

# Research on the Correlation Between rs2110385 Polymorphisms of the Visfatin Gene and Nonproliferative Diabetic Retinopathy

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**Abstract:** *Objective:* To investigate the association between rs2110385 polymorphisms of the visfatin gene and the risk of type 2 diabetic retinopathy (DR). *Methods:* 172 Han subjects were selected from Xi'an Shaanxi Province; 140 patients with type 2 diabetes mellitus (T2DM) and 32 normal controls (NC) were selected from our hospital. Patients with diabetes were divided into a non-DR group (T2DM) ( $n = 69$ ) and a nonproliferative diabetic retinopathy Group (DR) ( $n = 71$ ) after dilated fundus photography and fundus fluorescein angiography. rs2110385/Alu I genotypes were detected by standardized polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and the differences in the detection rates of different genotypes in the above populations were compared. *Results:* 1) The visfatin level in the DR Group was significantly higher than that in the NC and T2DM groups ( $P < 0.05$ ). 2) The frequency of GG genotype and G allele of rs2110385 in the DR Group were higher than those in the T2DM and NC groups (80.3, 69.6, 50.0, 86.6, 79, 65.6,  $P < 0.05$ ). 3) There were significant differences in allele frequency and genotype frequency distribution of rs2110385 between the DR Group and the NC group ( $P < 0.01$ ). *Conclusion:* Visfatin increased in the nonproliferative diabetic retinopathy group and could be a potential indicator for the clinical prediction of DR. The G allele of the rs2110385 polymorphic site may be related to the risk of DR.

**Keywords:** Visfatin; Diabetic retinopathy; Single nucleotide polymorphism; Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

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## 1. Introduction

With the rapid growth of China's social economy and changes in people's dietary structure and workload, the incidence of type 2 diabetes mellitus (T2DM) has increased yearly and poses a threat to human health. Further research on the pathophysiological mechanisms of T2DM has shown that the occurrence of diabetic complications was related to several adipocytokines. Visfatin has the effect of improving insulin sensitivity. It is

an adipokine that is mainly secreted by visceral adipose tissue. The human visfatin gene is 37,400 bp long and contains 10 introns and 11 exons. It has inflammatory and catabolic characteristics related to metabolic diseases such as obesity, insulin resistance, and T2DM <sup>[1]</sup>.

Researchers have found that the visfatin gene rs4730153 single nucleotide polymorphism (SNP) genotype AA was significantly associated with fasting blood glucose, fasting insulin, and HOMA-IR (homeostasis model assessment-insulin resistance), further confirming that different polymorphisms of the visfatin gene may affect obesity and glucose homeostasis <sup>[2]</sup>. Lu et al. <sup>[3]</sup> studied the role of the visfatin gene single nucleotide polymorphism (rs2110385) in patients with type 1 diabetes mellitus (T1DM) on the amount of insulin required to maintain glucose homeostasis. They found that the insulin dose used by the GG genotype to maintain glucose homeostasis was significantly lower than that of other genotypes <sup>[3]</sup>. Diabetic retinopathy (DR) is one of the common microvascular damages in diabetic patients. In developed countries, it is the most common cause of new blindness in adults aged 20–74 <sup>[4]</sup>. There are many related studies on visfatin and diabetes locally and internationally. However, research on the relationship between visfatin gene polymorphism and DR is relatively lacking. This study analyzed the visfatin rs2110385 polymorphisms in the Han diabetic population in Xi'an, Shaanxi Province, and whether it affects the occurrence and development of type 2 DR at the genetic level.

## 2. Objects and methods

### 2.1. Main experimental instruments and reagents

Polymerase chain reaction (PCR) thermal cycler (American BIO-RAD Company 580BR 10843); water-isolated incubator (Tianjin Taisite Instrument Co., Ltd. GH 3000); gel imager (American Gene Co., Ltd.); low-temperature high-speed centrifuge (American Gene Co., Ltd. Company Centrifuge 5424E); electrophoresis instrument (Beijing Liuyi Biotechnology); water bath (Tianjin Taisite Instrument Co., Ltd. DK-98-11); restriction endonuclease Alu 1 (Xi'an Runde Biotechnology Co., Ltd.); human Genomic DNA extraction kit (Omega, USA). Biochemical indicators were detected by enzymatic methods, insulin was detected by radioimmunoassay (RIA), glycated hemoglobin (HbA1C) was detected by high-pressure liquid chromatography (completed by DIASAT-20 detector of BIO-RAD Company); serum visfatin level was detected by ELISA (American R&D Company).

