Original Article

N-myc downstream-regulated gene 2 is related to insulin resistance through IL-6/STAT3 pathways in type 2 diabetic mice

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Abstract: N-myc downstream-regulated gene 2 (Ndrg2) is related to cell development. Previous studies have shown that Ndrg2 is highly expressed in pancreatic β cells. However, whether Ndrg2 participates in the pathogenesis of type 2 diabetic mellitus remains unknown. The current study hypothesized that Ndrg2 may participate in the pathogenesis of type 2 diabetic mellitus (T2DM) through inflammatory related IL-6/STAT3 pathways. Mice were divided into the diabetic and control group (normal mice). Diabetic mice were induced by a high-fat diet for 12 weeks, while the control group was fed with normal diet. Body weight, blood biochemical indexes, and IL-6 levels of the two groups were examined. Ultra-structures of the brain, kidneys, and retina tissues were tested by transmission electron microscopy (TEM). Expression and localization of Ndrg2, IL-6, STAT3, and p-STAT3 in pancreatic tissues were also identified by immunofluorescence, immunohistochemistry, or Western blotting. Results showed that body weight, blood glucose, blood lipids, and insulin sensitivity indexes of the two groups were very different, with statistically significant higher levels in high-fat fed mice, indicating the successful induction of T2DM. IL-6 levels were also upregulated in diabetic mice, contributing to insulin resistance and further micro-vessel ultrastructural changes observed in the brain, kidneys, and retina tissues. Ndrg2 was mainly located in the pancreatic islet and there were significantly higher levels of Ndrg2 in diabetic mice than in controls. Moreover, IL-6/STAT3 signaling pathways were activated in diabetic mice. Expression of Ndrg2 showed a positive linear correlation with IL-6 and p-STAT3 levels, indicating that Ndrg2 may participate in the pathogenesis of type 2 diabetic mellitus through IL-6/STAT3 pathways. Overall, this study indicates the possible regulation of Ndrg2 in insulin resistance and the pathogenesis of type 2 diabetes.

Keywords: Ndrg2, insulin resistance, type 2 diabetes, IL-6/STAT3 signaling pathways

Introduction

Type 2 diabetes mellitus (T2DM) is a prevalent metabolic disorder. It has many complications that affect people of all ages all over the world [1]. Prevalence and incidence of T2DM have increased during recent decades, becoming a serious issue for public health due to complications, including diabetic foot, diabetic retinopathy, and nephropathy [2, 3]. Conventional treatment of T2DM includes the use of insulin secretagogues, such as sulfonylureas, and insulin resistance suppressors, such as metformin and thiazolidinediones, and insulin [4]. However, these treatments have limitations and cannot prevent complications of T2DM. Therefore, understanding the pathogenesis of T2DM is necessary for effective therapy in clinic. Insulin resistance (IR) and pancreatic β-cell dysfunction, ultimately leading to insufficient insulin, have been considered two fundamental characteristics in the pathogenesis of T2DM.
Ndrg2 is involved in the development of T2DM and subsequent complications [5]. However, the mechanisms of insulin resistance in T2DM remain unclear, especially in the area of related genes.

N-myc downstream regulated gene 2 (Ndrg2), a member of the Ndrg gene family, is related to stress response, cellular development, and differentiation [6-8]. Previous studies have shown that Ndrg2 is involved in the pathogenesis of tumors, including neurotransmitter [9, 10], gastrointestinal neoplasms [11], and breast cancer [12]. It plays an important role in tumor suppression. Ndrg2 is also related to neurite outgrowth and can promote the neurite outgrowth of nerve growth factor differentiated PC12 cells, hypoxia stresses, and cell metabolic processes [13, 14]. It has been reported that Ndrg2 is highly expressed in pancreatic β cells and can protect them from lipo-toxicity through Akt-mediated pathways. This suggests that Ndrg2 may be involved in pancreatic β cell function and T2DM [8]. Ndrg2 was found to be an insulin-dependent phosphoprotein, involved in diabetic cognitive dysfunction [7, 15]. However, the physiological roles of Ndrg2 in type 2 diabetic mellitus remain to be established.

