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Nomilin Ameliorates Insulin Resistance via Modulating the Glucose Metabolism and PI3K/Akt/GLUT4 signaling pathway in Type 2 Diabetes

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ABSTRACT: Type 2 diabetes (T2D) became the most prevalent metabolic diseases worldwide characterized by insulin resistance (IR). Nomilin is a bioactive triterpene compound primarily found in citrus fruits and its anti-diabetes effect has been preliminary studied. This study combined network pharmacology, bioinformatics analysis and two insulin resistance cell models (HepG2 and THLE-2 cell line) were utilized to study the anti-diabetes effect of nomilin and its underlying molecular mechanisms. The network pharmacology and bioinformatics analysis showed that the PI3K/Akt signaling pathway is the critical signaling pathway to the anti-diabetes capability of nomilin. In both IR-HepG2 and IR-THLE-2 cell line, the nomilin can attenuate the high glucose and dexamethasone induced glucose consumption and uptake decrease, reactive oxygen species (ROS) and advanced glycation end-products (AGEs) generation, mitochondria pathological change, and mitochondria membrane potential loss. The Western Blot and In-cell Western results result showed that nomilin can achieve anti-diabetes effect through regulating the PI3K/Akt/GLUT4 signaling pathway and the phosphorylation of GSK3 β . This study illustrated that nomilin alleviated insulin resistance via activation of PI3K/Akt/GLUT4 signaling pathways, alleviating ROS accumulation and damage, and AGEs production, and improving the mitochondrial function. Accordingly, nomilin shows potential as a promising bioactive component for nutritional intervention and management of hepatic insulin resistance.

Keywords: Nomilin; Type 2 diabetes; Glucose consumption; Oxidative stress; PI3K/Akt/GLUT4 signaling pathway

1. Introduction

Diabetes mellitus (DM) has already become a common metabolic disease facilitating and leading to numerous complications, including nephropathy, neuropathy, and cardiomyopathy. This disease includes type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). While T1DM is characterized via insulin secretion insufficiency and T2DM is characterized via insulin sensitivity defection, also called insulin resistance (IR) ^[1]. T2DM is a complex metabolic disease, which could be attributed to insulin resistance, commonly alongside hyperlipidemia and hyperglycemia ^[2]. Over produced reactive oxygen species (ROS)

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generated under hyperglycemic conditions mediate accumulation of oxidative stress and are associated with the pathophysiology of DM and its complications [3]. Excessive ROS can damage cells, trigger insulin signaling via activation of cell death of β -cell, modify level of peripheral glucose uptake, and facilitates ascend gluconeogenesis [4]. Therefore, ROS scavenging and insulin resistance prevention may be the key to alleviate progression of T2D.

In the human body, comparing to other organs, the liver plays a pivotal role in the metabolism of carbohydrates, amino acids, lipids, and proteins [5]. Moreover, the liver plays a crucial role in maintenance in the hemostasis of blood glucose through gluconeogenesis and glycogenesis [6]. Insulin exerts its biological effects through insulin receptors of specific cells, thereby regulating blood glucose balance. The liver has traditionally been considered the primary target organ of insulin, playing a crucial role in regulating hepatic glucose homeostasis in patients with diabetes [7]. Hepatic insulin resistance can lead to disturbances in glucose balance and lipid deposition, potentially resulting in impaired glucose homeostasis. Given the common occurrence of liver damage in diabetes-related complications, treating hepatic insulin sensitivity defective is expected to become a promising and possible approach for treating diabetes [7].

Nomilin is a bioactive compound of the limonoid family, primarily found in citrus fruits, and it is also a triterpene compound. Moreover, nomilin has been widely demonstrated to possess multiple biological activities, including anticancer activity, anti-inflammatory activity, antioxidant activity, lipid-lowering activity, and antibacterial activity [8]. Regarding the anti-diabetes biological activity of nomilin, Ono et al. have preliminarily discovered in animal experiments that nomilin can achieve anti-diabetes biological activity by improving insulin resistance [9]. In addition, the results of an enzymatic study based on molecular dynamics by El-Feky et al. also indicate that nomilin is a potential anti-diabetes compound [10]. In summary, nomilin is a compound derived from citrus fruits with health benefits, perhaps great potential for anti-diabetes, but its underlying anti-diabetes mechanism is un-discovered or not yet studied. In this study, network pharmacology, bioinformatics, and two insulin-resistant cell models were utilized to determine the biological activity and discover related molecular mechanisms of nomilin about improvement on T2D by ameliorating insulin resistance.

2. Materials and Methods

2.1. Materials

The HepG2 and THLE-2 cell lines utilized in all cell experiments were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). All the materials were included and properly listed in Supplementary Table 1 with detailed experimental procedures.

2.2. Network pharmacology analysis

Overall, the methods and steps of network pharmacology analysis were based on our previous study [11]. The target of nomilin was predicted through SwissTargetPrediction and Pharmamapper databases [12, 13].

Disease-related protein targets were obtained from the GeneCards database based on the keywords of Insulin resistance and T2D [14]. The anti-diabetes targets of nomilin were obtained by intersecting nomilin targets with disease-related targets through Jvenn [15]. Protein-protein interactions (PPI) were analyzed through the String database [16]. Core target and central target were obtained through screening based on degree centrality (DC), closeness centrality (CC), betweenness centrality (BC), maximum neighborhood component (MNC), and maximal clique centrality (MCC), respectively [17]. KEGG and GO enrichment analysis were obtained through analysis using the Metascape database [18]. The GO enrichment analysis included the biological process (BP), cellular component (CC), and molecular function (MF). The KEGG and GO enrichment heatmaps were plotted by <https://www.bioinformatics.com.cn>, an online platform for data analysis and visualization [19].

2.3. Bioinformatics analysis

2.3.1. Data acquisition and processing

The transcriptome sequencing data used in this study were obtained from the Gene Expression Omnibus (GEO) database, with the GEO series accession number GSE76894 [20]. This dataset comprises RNA sequencing profiles from 103 human pancreatic islet samples isolated from cadaveric donors. Among them, 19 samples were from patients diagnosed with type 2 diabetes (T2D) (mean age: 51.2 years; mean BMI: 29.8 kg/m²; 10 males and 9 females; disease duration: 5-15 years; most cases were not on insulin therapy to avoid potential confounding effects of treatment), and 84 samples were from non-diabetic (ND) individuals with normal glucose homeostasis (mean age: 49.8 years; mean BMI: 25.4 kg/m²; 45 males and 39 females). Samples were processed to minimize batch effects, and rigorous quality control ensured high RNA integrity (RIN > 7). This dataset was specifically selected because it is one of the largest and highest-quality human islet RNA-seq cohorts available and directly focuses on islet dysfunction in T2D, which aligns closely with our emphasis on insulin resistance mechanisms. The raw data were downloaded and organized into a gene expression matrix using the R programming language.

2.3.2. Differential expression analysis of core genes

For the seven core genes, we performed differential expression analysis on the GSE76894 dataset using the limma package. Initially, a design matrix was constructed as Group (T2D vs ND). Subsequently, a linear model was fitted using `lmFit()`, and empirical Bayes robust estimation was conducted with `eBayes(trend = TRUE, robust = TRUE)`. Group comparisons were specified using `contrasts.fit()`, and the results were extracted with `topTable()`, with significance thresholds adjusted using the Benjamini–Hochberg method (`adjust.method = "BH"`) and set at FDR < 0.05. If only these seven genes were being validated, raw P values could be reported or Bonferroni correction could be applied. For visualization, boxplots were created using `ggplot2` (`geom_boxplot()`), with jittered points overlaid (`geom_jitter(width = 0.2, alpha = 0.6)`), and expression levels were uniformly transformed to $\log_2(x+1)$. Significance tests were performed using `stat_compare_means()` from `ggpubr`, with the option to select either the Wilcoxon rank-sum test (`method = "wilcox.test"`) or the t-test

(method = "t.test"). If batch effects were present, they were corrected using `removeBatchEffect()` to ensure robust results [21].

2.3.3. Immune infiltration analysis

For the analysis of immune infiltration, we used CIBERSORT (either the original R script or the CIBERSORTx platform) in combination with the LM22 gene signature matrix. The input data for the analysis was the expression matrix file of GSE76894 (with rows representing gene symbols and columns representing samples). For microarray data, we set `QN = TRUE`, while for RNA-seq data, we set `QN = FALSE`. The number of permutations (perm) was set to 1000 to ensure stable P values. The commonly used output format is relative proportion (relative), but if absolute abundance is required, `absolute = TRUE` or Absolute Mode can be enabled. The results provide the proportions of 22 immune cell types and their significance for each sample. Group differences were assessed using the Wilcoxon rank-sum test and adjusted using the Benjamini–Hochberg (BH) method. Visualization methods included stacked bar charts (to display the immune composition of samples), boxplots (to compare differences between T2D and ND), and heatmaps (sample $\times$ cell type, with column Z-score normalization) to present the overall patterns [22].

2.3.4. GSEA (Gene Set Enrichment Analysis), GSVA (Gene Set Variation Analysis), and WGCNA (Weighted Gene Co-expression Network Analysis)

GSEA was conducted using the `clusterProfiler` package (version, 4.6) in R software. A ranked gene list was first generated by sorting all genes in descending order based on $\log_2$ (Fold Change) values from differential expression analysis. Gene sets were retrieved from the Molecular Signatures Database (MSigDB, release 2025), including HALLMARK, KEGG, and other collections. The analysis was performed using the `GSEA()` function with the following key parameters: `pvalueCutoff = 0.05`, `minGSSize = 10`, `maxGSSize = 500`, and `nPerm = 1000`. Statistical significance was determined based on Normalized Enrichment Score (NES) and Benjamini-Hochberg adjusted *P*-values (`p.adjust`), with visualization performed using the `gseaplot2()` function [23].

GSVA was performed using the `GSVA` package (version, 1.52) in R software. The input consisted of a normalized expression matrix ($\log_2$ -transformed, with genes as rows and samples as columns). Gene sets were derived from pathway collections including GO Biological Process, KEGG, Reactome, etc. (MSigDB 2023). Pathway scoring was conducted using the `gsva()` function with the method set to "gsva", kernel set to the default Gaussian kernel, and parameters `min.sz = 10`, `max.sz = 500`, and `mx.diff = TRUE`. Differential analysis was subsequently performed on the resulting enrichment score matrix using the `limma` package to identify significantly enriched pathways [24].