### 2.2. Research subjects

Sixty-nine patients with a first-onset of T2DM who were hospitalized in the Endocrinology Department of the First Affiliated Hospital of Xi'an Medical University from April 2019 to January 2022 were selected, including 38 males and 31 females with an average age of  $(51.3 \pm 8.68)$  years; 71 patients had diabetic retinopathy, consisting of 43 males and 28 females, with an average age of  $(52.18 \pm 4.19)$  years. The control group consisted of 32 patients with normal glucose tolerance (17 males and 15 females), with an average age of  $(49.95 \pm 5.80)$  years. All subjects were not related by blood. Inclusion criteria: Meet the diagnostic criteria for T2DM based on the 2010 ADA: Medical Standards for the Diagnosis and Treatment of Diabetes <sup>[5]</sup>. Exclusion criteria: (1) Patients with acute complications of T2DM and severe infection; (2) severe liver and kidney damage and cardiopulmonary failure; (3) sequelae of severe cerebrovascular disease and are bedridden long-term; (4) usage of drugs that affect bone metabolism and treat osteoporosis; (5) diseases that affect retinopathy, such as macular degeneration and optic neuritis; (6) proliferative DR, diabetic maculopathy, or those requiring acute treatment through fundus examination according to the diagnostic criteria for DR in the Clinical Diagnosis and Treatment Guidelines for DR in China (2014) <sup>[6]</sup>.

### 2.3. Sample collection, DNA extraction, and biochemical index determination

All subjects fasted for 12 hours, and 6 ml of fasting venous blood was drawn early the following day. They were placed in 2 anticoagulant tubes with 3 ml each. One anticoagulant tube was immediately sent to the laboratory for automatic use. The serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were detected by a biochemical instrument; the fasting blood glucose (FBG) was detected by the glucose enzymatic method. The second anticoagulant tube was stored in a -80°C refrigerator for future inspection, and a human genomic DNA reagent extraction kit was used to extract leukocyte DNA from peripheral venous blood, and stored at 4°C for later use. The body mass index (BMI) and maximum heart rate (MHR) were calculated.

### 2.4. Target gene amplification (PCR reaction)

The primers were designed by Primer Premier 5.0 (PCR primer design software) and synthesized by Xi'an Runde Biotechnology Co., Ltd.; upstream 5'ATGCTGTTTCACATCCTCC3', 10 bp; downstream 5'TTTCCTCAGACCTGCTTGC3' 9.5 bp. All were prepared with sterile deionized water to 10 $\mu$ mol/L and kept at -20°C for later use.

PCR reaction conditions: The reaction system was set to be 25  $\mu$ L and each of the upstream and downstream primers were 1  $\mu$ L, Taq enzyme mix (12.5  $\mu$ L), template DNA (2  $\mu$ L), and double distilled water (ddH<sub>2</sub>O) of 8.5  $\mu$ L was added to make up the volume to 25  $\mu$ L.

Cycle parameters: After pre-denaturation at 95°C for 5 minutes, the main cycle was initiated (denaturation at 95°C for 30s, annealing at 58°C for 40s, extension at 72°C for 45s) for 30 cycles, with the final cycle at 72°C for 10 min, and the reaction was terminated by setting it to 4°C. 5  $\mu$ L of the PCR reaction product was obtained and detected by 1.8% agarose gel electrophoresis, and the product length was determined to be 187 bp.

### 2.5. Enzyme digestion reaction

PCR product enzyme digestion system (10  $\mu$ L): restriction endonuclease Alu I restriction endonuclease (0.5  $\mu$ L), 10 $\times$ NEB2 (1.0  $\mu$ L), rs2110385 site PCR product (2.0  $\mu$ L), with 6.5  $\mu$ L ddH<sub>2</sub>O added to make up the volume to 10.0  $\mu$ L. After centrifugation and mixing, the enzyme was placed in a 37°C incubator for overnight enzymatic hydrolysis.

### 2.6. Identification of enzyme digestion products

6  $\mu$ L of the digestion products were added to the wells of a 1.8% agarose gel and the naphthalene-2,3-dicarboxaldehyde (NDA) maker was used as a reference. Electrophoresis was carried out at 100 volts for 30 minutes and the genotype was observed under a gel imager and photographed simultaneously.

### 2.7. Statistical Analysis

The SPSS 27.0 statistical software was used. Measurement data are expressed as mean  $\pm$  standard deviation, and count data are expressed as frequency. Each group's allele and genotype frequencies were calculated. After the Hardy-Weinberg (HW) equilibrium, the chi-squared ( $\chi^2$ ) test was used to compare frequencies between groups. The *t*-test was used to compare the different genotypes within clinical data groups. Susceptibility analysis used unconditional logistic regression analysis. All significance tests were two-sided (test level  $\alpha < 0.05$ ). Results were considered statistically significant at  $P < 0.05$ .