Emerging studies have shown that inflammation is closely linked to type 2 diabetes [16]. Interleukin-6 (IL-6) is one of the proinflammatory cytokines that can attenuate insulin sensitivity and glucose regulation. It is related to insulin resistance of type 2 diabetes [17]. IL-6 mainly exerts its action through the activation of Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathways [18]. Previous studies have shown that the deletion of Ndrg2 decreased inflammatory response in a cortical stab injury model, while normal mice showed a higher inflammation status [19]. Therefore, the current study hypothesized that Ndrg2 may be involved in the pathogenic development of insulin resistance and type 2 diabetes through inflammation of related IL-6/STAT3 pathways. This study found that expression of Ndrg2 was significantly increased in the pancreatic islet of type 2 diabetic mice. Inflammatory response was also elevated in diabetic mice, leading to ultrastructure changes in main organs. The possible relation of Ndrg2 to inflammation and insulin resistance was also investigated. This study may provide the basis for further clarifying the function of Ndrg2 in T2DM, a novel area for clinical T2DM therapy.

Materials and methods

Experimental animals

All animal procedures were approved by the Wenzhou Medical University Animal Care and Use Committee. Forty healthy specific pathogen-free (SPF) male C57BL/6 mice, weighing 30-40 g, were purchased from the Experimental Animal Center of Wenzhou Medical University (license number: SYXK-2015-0009). All mice were maintained in the Animal Center at 25 ± 1°C under a 12-hour light-dark cycle.

Induction of diabetic animal model and grouping

Forty C57BL/6 male adult mice were randomly divided into the high-fat diet group (n=20) and normal diet group (n=20). The high-fat diet group was fed with a high-fat diet with 10% sucrose, 28% maltodextrin, 0.12% choline chloride, 10% lard, 27.5% casein, 0.24% methionine, and 0.1% salt from the SLAC Laboratory Animal Company in Shanghai, China. After 12 weeks, whole blood glucose obtained from the tail-veins was measured using a blood glucose monitor (Roche). Mice with fasting blood glucose levels ≥ 7 mmol/L and 2-hours postprandial blood glucose ≥ 11.1 mmol/L were considered diabetic mice. A total of 16 mice were successfully induced into diabetes after 12 weeks of high-fat feeding. Next, 10 randomly selected diabetic mice were used for the following study. Ten control mice were selected from the normal diet group. General conditions of mice were observed during the experimental period. Blood and tissue samples of the brain, kidneys, and retinas were collected for further detection.

Analysis of blood glucose, blood lipids, insulin levels, HOMA-IR, and oral glucose tolerance tests (OGTT)

After successful induction of the diabetic mice model, the two groups of mice were fasted for 12 hours. Blood was drawn for blood glucose, blood lipids, and insulin analysis using a standard vein blood collection technique. Serum samples were prepared by centrifuging blood
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for 10 minutes at 3000 rpm from both normal and diabetic rats. They were stored at -80°C. Fasting blood glucose and blood lipids were detected by the automatic biochemical analyzer. Fasting insulin levels were detected by the ELISA method. Insulin resistance was evaluated by calculating fasting blood glucose and fasting insulin levels using the following formula: insulin resistance index HOMA-IR = FPG (mmol/L) × FINS (ng/mL)/22.5 [20]. FPG means fasting plasma glucose and FINS means fasting insulin levels.

After 12 weeks of a high-fat diet, the diabetic group and control group were subjected to oral glucose tolerance testing. Briefly, mice fasted for 12 hours were intragastrically administrated with the glucose solution (2.0 g/kg). Blood glucose at 0, 10, 30, 60, 90, 120, and 180 minutes after treatment was detected (0 minutes means fasting blood sugar) and analyzed.

Enzyme-linked immunosorbent assay (ELISA) of IL-6

IL-6 levels from blood serum of rats were detected using an IL-6 ELISA kit. Briefly, protein samples, standards, or controls were added into each well and incubated for 2 hours at room temperature. After washing, 100 μL of IL-6 detection antibodies were added and incubated for another 2 hours. This was followed by washing and incubation with Streptavidin-HRP solution. The substrate solution was then added for color development and stop solution was finally added. The plate was read by a microreader at wavelength of 450 nm. Results are expressed in picograms of IL-6 per millimeter of blood serum.