WGCNA was conducted using the `WGCNA` package (version, 1.72) in R software. The expression matrix was first subjected to quality control by filtering low-expressed genes, and the `goodSamplesGenes()` function was employed to ensure that both samples and genes satisfied the analysis requirements. The optimal soft-thresholding power β was subsequently selected using the `pickSoftThreshold()` function based on scale-

free topology criteria; a β value of 5 was chosen for this study. Weighted co-expression networks were constructed using the `blockwiseModules()` function with the following primary parameters: power = 5, minModuleSize = 30, networkType = "signed", and mergeCutHeight = 0.25 [25].

2.4. Cell culture conditions and treatment

HepG2 and THLE-2 cell culture condition was following our previous study [17]. For IR-HepG2 or IR-THLR-2 cell modeling, cells were cultured in DMEM medium supplying high glucose (25 $\mu\text{mol/L}$, 4.5 mg/mL) and dexamethasone (20 $\mu\text{mol/L}$, 7.8 $\mu\text{g/mL}$) for 48 hours (Fig. S1.) [5, 6, 26–28], which are two common hepatic cell line studying IR. At the same time of modeling, different concentrations of nomilin or metformin were added to the culture medium for drug treatment or positive control treatment.

2.5. Cell viability measurement

The effect of nomilin on cell viability loss was measured via the CCK-8 method. HepG2 and THLE-2 cells were seeded, kept and cultured at a density of 5000 cells per well in a fully transparent 96-well plates and exposed to different concentrations (0, 8, 16, 32, 64, and 128 $\mu\text{mol/L}$) of nomilin (MCE, HY-N0547), at for 24-hour treatment before next step. After original culture medium removal, 100 μL of DMEM low glucose medium was introduced into each well, before adding 10 μL CCK-8 reagent inside, according to the user guide. The 96-well plate was next kept at 37 °C avoiding light for another 40 min incubation. Afterwards, the absorbance value at 450 nm was determined via utilizing the FLUOstar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany).

2.6. Measurement of intracellular glucose consumption by microplate reader

HepG2 and THLE2 cells were seeded at a density of 5000 cells per well in a 96-well plate for modeling treatment. After 48 hours of culture, the supernatant medium was aspirated. Before conducting the test, the working solution was prepared by mixing the Glucose Assay Buffer with Glucose Assay Reagent A, Glucose Assay Reagent B, and Enzyme Solution in the specified ratio, as instructed in the Glucose Colorimetric Assay Kit (GOD/POD Method) provided. The 160 μL pre-mixed working solution was introduced to every well without discrimination, and the reaction was allowed for 10 min maintenance at 37 °C in the dark. Then, the FLUOstar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany) was utilized to determine the absorbance value at 510 nm and proceed with data analysis.

2.7. Intracellular ROS detection by microplate reader

HepG2 and THLE2 cells were counted in advance and seeded at a density of 5000 cells/well in a fully black 96-well plate. Using the same modeling and drug administration methods, after 48 hours of culture, the ROS measurement kit was utilized, and the Carboxy-H₂DCFH-DA probe was diluted to 10 $\mu\text{mol/L}$ at final concentration. The 50 μL of pre-diluted working solution was introduced to each well to cover the cells, and incubated for 20 min in a 37 °C incubator. After incubation, the cells were washed thrice by PBS. Then, 50

μ L of PBS per well was added and detected via the FLUOstar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany) equipped with a 488 nm excitation wavelength and a 525 nm emission wavelength. The obtained data were normalized and analyzed.

2.8. Detection of intracellular AGEs via fluorescent signal and ELISA kit

HepG2 and THLE2 cells were counted in advance and seeded at a density of 5000 cells/ well in a fully black 96-well plate. The modeling and drug administration methods were the same as above. After 48 hours of culture, the cells were measured via a FLUOstar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany) equipped with a filter for excitation/emission wavelengths set at 355/405 nm ^[29]. The data were recorded for further analysis. Besides via fluorescence, AGEs of HepG2 and THLE2 were both also measured via ELISA kit (Cat. No. U96-1010E, YOBIBIO), following the user-guide provided by the manufacturer ^[5].

2.9. Mitochondrial depolarization level measurement

JC-1 was used as the professional mitochondrial depolarization (membrane potential) dye/indicator in this study, either at single cell level observation or semi-quantification under super-resolution microscope. Procedure with details was referring to methods conducted from our previous study ^[17].

2.10. Mitochondrial morphology observation and ROS measurement

Measurement of intracellular ROS level by Carboxyl-H₂DCFDA procedure is following our previous study, either at single cell level observation, semi-quantification under super-resolution microscope or bulk culture determination applying microplate reader ^[17]. Mitochondrial morphology change observation was achieved by HBmito Crimson and experimental procedure is following the user-guide provided.

2.11. Glucose uptake measurement

Glucose uptake measurement was achieved via following the user guide commercial kit by 2-NBDG, a fluorescently labeled deoxyglucose analog. Afterwards, cells seeded in confocal dish were observed under super-resolution microscope.

2.12. Super-resolution microscope & image analysis

Original fluorescent images representing ROS, mitochondrial morphology and depolarization, and glucose uptake level were all captured, re-constructed via ZEISS Elyra 7 Lattice SIM2 super-resolution microscope. Optical reconstruction and image processing were all conducted via ZEN black software (ZEISS; 3.6) following standard procedure. Image export was achieved by ZEN blue software (ZEISS; 3.4). Semi-quantification about fluorescent intensity of images were achieved by Fiji (Image J, 2.14.1)

2.13. Western blotting (WB) and in-cell Western (ICW) assays

All samples of ICW are prepared applying the same experimental procedure as immunofluorescence (IF) analysis from our previously built-up assay for fluorescent super-resolution microscope ^[11, 17]. The primary

antibodies against Akt, p-Akt, GSK3 β , p-GSK3 β , β -actin, and GLUT4 were used. IRDye 680RD Goat anti-Mouse IgG (H+L) and IRDye 800CW Goat anti-Rabbit IgG (H+L) were introduced as secondary antibody for signal detection, both of which are bought from LI-COR Biotechnology, USA. The following proteins GSK3 β , AKT and β -actin were detected after laser excitation at 680nm while the other proteins p-GSK3 β , p-Akt and Glut4 were detected after laser excitation at 700nm. Images of cells cultured in confocal dish were visualized using the Odyssey M multi-functional Imaging System (LI-COR Biotechnology, USA). Semi-quantification of protein expression level was achieved via ImageStudio Software 6.0 (LI-COR Biotechnology, USA). Image processing and export was achieved via Empiria Studio 3.3 (LI-COR Biotechnology, USA).

For conventional WB and related semi-quantification of protein amounts, experimental details were following our previous research [17, 22, 30]. Details of 1st and 2nd antibody were provided in the supplementary file.

2.14. Statistical analysis

All tests in this study have been conducted thrice at least. One-way analysis of variance (ANOVA) was applied for statistical analysis and data are shown via mean $\pm$ STD. Tukey's multiple range tests were applied for statistical significance analysis, setting at $P < 0.05$, via Graphpad Prism9.5.0 (Boston, MA, U.S.A) and SPSS v26.0 (IBM, Endicott, NY, U.S.A).

3. Results

3.1. Anti-diabetes target of nomilin

Network pharmacology method was utilized to primarily study and discover the anti-diabetes molecular mechanism of nomilin through regulating insulin resistance. The anti-diabetic protein target of nomilin was achieved through interacting target of insulin resistance, T2D, and nomilin (Fig. 1A, 1B). A total of 295 anti-diabetes target of nomilin was achieved.

3.2. Protein-protein interactions and core target screening

Generally, the targets analyzed by network pharmacology are used the core target which screening by the DC, CC, and BC in the PPI network. Therefore, the PPI network of the nomilin's anti-diabetes target was constructed based on the string database (Fig. 1C). After the topological analysis, the median of DC, BC, and CC was used for screening the core target of nomilin (Fig. 2A). Totally, 117 candidate core targets of nomilin were screened out and utilized in the following PPI network construction (Fig. 2B). The core targets with top 20 DC value were shown in Fig. 2C including the AKT1, ALB, CASP3, etc.

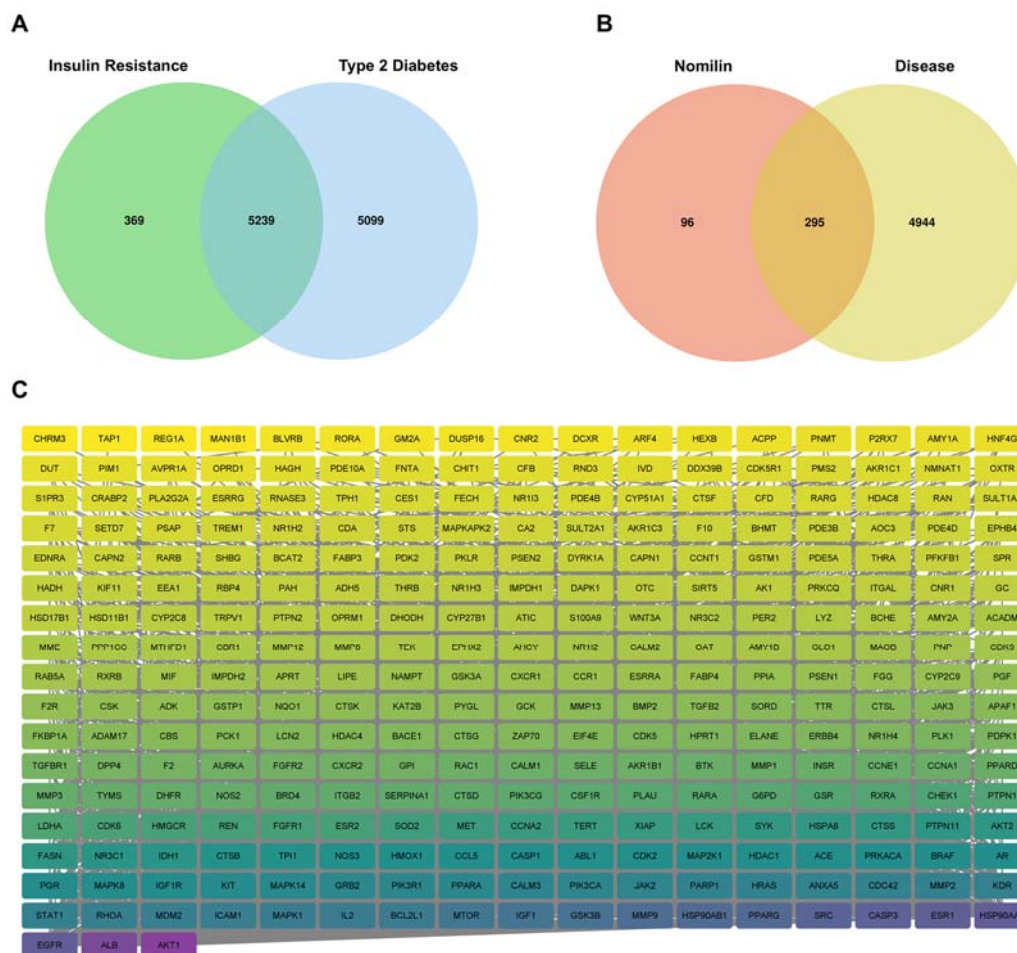


Fig. 1. The anti-diabetes targets of nomilin through improving the insulin resistance. (A) Venne diagram between insulin resistance targets and type 2 diabetes targets. (B) Venne diagram between nomilin target and disease related targets. (C) Protein-protein interactions network of the anti-diabetes targets of nomilin.

