM[2]. Diabetic kidney disease (DKD) is characterized by specific structural and functional changes in the kidneys of patients with DM. These changes result in a clinical presentation that includes hypertension, increased urinary albumin excretion and progressive deterioration in kidney function[1]. It has been estimated that DKD is the leading cause of end-stage renal disease (ESRD) and 30%-40% of patients with DKD are expected to develop ESRD[3]. More specifically, patients with higher levels of albuminuria, quick deterioration of glomerular filtration rate (GFR), uncontrolled hypertension, long duration of DM, presence of microvascular complications and positive family history of DKD are at higher risk of DKD progression to ESRD[4]. Importantly, DKD is associated with increased cardiovascular morbidity and mortality[4]. It has been shown that proteinuria and impaired GFR are independently associated with higher risk of adverse cardiovascular outcomes in patients with T2DM[5]. The main goals of treatment of DKD are to delay the deterioration of kidney function and to prevent cardiovascular events. Lifestyle measures (*i.e.*, diet and exercise), strict glycemic control and blood pressure control using renin–angiotensin–aldosterone system inhibitors are the cornerstone of DKD treatment[6].

Accumulating data suggest that glucagon-like peptide-1 receptor agonists (GLP-1 RAs) might have a role in the management of DKD. GLP-1 is secreted by the L-cells of small intestine after food intake and regulates glucose homeostasis[7]. GLP-1 RAs are divided into short-acting (exenatide, liraglutide and lixisenatide) or long-acting (albiglutide, dulaglutide, exenatide long-acting release and semaglutide)[8]. GLP-1 RAs induce substantial reductions in glucose levels without the risk of hypoglycemia and also reduce cardiovascular morbidity[9]. Notably, several randomized, placebo-controlled trials in patients with T2DM and established cardiovascular disease, CKD or multiple cardiovascular risk factors reported a beneficial effect on DKD. In the Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial (n = 9340), liraglutide reduced the incidence of the composite renal outcome (new-onset persistent macroalbuminuria, persistent doubling of the serum creatinine level and an estimated GFR ≤ 45 mL/min/1.73 m^2, the need for continuous renal-replacement therapy with no reversible cause of the renal disease, or death from renal disease) by 22% compared with placebo during a median follow-up of 3.8 years[10]. This reduction was driven by the lower incidence of new-onset persistent macroalbuminuria whereas the other endpoints did not differ between patients treated with liraglutide and placebo[10]. Liraglutide also reduced the incidence of new-onset microalbuminuria by 13%[10]. Even though GFR declined and albuminuria increased during follow-up in both groups, these changes were smaller in patients treated with liraglutide[10]. In the Trial to Evaluate Cardiovascular and Other Long-term Outcomes with Semaglutide in Subjects with Type 2 Diabetes (SUSTAIN-6, n = 3297), once-weekly semaglutide reduced the risk of new or worsening nephropathy (defined as a new onset of persistent macroalbuminuria, or persistent doubling of serum creatinine level and creatinine clearance) by 36% compared with placebo during a median follow-up of 2.1 years; this benefit was primarily due to the prevention of persistent macroalbuminuria[11]. In the Researching Cardiovascular Events with a Weekly Incretin in Diabetes (REWIND) trial (n = 9901), once-weekly dulaglutide reduced the incidence of the renal component of the composite microvascular outcome (defined as first occurrence of new macroalbuminuria, a sustained decline in estimated GFR ≥ 30% from baseline, or chronic renal replacement therapy) by 15% compared with placebo during a median follow-up of 5.4 years[12]. Again, this benefit was due to a decreased risk of new macroalbuminuria in patients treated with dulaglutide whereas the incidence of sustained decline in GFR and chronic renal replacement therapy did not differ between the 2 groups[12]. In a smaller randomized study in 577 patients with moderate-to-severe DKD, dulaglutide had

similar effects on albuminuria with insulin glargine but was associated with higher GFR at 52 wk[13]. In a recent meta-analysis of 7 placebo-controlled, cardiovascular outcome trials in patients with T2DM (n = 56004), treatment with GLP-1 RAs reduced the risk of the composite renal outcome by 17%; again, this benefit was only due to a reduction in the incidence of macroalbuminuria by 24%[14].

In addition to the glucose-lowering action of GLP-1 RAs, several other mechanisms appear to underpin the effects of these agents on renal function[15]. GLP-1RAs lower blood pressure both due to weight loss and due to direct effects on the kidney[15]. Indeed, it has been reported that GLP-1 RAs promote natriuresis and diuresis due to the inhibition of the sodium–hydrogen exchanger 3, which is located in the renal proximal tubular cells[16,17]. In addition, preclinical models suggest that GLP-1 RAs exert anti-inflammatory effects and decrease oxidative stress in the kidneys[18,19].

CONCLUSION

In conclusion, GLP-1 RAs appear to reduce the incidence of persistent macroalbuminuria in patients with T2DM. On the other hand, these agents do not appear to affect the rate of decline of GFR. However, this might be due to the relatively short duration of the trials that evaluated these effects. Moreover, these trials were not designed nor powered to assess renal outcomes. Given than macrolbuminuria is a strong risk factor for the progression of DKD[5,20], it might be expected that GLP-1 RAs will prevent the deterioration in renal function in the long term. However, this remains to be shown in appropriately designed randomized controlled trials in patients with DKD. The FLOW trial (NCT03819153) is currently evaluating the effects of semaglutide *vs* placebo on the progression of renal impairment in patients with DKD and is expected to be completed in 2024[21].

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ORIGINAL ARTICLE

Basic Study

Analysis of long noncoding RNA-associated competing endogenous RNA network in glucagon-like peptide-1 receptor agonist-mediated protection in β cells

Li-Juan Cui, Tao Bai, Lin-Ping Zhi, Zhi-Hong Liu, Tao Liu, Huan Xue, Huan-Huan Yang, Xiao-Hua Yang, Min Zhang, Ya-Ru Niu, Yun-Feng Liu, Yi Zhang

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Author contributions: Bai T, Zhi LP, and Yang HH performed the experiments; Cui LJ, Xue H, Niu YR, and Zhang M performed the data analysis and interpretation; Liu T, Liu ZH, and Yang XH edited and revised the manuscript; Zhang Y and Liu YF were involved in the study design, data analysis and interpretation, manuscript composition, and manuscript editing; all authors approved the final version of the manuscript.

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Abstract

BACKGROUND

Long noncoding RNAs (lncRNAs) and mRNAs are widely involved in various physiological and pathological processes. The use of glucagon-like peptide-1 receptor agonists (GLP-1RAs) is a novel therapeutic strategy that could promote insulin secretion and decrease the rate of β-cell apoptosis in type 2 diabetes mellitus (T2DM) patients. However, the specific lncRNAs and mRNAs and their functions in these processes have not been fully identified and elucidated.

AIM

To identify the lncRNAs and mRNAs that are involved in the protective effect of GLP-1RA in β cells, and their roles.

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Data sharing statement: The datasets (Series GSE138744) generated and analyzed during the current study are available in the (GEO) repository, (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138744). To review the data, please enter token: *opslyaomvhmlzkb* into the box. Our data in GSE138744 remains in private status now. You can review it through the pathway above, and we will make the data publicly available prior to the publication of this article.

METHODS

Rat gene microarray was used to screen differentially expressed (DE) lncRNAs and mRNAs in β cells treated with geniposide, a GLP-1RA. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed to assess the underlying functions of DE mRNAs. Hub mRNAs were filtered using the STRING database and the Cytoscape plugin, CytoHubba. In order to reveal the regulatory relationship between lncRNAs and hub mRNAs, their co-expression network was constructed based on the Pearson coefficient of DE lncRNAs and mRNAs, and competing endogenous RNA (ceRNA) mechanism was explored through miRanda and TargetScan databases.

RESULTS

We identified 308 DE lncRNAs and 128 DE mRNAs with a fold change filter of ≥ 1.5 and *P* value < 0.05. GO and KEGG pathway enrichment analyses indicated that the most enriched terms were G-protein coupled receptor signaling pathway, inflammatory response, calcium signaling pathway, positive regulation of cell proliferation, and ERK1 and ERK2 cascade. *Pomc*, *Htr2a*, and *Agtr1a* were screened as hub mRNAs using the STRING database and the Cytoscape plugin, CytoHubba. This result was further verified using SwissTargetPrediction tool. Through the co-expression network and competing endogenous (ceRNA) mechanism, we identified seven lncRNAs (NONRATT027738, NONRATT027888, NONRATT030038, *etc*.) co-expressed with the three hub mRNAs (*Pomc, Htr2a, and Agtr1a*) based on the Pearson coefficient of the expression levels. These lncRNAs regulated hub mRNA functions by competing with six miRNAs (*r*no-miR-5132-3p, rno-miR-344g, rno-miR-3075, *etc*.) *via* the ceRNA mechanism. Further analysis indicated that lncRNA NONRATT027738 interacts with all the three hub mRNAs, suggesting that it is at a core position within the ceRNA network.

CONCLUSION

We have identified key lncRNAs and mRNAs, and highlighted here how they interact through the ceRNA mechanism to mediate the protective effect of GLP-1RA in β cells.

Key Words: Type 2 diabetes; β cell; Long noncoding RNA; Competing endogenous RNA; Co-expression analysis; Glucagon-like peptide-1 receptor agonist

Core Tip: This study investigated the long noncoding RNA (lncRNA) regulatory network involved in the protective effects of geniposide, a glucagon-like peptide-1 receptor agonist (GLP-1RA), in pancreatic β cells using a microarray. We identified key lncRNAs and mRNAs, and highlighted how they interact through the competing endogenous RNA mechanism to mediate GLP-1RA-mediated protection in β cells. Our study has contributed to a deeper understanding of the molecular mechanism of β cell protection by GLP-1RA at the transcriptional level.

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INTRODUCTION

The impaired function and diminished mass of β cells in type 2 diabetes mellitus (T2DM) patients lead to insufficient insulin secretion and hyperglycemia[1]. Additionally, malfunction, de-differentiation, and apoptosis of β cells are the key characteristics of T2DM[2]. Impaired insulin secretion is a key contributor to chronic

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hyperglycemia in T2DM patients[2,3]. Hence, a strategy to block β cell apoptosis and restore β cell function is urgently needed. As a class of promising anti-diabetic drugs, glucagon-like peptide-1 receptor agonists (GLP-1RAs) have been shown to potentiate insulin secretion in a glucose-dependent manner, which can decrease blood glucose levels without the risk of hypoglycemia[4-6]. Moreover, studies have demonstrated that GLP-1RAs prevent β cells from premature apoptosis and promote their function[7,8]. Recent studies reported that geniposide, a monomer extracted from gardenia, is a novel GLP-1RA with multiple protective effects in human diseases, such as Alzheimer's disease[9], obesity-related cardiac injury[10], and myocardial ischemia[11]. We and other researchers found that geniposide potentiates insulin secretion, promotes proliferation, and decreases the rate of β cell apoptosis by stimulating the GLP-1 receptor[12-14].

