# STUDY ON THE MECHANISM OF ACTION OF GIGANTOL ON DIABETIC RETINA THROUGH MIRNA-21/PI3K/AKT SIGNALING PATHWAY

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***ABSTRACT***

***Introduction:*** *to clarify the mechanism of gigantol in preventing and treating diabetic retinopathy.*

***Materials and methods:*** *in this paper, the high glucose cell model was constructed, and the miRNA-21 was transferred into the high glucose environment cells. CCK8, qRT-PCR and western blot experiments were used for study.*

***Results:*** *gigantol inhibited AKT phosphorylation, PI3K phosphorylation and VEGFA expression in vitro. Compared with the normal group, the expression level of miRNA-21 in the high glucose environment significantly increased, which promoted the expression levels of P-PI3K, P-AKT and VEGFA. The expression levels of P-PI3K, P-AKT and VEGFA decreased significantly after the intervention of gigantol. The molecular docking results showed that there was potential interaction between gigantol and AKT.*

***Conclusion:*** *gigantol may play a therapeutic role in diabetic retina through miRNA-21/PI3K/AKT signaling pathway.*

***Keywords:*** *Diabetic retinopathy, gigantol, miRNA-21, AKT.*

*DOI: 10.19193/0393-6384_2022_5_526*

***Received*** *March 15, 2022;* ***Accepted*** *June 20, 2022*

## Introduction

According to the results of epidemiological investigation, with the increase of the incidence of diabetes, the World Health Organization has made a conservative estimation that the prevalence of diabetes will reach 592 million in 2035, up to 55% from the current 382 million[(1)]. In the 30 years from 1980 to 2010, after 7 national diabetes surveys, it was found that the number of diabetic patients in the Chinese mainland increased by 17 times, and diabetes and abnormal glucose regulation have become epidemic diseases in China[(2)]. Diabetic Retinopathy (DR) is one of the microvascular complications of diabetes. At present, DR has become the main blinding eye disease among working-age people all over the world. How to effectively prevent and treat DR has become an important public health problem. DR is a complex disease. It is found that the epigenetic factor may be a major factor in the occurrence and progress of DR. MicroRNAs (miRNAs) are single-stranded non-coding RNA molecules with a length of 19 nucleotides to 22 nucleotides. They have been proved to be an essential regulator of gene expression after transcription. Plenty of evidence shows that the key functions of miRNAs exist in a wide range of physiological and pathological processes, including cell survival, proliferation, differentiation, apoptosis, and immune response[(3, 4)]. The phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway is

an important pathway of cell membrane receptor signal transduction into cells, which plays an important role in cell proliferation, growth and differentiation[5]. VEGF/PI3K/AKT signaling pathway is the most classic signaling pathway in the process of promoting endothelial cell proliferation, survival and angiogenesis. The previous research carried out by our research group found that gigantol has antioxidant and anti-apoptosis effects on human retinal microvascular endothelial cells induced by high glucose. It can inhibit cell migration and the expression of HIF-1α/VEGF pathway under high glucose[6, 7]. In addition, VEGF can stimulate downstream PI3K-AKT signaling cascade molecules, inhibit endothelial cell apoptosis, and promote endothelial cell proliferation and angiogenesis; in the hypoxic-ischemic environment induced by high glucose, it can promote the increase of the phosphorylation level of AKT in PI3K /AKT signal pathway in endothelial cells, thus inducing the formation of new blood vessels[8, 9].

Gigantor, a plant of dendrobium in Orchidaceae, is a valuable medicinal material in China. It was first published in Shennong's Herbal Classic of Materia Medica, which said: "Gigantol is mainly used for treatment of the injury to the qi of middle-jiao energy. It can relieve arthralgia, removing moisture, tonify internal organs and marked emaciation and strengthen yin. Long-term administration of gigantol can strengthen intestines and stomach, maintain neat shape and prolong life". Ancient Chinese doctors believed that gigantol has the effects of nourishing yin, clearing heat, benefiting the stomach and promoting fluid production, and it can be used to treat diseases such as blurred vision. Modern pharmacological studies have found that gigantol contains polysaccharides, alkaloids, dibenzyl compounds and other effective components. Among them, dibenzyl compounds are important chemical components in gigantol, which have important biological activities such as anti-tumor activity and anti-mutagenesis activity[10].