3.3. KEGG&GO enrichment analysis

The core targets of nomilin were utilized to conduct the KEGG signaling pathway enrichment analysis of KEGG signaling pathway (Fig. 2D) and GO (Fig. 2E). The KEGG signaling pathway enrichment analysis showed that the PI3K-Akt signaling pathway, Ras signaling pathway, Estrogen signaling pathway, and Insulin signaling pathway may be the critical signaling pathway of anti-diabetes effect of nomilin.

To elucidate the biological functions of the 81 core targets of nomilin, we performed GO enrichment analysis across three ontologies: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) (Fig. 2E). Specifically, the most over-represented BP terms included cellular response to hormone stimulus, positive regulation of cell migration, and cellular response to lipid. At the cellular component (CC) level, the enriched terms included centrosome, side of membrane, and ficolin-1-rich granule. In the molecular function, the targets were significantly enriched for phosphotransferase activity, alcohol group as acceptor, kinase binding, and kinase regulator activity.

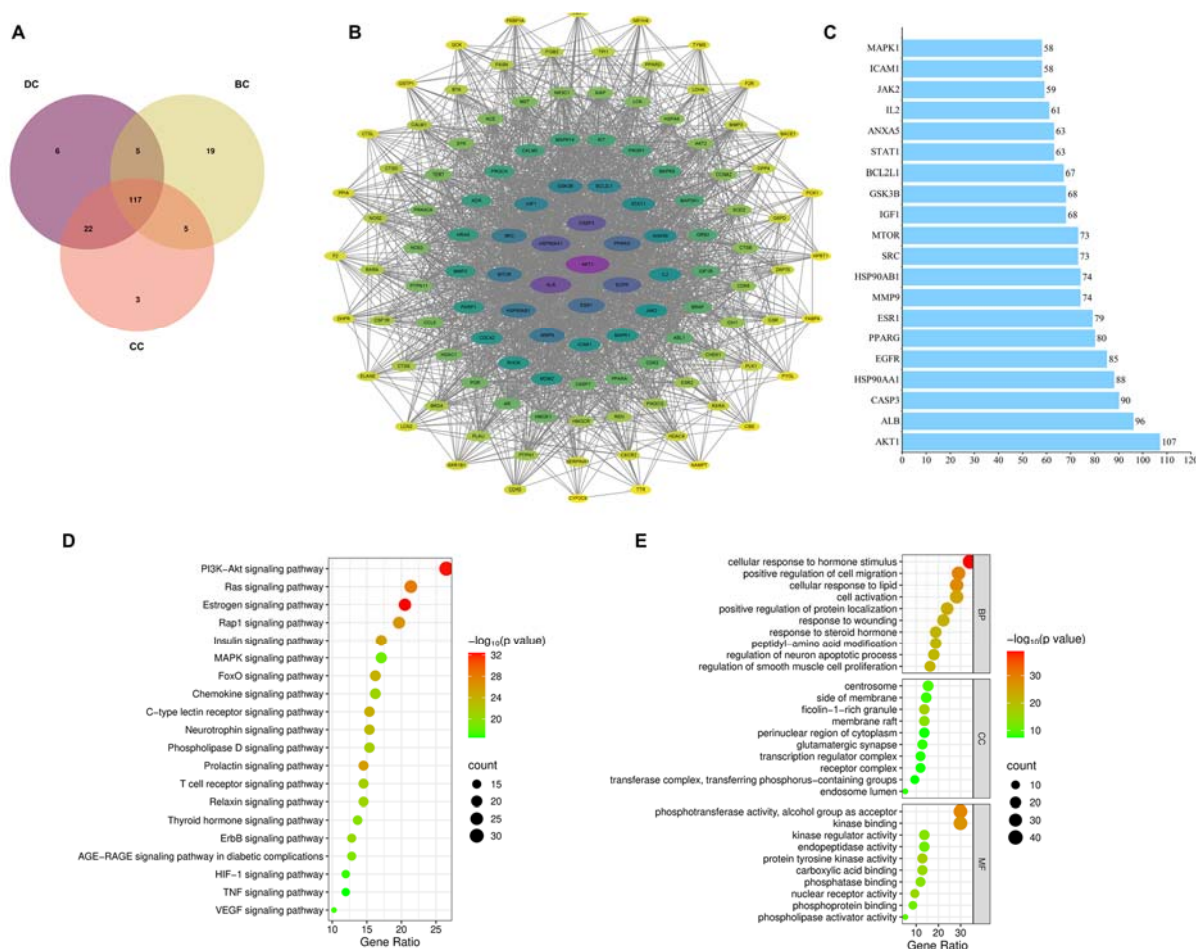


Fig. 2. Core target screening and the KEGG & GO enrichment analysis. (A) Venne diagram of targets with the top 20 DC, BC, and CC value. (B) Protein-protein interactions network of the anti-diabetes core targets of nomilin. (C) Top 20 DC value of core targets. (D) KEGG signaling pathway enrichment analysis. (E) GO enrichment analysis. DC, degree centrality; CC, closeness centrality; BC, betweenness centrality; BP, biological process; CC, cellular component; MF, molecular function.

3.4. Central target screening

Before the further bioinformatics analysis, the central targets were screened out as core genes. Moreover, the networks of targets with top 10 MCC (Fig. 3A), MNC (Fig. 3B), DC (Fig. 3C), and CC (Fig. 3D) values were built respectively. The Venny online tool was utilized to present the overlapping targets of four networks as central targets (core gene), including AKT1, CASP3, EGFR, SRC, HSP90AA1, ALB, and ESR1 (Fig.3E). The PPI network of the central targets was also constructed, and results are presented in Fig. 3F.

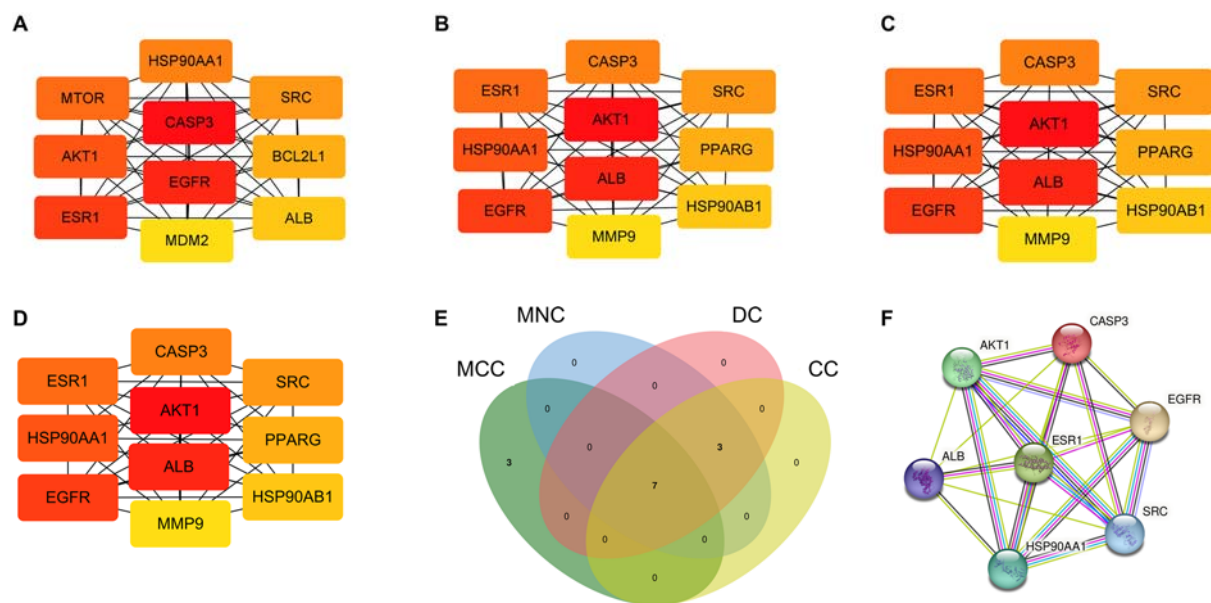


Fig. 3. The central target (hub gene) screening of nomilin. (A) The network of targets with top 10 MCC value. (B) The network of targets with top 10 MNC value. (C) The network of targets with top 10 DC value. (D) The network of targets with top 10 CC value. (E) Venn diagram of targets with top 10 MCC, MNC, DC and CC values. (F) The protein-protein interaction network of central targets. DC, degree centrality; CC, closeness centrality; BC, betweenness centrality; MNC, maximum neighborhood component; MCC, maximal clique centrality.

3.5. Differential expression analysis of core genes

Through differential expression analysis between individuals with T2D and those non-diabetics (ND), it was revealed that the target genes *AKT1*, *CASP3*, *ALB*, *EGFR*, *HSP90AA1*, and *SRC* are highly expressed in T2D. Additionally, the expression of *AKT1*, *ESR1*, *EGFR*, and *SRC* genes exhibited significant differences between the T2D and ND groups. Meanwhile, the *ESR1* gene was found to be downregulated in T2D and also showed significant differences in expression between the T2D and ND groups (Fig. 4). These findings suggest that these genes play important roles in the development and progression of T2D. Among them, *AKT1*^[31], *EGFR*^[32], and *SRC*^[33] may contribute to insulin resistance, while the downregulation of *ESR1* may weaken its protective effects.