Long noncoding RNAs (lncRNA) are some of the recently studied regulatory molecules[15]. These RNAs are transcripts that are longer than 200 nucleotides and do not code for proteins[16]. Mechanically, lncRNAs exert their regulatory effects through communication with other molecules. Growing evidence demonstrates that lncRNAs regulate mRNA expression by competing with microRNAs (miRNAs)[17,18]. The competition among lncRNAs, miRNAs, and mRNAs was termed the competing endogenous RNA mechanism or "ceRNA mechanism", which is widely involved in multiple biological processes, including insulin signal transduction that may affect diabetes development[19]. Currently, the mechanisms of the protective effect of GLP-1RA in β cells have been widely investigated, but their potential relationship with mRNAs and lncRNAs is yet to be explored.

We previously demonstrated that geniposide protects pancreatic β cells *via* GLP-1R[13]. In this study, we examined the expression profiles of lncRNAs and mRNAs in INS-1 cells treated with or without geniposide *via* microarray. We further explored, *via* biological information analysis, the interaction between lncRNAs and mRNAs, and whether the ceRNA network was involved in their regulatory relationship. We aimed to identify the roles of lncRNAs and mRNAs in mediating the protective effect of GLP-1RA in β cells.

MATERIALS AND METHODS

Cell culture and treatment

Rat pancreatic INS-1 cells were purchased from the National Infrastructure of Cell Line Resource (Identification number: 3111C0001CCC000378). The cells grew irregularly, polygonally, and adherently. Mycoplasma detection was negative. INS-1 cells were grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, 100 μg/mL penicillin, and 50 μmol/L β-mercaptoethanol at 37 °C in an atmosphere containing 5% CO_2. INS-1 cells were seeded in 12-well plates to approximately 80% confluence and treated with or without 10 μmol/L geniposide for 24 h. Three technical replicates were performed on each independent sample.

RNA extraction, purification, and quality control

Total RNA was extracted and purified using the RNeasy Mini Kit (Cat#74106, QIAGEN, GmBH, Germany) following the manufacturer's instructions and checked for an RIN number to inspect RNA integration with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, United States).

The initial sample of the chip experiment was total RNA that was subjected to quality inspection using a NanoDrop ND-2000 spectrophotometer and Agilent Bioanalyzer 2100 (Agilent Technologies). Then, quality-qualified RNA was subjected to subsequent chip experiments.

Microarray and data analysis

Rat microarray: The Rat microarray Agilent-074571 RAT_LNCRNA_20150413 was developed by Shanghai Bohao Company. The probe information was queried from the GEO database with platform number GPL27603. This microarray was used to profile the lncRNAs and mRNAs. The probe design was based on the latest version of the genome covering core lncRNA and mRNA databases, such as GENCODE V21, Ensembl, UCSC, NONCODE, LNCipedia, and lncRNAdb.

RNA labeling and array hybridization: Total RNA was amplified and labeled with the Low Input Quick Amp Labeling Kit, One-Color (Cat. # 5190-2305, Agilent

Technologies), following the manufacturer's instructions. Labeled cRNAs were purified with the RNeasy Mini Kit (Cat. # 74106, QIAGEN).

Each slide was hybridized using 600 ng Cy3-labeled cRNA and a Gene Expression Hybridization Kit (Cat. # 5188-5242, Agilent Technologies) in a hybridization oven (Cat. # G2545A, Agilent Technologies), according to the manufacturer's instructions. After 17 h of hybridization, slides were washed in staining dishes (Cat. # 121, Thermo Shandon, Waltham, MA, USA) with the Gene Expression Wash Buffer Kit (Cat. # 5188-5327, Agilent Technologies) according to the manufacturer's instructions.

Data acquisition and analysis: Slides were scanned with an Agilent Microarray Scanner (Cat. # G2565CA, Agilent Technologies) with default settings: Dye channel, green; scan resolution = 3 μm; PMT 100%; 20 bit. Data were extracted with Feature Extraction software 10.7 (Agilent Technologies). Raw data were normalized using Robust Multichip Average (RMA) algorithm and limma packages in R. Significantly differentially expressed (DE) lncRNAs and mRNAs between the two groups were selected if the fold changes of the threshold values were ≥ 1.5 and P value < 0.05.

Gene Ontology analysis

Gene Ontology (GO) analysis can be divided into three parts: Molecular function, biological process, and cellular component, which respectively describe the molecular functions of potential gene products, the biological processes involved, and the cellular environment in which they are located. Enrichment analysis was performed *via* David 6.8 (https://david.ncifcrf.gov/) database[20,21]. David 6.8 for annotation, visualization, and integrated discovery provides a comprehensive set of functional annotation tools to understand the biological meaning behind the long list of genes. The GO terms obtained in the drawing are arranged in descending order according to the –log10 (P value) of enrichment, and we take the first 10 if there are more than 10 results.

Pathway analysis

The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DE genes can enrich the significant pathways and help to find the biological regulatory pathways for significant differences in experimental conditions. The David 6.8 (https://david.ncifcrf.gov/) database also can be used for the enrichment of pathway. The KEGG pathway terms obtained in the drawing are arranged in descending order according to –log10 (P value) of enrichment, and we take the first 10 if there are more than 10 results.

Screening hub mRNAs

Based on the GO and KEGG analyses, a protein-protein interaction (PPI) network was constructed through the STRING (v11.0, https://string-db.org/) database for all DE mRNAs that were enriched in the GO and KEGG terms. STRING is a database of known and predicted protein-protein interactions, including direct (physical) and indirect (functional) associations, which stem from computational prediction, knowledge transfer between organisms, and interactions aggregated from other (primary) databases. Further improvements in version 11.0 include a completely redesigned prediction pipeline for inferring protein-protein associations from co-expression data, an API interface for the R computing environment, and improved statistical analysis for enrichment tests in user-provided networks. The co-expression scores in STRING v11.0 are computed using a revised and improved pipeline, making use of all microarray gene expression experiments deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus[22]. Since our data came from microarray, the protein-protein interactions could be reflected through the STRING database based on the co-expression relationship, and a confidence score ≥ 0.4 was set as the cut-off criterion.

Next, mRNAs in the PPI network were ranked with the Cytoscape (Cytoscape_v3.7.2) plugin CytoHubba, which provides 11 topological analysis methods, including Degree, Edge Percolated Component, Maximum Neighborhood Component, Density of Maximum Neighborhood Component, Maximal Clique Centrality, and six centralities (Bottleneck, EcCentricity, Closeness, Radiality, Betweenness, and Stress), based on the shortest paths. CytoHubba provides a user-friendly interface for exploring important nodes in biological networks[23]. The hub mRNAs were selected from the top three *via* integrated scores of the 11 algorithms.

Prediction of GLP-1 and geniposide targets

SwissTargetPrediction (http://www.swisstargetprediction.ch/) is an online analysis software for small molecule target prediction, which can predict the target of small molecule compounds based on the principle of molecular similarity. We used this tool to predict the GLP-1 and geniposide targets by converting GLP-1 and geniposide into the standard SMILES format (Canonical SMILES) *via* the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), importing the SMILES format file into the SwissTargetPrediction online analysis platform, setting the property to "Rattus norvegicus", and predicting the targets.

Construction of co-expression network

The Pearson coefficient was calculated based on the normalized chip expression matrix of DE lncRNAs and mRNAs. Molecules were considered with a strong correlation with the filters set at $P < 0.05$ and $|R| > 0.9$. These molecules including lncRNAs and hub mRNAs were constructed into a co-expression network.

Construction of ceRNA network

The ceRNA network was constructed based on the relationships among lncRNAs, miRNAs, and mRNAs. It is established that post-transcriptional regulation of mRNAs could be bound by single-stranded miRNAs, and lncRNAs can directly interact by invoking the miRNA sponge to regulate mRNA expression and activity[24]. The specific steps are as follows:

Prediction of hub mRNA-miRNA pairs: The MiRanda (http://www.microrna.org/) and TargetScan (http://www.targetscan.org/vert_71/) databases provide two algorithms for finding genomic targets for miRNAs. The input file included rat miRNA sequences and 3' untranslated region (UTR) sequences of hub mRNAs. The energy and score threshold filters set for MiRanda were -20 kcal/mol and 50, respectively, and the TargetScan binding type filters were set as 8-mer and 7-mer. The intersections of the results from both databases offered the final prediction of hub mRNA-miRNA pairs.

Prediction of lncRNA-miRNA pairs: Rat lncRNA sequences were downloaded from the NONCODE (http://www.noncode.org/index.php) database. The input file included 3' UTR sequences of lncRNAs from the co-expression network and miRNA sequences from last step, which could interact with hub mRNAs. MiRanda and TargetScan were used as described before to screen lncRNA-miRNA pairs. The intersection of the results from both databases offered the final lncRNA-miRNA pairs.

ceRNA network construction: Based on common miRNAs, the ceRNA network was constructed among lncRNAs, miRNAs, and hub mRNAs, indicating that these lncRNAs could co-express with and regulate hub mRNAs through miRNAs. Cytoscape v3.7.1 was used to construct and visualize the ceRNA network.

Statistical analysis

IBM SPSS 25.0 software was used to analyze all statistical data. The random variance model *t*-test was employed to identify DE mRNAs and lncRNAs between the control and geniposide-treated groups. Fisher's exact test was applied for the GO and pathway analyses. P values < 0.05 were considered statistically significant.

RESULTS

Microarray data profile

According to the microarray expression profiling data, a total of 167 upregulated and 141 downregulated lncRNAs were identified in the geniposide-treated group compared with those in the control group with a set filter fold change ≥ 1.5 and P value < 0.05. Meanwhile, 28 upregulated and 100 downregulated mRNAs were identified with the same filter settings (Figure 1A and B). This result showed that geniposide treatment induced differential expression of lncRNAs and mRNAs in β cells.