Gigantor is a dibenzyl compound monomer extracted and separated from dendrobe. It is recorded in Chinese Pharmacopoeia (2015 edition)[11] that gigantol is the quality control index of dendrobium fimbriatum hook with a molecular weight of 274.31. It not only contains active hydroxyl groups, but also has certain hydrophobicity and aromaticity. Thus, it has a wide range of pharmacological activities. In this paper, the model of high glucose human retinal vascular endothelial cells was constructed, and miRNA-21 was transfected into cells in high glucose environment. The expression levels of miRNA-21, PI3K and AKT were observed by CCK8, QRT-PCR and WB to provide a possible basis for the treatment of DR with gigantol.

## Materials and methods

### *Cell culture*

ECM culture medium containing 10% fetal bovine serum and 1% double antibody (mixture of streptomycin) was added in an incubator and cultured at 37°C and 5% $CO_2$. Microscopically, the cells were adherent cells. Cells in logarithmic growth phase were digested with trypsin and inserted into six-well plates at a density of 300,000 cells/well. The cells grew adhering to the wall for 48h.

### *Cell transfection*

Cells were cultured in a culture medium containing 10% fetal bovine serum and 1% double antibody (mixture of streptomycin) at 37°C and 5% $CO_2$. After reaching a certain cell density, the cells were digested by trypsin.

After counting under the microscope, they were spread into a 6-well culture plate at a density of 500,000 cells per well. After adding 2mL culture medium into each well, the cells were cultured in the incubator with 5% $CO_2$ at 37°C. After growing to 70%-80%, they were transfected.

• A) blank group: 250 ul Opti-mem serum-free medium;

• B) NC group: 5ul NC (about 100pmol) was dissolved in 245ul Opti-mem serum-free medium.

• C) mir-21-5p mimicry group: 5 ul mir-424-3p mimicry (about 100pmol) was dissolved in 245ulOpti-mem serum-free medium.

• D) 15ul lipo2000 was dissolved in 735ul Opti-mem serum-free medium. After mixing well, the solution stood at room temperature for 5min. After mixing A with 1/3D, B with 1/3D, and C with 1/3D, the solution stood at room temperature for 20min. After discarding the original culture medium, the mixture was rinsed once with sterile PBS. Then, 2mL serum-free culture medium was added. After that, the mixture in each tube was slowly added into the corresponding culture solution and shaken well. After keeping it in an incubator at 37°C for 6h, the serum-free transfection solution was sucked out and complete culture solution was added to continue the culture. Cells were collected after transfection for 24h for follow-up experiments.

***qRT-PCR***

Total RNA was extracted from cultured cells using TRIzol reagent. For mRNA detection, cDNA was synthesized from 1 μg total RNA using PrimeScript™ RT Master Mix (Takara Biotechnology, Japan). Then, qRT-PCR analysis was performed with SYBR Premix Ex Taq TM (Takara Biotechnology, Japan). The relative expression of GAPDH mRNA was measured by relative standard curve method ($2^{-\triangle\triangle CT}$) as follows: mmu-PTEN: forward, 5'-TTGGCCGGTGTCATAATGTCT-3', backward, 5'-GCAGAAAGACTTGAAGGCGTA-3'; and mmu-GAPDH: forward, 5'-GTGGCAAAGTGGAGATTGTTGCC-3' and backward, 5'-GATGATGACCCGTTTGGCTCC-3'.