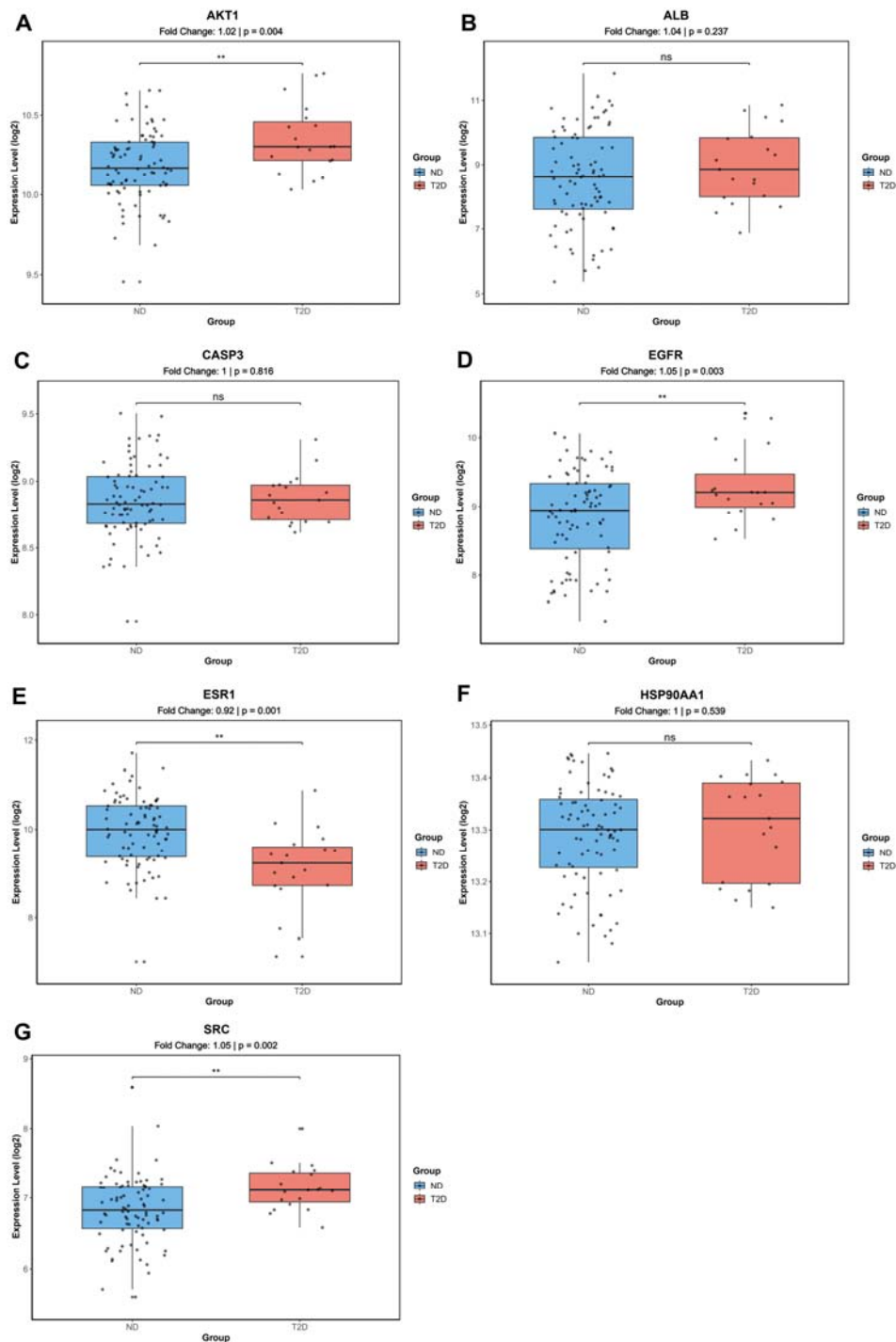


Fig. 4. Boxplots of differential expressions of core genes. Expression of AKT1 (A), CASP3 (B), ALB (C), EGFR (D), HSP90AA1 (E), and SRC (F) in the T2D and ND groups. Red denotes the T2D group, and blue denotes the ND group. T2D, type 2 diabetes; ND, non-diabetics.

3.6. Immune infiltration analysis of core genes

Next, to further study the potential associations between the target genes and the immune cell infiltration, we utilized the CIBERSORT R package (based on the LM22 signature gene matrix) for analysis about the immune cell composition in individuals with T2D and ND individuals. The results showed that the percentages of naive B cells, resting memory CD4⁺ T cells, regulatory T cells (Tregs), activated NK cells, monocytes, and eosinophils were significantly different between the T2D and ND groups (Fig. 5A). Further comparison about

immune cell abundance between the two groups revealed that naive B cells, resting memory CD4⁺ T cells, monocytes, and M0 macrophages had relatively high mean proportions in both groups (Fig. 5B, 5C). In the heatmap of immune cell correlations, we observed distinct negative correlations: activated NK cells had correlation coefficients of -0.71 to -0.78 with resting NK cells and activated mast cells, while resting NK cells and activated mast cells were significantly positively correlated (correlation coefficient of approximately 0.71) (Fig. 5D). These findings suggest that there may be antagonistic and cooperative relationships in the immune regulation between NK cell subsets and mast cells.

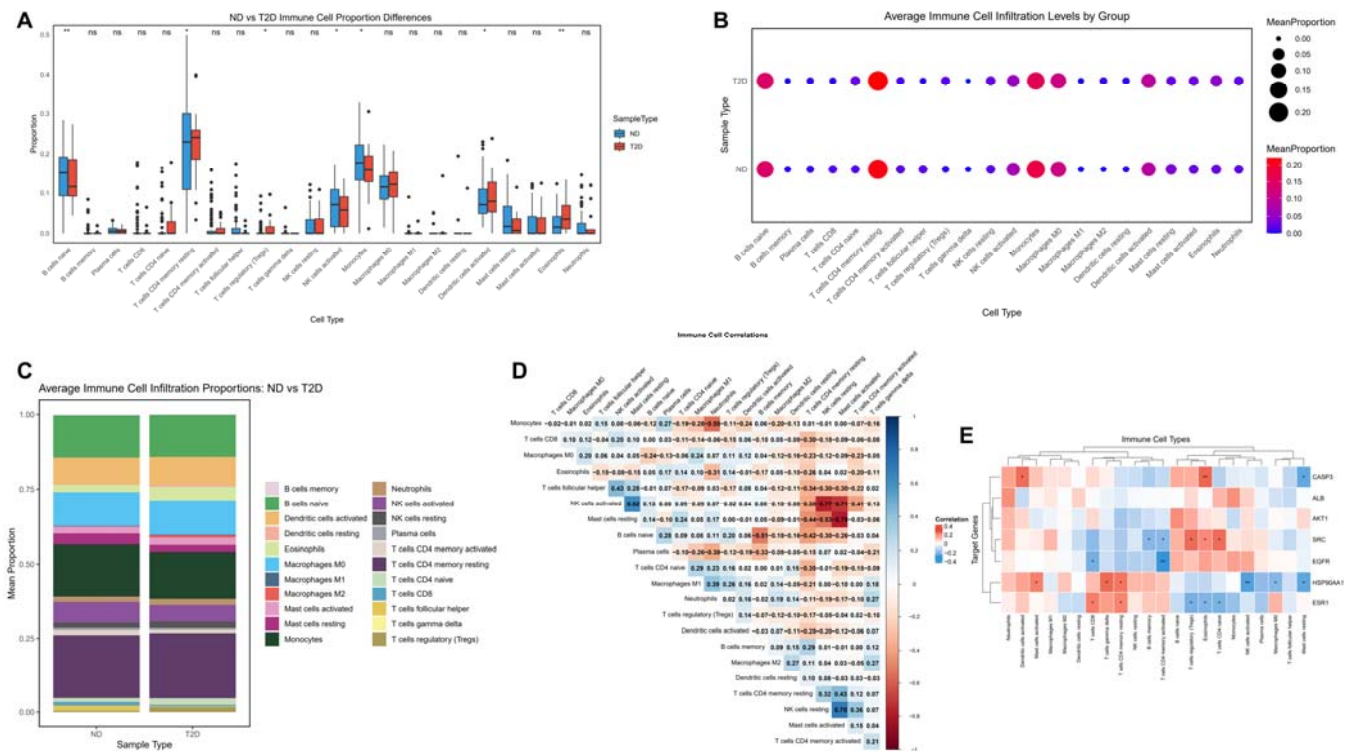


Fig. 5. Immune infiltration analysis of core genes. (A) Differential analysis of immune cell proportions. Red represents type 2 diabetes, and blue represents non-diabetes. (B) Bubble plot of immune infiltration levels. The redder the color and the larger the circle, the higher the average proportion of cells. (C) Bar plot of immune infiltration levels. Different colors represent different immune cell types. (D) Correlation analysis of immune cells. Correlation heatmap, with blue indicating positive correlation and red indicating negative correlation. (E) Correlation analysis between target genes and immune cells. Red represents positive correlation, and blue represents negative correlation. “*” indicates $P < 0.05$, and “***” indicates $P < 0.01$.

Subsequently, we systematically analyzed the correlations between the seven target genes and 22 immune cell types. The results showed that, except for *ALB* and *AKT1* genes which did not show significant correlations, the other five genes were significantly correlated with multiple immune cells (Fig. 5E). Specifically, *CASP3* was significantly positively correlated with activated dendritic cells and eosinophils, but significantly negatively correlated with resting mast cells; *SRC* was positively correlated with Tregs, eosinophils, and naive CD4⁺ T cells, but negatively correlated with memory B cells and activated memory CD4⁺ T cells; *EGF* was significantly negatively correlated with both activated memory CD4⁺ T cells and CD8⁺ T cells; *HSP90AA1* was significantly negatively correlated with activated NK cells and resting mast cells, but positively correlated with $\gamma\delta$ T cells and activated mast cells; *ESR1* was significantly positively

correlated with naive CD4⁺ T cells and eosinophils, but significantly negatively correlated with resting memory CD4⁺ T cells and CD8⁺ T cells.

In summary, these results not only revealed a characteristic immune infiltration pattern in T2D patients but also suggested that the target genes may involve in the progression and development of T2D by regulating the abundance and function of specific immune cell subsets. Among them, the immune-relatedness of genes such as *CASP3*, *SRC*, and *HSP90AA1* was particularly prominent, and they may involve in the immunopathological process of T2D with importance.

To further explore the deeper mechanisms, we conducted comprehensive bioinformatics analyses such as GSEA, GSVA, and WGCNA analysis.

