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ORIGINAL ARTICLE

Identification of Blood miR-216a, miR-377 and Their Target Genes ANGPTL4, GAP-43 and Serum of PPARG as Biomarkers for Diabetic Peripheral Neuropathy of Type 2 Diabetes

Ya Li, Wei-Guo Ma, Xiao-Cheng Li

Department of Endocrinology, The First Affiliated Hospital of Xi'an Medical University, Xi'an, China

SUMMARY

Background: **Diabetic peripheral neuropathy (DPN) is one of the most common and complex chronic complications of diabetes, but it is clinically lacking effective means for early diagnosis and early treatment. MicroRNA, in the occurrence and development of the disease, has an important regulatory role. Its role in diabetes has been reported more. However, specific research on microRNA in DPN is rare.**
Methods: **Based on the results of bioinformatics screening, miR-377 and miR-216a, their respective target molecules growth association protein 43 (GAP-43) and angiopoietin-like 4 protein (ANGPTL4), and related pathways peroxisome proliferator activated receptor gamma (PPARG) and chemerin were tested by RT-qPCR and ELISA in blood samples of DPN to analyze the correlation between these differentially expressed molecules and clinicopathological factors of DPN.**
Results: **In this study, we found that miR-377, miR-216a, GAP-43, ANGPTL4, and PPARG were significantly differentially expressed genes for DPN. The correlation analysis showed that they were closely related to the clinical indicators of DPN suggesting that they may be involved in the development of DPN. In addition, receiver operating characteristic (ROC) curves generated for miR216a, miR377, ANGPTL4, GAP43, PPARG revealed that they can be used as new molecular diagnostic markers of DPN.**
Conclusions: **miR-216a, miR-377, ANGPTL4, GAP-43, and PPARG could potentially be biomarkers of DPN.**
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Correspondence:
Ya Li
Department of Endocrinology
The First Affiliated Hospital of
Xi'an Medical University
Fenghao Street No 48
710077 Lianhu District
Xi'an, Shanxi province
China
Email: yaliya19@126.com

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KEY WORDS

DPN, miR-216a, miR-377, PPARG, biomarkers

INTRODUCTION

Diabetic neuropathy (DN) is a common and complex complication of diabetes [1]. Lesions can be involved in the central nervous system, peripheral nerves, and autonomic nerves [2]. Related studies have shown that 50% to 80% of diabetic patients have varying degrees of peripheral neuropathy. Severe lesions can lead to ulcers, gangrene, and even amputation. This is why DPN is one of the main causes of diabetes disability. However, its early clinical manifestations are not obvious, making diagnosis and prevention even more difficult. Therefore, the study of molecular markers for DPN is of great significance for the prevention and treatment of the dis-

ease.

MicroRNA is a class of small, non-coding RNA (about 20 - 25 nucleotides).The mechanism of action is in the post-transcriptional level, with the target mRNA having complete or not complete complementary pairs to reduce or inhibit its translation [3]. They are involved in regulating cell growth, differentiation and apoptosis and other physiological events, and the occurrence and development of the disease. In the more extensive oncology studies, miRNAs can be used as biomarkers of human tumors [4], which can be used as early diagnostic markers and potential therapeutic targets [5]. With the deepening of microRNA research, the study found that microRNA plays an important role in the pathogenesis of diabetes. Throughout the onset of diabetes, microRNAs can regulate insulin secretion [6]. The body produces insulin resistance, and damages the insulin target cells, including vascular endothelial cells, cardiomyocytes, etc. [7,8]. However, microRNAs in the pathogenesis of diabetes research are more concentrated on the muscle, liver, pancreas, heart, and kidney and other systems, and microRNA in DPN is rarely reported.

In our previous research, secondary study on microarray dataset (GSE24290) downloaded from Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI), we found that miR-216a and miR-377 expression is different in DPN patients sural nerve samples [9]. Angiopoietin-like 4 (ANGPTL4) and growth associated protein 43 (GAP-43) were the target genes of miR-216a and miR-377, respectively. The results of weighted gene co-expression network analysis suggested ANGPTL4 and GAP-43 are differentially expressed genes and are significantly associated with diabetes and that they are closely related to diabetes. In addition, the analysis of KEGG pathway enrichment showed that PPAR signal pathway is the most significant signal pathway associated with DPN. It is reported that the important constituent of PPAR signaling pathway peroxisome proliferators - activated receptor gamma (PPARG) - is associated with glucose metabolism [10]. So the detection of PPARG is particularly important.

Our previous study confirmed the key role of inflammation in DPN, as evidenced by the finding that inflammation was the most significant GO term associated with differentially expressed genes (DEGs) in the miRNA - gene network [9]. Intervention in inflammation may be an effective method of prevention and treatment of DPN. Chemerin is a new type of adipocytokine that was discovered in recent years that can exert a proinflammatory effect by altering the secretion and expression of inflammatory mediators to promote inflammatory response. Studies have shown that chemerin levels within the blood of obese patients with diabetes are significantly increased [11] and may play a role in the pathophysiological mechanisms of type 2 diabetes mellitus. Therefore, we hypothesize that chemerin is an important factor of DPN.