The qRT-PCR reaction was carried out using FastStart Universal SYBR Green Master Mix (Roche, Indianapolis, USA), miRNA specific forward primer and universal reverse primer (RiboBio, Guangzhou, China).

***Western blot***

The expression of related proteins was detected. After the cell treatment, the cells were washed twice with PBS. RIPA was used to split the cells, and protease inhibitor and phosphatase inhibitor (100: 1: 1) were used to split the cells. The total protein of the cells was extracted. The protein concentration was measured by BCA, and 10ul per well was quantified.

Electrophoresis with 10%SDS-PAGE gel at 80mv was carried out for 30min and at 120mv for 60min. Membrane transfer was carried out at 200mA and under constant pressure. After sealing (sealing with 10%BSA for phosphorylation, and sealing with 10% skim milk for non-phosphorylation), primary antibody was added for incubation overnight at 4°C. After that, the membrane was washed with TBST for 3 times. Then the second antibody IgG labeled with horseradish peroxidase was added for incubation at room temperature for 1h. TBST film washing followed by development and exposure. Photoshop software was used for analysis. The ratio of optical density of each group to β-tubulin optical density was calculated, and the relative expression level of protein was analyzed.

***Molecular docking***

The protein crystal structure of AKT (code 2UZT) has been analyzed (protein database; http://www.rcsb.org/). The chemical structural formula of gigantol was confirmed on the website https://pubchem.ncbi.nlm.nih.gov/ (Compound CID: 3085362), and then the 3D structural formula was downloaded and saved in SDF format. Finally, the protein structure and the 3D structure of gigantol were imported into the software (http://clab.labshare.cn/) to get the results.

***Statistical methods***

Statistical analysis: SPSS 25.0 statistical software was used for statistical analysis, and the data were expressed as mean±standard deviation. One-way ANOVA was used for comparison between groups. If the difference was statistically significant, LSD-t-test was further used for pairwise comparison. $P<0.05$ was used to judge whether the difference was statistically significant.

## Results

***Effect of gigantol on cell activity***

The results of CCK8 experiment are shown in Figure 1A. After setting different concentrations of gigantol gradient (1-150μmmol/L) and incubating cells in normal glucose concentration environment for 48 hours, the results showed that except for the induction of apoptosis at 150μmmol/L, the other groups had no significant cytotoxicity. In addition, when the gigantol concentration was 25μmmol/L, it had statistical significance compared with the control group ($p<0.05$). According to the experimental results above, gigantol (1-100μmmol/L) was added into 30mM high glucose environment. The results of CCK8 are shown in figure 1B. The cell activity of the high glucose group was higher than that of the normal group, indicating statistical significance ($p<0,05$). Compared with the high glucose group, the cell activities of the gigantol drug intervention groups were significantly inhibited, except that 1μmmol/L gigantol had no significant inhibition on cell proliferation induced by high glucose. When the drug concentration was 5μmmol/L, there was statistical significance ($p<0.05$). When the drug concentration reached 50μmmol/L, there was statistical significance ($p<0.001$). When the drug concentration reached 100ummol/L, there was statistical significance ($p<0.001$). The results suggested that the inhibitory effect of dendrophenol on HG-induced cell proliferation was not caused by cytotoxicity. Since the cell activity was lower than that in the normal group when the dendrophenol concentration reached 100μmmol/L, this concentration was not selected. The concentration of 50μmmol/L gigantol was selected for the subsequent experiment.