GSEA results revealed that the DEGs were significantly enriched in several key metabolism-related pathways, including PI3K-Akt-mTOR signaling, insulin signaling pathway, insulin resistance, mTORC1 signaling, and oxidative phosphorylation. All these pathways are closely associated with the pathogenesis and progression of T2D, suggesting that the research subjects may harbor critical molecular mechanisms underlying insulin signaling transduction disorders, energy metabolism dysregulation, and mitochondrial dysfunction (Fig. S2. A-E). GO Biological Process enrichment analysis based on GSEA indicated that vesicle-mediated transport in synapse, regulated exocytosis, synaptic vesicle cycle and synaptic vesicle exocytosis may be the related biological processes in T2D regulation (Fig. S2. F).

GSVA demonstrated that multiple pathways associated with cell adhesion, extracellular matrix (ECM) remodeling, and epithelial differentiation were significantly enriched. These pathways included cell substrate adhesion, positive regulation of cell–cell adhesion, collagen-containing extracellular matrix, epidermal cell differentiation, and epidermis development. These alterations suggest that cell-cell and cell-matrix interactions may undergo remodeling under T2D conditions, which aligns with the common ECM abnormalities and inflammation-associated structural changes in the T2D tissue microenvironment. Moreover, the significant enrichment of the neuron regulation-related pathway neuron-to-neuron synapse and the secretion regulation pathway calcium ion–regulated exocytosis implied that neuronal signal transduction and calcium ion-dependent vesicular secretion might be perturbed. This finding is consistent with the well-documented mechanisms in T2D, such as impaired calcium ion homeostasis in pancreatic β -cells and defective insulin secretion. Additionally, the enrichment of response to molecule of bacterial origin DNA-binding transcription activator activity indicated enhanced inflammation-related signaling responses and transcriptional regulatory activity. These observations further support the characteristic features of T2D accompanied by chronic low-grade inflammation and metabolic stress (Fig. S3. A-C).

The WGCNA performed in this study, a soft-thresholding power of 5 was selected, and a total of 12610 genes were finally incorporated and clustered into 37 co-expression modules (Fig. S4. A-C). In the module–trait correlation analysis involving 103 samples (84 ND vs. 19 T2D cases), multiple modules exhibited significant correlations with the T2D phenotype (all $P < 0.05$), including ME3, ME24, ME17, ME4, ME6, ME13, and ME29. These results suggest that these modules may exert potential roles in T2D-related molecular

regulation (Fig. S4. D). Statistical analysis of module feature genes revealed that the major module eigengenes were concentrated in Module 21, 1, 6, 22, 3, and 20 (Fig. S4. E). Hub gene-module eigengene correlation analysis further identified that multiple key genes associated with T2D metabolic imbalance or tissue remodeling showed distinct module affiliations: EGFR and SRC displayed the highest correlations with the ME2 module, with correlation coefficients of 0.70 and 0.65, respectively; AKT1 was most strongly correlated with the ME17 module ($r = 0.63$), which is closely implicated in insulin signaling and metabolic regulation; CASP3 showed the highest correlation with the ME23 module ($r = 0.62$), implying that apoptotic processes may be active in T2D-associated tissue pathology; ESR1 was significantly correlated with the ME4 module ($r = 0.74$), which may be involved in the regulation of inflammation, lipid metabolism, and energy homeostasis. Furthermore, EGFR also presented high correlations with the ME6 and ME13 modules ($r = 0.74$ and 0.72 , respectively), while SRC reached its highest correlation with the ME6 module ($r = 0.77$). The metabolism-related gene ALB exhibited the strongest correlations with the ME9 and ME1 modules ($r = 0.69$ and 0.66 , respectively), suggesting potential alterations in plasma protein metabolism or liver-related functions under T2D conditions (Fig. S4. F).

3.7. *Nomilin alleviates glucose consumption in IR-HepG2 cell model*

The CCK8 assay was applied to measure cytotoxicity of concentration-gradient of nomilin on HepG2 cells and accordingly $64 \mu\text{mol/L}$ was found with highest dose without significant cell viability loss (Fig. 6A). The glucose consumption and advanced glycation end products (AGEs) were utilized for primary test of anti-diabetic effect (Fig. 6B, 6C). It is obviously shown that by a concentration-dependent manner, comparing to metformin (positive control), nomilin significantly alleviated the descend in glucose consumption level and AGEs level. Moreover, this finding was also validated by ELISA experiments (Fig. 6D). Since results detected by fluorescent microplate readers show trends in bulk culture, this study also applied single cell level observation via fluorescent super-resolution microscope and related fluorescent semi-quantification (Fig. 6E, 6F). Similar to macroscopic level, green fluorescence representing 2-NBDG decreased (Glucose intake) for insulin resistance modeling stimulation but recovered significantly after nomilin introduction (Fig. 6E). And semi-quantification results also supported this observation (Fig. 6F).

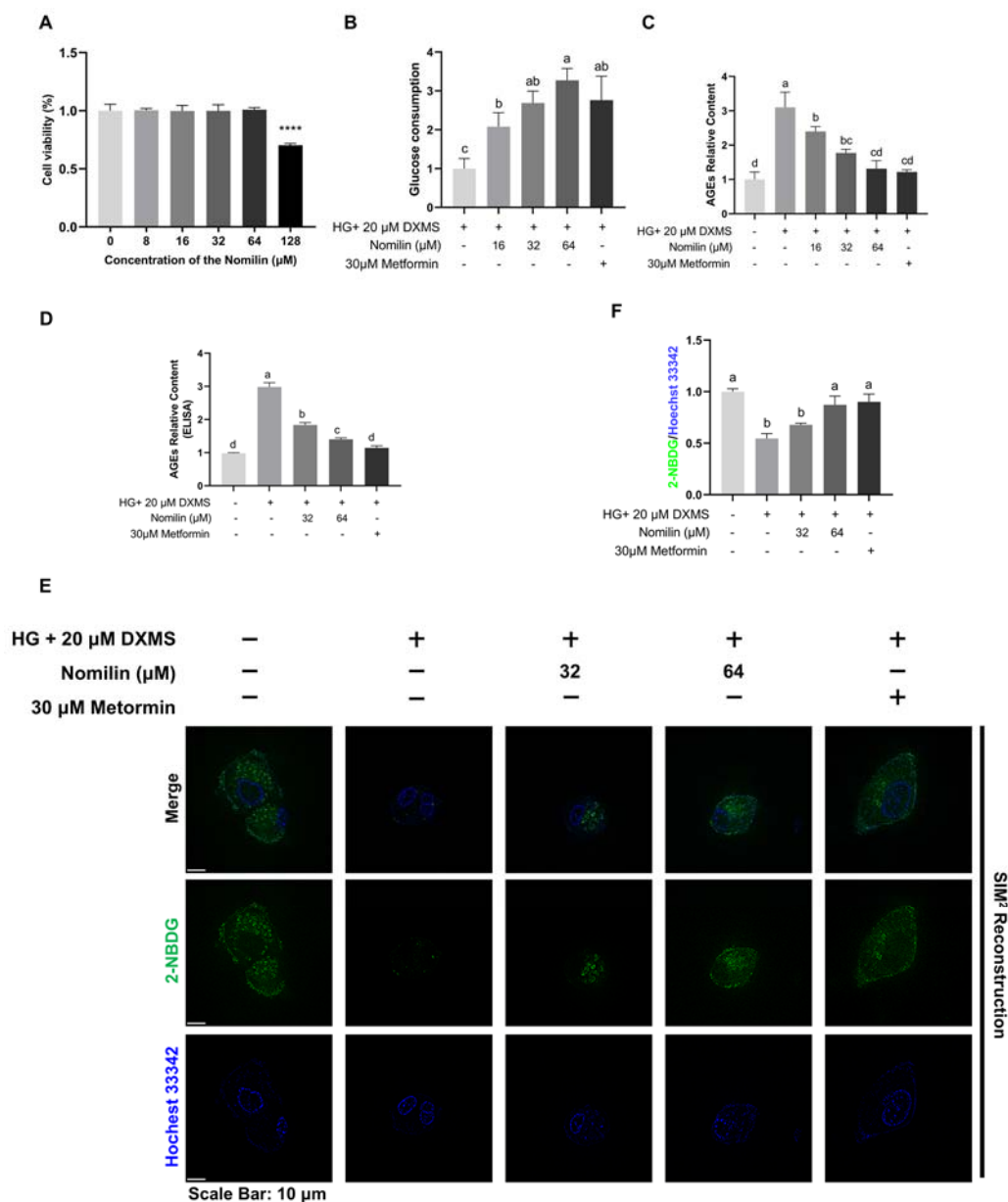


Fig. 6. Nomilin improves AGEs and Glucose uptake amount in high glucose and dexamethasone-induced IR-HepG2 cell model. (A) Cell viability test of nomilin via CCK8. (B-D) glucose consumption and AGEs measurement at bulk culture level via fluorescence or ELISA. (E-F) Fluorescent figure and semi-quantification of single cell observation after 2NBDG staining. Results are shown by mean $\pm$ S.D, and all are with thrice biological repeats. Different letters (a-d) above bars represent significant difference ($P < 0.05$) referring to ANOVA and Tukey's multiple range tests. DXMS, dexamethasone; HG, high glucose. AGEs, advanced glycation end-products. Scale bar 10 μmol/L.

3.8. Nomilin alleviates intracellular ROS accumulation and mitochondrial membrane potential loss in IR-HepG2 cell model

Roles and contribution of AGEs and ROS, which induced mitochondrial abnormality has already been reported in diabetes and IR-HepG2 cell model [34-35]. Accordingly, intracellular ROS accumulation and mitochondrial abnormality were measured in IR-HepG2 model to assess improvement effect of nomilin (Fig. 7).