GO and KEGG pathway analyses

To investigate the biological function and potential mechanism of DE mRNAs, GO and KEGG pathway enrichment analyses were performed *via* the DAVID database

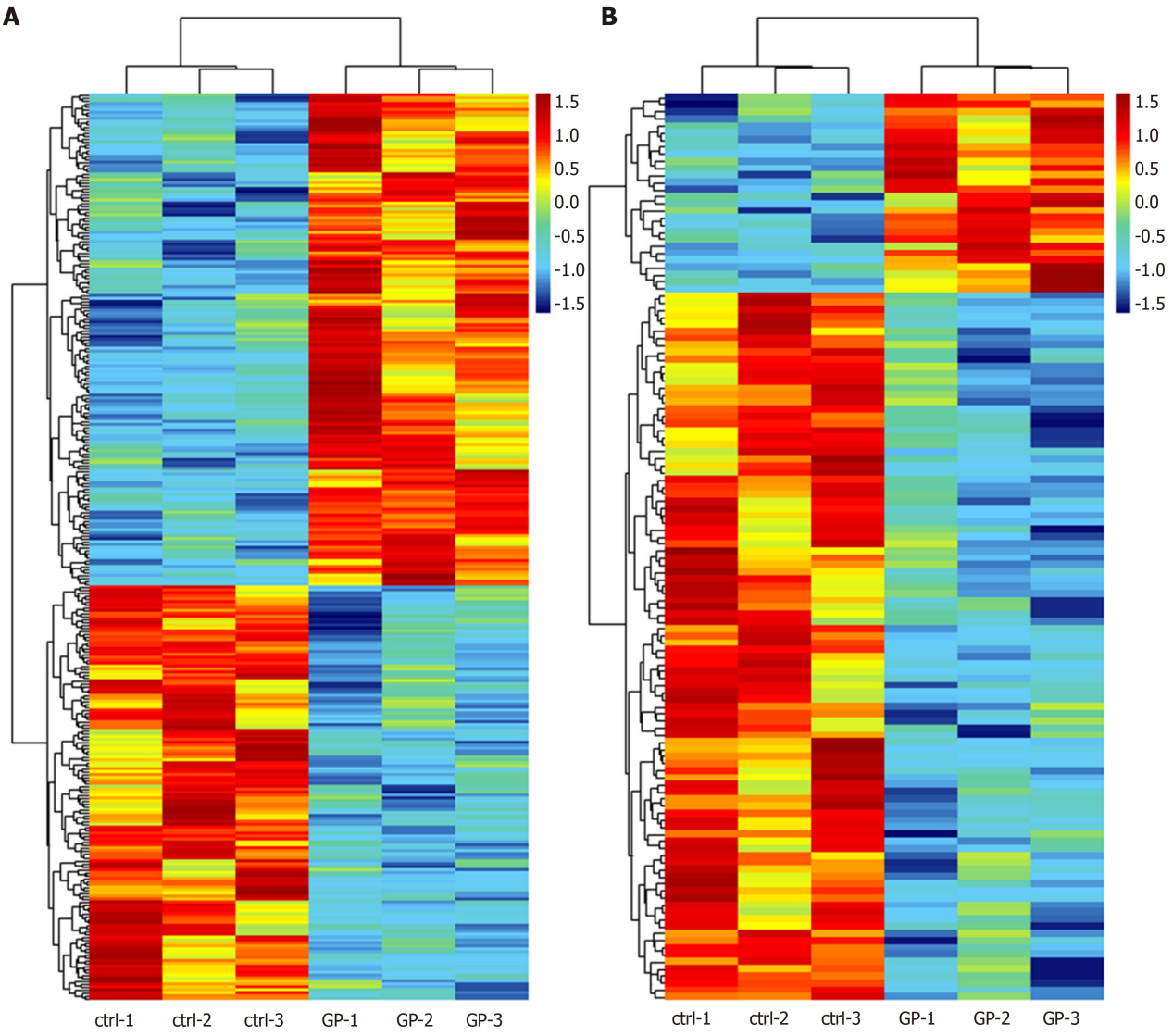

Figure 1 Heatmap of differentially expressed long noncoding RNAs (A) and mRNAs (B). A: A total of 167 upregulated and 141 downregulated long noncoding RNAs were identified in the geniposide-treated group (GP1 to GP3) compared to the control group (ctrl-1 to ctrl-3) with fold change filter set at ≥ 1.5 and *P* value < 0.05. B: A total of 28 upregulated and 100 downregulated mRNAs were identified with the same filter settings.

(Figure 2). Biological process analysis was mainly enriched in terms of inflammatory response and positive regulation of the ERK1 and ERK2 cascade. Molecular function analysis was primarily enriched in terms of G-protein coupled receptor (GPCR) activity, and the KEGG pathway was mainly enriched in terms of the calcium signaling pathway. Detailed information is presented in Table 1. These findings showed that DE mRNAs participated in biological functions that were closely related to insulin secretion and β cell viability (Figure 2). Next, we investigated the core mRNAs that perform these functions.

Construction of PPI network and screening hub mRNAs

To construct a DE mRNA interaction network, we studied the PPI relationship *via* the STRING database. This network was comprised of DE mRNAs enriched in the GO and KEGG terms, including 120 nodes and 52 edges (Figure 3). To discover the core mRNAs in this network, CytoHubba was used to screen the hub mRNAs. Based on the CytoHubba scores, the top three hub mRNAs, *Pomc*, *Htr2a*, and *Agtr1a*, were identified (Figure 4), indicating that they had more interactions with other mRNAs and participated in various functions and pathways than other hub mRNAs.

Verification of hub mRNA prediction

To confirm the accuracy of the hub mRNA prediction, we queried the GLP-1 and geniposide targets using SwissTargetPrediction tool. One hundred GLP-1 and 80 geniposide targets were predicted with SwissTargetPrediction. Among them, 22

Table 1 Significantly enriched Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway terms of differentially expressed long noncoding RNAs

Category	Term	–log10 (*P* value)
GOTERM_BP	G-protein coupled receptor signaling pathway	3.23
GOTERM_BP	Inflammatory response	2.73
GOTERM_BP	Neuropeptide signaling pathway	2.59
GOTERM_BP	Positive regulation of cell proliferation	2.55
GOTERM_BP	Detection of temperature stimulus involved	2.53
GOTERM_BP	Positive regulation of ERK1 and ERK2 cascade	2.32
GOTERM_BP	Regulation of blood coagulation	2.26
GOTERM_BP	Midgut development	2.26
GOTERM_BP	Phospholipase G-protein coupled receptor signaling pathway	2.26
GOTERM_BP	Nervous system development	2.19
GOTERM_CC	Integral component of plasma membrane	3.35
GOTERM_CC	Extracellular space	3.14
GOTERM_CC	Extracellular region	2.46
GOTERM_CC	External side of plasma membrane	1.67
GOTERM_CC	Secretory granule	1.60
GOTERM_CC	Plasma membrane	1.46
GOTERM_CC	Integral component of membrane	1.22
GOTERM_CC	Microvillus	1.19
GOTERM_CC	Dendrite	1.17
GOTERM_CC	Caveola	1.04
GOTERM_MF	G-protein alpha-subunit binding	2.96
GOTERM_MF	Growth factor activity	1.93
GOTERM_MF	G-protein coupled receptor activity	1.39
GOTERM_MF	Serotonin binding	1.18
GOTERM_MF	Cytokine activity	1.13
GOTERM_MF	L-amino acid transmembrane transporter activity	1.02
GOTERM_MF	G-protein coupled peptide receptor activity	1.02
GOTERM_MF	Olfactory receptor activity	1.01
KEGG PATHWAY	Neuroactive ligand-receptor interaction	1.97
KEGG PATHWAY	Calcium signaling pathway	1.51

common targets were shared by both GLP-1 and geniposide (Table 2). The prediction results showed that *Agtr1* and *Agtr1b* were among the most common targets. *Agtr1* and *Agtr1b*, as well as our hub mRNA *Agtr1a*, all belong to the *Agtr* family. Additionally, *Htr2a* was also present in the GLP-1 targets. This was consistent with our mining of *Htr2a* and *Agtr1a* as target mRNAs that mediate the functions of geniposide, confirming the accuracy of our analysis. On the other hand, *Pomc* was not found as a GLP-1 or geniposide target per SwissTargetPrediction, but was predicted as the hub mRNA with the highest score by CytoHubba, suggesting that *Pomc* was a potential GLP-1 and geniposide target.

Co-expression network among hub mRNAs and DE lncRNAs

To explore the key lncRNAs that are closely related to the hub mRNAs, a co-expression network was constructed based on the Pearson coefficient of DE lncRNAs and mRNA expression levels. The number of lncRNAs identified to be co-expressed

Table 2 Common molecular targets of glucagon-like peptide-1 and geniposide from SwissTargetPrediction

Common name	Uniprot ID	Target class
Ace	P47820	Ligand-gated ion channel
Adrb3	P26255	Electrochemical transporter
Agtr1	P25095	Family A G protein-coupled receptor
Agtr1b	P29089	Family A G protein-coupled receptor
App	P08592	Secreted protein
Ca2	P27139	Transferase
Chrna7	Q05941	Hydrolase
Ctsk	O35186	Protease
Ednra	P26684	Enzyme
Ednrb	P21451	Family A G protein-coupled receptor
Hdac1	Q4QQW4	Nuclear receptor
Hmgcr	P51639	Enzyme
Mme	P07861	Enzyme
Oprm1	P33535	Family A G protein-coupled receptor
Pparg	O88275	Nuclear receptor
Prkcg	P63319	Structural protein
Ptgs2	P35355	Family A G protein-coupled receptor
Pygl	P09811	Enzyme
Ren1	P08424	Family A G protein-coupled receptor
Slc6a3	P23977	Electrochemical transporter
Tert	Q673L6	Enzyme
Trpv1	O35433	Voltage-gated ion channel

with *Pomc*, *Htr2a*, and *Agtr1a* was 21 (Figure 5A and 5B). The fold change and the Pearson coefficient of each lncRNA are shown in Table 3.

The expression of the three hub mRNAs (*Pomc*, *Htr2a*, and *Agtr1a*) was downregulated in geniposide-treated INS-1 cells compared to that in untreated cells. Among the 21 co-expressed lncRNAs, 11 (NONRATT002662, NONRATT024273, NONRATT012943, NONRATT027738, NONRATT005090, NONRATT012881, NONRATT005619, NONRATT019513, NONRATT027888, NONRATT006762, and NONRATT030038) were downregulated and positively correlated with hub mRNAs. The correlation between the expression levels of the 11 lncRNAs and hub mRNAs prompted us to explore whether they are functionally related.