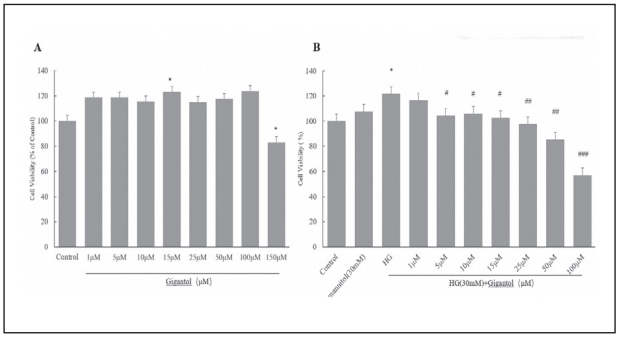


**Figure 1:** Effect of gigantol on cell activity (A) Cell activity under different concentrations of gigantol at normal glucose concentration for 48h. (b) Cell activity under different concentrations of gigantol at high glucose concentration for 48h.
**: p<0.05. vs control group #: p<0.05, ##: p<0.01, ##: p<0.001. vs high glucose group.*

### *Gigantor can inhibit RNA expression of VEGFA and AKT induced by miRNA-21 in high glucose environment*

It was found that the RNA level of miRNA-21 significantly increased under the stimulation of high glucose. Compared with the normal group, the protein expressions of AKT and VEGFA in high glucose group were higher, and the difference was statistically significant (p<0.05). Compared with the high glucose group, the RNA expression levels of AKT and VEGFA in the high glucose group of miRNA-21 groups were higher, and the difference was statistically significant (p<0.05).

Compared with anti-miRNA-21 group, it was found that the expression of AKT and VEGFA RNA in anti-Mirna-21 group increased and decreased, and the difference was statistically significant (p<0.05). The RNA expression of AKT and VEGFA after gigantol intervention was significantly lower than that of miRNA-21 group. In addition, there was no significant difference between mannitol group and normal group (p>0.05).

### *Gigantor can inhibit the protein expressions of P-PI3K, P-AKT, and VEGFA induced by miRNA21*

The results showed that there were differences in the expression of miRNA21, P-PI3K, P-AKT and VEGFA proteins among the groups (Figure 3b, C, D). Compared with the normal group, the protein expressions of PI3K, AKT and VEGFA in the high glucose group were significantly higher (p<0.05). Compared with the high glucose group, the protein expressions of PI3K, AKT and VEGFA in the high glucose group of miRNA-21 group were significantly higher (p<0.05). Compared with the anti-miRNA-21 group, the expression of PI3K, AKT and VEGFA in anti-Mirna-21 group increased and decreased, and the difference was statistically significant (p<0.05). The protein expression of PI3K, AKT and VEGFA after the intervention of gigantol drugs was significantly lower than that of miRNA-21 group. In addition, there was no significant difference between mannitol group and normal group (p>0.05).

The results showed that there was no significant difference in protein expression of AKT and PI3K in each group (Figure 3e, F), and there was no statistical significance (p>0.05).