Based on the preliminary bioinformatics prediction results, the expression levels of miR-216a and miR-377 and corresponding target genes ANGPTL4 and GAP-43 and the expression of PPARG and chemerin were detected by real-time quantitative real-time PCR (qRT-PCR) and ELISA. The goal was to further explore the correlation between these molecules and DPN to determine the possibility of becoming a specific molecular marker of DPN, and to provide new ideas about the diagnosis and treatment of DPN.

MATERIALS AND METHODS

Study Population

In this study, 80 patients were selected, including 24 cases of type 2 diabetes without neuropathy (control group) and 56 cases of type 2 diabetes mellitus with neuropathy (DPN group). Patients were recruited from endocrinology outpatients and inpatients at Xi'an Medical University First Affiliated Hospital from December 2016 to March 2017. The controls were diagnosed according to American Diabetes Association (ADA) diagnosis criteria for diabetes [12]. The inclusion criteria for DPN were: comply with DPN diagnostic criteria developed in 2010 ADA Guide to Diabetes [13]. For clinical diagnosis of diabetic peripheral neuropathy, symptoms of DSPN (such as pain, numbness, paresthesia, etc.) or signs (ankle reflex, pressure, vibration, tingling, temperature), as well as abnormal nerve conduction study (NCS) were used.

Individuals with any other clinically systemic acute or chronic inflammatory diseases, renal failure, dementia, cancers and other specific types of diabetes, e.g., type 1 diabetes or gestational diabetes mellitus (GDM) were excluded. The study was performed in accordance with the Declaration of Helsinki and approved by the Medical Ethics Committee of Xi'an Medical University. Written informed consent was obtained from all subjects. Standardized techniques were used for anthropometric measurements, including height (m) and weight (kg). Body mass index (BMI) was calculated as [weight (kg)/height $(m)^2$]. All subjects underwent a general physical exam the morning after an overnight fast.

Laboratory measurements

Blood levels of total and HDL cholesterol, triglycerides, fasting glucose, HbA1c, fasting insulin, creatinine, and hs-CRP were measured by standard procedures.

RNA extraction and RT-qPCR assay

Fasting blood samples were collected from each subject and anticoagulated with EDTA. Total RNA was isolated from 250 μL of blood using TRIzol® LS (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions with modification. Approximately 2 μg of total RNA was used for the first strand cDNA synthesis using the PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China). Real-time PCR was performed with Power

Table 1. The primer sequences of genes.

Genes	The primer sequences (5'-3')
GAPDH-hF	TGACAACTTTGGTATCGTGGAAGG
GAPDH-hR	AGGCAGGGATGATGTTCTGGAGAG
ANGPTL4-hF	GGCTCAGTGGACTTCAACCG
ANGPTL4-hR	CCGTGATGCTATGCACCTTCT
GAP-43-hF	GGCCGCAACCAAAATTCAGG
GAP-43-hR	CGGCAGTAGTGGTGCCTTC
GAP-43-hR	CGGCAGTAGTGGTGCCTTC
MiR-216a-jh-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAG
MiR-377-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAAAA
MiR-377-F	GCGCATCACACAAAGGCAAC
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
U6-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATG

SYBR Green PCR Master Mix (Applied Biosystems Inc.). Relative expression of genes was analyzed using the $2^{-\Delta\Delta Ct}$ method [14]. Two reference genes, U6 and GAPDH (glyceraldehyde-3-phosphatedehydrogenase) were selected based on expression stability. II primer sequences were described in Table 1.

Serum analysis

Blood samples were collected and left at RT in the centrifuge tube for 2 hours to make serum precipitation (preferably the centrifuge tube tilted, so that the cross-section of the liquid surface increases, contributing to the precipitation of serum). The sample was then separated by centrifugation at 3,000 g for 20 minutes at 4°C to retrieve serum. The serum was then stored at -80°C until assay to avoid repeated freezing and thawing. PPARG and chemerin were performed by ELISA (R&D System, UK).

Statistical analysis

The data are expressed as the mean ± SEM. Normality of the two groups of measured data was tested using the Shapiro-Wilk method. Differences between groups were compared by *t*-test or Mann-Whitney test. If the two groups were qualitative data, the chi-square test was used to compare the differences between groups. To determine the relationship between biomolecules and clinical records, Pearson's correlation analysis was used for normal variables, if the variables do not meet the normality, Spearmen's correlation analysis was used for statistical analysis. The statistical software used was SPSS 17.0 (SPSS Inc., Chicago, IL, USA), mapping software for Graphpad prism 5 (Graphpad Software, San Diego, CA, USA), and $p < 0.05$ and $p < 0.01$ were significant differences and significant differences as screening criteria, respectively.