### 3. Results

#### 3.1. Comparison of general clinical indicators

As shown in **Table 1**, Compared with the NC group, the blood visfatin, TC, FBG and diastolic blood pressure (DBP) of the DR group were significantly higher as compared to the NC group ( $P < 0.05$ ). The systolic blood pressure (SBP), FBG, TC, HDL, and LDL in the T2DM group were significantly higher than those in the NC group ( $P < 0.05$ ). The blood visfatin level of patients in the DR group was significantly higher as compared to those in the NC group ( $P < 0.05$ ).

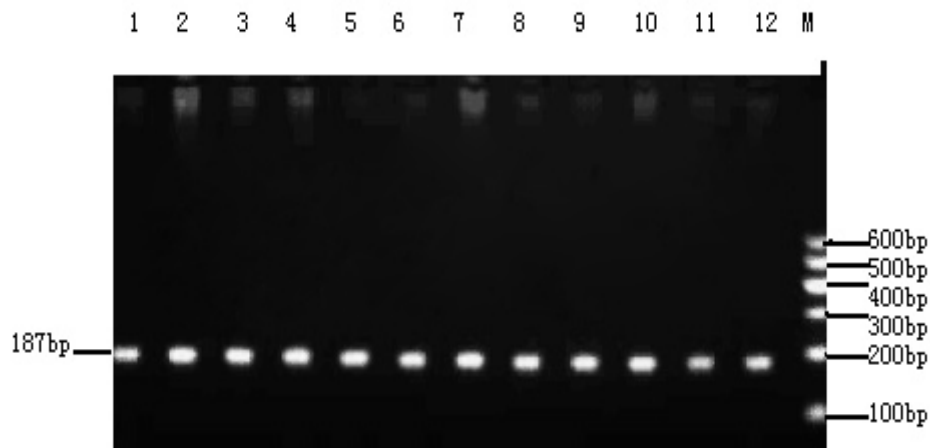
**Table 1.** Comparison of general clinical data (mean  $\pm$  standard deviation, ng/L)

Group	Cases, <i>n</i> (men and women)	Age (Year)	BMI (kg/m <sup>2</sup> )	WHR	SBP (mmHg)	DBP (mmHg)	FBG (mmol/l)	TC (mmol/L)	TG (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)	Visfatin (pmol/l)
NC	32(17/15)	49.95 $\pm$ 5.80	23.73 $\pm$ 1.53	0.88 $\pm$ 0.05	117.6 $\pm$ 7.81	79.7 $\pm$ 7.10	5.22 $\pm$ 0.82	4.14 $\pm$ 0.99	1.68 $\pm$ 1.04	2.65 $\pm$ 0.73	1.17 $\pm$ 0.31	76.39 $\pm$ 8.83
T2DM	69(38/31)	51.3 $\pm$ 8.68	24.38 $\pm$ 3.09	0.90 $\pm$ 0.067	130.7 $\pm$ 20.08 $\blacksquare$	82.33 $\pm$ 10.83	7.76 $\pm$ 1.65 $\blacksquare$	4.85 $\pm$ 1.04 $\blacksquare$	1.94 $\pm$ 1.17	3.26 $\pm$ 0.79 $\blacksquare$	1.00 $\pm$ 0.21 $\blacksquare$	81.66 $\pm$ 9.25 $\blacksquare$
DR	71(43/28)	52.18 $\pm$ 4.19	24.27 $\pm$ 3.52	0.89 $\pm$ 0.08	138.3 $\pm$ 20.34	81.66 $\pm$ 9.25 $\blacksquare$	8.56 $\pm$ 3.32 $\blacksquare$	4.85 $\pm$ 1.04 $\blacksquare$	1.20 $\pm$ 1.27	3.19 $\pm$ 1.30	1.08 $\pm$ 0.27	90.89 $\pm$ 10.74 $\blacksquare\blacktriangle$

Note: Compared with the healthy control group,  $\blacksquare$  represents  $P < 0.05$ ; compared with the T2DM group,  $\blacktriangle$  represents  $P < 0.05$ .