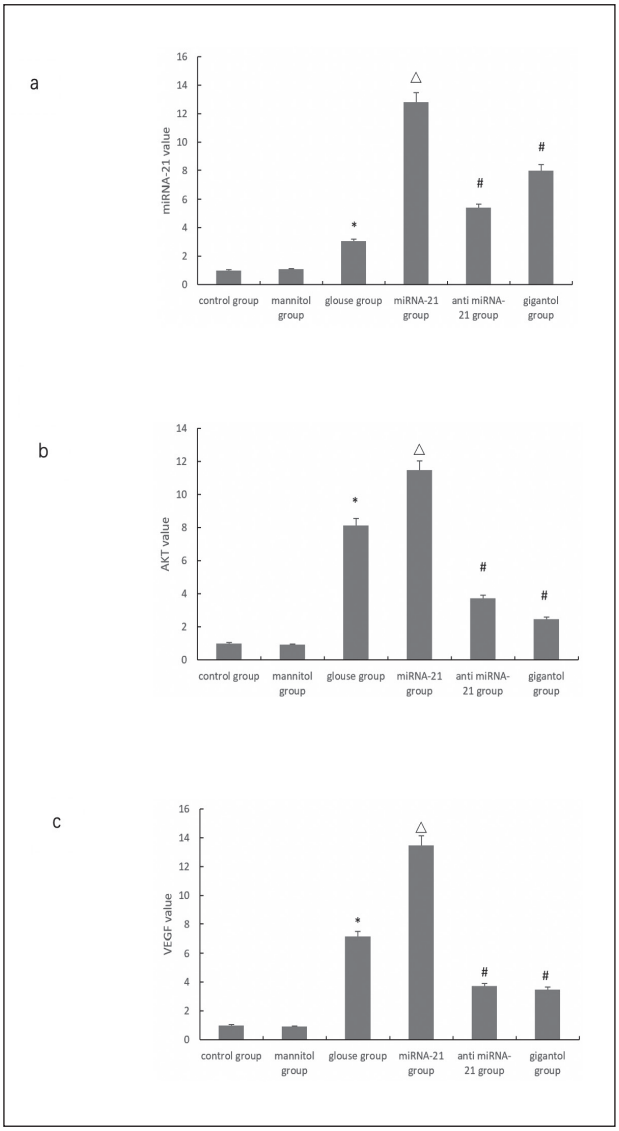


**Figure 2:** (a) Expression of miRNA-21 induced by high glucose. (b) gigantol reduced the RNA expression of AKT induced by miRNA21 and high glucose. (c) gigantol decreased the RNA expression of VEGFA induced by miRNA21 and high glucose.
**p<0.05 vs normal group; △p<0.05 vs miRNA-21 group, #p<0.001 vs high glucose group.*

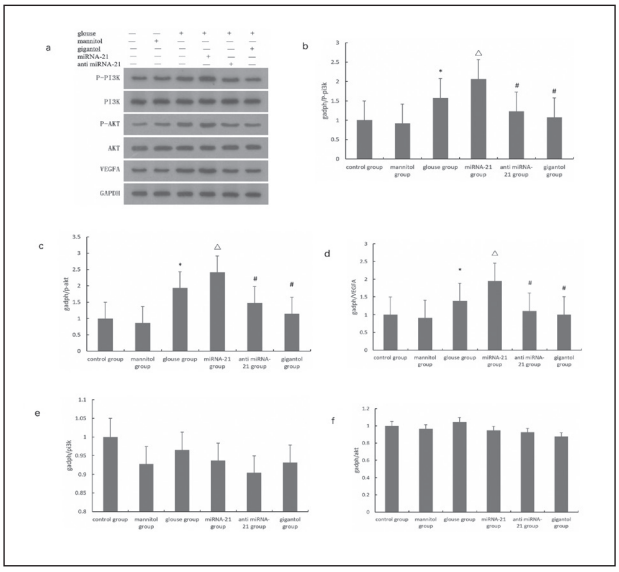


**Figure 3:** Gigantol inhibits the protein expression of P-PI3K, P-AKT and VEGFA induced by miRNA21 in cells induced by high glucose. (a) Gigantor decreased the protein expression of P-PI3K, P-AKT and VEGFA induced by miRNA21 and high glucose. (b) Optical density analysis of P-PI3K protein protein quantification. (c) Optical density analysis of P-Akt protein quantification. (d) Optical density analysis of d)VEGFA protein quantification. (e) Optical density analysis of PI3K protein quantification. (f) Optical density analysis of Akt protein quantification.
*p<0.05 vs normal group; △p<0.05 vs miRNA-21 group and #p<0.001 high glucose group.

***Gigantor has strong ability of binding with AKT***

The 3D and 2D interaction maps of gigantol and AKT molecule docking experiment (Figure 4) show that gigantol interacts with AKT at the interface binding site of AKT, and the absolute value of gigantol binding energy is 8.1kcal/mol.