RESULTS

Characteristics of study subjects

Statistical analysis showed that the differences in diabetes-related clinicopathological factors between the DPN group and the control group (Table 1). The results showed that age ($p = 0.001$), duration of disease ($p < 0.001$), systolic blood pressure ($p = 0.007$), lymphocytes ($p = 0.037$), HbA1c (%) ($p = 0.045$), postprandial half an hour C peptide ($p = 0.023$), one-hour postprandial C peptide ($p = 0.004$), two-hour postprandial C peptide ($p = 0.006$), high-density lipoprotein cholesterol ($p = 0.016$), FT3 ($p = 0.028$), T3 ($p = 0.037$), gamma-glutamyltranspeptidase ($p = 0.007$), urinary albumin-to-creatinine ratio ($p = 0.034$), 25-(OH)2-VitD3 ($p < 0.001$), and DPN ($p = 0.015$) had statistically significant differences between the two groups.

The levels of miR-216a and miR-377 are increased and their target genes ANGPTL4 and GAP-43 are decreased in patients with DPN.

We used RT-qPCR to detect the expression of miR-216a and target gene ANGPTL4 in 56 patients with progressive DPN and 24 patients with non-progressive DPN. The results showed that the expression of miR-216a in neurologic blood samples (8.961 ± 11.694) was significantly higher than that in non-neuropathic blood samples (2.437 ± 3.794), and the difference was statistically significant ($p < 0.01$, Figure 1A). In contrast, the expression level of the target gene ANGPTL4 in neurological blood samples (0.470 ± 3.905) was significantly lower than that in control group (1.021 ± 0.181) ($p < 0.001$, Figure 1B). Similarly, RT-qPCR was used to detect the expression of miR-377 and target gene GAP43 in 56 patients with progressive DPN and 24 patients with non-progressive DPN. The results showed that the expression level of miR-377-3p in neuropathy

Table 2. Analysis of the difference between cases with progressive DPN and controls (non-progressive DPN).

Variables	non- DPN (n = 24)	DPN (n = 56)	Z/t	p-value
Age (years)	50.170 ± 13.915	61.590 ± 13.827	3.397	0.001 **
Duration of diabetes (years)	3.921 ± 5.922	9.853 ± 6.926	3.634	< 0.001 **
BMI (kg/m^2)	24.969 ± 2.792	23.997 ± 2.697	1.539	0.124
Systolic blood pressure (mmHg)	121.830 ± 12.328	134.480 ± 25.325	2.681	0.007 **
Lymphocyte (cell/μL)	2.498 ± 1.035	1.973 ± 0.706	2.132	0.037 *
Two-hour postprandial blood glucose (mmol/L)	13.365 ± 3.505	15.078 ± 4.693	1.610	0.111
HbA1c (%)	8.847 ± 2.115	9.616 ± 2.654	1.986	0.045 *
30 minutes postprandial C peptide (mmol/L)	3.292 ± 1.917	2.365 ± 0.961	2.268	0.023 *
One-hour postprandial C peptide (mmol/L)	5.669 ± 3.460	3.330 ± 1.367	3.209	0.004 **
Two-hour postprandial C peptide (mmol/L)	7.575 ± 4.316	5.371 ± 2.580	2.758	0.006 **
HOMA-IR	3.000 ± 2.106	3.448 ± 4.258	0.922	0.356
TC (mmol/L)	4.087 ± 0.774	4.576 ± 1.228	1.636	0.102
TG (mmol/L)	1.908 ± 1.020	2.056 ± 1.376	0.061	0.951
HDL-C (mmol/L)	1.217 ± 0.866	1.237 ± 0.317	2.406	0.016 *
LDL-C (mmol/L)	2.275 ± 0.436	2.513 ± 0.805	1.585	0.113
FT3 (pmol/L)	4.823 ± 0.601	4.484 ± 0.806	2.197	0.028 *
T3 (nmol/L)	1.434 ± 0.228	1.323 ± 0.332	2.086	0.037 *
GGT (IU /L)	2.794 ± 1.042	3.324 ± 0.709	2.681	0.007 **
UACR (mg/g)	20.375 ± 7.113	25.069 ± 9.801	2.122	0.034 **
25-(OH)2-VitD3 (mmol/L)	18.983 ± 6.998	10.428 ± 5.551	4.701	< 0.001 **
Gender (male)	11 (45.8%)	31 (53.4%)	0.394	0.530
DPN (%)	24 (100%)	0 (0%)	82.000	< 0.001 **

Data are presented as number (percentage) for categorical data, mean (standard deviation) for parametrically distributed data, or median (interquartile range) for nonparametrically distributed data.
Abbreviations: HOMA-IR - homeostasis model assessment of insulin resistance, HOMA-β - homa beta cell function index, TC - total cholesterol, TG - triacylglycerol, HDL-C - high-density lipoprotein cholesterol, LDL-C - low-density lipoprotein cholesterol, T4 - thyroxine, FT4 - free thyroxine, T3 - triiodothyronine, FT3 - free triiodothyronine, TSH - thyroid stimulating hormone, TPOAb - anti-thyroid peroxidase autoantibody, TGAb - anti-thyroglobulin antibodies, Hs-CRP - high-sensitivity C-reactive protein, GGT - gamma-glutamyl transpeptidase, UACR - urinary albumin-to-creatinine ratio, DPN - diabetic peripheral neuropathy.