Ultra-structures of the brain, kidneys, and retina tissues

Ultra-structures of the brain, kidneys, and retina tissues from diabetic and control mice were examined by transmission electron microscopy (TEM). Tissue samples were carefully excised and immediately fixed in 2.5% glutaraldehyde for 24 hours. They were then treated according to general protocol for TEM observation. Briefly, samples were fixed with 1% osmium tetroxide for 2 hours, routinely dehydrated through a graded ethanol series, and embedded in epoxy resin. Resin-embedded blocks were cut into 60 nm to 80 nm ultrathin sections using an ultramicrotome. Ultrathin sections were placed on carbon-coated nickel grids and examined with an H-600 TEM (H-600; Tokyo, Japan), operating at 80 kV.

Immunofluorescence or immunohistochemical staining of Ndrg2, insulin, STAT3, and p-STAT3

For immunofluorescence staining of insulin and Ndrg2, the sections were stained with primary antibodies: guinea pig anti-insulin (ab7842, 1:100) and rabbit anti-Ndrg2 (ab174850, 1:100, Abcam) at 4°C overnight, respectively. Next, the slides were washed and incubated with Alexa Fluor 488-labeled Goat Anti-Rabbit IgG or Alexa Fluor 555-labeled Donkey Anti-Mouse IgG and for 1 hour at 37°C. Finally, cell nuclei were visualized by DAPI (Beyotime Institute of Biotechnology). All fluorescent images were taken using a fluorescent microscope.

For immunohistochemical staining of Ndrg2, STAT3, and p-STAT3, tissue samples were deparaffinized and rehydrated. They were then immersed in 3% H2O2 and 80% carbinol for 15 minutes at room temperature to block endogenous peroxidase activity. To recover the antigen, slides were put into 10 mM citrate buffer and heated in a microwave oven twice. Non-specific binding sites were blocked with 5% bovine serum albumin (BSA) (Beyotime) in PBS for 1 hour at room temperature. Primary antibodies of anti-Ndrg2 (ab174850, 1:100, Abcam), anti-STAT (#9139, 1:300, CST), and anti-p-STAT (#9145, 1:200, CST) were then added and incubated with tissues at 4°C overnight. After washing, slides were then added with HRP-linked secondary antibodies for 1 hour. Antibody binding sites were visualized by incubation with a DAB chromogen kit (ZSGB-BIO, Beijing, China), then counterstained with hematoxylin (Beyotime Institute of Biotechnology, China) for 5 minutes. All slices were observed with a Zeiss 40FL Axioskop fluorescent microscope.

Western blotting

The tissues were completely homogenized in RIPA lysis buffer using a tissue grinding machine. Protein extracts were first quantified with BCA reagents. Samples containing 80 μg protein were separated on SDS-PAGE and transferred to PVDF membranes. Afterward,
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the membranes were blocked with 5% skim milk for 2 hours and incubated at 4°C overnight with anti-IL-6 (#12912, 1:1000, CST), anti-Ndrg2 (ab174850, 1:1000, Abcam) primary antibody, anti-STAT (#9139, 1:1000, CST), and anti-p-STAT (#9145, 1:2000, CST). This was followed by incubating with horseradish peroxidase-conjugated secondary antibodies for 2 hours. The bands were detected by electrochemiluminescence reagent (Invitrogen) and the signals were visualized by a ChemiDocXRS+ Imaging System (Bio-Rad).

Statistical analysis

Data from at least three individual experiments are presented as mean ± standard deviation (mean ± SD). Statistical trends were analyzed by t-tests (Graph Pad Prism 6.0) and correlation analysis. Statistical significance is set at *P < 0.05 and **P < 0.01 versus the indicated group.

Results

Analysis of body weight, blood glucose, insulin, blood lipids, and OGTT

Mice fed with a high-fat diet showed fluffy hair, polydipsia, polyphagia, polyuria, and reduced activity, compared with normal diet mice. After 12 weeks of treatment, body weight and blood glucose levels of the high-fat diet group were measured. The mice were significantly hyperglycemic (p < 0.01) and showed an increase in body weight and fasting insulin levels during this period (p < 0.01) (Figure 1A-C), indicating that type 2 DM was successfully induced in these mice. Blood lipid levels, including total cholesterol and triglycerides, were also significantly increased in the DM group, compared with controls (Figure 1D, 1E). OGTT results in diabetic and normal mice are shown in Figure 1F. After intragastrical administration of the glucose solution, blood glucose levels in both groups were significantly increased with the prolongation of time, peaking at 30 minutes, which achieved about 26.03 mmol/L in diabetic mice and 13.28 mmol/L in controls, respectively. Afterward, blood glucose levels rapidly declined in both groups within 60 minutes. They then showed a mild reduction trend from 60 to 180 minutes. Blood glucose levels of control mice became normal again within 2 hours after treatment. However, diabetic mice still showed higher blood glucose levels, even after 3 hours of treatment, suggesting that the metabolism of glucose in diabetic mice was abnormal, compared with normal mice.