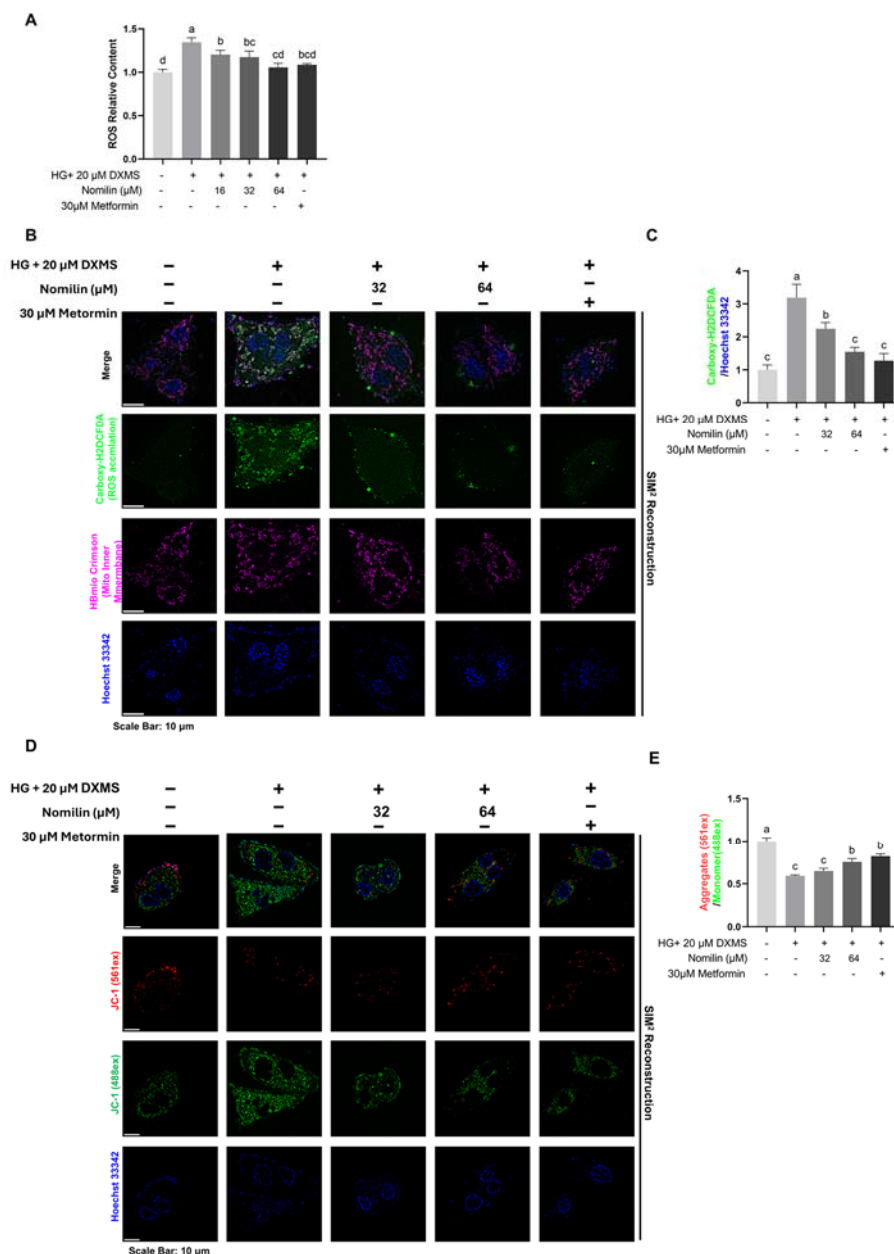


Fig. 7. Nomilin alleviates high glucose and dexamethasone-facilitated ROS accumulation, mitochondrial morphological change and mitochondrial potential loss in IR-HepG2 cell model. (A) ROS accumulation at bulk culture. (B-C) Fluorescent figure and semi-quantification of single cell observation after Carboxy-H₂DCFDA & HBmito Crimson co-staining. (D-E) Fluorescent figure and related semi-quantification of single cell observation after JC-1 staining. Results are shown by mean $\pm$ S.D, and all are with thrice biological repeats. Different letters (a-c) above bars represent significant difference ($P < 0.05$) referring to ANOVA and Tukey's multiple range tests. DXMS, dexamethasone; HG, high glucose. Scale bar 10 μ mol/L.

Either at bulk culture (Fig. 7A) or single cell level (Fig. 7B, 7C), nomilin was detected with significant alleviation effect against modeling-facilitated intracellular ROS accumulation by a concentration-dependent manner in IR-HepG2 cell model, comparing to metformin (positive control). Meanwhile, co-staining of ROS and mitochondrial membrane results indicated that modeling-facilitated mitochondrial morphological deterioration was alleviated by nomilin alongside ROS level decrease. So, besides morphological improvement, further measurement of mitochondrial membrane potential was also conducted via JC-1, the sensitive dye for mitochondrial membrane potential. Nomilin improved modeling-facilitated mitochondrial membrane potential loss by a concentration-dependent manner (Fig. 7D), which is also verified by fluorescent semi-quantification results (Fig. 7E).

3.9. Nomilin alleviates glucose consumption in IR-THLE-2 cell model

Since HepG2 cell is not a normal hepatic cell with differences in metabolic characteristics [27, 36], this study also included a normal hepatic cell, THLE-2 cell, for all experimental procedure. The CCK8 assay implied that 64 $\mu\text{mol/L}$ is the highest non-toxic dose of nomilin on THLE-2 cells (Fig. 8A). Similarly, further study showed significant mitigating effect of nomilin in glucose consumption decrease (Fig. 8B) and AGEs generation, via either fluorescence or ELISA (Fig. 8C-D) on IR-THLE-2 cells. Besides bulk culture, both fluorescent observation (Fig. 8E) and related semi-quantification results (Fig. 8F) supported the improvement effect of nomilin in glucose intake on modeling facilitated IR-THLE-2 cell model, which is similar as above in HepG2 (Fig. 6).

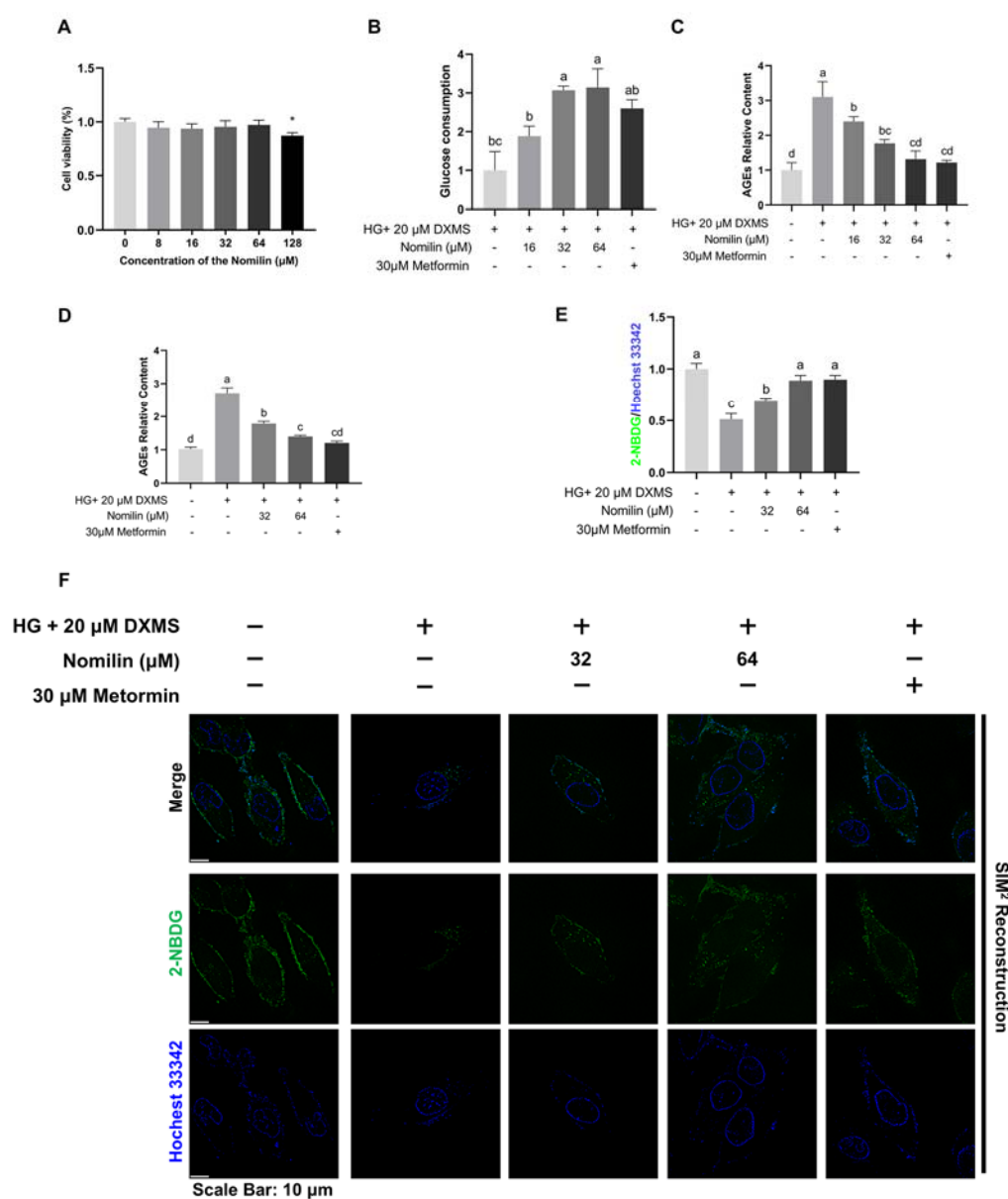


Fig. 8. Nomilin improves AGEs and Glucose uptake amount in high glucose and dexamethasone-induced IR-THLE-2 cell model. (A) Cell viability test of nomilin via CCK8. (B-D) glucose consumption and AGEs measurement at bulk culture level either via fluorescence or ELISA. (E-F) Fluorescent figure and semi-quantification of single cell observation after 2NBDG staining. Results are shown by mean $\pm$ S.D, and all are with thrice biological repeats. Different letters (a-d) above bars represent significant difference ($P < 0.05$) referring to ANOVA and Tukey's multiple range tests. DXMS, dexamethasone; HG, high glucose. AGEs, advanced glycation end-products. Scale bar 10 $\mu\text{mol/L}$.

3.10. Nomilin alleviates intracellular ROS accumulation and mitochondrial membrane potential lose in IR-THLE-2 cell model

Same as above, intracellular ROS accumulation and mitochondrial membrane potential lose was also measured in IR-THLE-2 cell model. Similarly, nomilin also alleviated ROS either at bulk culture (Fig. 9A) or single cell level, which is shown by fluorescent figure (Fig. 9B) and semi-quantification (Fig. 9C). Also, morphological collapse was partly alleviated via nomilin introduction alongside ROS accumulation decrease. So, accordingly, mitochondrial membrane potential loss was also measured. Both single cell observation (Fig. 9D) and semi-quantification (Fig. 9E) results supported that nomiline partly rescues the phenotypic change about modeling-facilitated mitochondrial membrane potential lose, which is also similar as above on HepG2 cell (Fig. 7D, 7E).