Construction of ceRNA network

A ceRNA network was constructed based on the common miRNAs that could bind to the 11 lncRNAs, as well as the three hub mRNAs. Based on the intersection of the prediction results from MiRanda and TargetScan, we obtained 77, 16, and 23 miRNAs that could bind to the 3' UTR of *Htr2a*, *Pomc*, and *Agtr1a*, respectively. Among these miRNAs, we found that rno-miR-449a-5p could interact with both *Htr2a* and *Pomc*, while rno-miR-5132-3p, rno-miR-344g, rno-miR-3075, rno-miR-378a-5p, and rno-miR-874-3p could interact with both *Htr2a* and *Agtr1a*.

Then, we screened lncRNAs that could be bound by these six miRNAs from the 11 co-expressed lncRNAs and found seven such lncRNAs. Based on this analysis, a ceRNA network composed of three hub mRNAs, six miRNAs, and seven lncRNAs was constructed (Figure 6A). This network suggested that lncRNAs (NONRATT002662, NONRATT005090, NONRATT005619, NONRATT019513, NONRATT027738, NONRATT027888, and NONRATT030038) may competitively bind to miRNAs (rno-miR-449a-5p, rno-miR-5132-3p, rno-miR-344g, rno-miR-3075, rno-miR-378a-5p, and rno-miR-874-3p), and thereby affect the expression and function of

Table 3 Long noncoding RNAs co-expressed with *Pomc*, *Htr2a*, and *Agtr1a*

Accession No.	Fold change	Pearson coefficient		
		*Pomc*_R	*Htr2a*_R	*Agtr1a*_R
NONRATT002662	0.1959	0.9090	0.9755	0.9817
NONRATT024273	0.2602	0.9154	0.9771	0.9545
NONRATT012943	0.3411	0.9204	0.9472	0.9517
NONRATT027738	0.3471	0.9406	0.9727	0.9301
NONRATT005090	0.3603	0.9784	0.9478	0.9135
NONRATT012881	0.4662	0.9546	0.9744	0.9543
NONRATT005619	0.5677	0.9246	0.9325	0.9156
NONRATT019513	0.5766	0.9175	0.9455	0.9540
NONRATT027888	0.6136	0.9008	0.9814	0.9888
NONRATT006762	0.6532	0.9681	0.9586	0.9307
NONRATT030038	0.6647	0.9150	0.9693	0.9579
NONRATT018166	1.5013	-0.9275	-0.9451	-0.9448
NONRATT009725	1.5275	-0.9611	-0.9396	-0.9083
NONRATT029435	1.5916	-0.9026	-0.9437	-0.9207
NONRATT011324	1.6616	-0.9657	-0.9531	-0.9340
NONRATT028536	1.7515	-0.9181	-0.9419	-0.9257
NONRATT022232	1.8792	-0.9313	-0.9539	-0.9562
NONRATT008800	1.9416	-0.9273	-0.9485	-0.9313
NONRATT019269	2.1925	-0.9377	-0.9152	-0.9126
NONRATT011068	2.4783	-0.9561	-0.9828	-0.9551
NONRATT018149	3.9636	-0.9114	-0.9435	-0.9337

Pearson coefficient < 0 means a negative correlation, and Pearson coefficient > 0 means a positive correlation.

hub mRNAs (*Pomc*, *Htr2a*, and *Agtr1a*). Further analysis of the network indicated that *NONRATT027738* can regulate all the three hub mRNAs (*Pomc*, *Htr2a*, and *Agtr1a*) through miRNAs (rno-miR-449a-5p, rno-miR-5132-3p, and rno-miR-378a-5p) (Figure 6B).

DISCUSSION

GLP-1RA increases β cell sensitivity to glucose and protects β cells from apoptosis[25]. The insulinotropic effects of GLP-1RA are glucose-dependent, posing a low risk for hypoglycemia[26]. To further understand the functions of GLP-1RA in β cells, it is essential to identify the molecular mechanisms involved. Increasing evidence indicates that lncRNAs play key roles in many biological processes, including insulin secretion and cell proliferation by the ceRNA mechanism[27]. In this study, we analyzed the microarray data of INS-1 cells treated with geniposide, which is a GLP-1RA confirmed by plenty of studies[10,13,14]. Our input data were analyzed using bioinformatic tools, including analyses of GO/KEGG pathway, PPI network, co-expression network, and the ceRNA network. With the bioinformatic tools, we identified three hub mRNAs, seven lncRNAs, and six miRNAs that mediated the protective effects of GLP-1RA in β cells through the ceRNA mechanism.

Pomc, *Htr2a*, and *Agtr1a* were also identified as hub mRNAs in the ceRNA network of pancreatic islet-like cell clusters. *Pomc* expression was downregulated in T3pi cells, which could increase β cell proportions and insulin synthesis[28]. Dominguez *et al*[29] compared the mRNA expression in pancreatic islets from type 2 diabetic and non-diabetic patients. The mRNA expression levels were higher in T2DM patients;

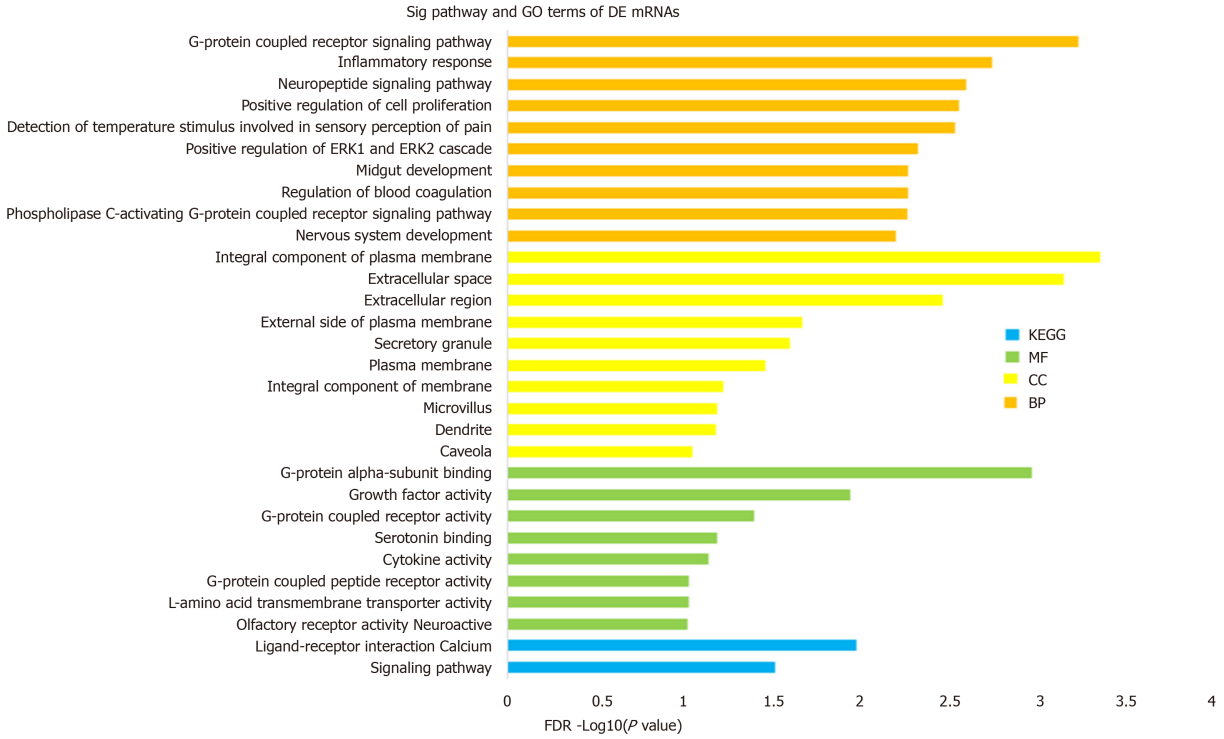

Figure 2 Gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment of differentially expressed mRNAs. Gene Ontology (GO) analysis can be divided into three parts: Molecular function, biological process, and cellular component, which respectively describe the molecular functions of potential gene products, the biological processes involved, and the cellular environments in which they are located. Enrichment analysis was performed *via* the DAVID 6.8 database. The enriched terms of GO and Kyoto Encyclopedia of Genes and Genomes pathway analysis are arranged in descending order according to -log10 (*P* value). BP: Biological process; MF: Molecular function; CC: Cellular component; KEGG: Kyoto Encyclopedia of Genes and Genomes.

however, their findings confirmed that the lower expression of *Pomc* was protective to β cells. It was reported that the expression of 5-hydroxytryptamine (5-HT) participates in the regulation of insulin secretion, and overexpression of *Htr2a* is associated with islet dysfunction in T2DM[30]. Testosterone was shown to prevent pancreatic β cell apoptosis by suppressing *Agtr1a* expression[31]. These studies further confirm our finding that the downregulation of *Pomc*, *Htr2a*, and *Agtr1a* is protective to β cells.

Based on the GO and KEGG analyses, *Pomc*, *Htr2a*, and *Agtr1a* were enriched in the G-protein-coupled receptor signaling pathway, serotonin receptor signaling pathway, inflammatory response, positive regulation of cell proliferation, ERK1 and ERK2 cascade, and cytosolic calcium ion concentration. More studies have shown that the ERK signaling pathway[32], calcium ion concentration[33], inflammatory response[34], and cell proliferation are essential for augmenting insulin secretion and β cell mass protection from premature apoptosis.

Additionally, through the SwissTargetPrediction database, we verified that *Htr2a* and *Agtr1a* are GLP-1 and geniposide targets. This result strongly confirmed the accuracy of our screening for hub mRNAs. Specifically, *Pomc* scored highest among the hub mRNAs based on our CytoHubba analysis, suggesting that *Pomc* is a potential GLP-1RA target.

Our analysis showed that six miRNAs were involved in mediating GLP-1RA function within the ceRNA network. In support of our results, four of the six miRNAs (*miR-449a*, *miR-378a*, *miR-344*, and *miR-874*) have already been implicated in regulating insulin signaling, improving metabolic dysregulation, and activating insulin synthesis[35-38]. The other two miRNAs (*miR-5132-3p* and *miR-3075)* have been suggested to play a regulatory role in the proliferation and migration of osteoblasts and Schwann cells[39,40]. Hence, we propose that *miR-5132-3p* and *miR-3075* may act as new effector molecules in β-cell regulation.