HOMA-IR and IL-6 levels

HOMA-IR, an index of insulin resistance, was tested. Results are shown in Figure 2A.
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NOMA-IR was significantly promoted in diabetic mice, indicating that insulin resistance levels can be significantly upregulated by high-fat diet feeding, leading to diabetic mellitus.

Levels of IL-6 in blood serum of both normal and diabetic rats were detected by ELISA. As shown in Figure 2B, diabetic rats showed significantly higher level of IL-6s, achieving about 101 pg/mL, compared to 50.7 pg/mL in normal rats. Results indicate that a chronic inflammatory response occurred, possibly leading to other pathology changes in type 2 diabetic rats.

In addition, the current study evaluated the linear correlation between IL-6 levels and insulin resistance indexes in diabetic mice. Figure 2C shows a positive correlation between IL-6 levels and insulin resistance indexes in diabetic mice, suggesting that with increased IL-6 levels, insulin resistance levels would also be enhanced. IL-6 may be an indicator for severity of insulin resistance in type 2 diabetic rats.

Ultra-structure of mice brains, kidneys, and retinas

Pathological ultrastructural changes of major organs of diabetic rats that could be impaired by diabetes were examined using TEM. Figure 3 shows the brain, renal, and retinal tissue ultrastructure from rats. For brain tissue, in diabetic mice, the nuclear membrane of the brain tissue was complete. However, the microvascular endothelial cell nucleus was swollen with a smaller cavity. The mitochondrial structure was swollen with an incomplete bilayer membrane. In the control group, the nuclei of the brain tis-
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sues were clear with uniform distributed chromatin. The cytoplasm was abundant. Mitochondria were round and formed into a double layer. The structure of the microvascular was normal, with smooth lumen and abundant axons around them.

For renal tissue, in the diabetic group, glomerular capillary basement membranes were thick and their three-layered microstructure disappeared. The mitochondrial structure was swollen with a deficient bilayer membrane. In the control group, the micro-vessel structure of the glomerular capillary basement membrane was clear with a clear nucleus membrane. The mitochondria were round and had a double layer structure. The retinal ultrastructure was also observed. The retinal endothelial cells of the diabetic mice showed edema and irregular nuclear morphology with nuclear chromatin aggregation and sideling. The mitochondria were swollen and degenerated. Basement membrane degeneration, as well as lumen narrowing or occlusion, could be observed in diabetic mice. In contrast, the retinal basement membranes in control mice exhibited a continuous uniform membrane structure around the endothelial cells and peripheral cells. The mitochondria were intact with a round and double layer structure.

Present results show the ultrastructural changes of micro-vessels in the brain, kidneys, and retinas, confirming that the chronic inflammatory status in type 2 diabetes may impair micro-vessels in major organs.

**Immunofluorescence of Ndrg2 expression in pancreatic tissue**

Immunofluorescence images in Figure 4 show the distribution of Ndrg2 (green) and insulin (red) in mice pancreatic tissue. Ndrg2 was mainly distributed in the islet of pancreatic tissue in diabetic mice. In contrast, Ndrg2 positive stained tissue can be barely seen in the control group, suggesting that Ndrg2 level were upregulated in type 2 diabetic mice.

**Immunohistochemistry of Ndrg2, STAT3, and p-STAT3 and correlation between IL-6, Ndrg2, and p-STAT3 in pancreatic tissue**

Immunohistochemistry was used to detect expression of Ndrg2 in pancreatic tissue of mice. Figure 5A shows that Ndrg2 was mainly distributed in the islet of pancreatic tissue. The diabetic group exhibited significantly higher expression of Ndrg2 than the control group (P <0.01, Figure 5B), which confirmed immunofluorescence results of Ndrg2 in Figure 4.