Abbreviation: Body mass index, BMI; systolic blood pressure, SBP; diastolic blood pressure, DBP; total cholesterol, TC; triglycerides, TG; glycosylated hemoglobin, HbA1C; fasting blood glucose, FPG; type 2 diabetes, T2DM; healthy control, NC; diabetic retinopathy, DR; waist-to-hip ratio, WHR

#### 3.2. PCR amplification product results

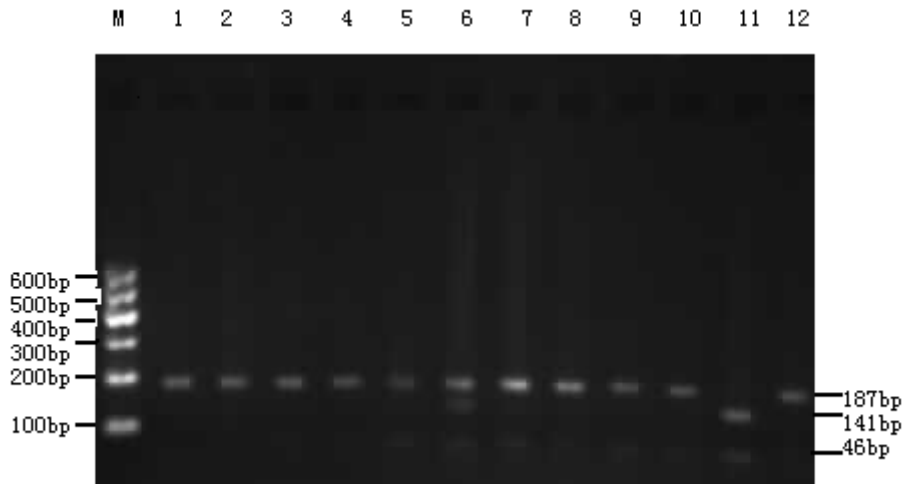


**Figure 1.** PCR product at rs2110385 of visfatin gene by 1.8% agarose gel electrophoresis

Lane M indicated the DNA marker of 600, 500, 400, 300, 200, and 100 bp from top to bottom. Lanes 1–12 were 187 bp of PCR product containing rs2110385.

#### 3.3. Visfatin gene polymorphism (SNP) analysis

After the PCR product of the visfatin gene rs2110385 site was digested with Alu I restriction endonuclease, the electrophoresis results showed that the band with only 187 bp was G/G homozygous, and the band with 46 and 141 bp was T/T homozygous; the band with 46, 141, 187 bp were G/T heterozygotes.



**Figure 2.** Genotype analysis of rs2110385 polymorphisms of visfatin gene

Lane M indicated the DNA marker; lanes 1, 2, 3, 4, 5, 7, 8, 9, 10, and 12 were G/G genotype; lane 6 was G/T genotype; lane 11 was T/T homozygous.

### 3.4. Genetic fit test (HW equilibrium test) results of the research subjects

There were no significant differences between the HW balance and the theoretical frequency of genotype distribution in this study population ( $P > 0.05$ ). This indicated that the sample in this study was representative of the population.

### 3.5. Comparison of genotype distribution and allele frequency between groups

As shown in **Table 2**, The GG genotype and G allele frequency of the visfatin gene rs2110385 locus showed an increasing trend in the NC group, T2DM group, and DR group. There were significant differences in the genotype frequency and allele frequency distribution of rs2110385 among the three groups ( $P < 0.01$ ).

**Table 2.** Comparison of genotype and allele frequency distribution among the three groups

Group	Cases, <i>n</i>	Genotype (%)			Allele (%)	
		GG	TT	GT	G	T
NC	32	16 (50.0)	6 (18.6)	10 (31.2)	42 (65.6)	22 (34.4)
T2DM	69	48 (69.6)	8 (11.6)	13 (18.8)	109 (79.0)	29 (21.0)
DR	71	57 (80.3)	5 (7.0)	9 (12.7)	123 (86.6)	19 (13.4)

Comparison of genotype distribution between groups,  $\chi^2 = 9.747$ ,  $P = 0.045$ .

Comparison of allele frequencies between groups,  $\chi^2 = 12.06$ ,  $P = 0.002$ .

As shown in **Table 3**, there were no significant differences in the frequency distribution of rs2110385 genotypes and alleles between T2DM and NC groups, and T2DM and DR groups ( $P > 0.016$ ). There was a significant difference in the allele frequency distribution of the rs2110385 genotypes and alleles between the DR and NC group ( $P < 0.016$ ).

**Table 3.** Pair-to-pair comparison between groups (Bonferroni method, calibration test level  $\alpha = 0.05/3 = 0.016$ )

Contrast group	1 $\chi^2$	1P	2 $\chi^2$	2P
NC vs T2DM	3.607	0.165	4.135	0.042
NC vs DR	9.810	0.007	12.198	< 0.001
T2DM vs DR	2.163	0.339	2.872	0.090

Note: 1 $\chi^2$  and 1P represent pairwise comparisons of genotype distributions between groups; 2 $\chi^2$  and 2P represent pairwise comparisons of allele frequencies between groups.