(5.051 ± 7.205) was significantly higher than that in non-neuropathic blood samples (1.669 ± 1.508) $p < 0.05$, (Figure 1C). In contrast, the expression level of target gene GAP43 in neurological blood samples (0.821 ± 0.561) was significantly lower than that in the control group (1.064 ± 0.367) ($p < 0.05$, Figure 1D).

Expression of serum inflammatory response factor PPARG and adipocytokine chemerin in serum of DPN

The expression of PPARG and adipocytokinechemerin in the control group and DPN group were detected by ELISA. PPARG was found to be higher in the DPN group (496.784 ± 67.352) than in the control group (408.219 ± 53.328), and the difference was significant (Figure 2A, $p < 0.001$). However, there was no significant difference in adipocytokinechemerin (Figure 2B, $p > 0.05$).

Correlation between clinicopathological factors with miR-216a, miR377 and their target genes ANGPTL4 and GAP-43 in DPN

Statistical analysis of the relationship between clinical and pathological variables with expression of miR-216a and ANGPTL4. The results showed that the expression level of miR-216a was significantly correlated with HbA1c (%) ($p = 0.040$), the expression level of ANGPTL4 was significantly correlated with age

Table 3. The correlation between expression of miR-216a and ANGPTL4 and clinical parameters in diabetic peripheral neuropathy.

Variables	ANGPTL4		miR-216a	
	r	p	r	p
Age (years)	0.602	0.002 **	0.212	0.320
Duration of diabetes (years)	0.510	0.011 *	-0.049	0.819
DPN	0.602	0.002 **	-0.361	0.083
Two-hour postprandial blood glucose (mmol/L)	0.554	0.005 **	0.066	0.758
HbA1c (%)	0.210	0.325	-0.421	0.040 *
One-hour postprandial C peptide (mmol/L)	-0.525	0.008 **	0.187	0.383
Two-hour postprandial C peptide (mmol/L)	-0.461	0.023 *	0.016	0.940
TC (mmol/L)	-0.415	0.044 *	0.150	0.485
TG (mmol/L)	-0.413	0.045 *	0.126	0.558
LDL-C (mmol/L)	-0.407	0.048 *	0.009	0.965
FT3 (pmol/L)	-0.404	0.050 *	0.131	0.541
T3 (nmol/L)	-0.603	0.002 *	0.276	0.192

miR-216a levels in progressive DPN patients (n = 56) and in control group (non-progressive DPN) (n = 24). ANGPTL4 levels in progressive DPN patients (n = 12) and in control group (non-progressive DPN) (n = 12). Simple correlations between miR-216a, ANGPTL4 values, and various parameters.
Abbreviations: TC - total cholesterol, TG - triacylglycerol, LDL-C - low-density lipoprotein cholesterol, T3 - triiodothyronine, FT3 - free triiodothyronine, DPN - diabetic peripheral neuropathy.

Table 4. The correlation between expression of miR-377 and GAP-43 and clinical parameters in diabetic peripheral neuropathy.

Variables	miR-377		GAP-43	
	r	p	r	p
Gender	0.465	0.022 *	0.033	0.802
DPN	-0.596	0.002 **	0.276	0.030 *
HOMA-IR	0.473	0.020 *	-0.213	0.096
FT3 (pmol/L)	0.277	0.190	-0.322	0.011 *

miR-377 levels in progressive DPN patients (n = 56) and in control group (non-progressive DPN) (n = 24). GAP-43 levels in progressive DPN patients (n = 12) and in control group (non-progressive DPN) (n = 12). Simple correlations between miR-377, GAP-43 values, and various parameters.
Abbreviations: HOMA-IR - homeostasis model assessment of insulin resistance, FT3 - free triiodothyronine, DPN - diabetic peripheral neuropathy.

(p = 0.002), duration of diabetes (p = 0.011), DPN (p = 0.002), two hours postprandial blood glucose (p = 0.005), one-hour postprandial C peptide (p = 0.008), two-hour postprandial C peptide (p = 0.023), TC (p = 0.044), TG (p = 0.045), LDL-C (p = 0.048), FT3 (p = 0.05), and T3 (p = 0.002) (Table 2). As shown in Table 3, miR377 was significantly associated with gender (p = 0.022), DPN (p = 0.002), and HOMA-IR (p = 0.020). The expression level of GAP-43 was significantly correlated with DPN (p = 0.030) and FT3 (p = 0.011) (Table 3).