LncRNAs can act as miRNA sponges to regulate mRNA expression and activity *via* the ceRNA mechanism[24]. Recently, studies have revealed that lncRNAs are involved in the process of insulin secretion and β cell apoptosis through the ceRNA mechanism[41,42]. In this study, we identified seven lncRNAs (NONRATT002662, NONRATT005090, NONRATT005619, NONRATT019513, NONRATT027738, NONRATT027888, and NONRATT030038) that competitively bind to six miRNAs

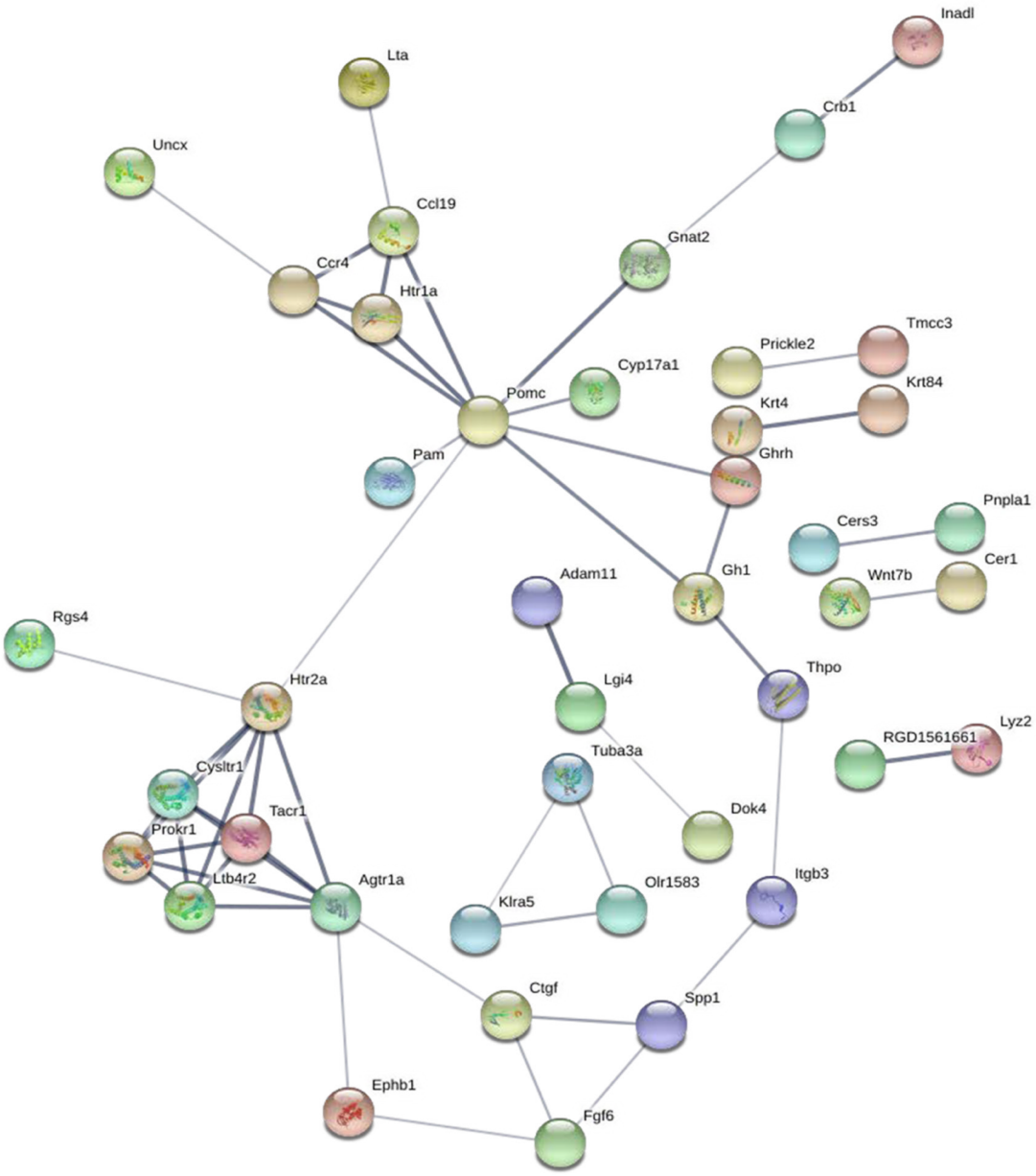

Figure 3 Protein-protein interaction network of differentially expressed mRNAs. The protein-protein interaction network was constructed through the STRING database for all differentially expressed mRNAs that were enriched in the Gene Ontology and Kyoto Encyclopedia of Genes and Genomes terms. This network includes 120 nodes and 52 edges.

(rno-miR-449a-5p, rno-miR-5132-3p, rno-miR-344g, rno-miR-3075, rno-miR-378a-5p, and rno-miR-874-3p), thereby influencing the expression and function of hub mRNAs (*Pomc*, *Htr2a*, and *Agtr1a*). Deciphering this lncRNA-miRNA-mRNA network deepens our understanding of the ceRNA mechanism in the protective effect of GLP-1RA in β cells.

Considering that therapeutic RNAi technology has now been tested in humans[43,44], we believe that our report provides novel RNAs as potential therapeutic targets in the GLP-1RA-mediated protection of β cells. However, further studies are required to better understand and confirm the specific function of these RNAs in β cells.

In summary, this study revealed the expression profiles of lncRNAs and mRNAs in geniposide-treated INS-1 cells. Further exploration *via* biological information analysis demonstrated that the ceRNA mechanism is involved in the regulatory relationship between lncRNAs and mRNAs in β cells. These findings provide significant insight in understanding the mechanisms of GLP-1RA function at the transcription level.

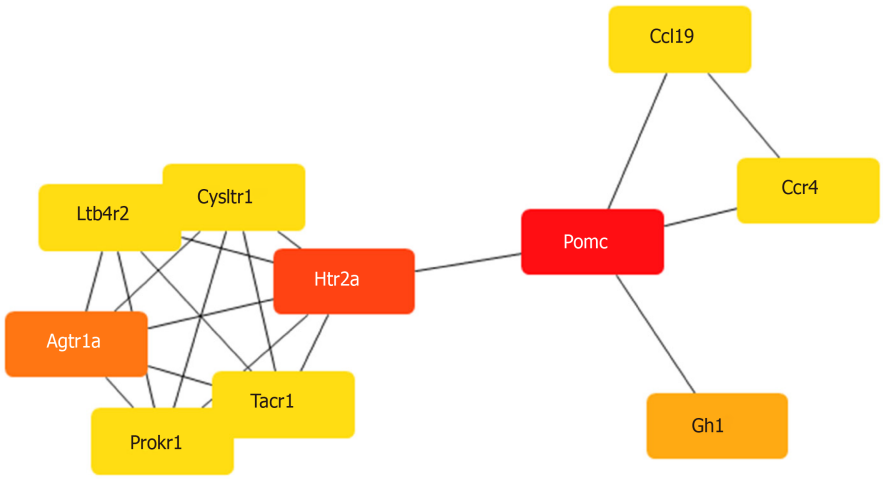

Figure 4 Hub mRNAs. The Cytoscape plugin, CytoHubba, was used to identify hub mRNAs of the complex network. The mRNAs were colored according to their scores in CytoHubba. The darker the color, the higher the score. By combining the scores of the 11 algorithms of CytoHubba, the mRNAs *Pomc*, *Htr2a*, and *Agtr1a* got the top three scores and were thus considered to be hub mRNAs.

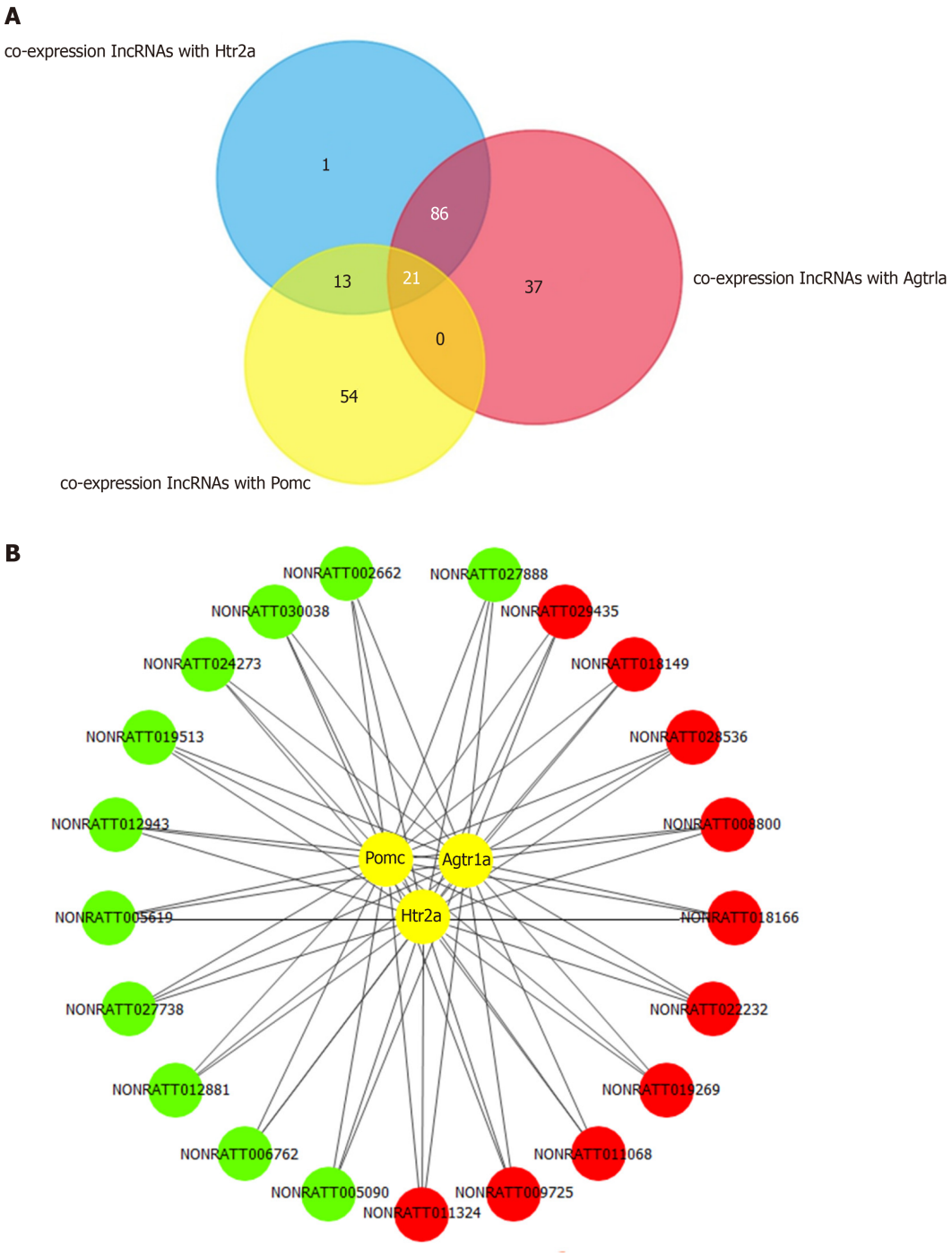

Figure 5 Co-expression network of long noncoding RNAs and hub mRNAs. A: Venn diagram of co-expressed long noncoding RNAs (lncRNAs) with *Pomc*, *Htr2a*, and *Agtr1a*. A co-expression network was constructed based on the Pearson coefficient with the filter condition as *P* value < 0.05 and |R| > 0.90. Twenty-one common lncRNAs were obtained by intersecting the co-expressed lncRNAs of *Pomc*, *Htr2a*, and *Agtr1a*. B: The 21 common lncRNAs co-expressed with *Pomc*, *Htr2a*, and *Agtr1a*, including 11 downregulated and 10 upregulated lncRNAs. Red circles represent upregulated lncRNAs, whereas the green circles represent downregulated lncRNAs.