#### 4. Discussion

Visfatin is an adipokine mainly produced and secreted by adipose tissue. It has pro-inflammatory properties and can reduce insulin sensitivity and promote the development of insulin resistance. Studies have shown that visfatin induces Hep G2 cells to produce pro-inflammatory cytokines through the STAT3 and nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathways and inhibits the insulin signaling pathway [7]. Elevated blood visfatin levels in patients with T2DM or metabolic syndrome are related to coronary and carotid atherosclerosis [8]. Some researchers also believe that serum visfatin levels in T2DM patients were significantly elevated, especially in T2DM GG genotype patients with cardiovascular disease (CVD) [9]. The results of this study showed that the blood visfatin levels of patients in the DR group were significantly higher as compared to those in the NC group and the simple T2DM group. A recent study reported that visfatin was highly expressed in the serum and vitreous bodies of patients with DR [10]. Under high glucose conditions, visfatin promotes the proliferation, migration, and tube formation of RF/6A cells, suggesting that the visceral fat hormone strongly affects retinal neovascularization.

Studies have reported that glucose and lipid metabolism and obesity in the body were affected to a certain extent by genetic locus variations of visfatin, where visfatin gene polymorphisms may significantly increase one's susceptibility to obesity [1]. Li et al. [11] reported that lipid metabolism disorder in T2DM in Chinese patients was related to the -1535C/T polymorphism of the visfatin gene promoter. In diabetic Egyptians, both the NAMPT-948G/G genotype and G allele were significantly associated with T2DM and CVD. The genetic variation of NAMPT-948G/T may be associated with T2DM and its CVD complications in Egyptian patients [9]. Zhang et al. [12] analyzed the visfatin gene promoter rs2110385 polymorphism in the Han population in Gansu, China, and suggested that IT may be associated with T2DM in the Chinese Han population. Yan et al. [13] selected the Han population in Xi'an, Shaanxi Province for research and conducted polymorphism analysis at multiple sites of the visfatin gene, including the rs2110385 site. The results showed that the GT genotype and T allele frequency at rs2110385 were higher in the T2DM group as compared to the control group, suggesting that the T allele at this locus may have a certain relationship with the risk of T2DM [13]. Although there have been relevant studies on visfatin gene polymorphisms, there are insufficient studies on visfatin gene polymorphisms in DR patients. This study compared the genotype and allele frequency of visfatin rs2110385 locus between the DR and NC group. It was found that the GG genotype frequency and G allele frequency of the rs2110385 locus in the DR group were higher as compared to those in the T2DM and NC groups; There were significant differences in the rs2110385 genotype and frequency distribution between DR and NC groups. The G allele of the visfatin rs2110385 polymorphism may be associated with the risk of type 2 DR. Multi-center and multi-sample studies are needed to further clarify this.

Research theories on the relationship between visfatin gene polymorphisms and abnormal glucose and lipid metabolism are also somewhat controversial [14]. Paschou et al. [15] studied 12 SNP sites of visfatin and found that it does not correlate with diabetes; Liu et al. [16] found that the rs4730153 polymorphism may affect the

occurrence and development of acute coronary syndrome (ACS) by regulating the level of visfatin and related factors and regulating the expression of inflammation or metabolism. In addition, Marjani et al.<sup>[17]</sup> also found that the rs4730153 polymorphism was associated with lipid metabolism and insulin resistance but not with non-alcoholic fatty liver disease.

T2DM is a complex polygenic genetic disease where complications gradually appear as the disease progresses. Zhang<sup>[18]</sup> showed that vascular endothelial adhesion molecule-1 (VCAM-1) was a marker of impaired vascular endothelial function. Visfatin can induce an increase in VCAM-1, suggesting that visfatin is related to the vascular complications of diabetes and is the cause of the impairment of vascular endothelial function in diabetes. This study only selected the rs2110385 locus of visfatin to study among the local population in Xi'an, Shaanxi. Genetic variation was found at this locus, and the visfatin level and G alleles were expected to become early indicators for predicting the occurrence and development of DR. However, this study only analyzed one gene locus of visfatin. Since T2DM is a polygenic genetic disease, the sample size needs to be increased to reveal the correlation between each gene locus and the disease. Comprehensive research on multiple genetic loci should be conducted to provide a reliable theoretical basis for the clinical diagnosis and treatment of diabetes and its complications.

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## Disclosure statement

The authors declare no conflict of interest.

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