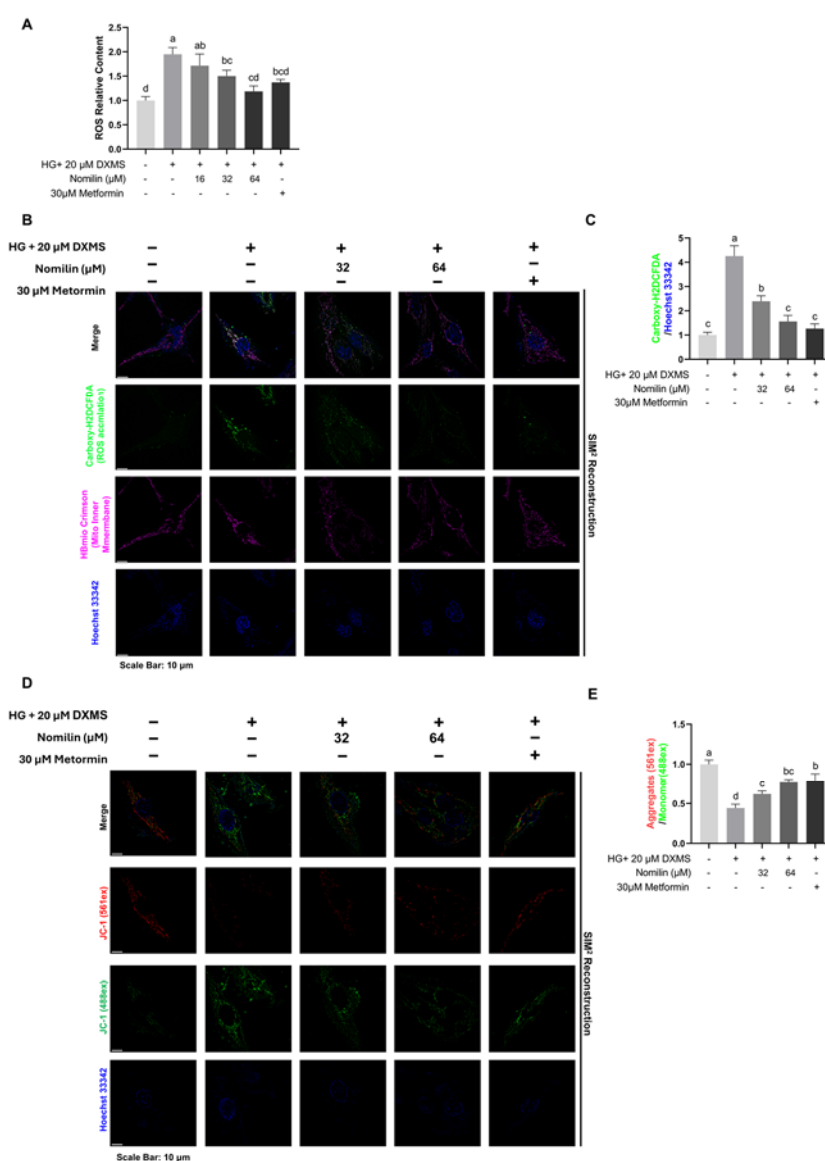


Fig. 9. Nomilin alleviates high glucose and dexamethasone-facilitated ROS accumulation, mitochondrial morphological change and mitochondrial potential loss in IR-THLE-2 cell model. (A) ROS accumulation at bulk culture. (B-C) Fluorescent figure and related semi-quantification of single cell observation after Carboxy-H₂DCFDA & HBmito Crimson co-staining. (D-E) Representative figure and semi-quantification of single cell observation after JC-1 staining. Results are shown by mean $\pm$ S.D, and all are with thrice biological repeats. Different letters (a-d) above bars represent significant difference ($P < 0.05$) referring to ANOVA and Tukey's multiple range tests. DXMS, dexamethasone; HG, high glucose. Scale bar 10 μ m/L.

3.11. Nomilin achieves anti-diabetes effect through regulating PI3K/Akt/GLUT4 signaling pathway

Referring to the ICW results, comparing to normal control, co-treatment of dexamethasone and high glucose facilitates decrease in protein expression level of p-PI3K (Fig. 10A), p-Akt (Fig. 10C), p-GSK3 β (Fig. 10E) and GLUT4 (Fig. 10G) especially referring to PI3K, Akt, GSK3 β and β -actin. However, due to nomilin introduction (at 64 μ mol/L), it is obviously shown that p-PI3K, p-Akt, p-GSK3 β and GLUT4 protein expression level were all improved in IR-THLE-2 cell model. Also, semi-quantification via fluorescence level also supported the scanning results above and indicating the improvement effect of nomilin is significant compared between model group (dexamethasone and high glucose) and treatment group (nomiline) (Fig. 10B, 10D, 10F, 10H).

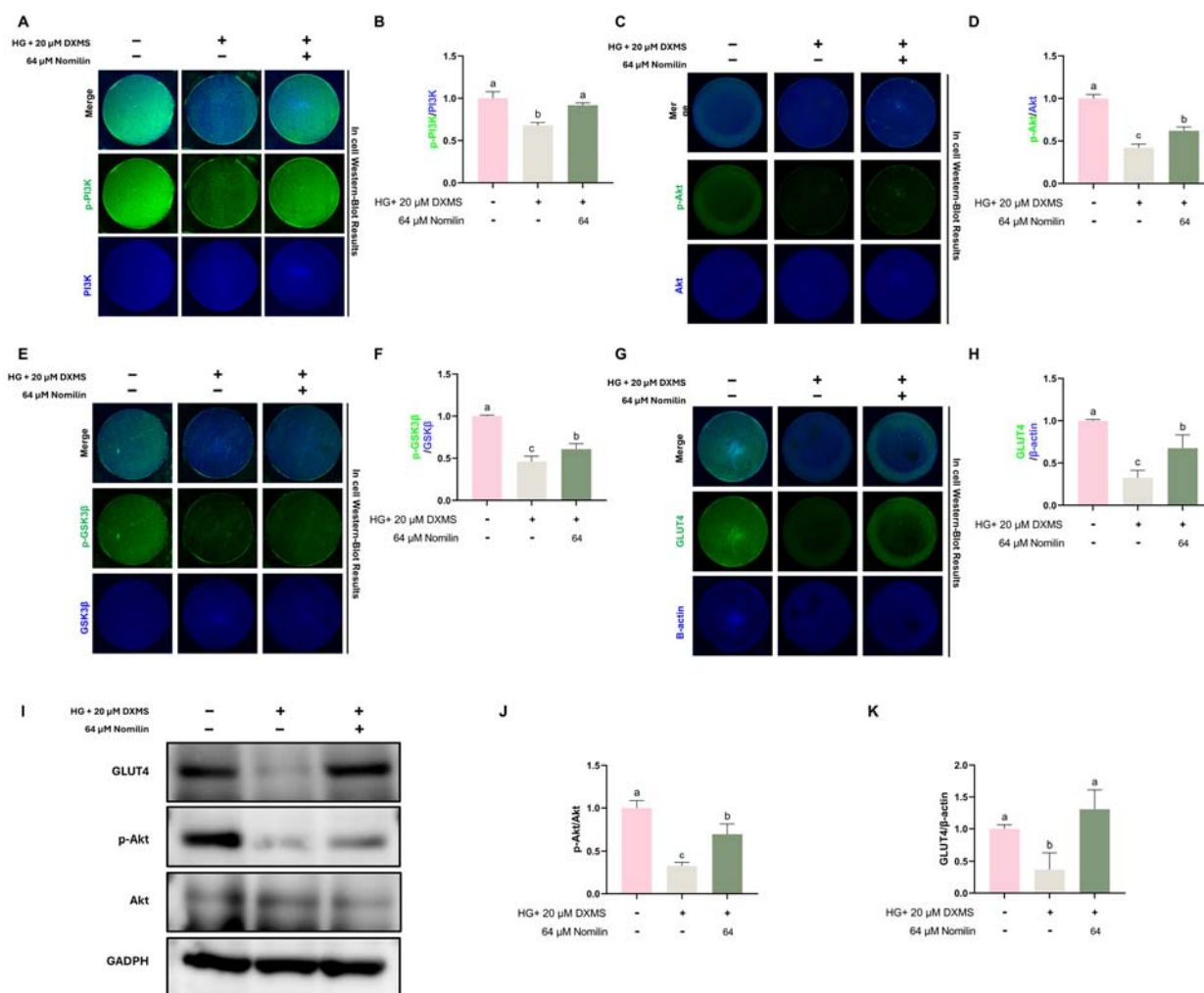


Fig. 10. Nomilin achieved anti-diabetes effect through regulating the PI3K/Akt/GLUT4 signaling pathway in high glucose and dexamethasone-treated THLE-2 cell model, detected via In-cell Western-blot assay. (A-B) In-cell Western-blot result of PI3K and p-PI3K, and semi-quantification of p-PI3K/PI3K. (C-D) In-cell Western-blot result of Akt and p-Akt, and semi-quantification of p-Akt/Akt. (E-F) In-cell Western-blot result of GSK3 β and p-GSK3 β , and semi-quantification of p-GSK3 β /GSK3 β . (G-H) In-cell Western-blot result of GLUT4 and β -actin, and semi-quantification of GLUT4/ β -actin. (I) The Western Blot analysis of p-Akt, Akt, GLUT4, and β -actin. (J) The semi-quantification of p-Akt/Akt based on the Western Blot (K) The semi-quantification of GLUT4/ β -actin based on the Western Blot. Results are shown by mean $\pm$ S.D, and all are with thrice biological repeats. Different letters (a-c) above bars represent significant difference ($P < 0.05$) referring to ANOVA and Tukey's multiple range tests. DXMS, dexamethasone; HG, high glucose.

Beside ICW, we also conducted WB and related semi-quantification of targeted proteins (p-Akt, Akt, GLUT4 and β -actin) (Fig. 10I, 10J, 10K). Nomilin alleviated the decrease of phosphorylation of Akt and

GLUT4 protein level decrease mediated by high glucose/dexamethasone co-inducement (Fig. 10I), which is also supported by the semi-quantification results (Fig. 10J, 10K) and consistent with ICW results above.

Therefore, nomiline might achieve anti-diabetic effect in IR-THLE2 cell model through regulating PI3K/Akt/GLUT4 signaling pathway.