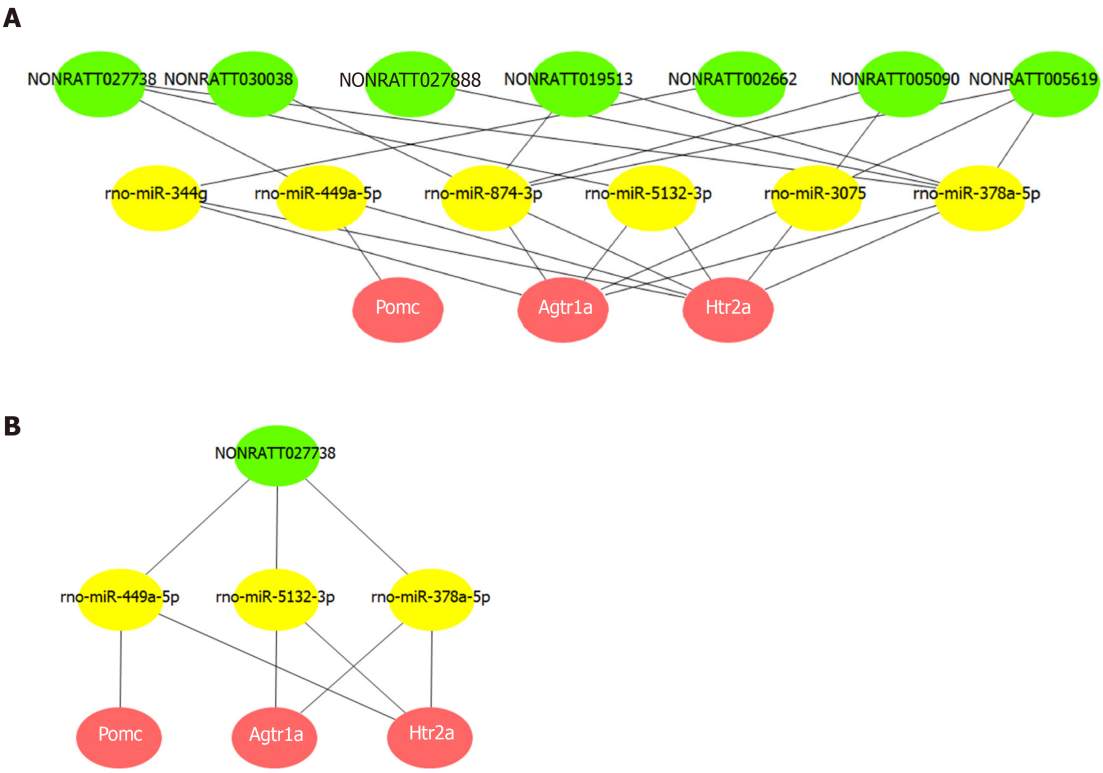

Figure 6 Competing endogenous RNA network of key long noncoding RNAs and *Pomc*, *Htr2a*, and *Agtr1a*. A: The competing endogenous RNA network was constructed *via* common miRNAs that could bind to these 11 long noncoding RNAs (lncRNAs), as well as the three hub mRNAs. Based on the intersection of the predictions from miRanda and TargetScan, seven lncRNAs were found to competitively bind six miRNAs, thereby affecting the expression and function of the hub mRNAs. B: Further network analysis indicated that NONRATT027738 can regulate all the three hub mRNAs (*Pomc*, *Htr2a*, and *Agtr1a*) through miRNAs (rno-miR-449a-5p, rno-miR-5132-3p, and rno-miR-378a-5p).

ARTICLE HIGHLIGHTS

Research background

As a class of promising anti-diabetic drugs, glucagon-like peptide-1 receptor agonists (GLP-1RAs) have been shown to prevent β cells from apoptosis and potentiate insulin secretion in a glucose-dependent manner, which can decrease blood glucose levels without the risk of hypoglycemia. Long noncoding RNAs (lncRNA) are transcripts that are longer than 200 nucleotides and do not code for proteins. Growing evidence demonstrates that lncRNAs regulate mRNA expression by competing with miRNAs, which was termed as "ceRNA mechanism". Studies have demonstrated that ceRNA mechanism is widely involved in multiple biological processes, including insulin signal transduction that may affect diabetes development. Currently, the mechanisms of the protective effect of GLP-1RA on β cells have been widely investigated; however, the specific lncRNAs and mRNAs and their functions in these processes have not been fully identified and elucidated.

Research motivation

Is there any specific lncRNAs that participate in the protective effect of GLP-1RAs in β cells? What is the mechanism of lncRNAs involved in this process? Answering these questions will provide significant insight in understanding the mechanisms of GLP-1RA function at the transcription level.

Research objectives

We and other researchers found that geniposide potentiates insulin secretion, promotes proliferation, and decreases the rate of β cell apoptosis by stimulating the GLP-1 receptor. In this study, we further identified the lncRNAs and mRNAs that were involved in the protective effect of geniposide on β cells, and their roles. This will be helpful for in-depth exploration of the mechanism of GLP-1RAs function in β cells.

Research methods

Rat gene microarray was used to screen differentially expressed (DE) lncRNAs and mRNAs in β cells treated with geniposide, a GLP-1RA. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed to assess the underlying functions of DE mRNAs. Hub mRNAs were filtered using the STRING database and the Cytoscape plugin, CytoHubba. In order to reveal the regulatory relationship between lncRNAs and hub mRNAs, their co-expression network was constructed based on the Pearson coefficient of DE lncRNAs and mRNAs, and competing endogenous (ceRNA) mechanism was explored through miRanda and TargetScan databases.

Research results

We identified 308 DE lncRNAs and 128 DE mRNAs with a fold change filter of ≥ 1.5 and P value < 0.05. GO and KEGG pathway enrichment analyses indicated that the most enriched terms were G-protein coupled receptor signaling pathway, inflammatory response, calcium signaling pathway, positive regulation of cell proliferation, and ERK1 and ERK2 cascade. *Pomc*, *Htr2a*, and *Agtr1a* were screened as hub mRNAs using the STRING database and the Cytoscape plugin, CytoHubba. This result was further verified using SwissTargetPrediction tool. Through the co-expression network and competing endogenous (ceRNA) mechanism, we identified seven lncRNAs (NONRATT027738, NONRATT027888, NONRATT030038, *etc*.) co-expressed with the three hub mRNAs (*Pomc, Htr2a, and Agtr1a*) based on the Pearson coefficient of the expression levels. These lncRNAs regulated hub mRNA functions by competing with six miRNAs (rno-miR-5132-3p, rno-miR-344g, rno-miR-3075, *etc*.) *via* the ceRNA mechanism. Further analysis indicated that lncRNA *NONRATT027738* interacts with all the three hub mRNAs, suggesting that it is at a core position within the ceRNA network.

Research conclusions

We have identified key lncRNAs and mRNAs, and highlighted here how they interact through the ceRNA mechanism to mediate the protective effect of GLP-1RA in β cells.

Research perspectives

The "ceRNA mechanism", which is widely involved in multiple biological processes, mediates the protective effect of GLP-1RAs in β cells. The value of bioinformatics allows scientists to create comprehensive databases of biological and health information that can be used to test theories and generate solutions to medical problems that affect us all.

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SYSTEMATIC REVIEWS

Lower extremity amputations and long-term outcomes in diabetic foot ulcers: A systematic review

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Abstract

BACKGROUND

Diabetes mellitus causes a large majority of non-traumatic major and minor amputations globally. Patients with diabetes are clinically complex with a multifactorial association between diabetic foot ulcers (DFU) and subsequent lower extremity amputations (LEA). Few studies show the long-term outcomes within the cohort of DFU-associated LEA.

AIM

To highlight the long-term outcomes of LEA as a result of DFU.

METHODS

PubMed/MEDLINE and Google Scholar were searched for key terms, "diabetes", "foot ulcers", "amputations" and "outcomes". Outcomes such as mortality, re-amputation, re-ulceration and functional impact were recorded. Peer-reviewed studies with adult patients who had DFU, subsequent amputation and follow up of at least 1 year were included. Non-English language articles or studies involving children were excluded.

RESULTS

A total of 22 publications with a total of 2334 patients were selected against the inclusion criteria for review. The weighted mean of re-amputation was 20.14%, 29.63% and 45.72% at 1, 3 and 5 years respectively. The weighted mean of

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mortality at 1, 3 and 5 years were 13.62%, 30.25% and 50.55% respectively with significantly higher rates associated with major amputation, re-amputation and ischemic cardiomyopathy.

CONCLUSION
Previous LEA, level of the LEA and patient comorbidities were significant risk factors contributing to re-ulceration, re-amputation, mortality and depreciated functional status.

Key Words: Lower extremity amputations; Long-term outcomes; Diabetic foot ulcers; Quality of life; Re-amputation; Diabetes

Core Tip: There was a significantly higher degree of re-amputation and mortality in those who undergo amputations due to diabetic foot ulcers in addition to impact on quality of life. Data on long-term outcomes in these patients were limited and requires further research to better understand the long-term outcomes in this subset of patients.