Present results show that Ndrg2 was highly expressed in type 2 diabetic mice. However, whether Ndrg2 is related to IL-6 and insulin resistance and further contributes to develop-
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Figure 5. Immunochemistry staining results of Ndrg2 (A, B), STAT3 (A, D), and p-STAT3 (A, E) in two groups of mouse pancreatic tissue and the linear correlation between Ndrg2, IL-6, and p-STAT3 (C, F).
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The current study evaluated linear correlations between IL-6 and Ndrg2. As shown in Figure 5C, there was a positive correlation between IL-6 and Ndrg2 in diabetic mice, with an increase of IL-6 expression. Ndrg2 levels were also enhanced, which suggested that Ndrg2 is closely related to IL-6 and insulin resistance and may contribute to the pathogenesis of diabetic mellitus through IL-6 related pathways.

Present results also show upregulated IL-6 levels and ultrastructural changes of micro-vessels of the brain, renal, and retinal tissue in diabetic mice. To test the hypothesis that Ndrg2 may be involved in insulin resistance of type 2 diabetes through inflammation related IL-6/STAT3 pathways, this study detected expression of STAT3 and p-STAT3 in islet tissues. For STAT3 levels, there were no obvious changes between diabetic and normal mice. In terms of p-STAT3, significantly higher levels were observed in diabetic mice, compared to controls. This indicated that the phosphorylation of STAT3 was activated with the development of diabetes. Moreover, there was a positive correlation between Ndrg2 and p-STAT3 in diabetic mice, suggesting that with an increase of Ndrg2 expression, p-STAT3 levels would also be enhanced. This may be related to the pathogenesis of diabetic mellitus.

Western blot analysis

Western blot was performed to further confirm whether Ndrg2/IL-6/STAT3 pathways were activated in diabetic mice. Protein levels of Ndrg2 were detected by Western blot. Figure 6 shows protein levels of IL-6, Ndrg2, STAT3, and p-STAT3 in both types of mice. The relative band intensities of IL-6, Ndrg2, STAT3 and p-STAT3 in diabetic mice were significantly higher than controls, confirming the above immunostaining results in Figure 5. Results suggest that Ndrg2 may be a contributor to development of type 2 diabetic mellitus through activating inflammation related IL-6/STAT3 signaling pathways.

Discussion

The Ndrg family, including Ndrg1, Ndrg2, Ndrg3, and Ndrg4, is a new gene family found in recent years. This family is related to the proliferation and differentiation of cells [21]. Ndrg2 is widely expressed in a variety of tissues, including salivary glands, nervous tissue, myocardium, and skeletal muscle tissue. It plays vital roles in cell growth, stress response, and apoptosis [14]. In this study, the type 2 diabetic mice model was successfully induced through the high-fat diet feeding method. It was found that Ndrg2 was mainly distributed in the islet of the pancreatic tissue in T2DM mice. Levels of Ndrg2 were significantly upregulated in diabetic mice, compared with normal mice. In addition, expression levels of Ndrg2 were positively correlated with IL-6 levels, suggesting that Ndrg2 is related to insulin resistance levels and may participate in the pathogenesis of type 2 diabetic mellitus.

High-fat diet induced obesity and the T2DM mice models have been widely used. This method can establish proper etiological, pathological, and treatment options for researchers to investigate the phenotypic mechanisms and therapeutic possibilities [22]. This study used the high-fat diet feeding method to induce T2DM mice. After 12 weeks of feeding, 80% mice exhibited typical T2DM symptoms, such as high glucose levels (fasting status ≥ 7 and postprandial blood glucose ≥ 11.1 mmol/L), hyperlipemia, and micro-vessel pathological changes in the brain, kidneys, and retina tissues. These factors demonstrated that T2DM mice were successfully induced by the high-fat diet feeding method. Successfully induced diabetic mice were then used in subsequent experiments.