4. Discussion

Nomilin is a naturally sourced triterpene compound with health benefits and primarily found in citrus fruits, which has been reported to have multiple biological effects, including anticancer effect, anti-inflammatory effect, antioxidative effect, lipid-lowering effect, etc. [8]. About the anti-diabetes effect of nomilin, both *in vitro* [10] and *in vivo* [9] have preliminarily studied its anti-diabetes effect. However, to the best of our knowledge, its underlying molecular mechanism is still kept undiscovered. This study first performed the network pharmacology to predict its underlying molecular mechanism in attenuating insulin resistance in HepG2 cells, which may contribute to the development of new strategies for T2D intervention. And a total of 205 anti-diabetes targets and 117 anti-diabetes core targets were identified. Also 7 central targets were found as core genes for further bioinformatic analysis. Moreover, the KEGG signaling pathway enrichment analysis showed that the PI3K-Akt signaling pathway is the most critical pathway to the anti-diabetes effect of nomilin through regulating insulin resistance. The PI3K/Akt signaling pathway has already been thoroughly and widely recognized as a promising and potential therapeutic target for the treatment of T2D [37]. Therefore, PI3K/Akt signaling pathway has become a key verification target to discover the molecular mechanism section of this study.

Our bioinformatics analysis and results pointed out that *AKT1*, *EGFR*, and *SRC* are all highly expressed in T2D groups, suggesting these genes with high possibility in close relationship or even involvement in T2D' progression. AKT1, also named as the protein kinase B, is an important regulatory kinase involving in the signal transduction via the phosphatidylinositol 3-kinase (PI3K)-AKT signaling cascade to properly regulate the cell growth and survival. Previous literature has shown that the PI3K-Akt-FoxO3a pathway with prevention capability against STZ-induced cardiac dysfunction and structural cardiomyopathy in diabetic rats and alleviation effect on apoptosis of cardiomyocytes in diabetic hearts [38]. The phosphorylation level of AKT-Foxo1 has been proved with close relation on the progression of insulin resistance and apoptotic cell death in diabetic cardiomyopathy [39]. Epidermal growth factor receptor (EGFR) originates from the ErbB receptor family [40]. EGFR tyrosine kinase inhibitors can reduce the obesity rate in leptin receptor-deficient T2D mouse models [41]. In a diabetic lung cancer patient, introduction of EGFR tyrosine kinase inhibitors benefits on health via glycemic control [42]. Studies have shown that a high-fat diet increases EGFR protein expression amount and its ligand amphiregulin in adipose tissue macrophages (ATMs). Selective deletion of EGFR in ATMs inhibits the proliferation of resident ATMs and the infiltration of monocytes into adipose tissue, thereby reducing the development of obesity and insulin resistance [43]. Studies have shown that in cardiomyopathy, hyperglycemia activates FGFR1 via the well-studied TLR4 and c-Src axis, facilitates

MAPKs-mediated NF κ B activation, increases inflammation, and subsequently leads to fibrosis and hypertrophy, driving diabetic cardiomyopathy [44].

Mitochondrial, the mostly well-studied cell “power bank”, plays an important role in the intracellular energy supply and consumption balance, oxidation, signaling and programmed cell death, of which its dysfunction has been proved direct relation to T2D pathophysiology and progress for its importance in normality and functionality in adipocytes, hepatocytes and peripheral tissues [45–47]. Mitochondrial inherent abnormality for diabetes can facilitate metabolic chaos, and ROS and AGEs accumulation, which further facilitates hyperglycemia, a classical hallmark of DM and obesity progression [48–50]. Accordingly, mitochondrial dysfunction, especially alongside ROS accumulation, in membrane potential loss and morphological change has been regarded as hallmark of T2D [51–53]. Previous study has demonstrated oxidative stress scavenging of mitochondrial alleviates insulin resistance, clearly indicating clinical potential and role of mitochondrial abnormality rescues in treatment against insulin resistance [49, 54–55]. Our results suggest that nomilin attenuates ROS accumulation, as shown by both qualitative imaging and semi-quantitative data. This effect was consistently seen across IR-HepG2 and THLE-2 cell models induced by high glucose and dexamethasone (Fig. 7, 9). Meanwhile, it is also observed that mitochondrial morphological change and potential loss was rescued via nomilin introduction alongside ROS decrease (Fig. 7, 9). Moreover, this study also showed that nomilin can inhibit the AGEs generation, either detected via fluorescence or ELISA, and improve the glucose consumption and glucose uptake, with supportive quantitative results in macro- and micro-dimensions (Fig. 6, 8). These results collectively demonstrate the antioxidative properties of nomilin in both cellular IR models, highlighting its developmental potential as a bioactive candidate for T2D intervention in future studies.

PI3K/Akt signaling pathway, a canonical and conserved signal transduction signal pathway since 1985, has been found and well-studied on regulation on various biological process like cell destiny, cell cycle, proliferation, differentiation, communication, metabolism, programmed cell death and et al, which links to many diseases including T2D [37, 56–57]. Role of PI3K/Akt/GLUT4 has been well proved with association with T2D progression in involvement on glucose consumption and glycogen synthesis [37, 58–59]. Akt regulates intracellular hemostats is of glucose and lipid consumption and absorption balance [37, 60–61]. To be specific, AKT involves insulin signaling pathway regulation with a crucial role via phosphorylation on various metabolically important proteins. For example, by activating AKT2, it directly contributes to GLUT4 transcription and translation, especially in insulin-responsive tissues [37, 60]. The GLUT4 can improve the insulin resistance and increase the glucose uptake [62]. GSK3 β , its phosphorylation has been demonstrated with importance on glycogen synthesis regulation in normal conditions, which is also a therapeutic target for treating metabolic diseases [63]. However, it was suppressed under diabetic progression, which significantly blocks glycogen synthesis. To attribute, its upstream phosphorylation of PI3K and Akt was also significantly restrained [6, 64]. Accordingly, various drug developments focus on activation of PI3K/AKT/GLUT4 to combat against obesity or T2MD, for its vital role in cell metabolism regulation [55, 59, 65–67]. In this study, high glucose

and dexamethasone-induced IR hepatic cell model, the protein phosphorylation level of PI3K, Akt and GSK3 β , and the GLUT4 protein expression level was found decreased significantly comparing to the normal group, which is similar as previous studies [5-6]. However, after nomilin introduction, phosphorylation level of PI3K, Akt, GSK3 β and protein level of GLUT4 were significantly improved (Fig. 10), either measured via ICW or WB. Although the detailed mechanistic validation was performed in hepatic models, it must be emphasized that the PI3K/Akt/GLUT4 signaling pathway is evolutionarily conserved and plays a central role in glucose metabolism in liver, skeletal muscle, and adipose tissue alike.

Our study majorly focuses on the cellularly molecular mechanisms, the anti-diabetic potential of nomilin is indirectly supported by previous *in vivo* evidence. Ono and et al. found that dietary nomilin intervention (0.2% w/w) significantly improved glucose tolerance and alleviated insulin level in the HFD mice, via TGR5 receptor activation [9]. Meanwhile, our finding regarding PI3K/Akt/GLUT4 axis echo the recent research on other citrus limonoids, such as limonin, which was reported with mitigation effect against metabolic syndrome *in vivo* via IRS-1/GLUT4 axis [68]. Also, it is worth noting that in a double-blind, placebo-controlled, clinical trial (ClinicalTrials.gov: NCT03215043), the commercial citrus extracts like Eriomin (rich in flavonoids like eriocitrin) have been reported with modulation effects in hyperglycemia and reversal of prediabetes condition, including decrease in blood glucose, insulin resistance, glucose intolerance, systematic inflammation and oxidative stress [69]. Our finding that nomilin directly modulates the PI3K/Akt pathway in hepatocytes provides a complementary cellular mechanism that helps in explaining the holistic anti-hyperglycemic effects of citrus-derived bioactive compounds. Accordingly, these results together implied that nomilin may attenuate insulin resistance *in vitro* by regulating the PI3K/Akt/GLUT4 canonical cascade. Future studies should focus on evaluating its anti-diabetic efficacy *in vivo* and determining whether the same signaling mechanisms are involved.

Besides positive results presented above, there are still some limitations should be stated. Firstly, our investigation was primarily conducted applying two *in vitro* cell models (HepG2 and THLE-2 cells). Although dual-model approach successfully confirmed the molecular mechanism, specifically via modulation of the PI3K/Akt/GLUT4 signaling pathway and mitochondrial dynamics, cell-based measurement cannot fully cover and replicated the complexity of systematic and dynamic metabolic interplay, bioavailability, and pharmacokinetics of nomillin in a whole organism, either the mouse or the human. We applied advanced imaging technique and *in situ* protein measurement (*in-cell* western blot) to maximize the mechanistic depth at the cellular level, however, these findings require *in vivo* validation in the next-stage. Therefore, our future research would focus on evaluation on the therapeutic efficacy and safety of nolimin in a T2D animal model like high fat diet (HFD)-induced diabetic mice to bridge the gap from cellular mechanism study to clinical applications.

5. Conclusion

This study primarily provided compelling evidence that nomilin, a natural triterpene compound from citrus fruits, represents a promising candidate of nutritional supplement or lead compound for the management

of T2D through multifaceted mechanisms of action. However, further *in vivo* experiments and clinical trials are required for further establishment of therapeutic efficacy and safety. Our integrated approach combining network pharmacology, bioinformatics, and dual cell model validation reveals that nomilin exerts its anti-diabetic effects primarily through activation of the PI3K/Akt/GLUT4 signaling pathway, a critical pathway in glucose metabolism. The attenuation efficacy of nomilin was demonstrated through its ability to significantly attenuates insulin resistance in both HepG2 and THLE-2 cell models. Mechanistically, nomilin not only enhanced glucose consumption and uptake but also conferred protective effects against diabetes-associated complications, including oxidative stress reduction, advanced glycation end-product inhibition, and mitochondrial function preservation. Moreover, nomilin can maintain mitochondrial membrane potential and prevent pathological mitochondrial changes. For the molecular mechanism, nomilin can achieve the anti-diabetic effect through regulation on the PI3K/Akt/GLUT4 signaling pathway. In summary, our results highlight nomilin as a potential candidate for the development of functional food ingredients, offering a novel perspective on managing cellular insulin resistance. Future research should focus on *in vivo* validation and pharmacokinetic optimization to translate these promising preclinical results into clinical applications

Supporting Information

Information of materials used in the experiment (Supplementary Figures 1, 2, 3, 4, and Table 1).

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

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