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INTRODUCTION

Diabetes mellitus is a leading cause for non-traumatic major and minor amputations worldwide. The global diabetes prevalence is expected to rise to 10.2% by 2030, reflecting a considerable health and financial burden across the world due to its recognised aetiology in lower extremity amputations (LEA)[1]. The combination of peripheral neuropathy and vascular disease gives rise to diabetic foot ulcers (DFU) subsequently leading to a higher rate of LEA. LEA is defined as surgical removal of bones and soft tissue by transecting at any level of the lower extremity and can be classified into minor and major amputation. The globally accepted definition of minor amputation is below ankle joint encompassing forefoot and toe while major amputations are at or proximal to the ankle joint such as below or above knee amputation.

The associated immunosuppression as well as impaired blood flow to DFU make conservative treatment with antibiotics difficult and usually mandate extensive, repeated debridement or eventual amputation. Patients with diabetes have a varied, yet overall increased risk of LEA with an incidence of 50-500 per 100000[2]. The short- and long-term outcomes as a result of LEA are profound, as patients with diabetes often have complex comorbidities and socioeconomic backgrounds. Short-term outcomes following LEA are poor, with early post-operatively mortality up to 22%[3]. There is currently no systematic review nor meta-analysis published in this field.

The objective of this systematic review and meta-analysis was to elucidate the long-term outcomes in diabetic patients who have LEA as a result of an ulcer. Outcomes such as mortality, re-amputation, re-ulceration and functional return are discussed with an emphasis of associated risk factors.

MATERIALS AND METHODS

The authors performed a systematic review of electronic databases and peer-reviewed sources including PubMed/MEDLINE up to March 25, 2020. Key search terms "Diabetes", "Foot Ulcers", "Amputations" and "Outcomes" were used and the authors hand searched each identified manuscript for pertinent references. The inclusion criteria were as follows: (1) Age > 18 years; (2) Presence of DFU prior to undergoing an amputation; and (3) Outcomes measured at longer than at least 12 mo. The exclusion

criteria was limited to studies relating to paediatric patients. Only English language studies with original data were included with no restriction on publication date. Duplicate results were excluded. Each study was assessed for selection by two individual authors with a plan for escalation to corresponding authors, should there be a disagreement. No studies required further consensus. Statistical analysis looking at weighted mean age, rates of mortality at 1, 3 and 5 years and re-amputation were calculated. Individual studies were examined for bias, in particular, selection, attrition and recall bias. Significant loss to follow up, degree of heterogeneity and presence of confounding factors were examined. The PRISMA 2009 Checklist was followed. No conflict of interests are declared with no external funding sought for this study.

RESULTS

Key search terms yielded a total of 125 results of which 22 publications were selected against the inclusion and exclusion criteria for review. Of the selected studies, 11 studies were prospective, 8 retrospective, 3 systematic review and meta-analyses (Table 1). Overall, the majority of selected articles were level III evidence but ranged from level II-V and no randomized controlled trials were found. Sixteen of the 18 studies conducted standardisation of the patient cohort with regards to co-morbidities. In the 9 prospective studies, only three studies had any loss of follow up in patients (range 5%-20%).

Rates of re-ulceration were reported to be higher in patients with history of LEA and DFU compared to conservative management in both ipsilateral and contralateral limbs. The weighted mean was calculated for both re-amputation and mortality. A total of 9 articles reported rates of re-amputation of which only 2 articles specified rates at 1, 3 and 5 years. With the exclusion of 2 publications, all articles were published after 2001 and the calculated weighted mean of re-amputation was 20.14%, 29.63% and 45.72% at 1, 3 and 5 years respectively (Table 2). A total of 8 articles specify mortality outcomes at 1, 3 and 5 years, of which, only 2 articles were published prior to 2001 (Table 3). The cumulative total of 994 patients found a mean age of 70.2 years and exhibited a male predominance. The weighted mean mortality at 1, 3 and 5 years were 13.62%, 30.25% and 50.55% respectively and were only calculated using the studies which specified each interval's long-term outcome.

DISCUSSION

There is a paucity of data pertaining to long-term outcomes of patients with DFU-associated LEA. Few studies exceed 5 years with the longest study assessing outcomes just over 10 years. The main outcomes of this systematic review and meta-analysis found a weighted mean of mortality at 1, 3 and 5 years were 13.62%, 30.25% and 50.55% respectively and weighted mean of re-amputation at 1, 3 and 5 years were 20.14%, 29.63% and 45.72% respectively. Major amputation was found to be a negative prognostic factor for return to activities of daily living.

Re-ulceration

Patients with a previous LEA for DFU have a higher risk of re-ulceration than patients undergoing conservative treatment for DFU. In a 4-year follow-up study, 40% of patients with previous LEA had re-ulceration at 1 year compared to 30% of the non-LEA group. At the 3 and 5-year mark, the ulcer recurrence was 70% and 75% in the LEA group compared to 52% and 60% in the non-LEA group[4]. Following LEA, there is a high risk of re-ulceration of both the ipsilateral and contralateral limb with the former being higher. In a 5-year follow-up study of 245 patients who had undergone toe amputation for DFU, the cumulative incidence of a new foot ulcer at 1,3 and 5 years was 27.3%, 57.2% and 74.4% respectively. The rate of ulceration in the contralateral limb has been shown to be as high as 23%[5].

Re-amputation

Previous LEA is an important, independent risk factor for further amputations. The earliest studies in the late 1990s examining re-amputations in patients involved rates as high as 60% over 10 years in 90 patients with a Swedish study of 189 patients reporting re-amputation rates at 14%, 30% and 49% at years 1, 3 and 5 after the index LEA[6,7].

Table 1 Follow-up, nature, and presence of standardisation in selected studies

Ref.	Number of patients lost to follow up (5)	Nature of study	Standardisation for comorbidities
Chu *et al*[12]	17 (6.9)	Prospective	Standardised
Larrson *et al*[7]	0	Prospective	Not standardised
Schleiffer *et al*[23]	0	Prospective	Standardised
Faglia *et al*[20]	0	Prospective	Standardised
Van Damme *et al*[13]	0	Prospective	Standardised
Goldner *et al*[5]	NS	Prospective	Standardised
Morbach *et al*[16]	0	Prospective	Standardised
Ghanassia *et al*[8]	5 (5)	Prospective	Standardised
Skoutas *et al*[10]	0	Prospective	Standardised
Adler *et al*[24]	155 (2)	Prospective	Standardised
Ohsawa *et al*[25]	0	Prospective	Standardised
Izumi *et al*[9]	N/A	Retrospective	Standardised
Murdoch *et al*[6]	N/A	Retrospective	Standardised
Uzzaman *et al*[26]	N/A	Retrospective	Standardised
Acar *et al*[11]	N/A	Retrospective	Standardised
Nerone *et al*[17]	N/A	Retrospective	Standardised
Aulivola *et al*[27]	N/A	Retrospective	Not standardised
Jeyaraman *et al*[18]	N/A	Retrospective	Standardised
Jeyaraman *et al*[21]	N/A	Retrospective	Standardised
Thorud *et al*[15]	N/A	Meta-analyses	Standardised
Albers *et al*[28]	N/A	Meta-analyses	Standardised
Borkosky *et al*[14]	N/A	Systematic Review	Standardised

NS: Not specified; N/A: Not applicable.

More recently, in a study of 39 patients over 6.5 years, the re-amputation rate remained high at 55.6%[8]. This high rate was also observed over time in another study of 277 patients over 10 years where re-amputation at 1, 3 and 5 years was 26.7%, 48.3% and 60.7%[9]. Other studies show a lower re-amputation rate. A re-amputation rate of 21.5% in a study of 121 patients , 16.7% in 132 patients following DFU-related LEA and 12.5%, 22.3% and 47.1% in 245 patients at 1, 3 and 5 years[10-12].

There is conflicting evidence regarding the role of the index LEA in the risk of subsequent re-amputation. Murdoch *et al*[6] in the 1990s first illustrated that a large proportion of patients with an LEA at the level of the great toe or ray amputation received a higher-level amputation in the first 12 mo[6]. In 146 patients over a 5-year follow up period, a higher rate of re-amputation was observed in primary toe compared to a more proximal forefoot index LEA (28% *vs* 24%)[13].

A systematic review conducted by Borkosky *et al*[14] of 435 index ray amputations showed a re-amputation incidence of 19.8% over a mean follow-up of 26 mo. In response to this high rate of re-amputation, Throud *et al*[15] assessed the viability of a more proximal transmetatarsal amputation. A higher rate of 29.7% was observed in this systematic review of 1453 patients. The most statistically significant difference, however, was seen in 121 patients assessed by Skoutas *et al*[10] In this study with a follow-up of 18 mo, re-amputation rate following a toe and ray amputation were significantly higher compared to an index major LEA at all 1, 3 and 5 years. Overall, the rate of ipsilateral re-amputation significantly reduced by 34% as the level of the original LEA went higher.

However, the Swedish study in 1998 showed no difference in rate of re-amputation following an index major or minor LEA[7]. Similarly, in another study of 247 patients, there was no statistical difference in re-amputation rate regardless of the nature of

Table 2 Re-amputation at 1, 3 and 5 years in patients who previously had had a lower extremity amputation due to diabetes foot ulcer

Ref.	Year	*n*	Re-amputation, %		
			1 yr	3 yr	5 yr
Acar *et al*[11]	2017	132	NS	22	N/A
Uzzaman *et al*[26]	2016	79	NS	NS	13.9
Chu *et al*[12]	2014	245	12.5	22.3	47.1
Skoutas *et al*[10]	2009	121	21.5	N/A	N/A
Ghanassia *et al*[8]	2008	39	NS	NS	55.6
Izumi *et al*[9]	2006	277	26.7	48.3	60.7
Faglia *et al*[20]	2001	80	0	0	0
Murdoch *et al*[6]	1997	90	NS	NS	60
Hosch *et al*[29]	1997	35	63	N/A	N/A
Weighted, mean ± SD			20.14 ± 3.55	29.63 ± 8.35	45.72 ± 9.09

NS: Not specified; N/A: Not applicable.

Table 3 Mortality at 1, 3 and 5 years in patients who previously had had a lower extremity amputation due to diabetes foot ulcer

Ref.	Year	*n*	Mean age	Male, %	Mortality, %		
					1 yr	3 yr	5 yr
López-Valverde *et al*[30]	2018	203	72	78	9.4	27.2	44.5
Uzzaman *et al*[26]	2016	79	75	64.5	15.6	25	27
Chu *et al*[12]	2014	245	69.27	53	5.8	15.1	32.7
Morbach *et al*[16]	2012	38	68.8	58.7	15.4	33.1	45.8
Ghanassia *et al*[8]	2008	39	63.8	69.7	12	35	44
Faglia *et al*[20]	2001	80	63.4	73	33.3	51.9	74.1
Larrson *et al*[7]	1998	187	72	56	15	38	68
Apelqvist *et al*[4]	1993	123	70	55	20	41	73
Weighted, mean ± SD					13.62 ± 0.92	30.25 ± 2.12	50.55 ± 4.13

index LEA in the follow up period of 10 years[16]. With regards to contralateral re-amputation, the level of index LEA was also not statistically significant. The effect of the level of index LEA on subsequent re-amputation can be extremely useful in clinical decision making and have a significant effect on patient outcomes. Larger cohorts are needed to establish a meaningful association or lack thereof between index level LEA and re-amputation. In addition, recording the time of subsequent amputations may provide further insight into differences between type of index amputation and the interval time to re-amputation.