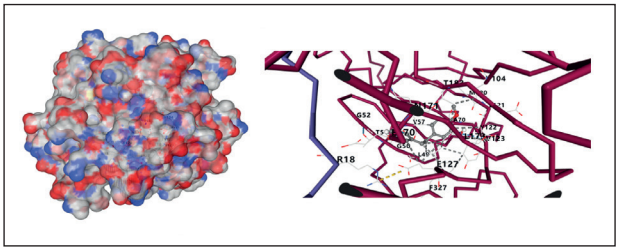


**Figure 4:** Docking between gigantol and AKT molecule. Absolute value=8.1

## Discussion

DR is a serious diabetic microangiopathy. Clinically, DR can be divided into two types: No Proliferative Diabetic Retinopathy (NPDR) and Proliferative Diabetic Retinopathy (PDR). It is found that the earliest response of retinal blood vessels in diabetic patients to hyperglycemia is the expansion of blood vessels and the change of blood flow.

These changes are caused by the body function of diabetic patients, and their purpose is to increase retinal metabolism[12]. Hyperglycemia plays an important role in the pathogenesis of retinal microvascular injury. High glucose mainly damages retinal blood vessels through the polyol pathway, accumulation of advanced glycation end products (AGEs), protein kinase C(PKC) pathway and hexosamine pathway[13, 14]. Pericytes are responsible for providing structural support for capillaries. Through in vitro and in vivo experimental studies of apoptosis, it is found that high glucose can promote pericytes' apoptosis during the onset of DR, and the early local damage of capillary wall is caused by pericytes' loss. This process is related to the clinical formation of fundus microaneurysm, which is the earliest clinical feature of DR[15, 16]. In addition to the loss of pericytes, the apoptosis of endothelial cells and the thickening of basement membrane together lead to the damage of blood-retinal barrier[17]. Moreover, capillary occlusion and ischemia are also caused by the injury of pericytes and endothelial cells. Ischemia/hypoxia in the retina leads to the up-regulation of vascular endothelial growth factor (VEGF) by activating hypoxia-inducible factor-1 (HIF-1)[18]. Other evidence shows that the increase of phospholipase A2 (PLA 2) in diabetes also triggers the up-regulation of VEGF[19, 20].

Mirna molecules are important regulators of gene expression, and can participate in a wide range of abnormal regulation processes. Previous studies have proved the importance of miRNA for retinal nerve cell differentiation and retinal precursor cell survival[11]. In addition, some researchers began to identify specific miRNAs. They identified the target genes of PDR from the early stage of NPDR. There are MiRNA-21, miRNA-181c, miRNA-1179 and miRNA-200 families in serum of patients with NPR and PDR. These miRNAs highly regulate oxidative stress and play an important role in endothelial abnormalities and pathogenesis-related to oxidative stress in DR[13]. Several miRNAs, including miRNA-21, have become important regulators of the development of Diabetic Nephropathy (DN). It was found that the expression of miRNA-21 increased in the serum and kidney tissues of DN patients, and the expression of miRNA-21 was up-regulated in kidney tissues of STZ-induced DN rats and podocytes treated with HG. Protein blot analysis

showed that the expression of Bax protein in kidney tissue of STZ-treated DN rats was significantly up-regulated, and the level of Bcl-2 protein was significantly reduced. However, inhibiting the expression of miRNA-21 with miRNA-21 inhibitor can greatly weaken these changes.

The reduction of the secretion of pro-inflammatory factors (IL-1β, TNF-α) can alleviate the kidney injury induced by STZ[21]. Angiogenesis, that is, the formation process of new blood vessels, refers to the generation of new blood vessels from endothelial cells, which is crucial in the process of development and subsequent physiological dynamic balance. In this study, it was found that gigantol could inhibit the expression of PI3K and AKT by inhibiting miRNA-21. Thus, we suspect that gigantol may exert its therapeutic effect on DR through miRNA-21/PI3K/AKT signaling pathway.

## Conclusion

This study indicated that gigantol, as one of the important quality indicators of gigantol, may inhibit the formation of DR neovascularization by inhibiting miRNA-21/PI3K/AKT signaling pathway. Therefore, it may play a therapeutic role in dr.

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*Funds:*
*This work was supported by the Shanghai Hongkou District Health Commission (Grant No. HongWei1901-1).*

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