Correlation between the level of PPARG with clinicopathological factors of DPN

The expression level of PPARG was significantly correlated with gender (p = 0.011), DPN (p = 0.001), HOMA-IR (p = 0.045), and TG (p = 0.037) (Table 4).

Table 5. The correlation between expression of PPARG and clinical parameters in diabetic peripheral neuropathy.

Variables	PPARG	
	r	p
Gender	-0.327	0.011 *
DPN	0.407	0.001 **
HOMA-IR	-0.260	0.045 *
TG (mmol/L)	-0.270	0.037 *

PPARG levels in progressive DPN patients (n = 12) and in control group (non-progressive DPN) (n = 12). Simple correlations between PPARG values and various parameters.

Table 6. The diagnostic value of miR-216a, miR-377, ANGPTL4, GAP-43, and PPARG in serum from DPN patients.

Variables	AUC	Std. error	p	95% CI	Cutoff	Sensitivity	Specificity
miR216a	0.792	0.078	0.002	0.639 to 0.944	1.211	0.783	0.750
miR377	0.709	0.078	0.032	0.555 to 0.862	2.744	0.592	0.909
ANGPTL4	0.840	0.096	0.005	0.560 to 1.000	0.694	1.000	0.750
GAP43	0.708	0.115	0.083	0.482 to 0.934	0.834	0.917	0.667
PPARG	0.847	0.079	0.004	0.692 to 1.000	488.837	0.667	0.917
Combined	1.000	0	< 0.001	1.000 to 1.000	NO	1.000	1.000

Abbreviations: AUC - area under the curve, CI - confidence interval.

The diagnostic value of miR-216a, miR-377, ANGPTL4, GAP-43, and PPARG in serum from DPN patients

Using ROC curve analysis of the diagnostic value for five indicators of DPN, the results are shown in Figure 3 and Table 5. The area under the curve for miR-216a was 0.792 with a sensitivity of 0.783 and a specificity of 0.750 (Figure 3A). When miR-377 was used to diagnose DPN, the area under the curve was 0.709 with a sensitivity of 0.592 and a specificity of 0.909 (Figure 3B). ANGPTL-4 diagnosis is better, the area under the curve is 0.840 with a sensitivity of 1.000 and a specificity of 0.750, as shown in Figure 3C. The diagnostic value of GAP-43 showed an area under the curve of 0.708, a sensitivity of 0.834, and a specificity of 0.917. Note that the p-value was 0.083 (Figure 3D). Assessment of the diagnostic value of PPARG showed an area under the curve of 0.847 with a sensitivity of 0.667 and a specificity of 0.917 (Figure 3E). In addition, although there is some data loss, the combined diagnostic value of the five factors should be optimal (Figure 3F).

DISCUSSION

In this study, miR-216a, miR-377, ANGPTL4, GAP43, and serum inflammatory response factor PPARG was significantly different in DPN and non-neuropathy. miR-216a, miR-377, and PPARG showed an upward trend, whereas ANGPTL4, GAP43 showed a downward trend. At the same time, the area under the curve (AUC) using receiver operating characteristic (ROC) analysis show those biomarkers have predictive value. This discovery partially confirmed our previous bioinformatics predictive results [9], while suggesting that these molecules can be used as potential markers of DPN.

Relatively speaking, miR-216a in diabetic research reports are rare, but more common in tumor research. It has been reported that miR-216a is expressed abnormally in a variety of tumor tissues. The low expression of miR-216a in normal tissues was detected by high-throughput sequencing [15]. In pancreatic cancer cells, miR-216a inhibits pancreatic cancer cell growth and promotes apoptosis by acting on JAK2 gene [16]. So far, it has been reported that miR-216a may be involved in the pathogenesis of diabetes [17]. Studies have shown that high levels of urinary miRNA-216a may be a risk factor promoting the development and progres-