The presence and severity of peripheral arterial disease (PAD) was also seen as a significant risk factor in the need for re-amputation. In a study of 163 patients with an index DFU-associated LEA, rates of re-amputation were significantly related to the presence and severity of PAD[17]. Over a mean follow-up of 3.65 years, these patients either had a subsequent major or minor re-amputation. A higher proportion of the major group (111 patients) had PAD compared to in the minor (52 patients) group (71.15% *vs* 22.23%; $P < 0.001$). Furthermore, there was a significantly decreased interval to a major amputation if PAD was present and whether it was mild to moderate (1.62 years) or severe (1.53 years) than if no PAD (3.24 years).

An Australian study done by Jeyaraman *et al*[18] of 513 patients with DFU with a mean follow-up of 5.8 years showed prior LEA was an independent factor to subsequent re-amputation. In the 263 patients who had a LEA, 85 (32.3%) had a prior LEA which was statistically significant. The odds ratio (OR) for any subsequent LEA

was 4.49 (95% confidence interval 1.69-11.9), further broken down into 4.84 in the minor group and 3.06 for a subsequent major LEA.

Mortality

We found a total of 8 articles which had reported on up to 5-year mortality in patients undergoing major and minor amputations secondary to DFU with interval data at 1, 3 and 5 years (Table 2). The weighted mean mortality at 1, 3 and 5 years were 13.62%, 30.25% and 50.55% respectively (Table 3). Furthermore, six other studies illustrated similar mortality at varied follow-up periods ranging from 1 to 10 years. Comparable 3-year mortality was reported by Nerone *et al*[17] and Ramsey *et al*[19] of 28.85% and 28% respectively.

Chu *et al*[12] (2014) is the largest study to date which observed 245 patients for 5 years post DFU-associated LEA and reported the cumulative mortality of 5.8%, 15.1% and 32.7% at 1, 3 and 5 years respectively. The overall all-cause mortality in this study was 37.8% with an average survival time for deceased patients of 3.8 years and a longer duration of survival in females (4.1 years). Cause of death varied including foot-related deaths (25.7%), renal failure (22.9%), heart failure (18.6%) and malignancy (17.1%). Age > 70, poor glycemic control (HbA1c > 9, $P < 0.01$), critical limb ischemia (OR = 5.60; 95%CI: 2.41-12.98, $P < 0.01$), diabetic nephropathy (OR = 3.86; 95%CI: 1.65-9.03; $P < 0.01$), level of amputation and re-amputation were identified as independent risk factors for impaired wound healing, re-ulceration, re-amputation and mortality.

The level and previous history of amputation were observed as risk factors. In Larson *et al*[7], a statistically significant higher mortality was observed following major index LEA than minor. Overall, mortality rates at 1, 3 and 5 year were also higher than previous studies (15%, 38% and 68%) respectively. Apelqvist *et al*[4] (1993) reported a higher long-term (1, 3 and 5 year) mortality rate among patients with a previous amputation from a diabetic foot ulcer compared to new, primary amputation (20%, 41%, 73% *vs* 8%, 27%, 42%).

Faglia *et al*[20] reported higher rates of mortality at each interval 33.3%, 51.9% and 74% of which ischemic cardiomyopathy was identified as the most frequent cause of death. A large proportion (47%) of this study population were affected with ischemic cardiomyopathy unlike a much smaller percentage reported in previous studies. Subsequently, it is an important independent patient characteristic to consider when assessing risk in patients undergoing LEA with DFU given there were no re-amputations in this cohort. Chu *et al*[12] also reported ischemic heart disease (HR = 1.6, 95%CI: 1.1-2.4) in addition to deeper ulcers with bone involvement (HR = 1.5, 95%CI: 1.2-1.7) as positive predictors for death.

In the Australian study of 513 patients conducted by Jeyaraman *et al*[21], there were 199 deaths during a mean follow-up of 5.8 years. The 5-year mortality was recorded at 24.6%, increasing to 45.4% at the 10-year mark. Of note, these patients died at an average age of 64.6 years, significantly lower than the Australian average of 80.4 years males and 84.5 years in females.

The 14 studies describing mortality post DFU-associated LEA fall within comparable ranges, however, some variability noted is due to smaller cohort sizes and presence of ischemic cardiomyopathy in particular.

Effect on the activities of daily living

There is limited data available describing the functional outcomes in patients undergoing DFU-associated LEA. Although such a cohort of patients invariably have complex socioeconomic backgrounds and variable demographics, our search yielded two studies with relatively objective measures of functional status.

Re-amputation has been noted to play an important role in return to functional status in long-term studies. Chu *et al*[12] examined the long-term impact on activities of daily living based on Barthel Index Classification (BIC). The Barthel Index of Activities of Daily Living is a simple tool measuring functional independence[22]. It comprises 10 separate sections of assessment with a total score of 100 and can be easily administered by health-care professionals. This is a world-wide accepted tool and has been utilized in a large number of studies. Chu *et al*[12] reported 31.9% of patients having moderate to severe dysfunction, assessed by the BIC, of the activities of daily living at 5 years. Furthermore, 77.9% of these patients had undergone re-amputation and 54.2% had died. In contrast, the remaining 126 patients considered to have no or mild dysfunction of activities of daily living had lower rates of re-amputation (34.9%) and mortality (30.2%). Of note, there was a higher ratio of major to minor amputations in the moderate to severe dysfunction group compared to no or mild dysfunction group [24 (major):22 (minor) *vs* 12 (major):32 (minor)].

Van Damme *et al*[13] reported that only 63% of the major amputations (n = 143)

regained an autonomic walking capability with their prosthesis suggesting poor functional recovery after major amputations undertaken for DFUs. As well as functional status, an important measure of lifestyle change in hospitalized patients is discharge destination. Larson J *et al*[7] reported a statistically significant difference in discharge following minor and major patients in 187 patients. In the minor group, 93% of patients returned home compared to only 62% in the major group ($P < 0.001$). Patients who underwent minor amputations also had a higher chance of satisfactory return to baseline function. In patients with a considerable walking capacity (> 1 km) prior to amputation, 49 of 68 patients (72.1%) who had a minor amputation regained this capacity compared to only 5 of the 28 patients (17.8%) with major amputation ($P < 0.001$).

The present study has several limitations. As this systematic review examined studies from a myriad of social backgrounds with variable demographics, heterogeneity in the studied population could not be avoided. The variable duration of follow-up also added to this heterogeneity, adding to the challenge to derive conclusions to specific groups of patients. Approximately 50% of the studies in our systematic review were retrospective, predisposing outcomes to historical or recall bias. Patients lost to follow-up could also potentially underestimate adverse outcomes leaning from the hypothesis of selection bias that such patients are less likely to be compliant with glucose monitoring. Similarly, non-participation at the start in prospective studies also represents a gap in outcomes from that particular cohort. The indications for re-amputation were not clearly noted in the examined studies making the cause-and-effect relationship from a previous amputation less reliable. Reporting of comorbidities was also patchy across studies making standardised conclusions difficult.

In conclusion, there was a significantly higher degree of re-amputation and mortality in the long-term in the population who undergo amputations due to diabetic foot ulcers. There was also a significant impact on the overall functional status of patients and quality of life. This systematic review and meta-analysis affirms the need for regular review and follow-up in this vulnerable group owing to this high risk of adverse outcomes. The effect of comorbidities was not clear and therefore further studies in a subset of patients with particular coexisting illnesses such as chronic kidney failure or peripheral vascular disease are needed.

ARTICLE HIGHLIGHTS

Research background

There is no previous systematic review and meta-analysis undertaken particularly in long-term outcomes of patients who undergo lower limb amputation (LEA) for diabetic foot ulcers (DFU). Although multiple studies describing short-term outcomes under 12 mo are available, conclusions for long-term outcomes are needed to support clinical decision-making in relation to patient characteristics.

Research motivation

Since DFU account for significant complications in patients with diabetes mellitus, the assessment of their long-term outcomes is necessary. The review of long-term outcomes following LEA is essential for decision-making and risk stratification for individual patients.

Research objectives

The aim of this paper is to establish a systematic review of long-term studies undertaken in patients who underwent LEA as a treatment modality for DFU. The focus of the review is on re-ulceration, re-amputation and the impact on the quality of life of patients. These parameters, particularly in the longer-term setting pave way for future research in larger cohorts, various demographics and relation to co-morbidities.

Research methods

Key search terms such as "diabetes", "foot ulcers", "amputations" and "outcomes" were searched on PubMed/MEDLINE and Google Scholar. A follow-up of 12 mo, age > 18 and LEA post DFU were inclusion criteria. Paediatric patients were excluded. Two co-authors selected studies based on the inclusion criteria and search results were limited to the English language. A total of 22 publications with a total of 2334 patients were selected. There were no randomised controlled trials with the majority of studies

being cohort studies.

Research results

Our results show a significant re-amputation and mortality rates at 1, 3 and 5 years after initial LEA for DFU. A positive correlation was also noted for previous other major amputation and ischemic cardiomyopathy. We attempted to standardise patients for co-morbidities, however, this was not possible in a minority of studies. Therefore, future research should be aimed at delineating the nature of association between LEA post DFU and patient co-morbidities.

Research conclusions

Our systematic review and meta-analyses support our key hypotheses of a significant positive association of re-amputation, mortality and quality of life in our set of patients on a long-term basis. The pivotal purpose of this study is data to assist patient selection and decision-making. It also supports the uniformity of similar rates of re-amputation and mortality in various studies globally with no significant outliers.

Research perspectives

Future research should be aimed at assessing the significance of co-morbidities on patients with DFU undergoing LEA. This will allow a closer risk stratification and aid patient decision-making individualised to their situation. In addition to this, as outcomes in diabetes mellitus often depend on patient compliance influenced by their socio-economic or cultural backgrounds, further studies are needed in these groups. The best methods for future studies would be larger, multi-center prospective studies.

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