In recent years, expression of Ndrg2 in pancreatic tissues and its roles in anti-lipid toxicity through Akt-dependent signaling have been studied. Results have indicated that Ndrg2 may
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be an important biological regulator in pancreatic β cells [14]. Present results show that Ndrg2 positive staining was mainly expressed in the cytoplasm and pancreas of pancreatic islet. Expression of Ndrg2 in pancreatic islets was significantly higher than that in normal mice (P < 0.05), suggesting that Ndrg2 may be associated with the pathogenesis of diabetes mellitus as a differentiation gene. Moreover, no discernible distribution among the nuclei of pancreatic cells could be observed, suggesting that Ndrg2 may be associated with abnormal insulin secretion in pancreatic cells. On the other hand, it was also found that decreased expression of Ndrg2 will attenuate the protective effects of Akt signaling pathways on pancreatic β cells [14]. Another study found that, as a protein kinase Akt substrate, Ndrg2 was involved in insulin signaling pathways and that protein kinase C can reduce the Akt-induced phosphorylation of Ndrg2 to inhibit secretion of insulin [7, 23]. This reminds us that Ndrg2 may have the potential to be used as an insulin secretion regulator in the future.

Insulin resistance and pancreatic β cell dysfunction are the two main reasons of the pathogenesis of type 2 diabetic mellitus [24]. Insulin resistance is a pathological status in T2DM patients whose insulin-sensitive tissues and cells, such as liver, skeletal muscle, and fat, have reduced insulin-mediated glucose uptake and utilization ability, failing to maintain normal glucose levels [25]. In addition, insulin resistance is also involved in oxidative stress and inflammatory response, causing cell and vascular damage, leading to the development of diabetic microvascular disease [26-28]. The current study found that the HOMA-IR index in type 2 diabetic mice was significantly higher than in normal mice, which confirmed that insulin resistance obviously existed in diabetic mice and played an important role in the pathogenesis of T2DM. Moreover, IL-6 levels, which are pro-inflammatory cytokines, were also upregulated and showed a linear relation to insulin resistance. This may also lead to the pathological changes of micro-vessels due to the chronic inflammatory status in diabetic mice [29]. Previous studies have shown that, with the prolongation of type 2 diabetes mellitus, patients at a high risk will suffer from diabetic complications, including neuropathy, nephropathy, myopathy, and retinopathy [30, 31]. In this study, TEM observation results confirmed the distinct pathological changes in micro-vessel ultrastructures of the brain, kidneys, and retina tissues, compared with normal mice. This may be the consequence of chronic inflammation characterized with high IL-6 levels in diabetic mice. Taken together, results suggest that IL-6 pathways are closely related to insulin resistance and can be activated in the development of type 2 diabetic mellitus.

Additionally, the current study found that Ndrg2 levels are positively correlated to IL-6 and further insulin resistance, indicating that Ndrg2 may exert its biological function and participate in the pathogenesis of type 2 diabetic mellitus through IL-6 pathways. IL-6/STAT3 is one of the main signaling pathways that IL-6 can activate cells and exert its function on inflammation [18, 32, 33]. This study found that protein levels of IL-6, STAT3, and p-STAT3 were all upregulated in diabetic mice, compared with controls. Ndrg2 showed a positive correlation to p-STAT3 levels in islet issue. Present results suggest that Ndrg2 may participate in the pathogenesis of type 2 diabetic mellitus through IL-6/STAT3 signaling pathways. However, the detailed function of Ndrg2 in T2DM and other possible involved signaling pathways should be investigated in further studies.

Conclusion

The current study successfully induced type 2 diabetic mice through the high-fat diet feeding method. Diabetic mice showed significantly higher weight, blood glucose, fasting insulin, and blood lipids levels. They also exhibited abnormal glucose metabolism and increased insulin resistance levels, compared with normal mice. IL-6 levels, positively related to insulin resistance, were upregulated in the diabetic mice. This may also lead to the pathology changes of micro-vessel ultra-structures of the brain, kidneys, and retina tissues. In addition, Ndrg2, which is mainly distributed in the islet of the pancreatic tissue, was significantly upregulated in diabetic mice. Ndrg2 also showed a positive correlation with IL-6 and further insulin resistance. IL-6/STAT3 signaling pathways were activated in type 2 diabetic mice, indicating that Ndrg2 may participate in the pathogenesis of diabetic mellitus through IL-6/STAT3 pathways. This study suggests that Ndrg2 may be
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related to the pathology of diabetes and may become a therapeutic target of type 2 diabetic mellitus.

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Disclosure of conflict of interest

None.

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