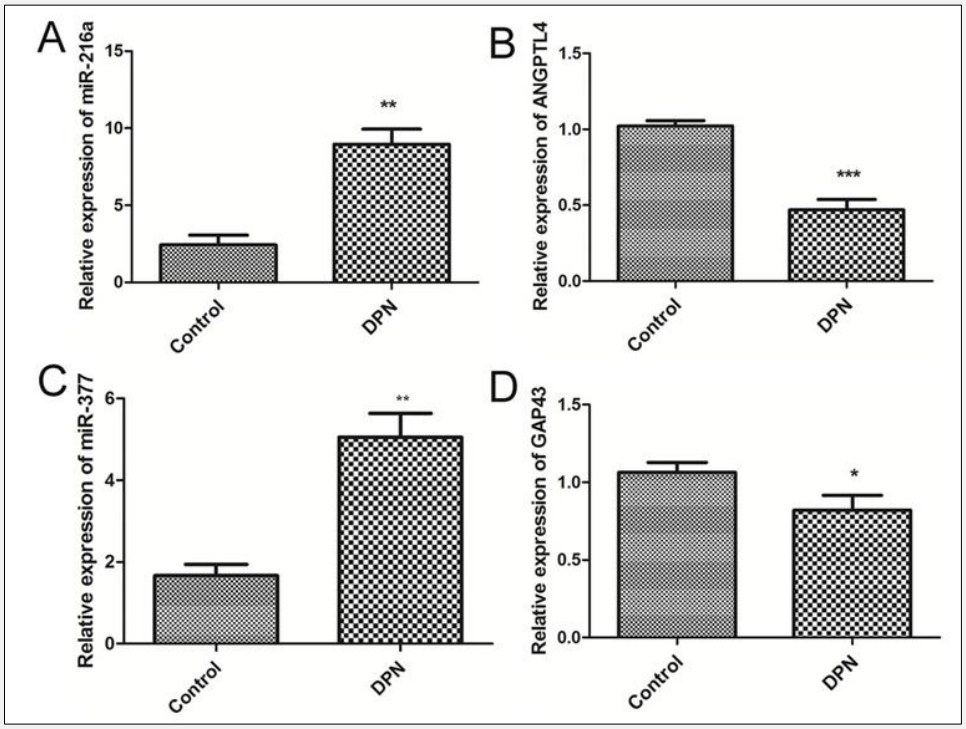

Figure 1. The expression of miR-216a and miR-377 were upregulated, and their target genes ANGPTL4 and GAP43 were decreased in patients' blood with progressive DPN.

Total RNA was extracted from patients' blood, RT-qPCR was then performed to evaluate the relative expression of miR-216a (A), ANGPTL4 (B), miR-377, (C) and GAP43 (D) in progressive DPN patients (n = 56) in comparison to the control group (non-progressive DPN) (n = 24). Values are represented as mean ± SD. * - p < 0.05 vs. control, ** - p and * - p < 0.01 vs. control.**

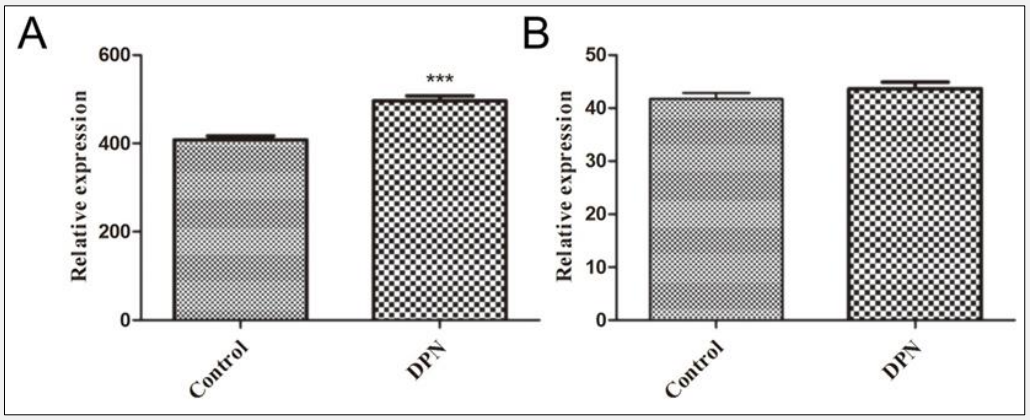

Figure 2. Expression of serum inflammatory response factor PPARG and adipocytokine chemerin in serum of DPN.

Sera of progressive DPN patients (n = 12) in comparison to non-progressive DPN group (n = 12). The levels of PPARG (A) and adipocytokine chemerin (B) were analyzed by ELISA. Values are represented as mean ± SD. * - p < 0.01 vs. control.**

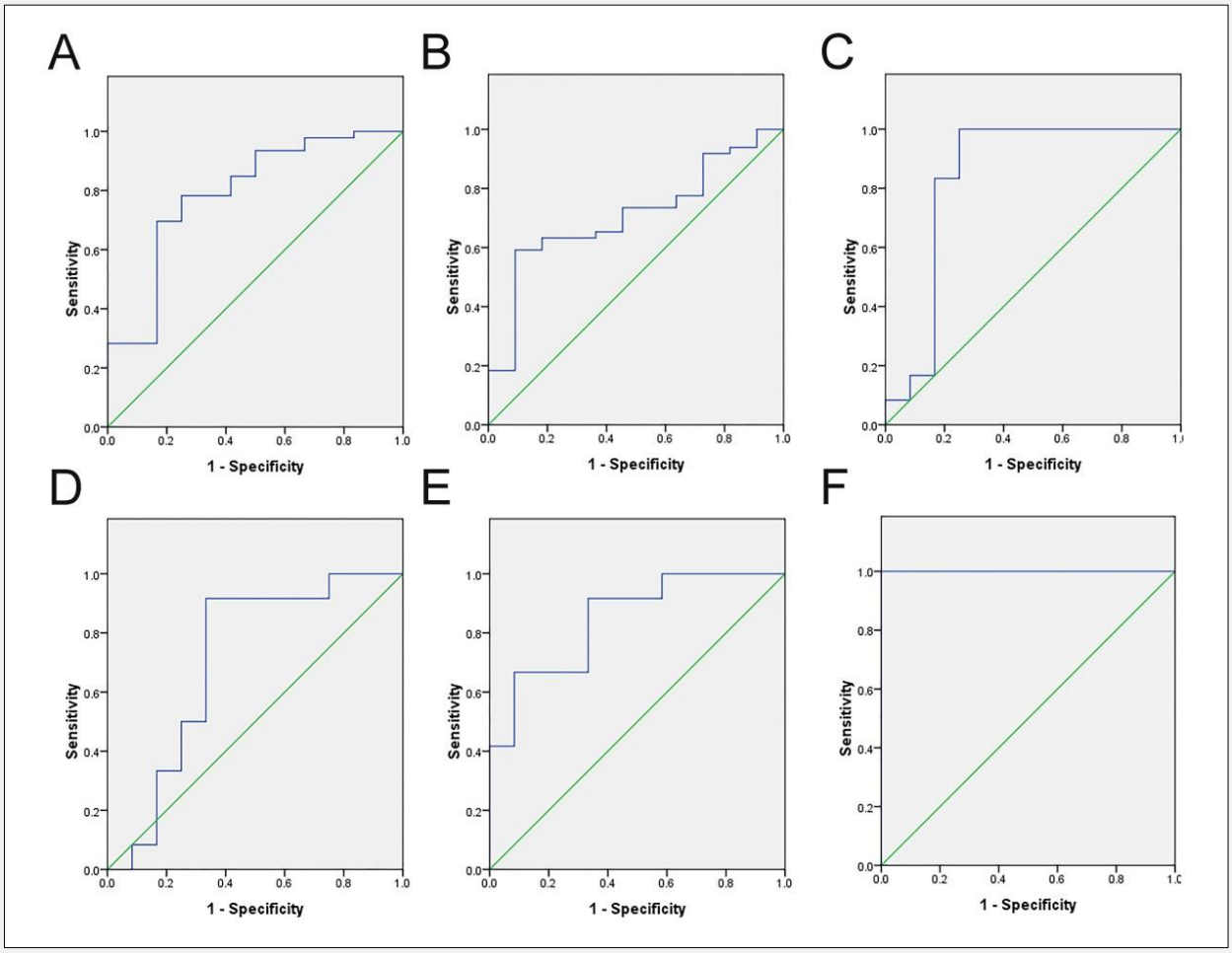

Figure 3. ROC curves and corresponding AUCs for diabetic peripheral neuropathy of Type 2 Diabetes.

(A) ROC curve of miR-216a for prediction of DPN, the AUC was 0.792 (95% CI, 0.639 to 0.944). (B) ROC curve of miR377 for prediction of DPN, the AUC was 0.709 (95% CI, 0.555 to 0.862). (C) ROC curve of ANGPTL4 for prediction of DPN, the AUC was 0.840 (95% CI, 0.560 to 1.000). (D) ROC curve of GAP43 for prediction of DPN, the AUC was 0.708 (95% CI, 0.482 to 0.934). (E) ROC curve of PPARG for prediction of DPN, the AUC was 0.708 (95% CI, 0.692 to 1.000). (F) ROC curve of combined for prediction of DPN, the AUC was 1.000 (95% CI, 1.000 to 1.000).
Abbreviations: AUC - area under the curve, CI - confidence interval, ROC - receiver operating characteristic.

sion of type 1 diabetic nephropathy [18].

However, miR-216ia is not reported in DPN. In this study, we found that the expression of miR-216a was significantly different in the two samples by measuring its expression in clinical DPN and non-lesion samples. The results showed that miR-216a and miR-377 may be involved in the development of DPN.

Results of correlation analyses showed that the expression level of miR-216a was significantly correlated with HbA1c. As is well known, glucose concentration and glycated hemoglobin are positively correlated. High HbA1c concentrations have been reported associated with an increased risk of developing DPN and may also impose risks of developing diabetic retinopathy and nephropathy in due course of time [19]. Interestingly, at the same time, studies have shown that moderate aerobic exercise can improve diabetic neuropathy by reducing HbA1c [20]. This result suggests that miR-216a may affect the pathological process of DPN through HbA1c.

ANGPTL4 is also known as fasting induced adipokines, modified by oligomerization, glycosylation, and other types of modifications, which are secreted by the cells into the blood circulation. They are involved in the process of glycolipid metabolism, angiogenesis and apoptosis [21]. Animal studies have shown that ANGPTL4

can improve its tolerance to glucose [22]. Human studies have shown that ANGPTL4 is one of the important factors in regulating the balance of glucose and lipid metabolism, which has a certain effect on improving glucose metabolism. The expression of ANGPTL4 in serum of many diabetic patients is abnormal. Lu et al. reported that the expression of ANGPTL4 was reduced in the serum of patients with proliferative diabetic retinopathy, while it was elevated in the vitreous humor [23].

These studies have shown that ANGPTL4 is involved in the development of diabetes. In this study, we examined the expression of ANGPTL4 in the blood of patients with DPN and the control group. The results showed that there was a significant difference in ANGPTL4 expression between the two groups. At the same time, our previous study shows that ANGPTL4 is the target gene of miR-216a, which further suggests that miR-216a may affect the occurrence and development of DPN through regulation of ANGPTL4. But the question of how ANGPTL4 is involved in the pathogenesis of DPN and how it is regulated by miR-216a requires further study. In addition, our study also found that ANGPTL4 expression levels were significantly associated with age, duration of diabetes, DPN, two-hour postprandial blood glucose, one-hour postprandial C peptide, two hour postprandial C peptide, TC, TG, LDL-C, FT3 and T3, and ANGPTL4. Diabetic peripheral neuropathy still has many uncertainties, but it is certain that it can regulate the pathological process of diabetic neuropathy by affecting these metabolic indicators.

Relatively speaking, miR-377 associated with diabetes has been reported in many research reports, but they are more focused on diabetic nephropathy. Studies have shown that miR-377 co-miR-192 can accelerate the development of glomerulosclerosis by up-regulating the expression of extracellular matrix proteins in the glomerular mesangial cells [24]. The level of miR-377 is abnormally high in patient's blood with diabetic nephropathy. Bioinformatics analysis showed that the target gene of miR-377 in diabetic nephropathy was smad. Molecular mechanism studies have shown that upregulation of miRNA-377 increases fibronectin (FN) expression by inhibiting p21 activation kinase (PKA1) and superoxide dismutase (SOD) gene expression. These studies suggest that miRNA-377 is involved in the development and progression of diabetic nephropathy. Therefore, studies have shown that urine miR-377 may be an important marker for type 1 diabetes [25]. However, so far, the study of whether miR-377 is associated with DPN is rare. The results of this study show that the expression of miR-377 in the blood of patients with DPN is significantly higher. The correlation analysis showed that miR-377 expression was significantly associated with gender, DPN, and HOMA-IR. It can be seen that miR-377 is closely related to DPN and plays an important role in the development and progression of DPN and may become a potential target for intervention.

GAP-43 is a target gene for miR-377, which is involved in the growth and transport of axons, the identification of target cells, the release of neurotransmitters, the establishment of synapses, and the regulation of regeneration and function. The expression of this molecule affects the regeneration function of the injured nerve. In addition, GAP-43 has a certain correlation with the sympathetic nerve density. Studies have shown that GAP-43 can be used as a marker of nerve injury and repair in sympathetic remodeling. So, GAP-43 can participate in the regulation of learning and memory. Studies of skin nerve fibers of patients with type 2 diabetes had shown that a decrease in GAP-43 expression can be a marker of early neurological damage in patients with type 2 diabetes [26]. However, whether GAP-43 is associated with DPN has not been reported. In this study, we have not only found that GAP-43 is significantly reduced in DPN, GAP-43 was also found to be a target gene for miR-377, which further suggests that GAP-43 may be involved in the development of DPN under the control of miR-377. Improving the level of GAP-43 may play an intervention role in the occurrence and development of DPN, which provides new research directions for the treatment of DPN. However, how GAP-43 functions under miR-377 regulation requires further study. In addition, the correlation analysis showed that the expression level of GAP-43 was significantly correlated with DPN and FT3. This result suggests that GAP-43 plays an important role in the pathogenesis of DPN, but the specific mechanism needs further study. Our previous study shows that DPN enriches multiple signaling pathways, of which the PPAR signal pathway is the most significant. Its main physiological functions are related to fatty acid metabolism, glucose metabolism, cell proliferation and differentiation. PPARG is one of the most important subtypes of the PPAR signaling pathway and is closely related to diabetes. Previous studies found that PPARG is widely present in adipocytes. In fact, many studies have found that PPARG is involved in glycolipid metabolism and mitochondrial energy metabolism regulation [27,28]. Although studies have shown that PPARG may be associated with the development of diabetes, especially type 2 diabetes, but with the DPN research rarely reported. The results of this study show that compared with the simple diabetic group, the expression of PPARG in DPN showed a significant difference. In addition, correlation analysis showed that PPARG had a significant correlation with gender, DPN, HOMA-IR and TG. The results further clarify that the PPARG gene is associated to the development of DPN and may serve as a diagnostic marker for the diagnosis of DPN and can be used as an indicator of the severity of DPN. However, the specific molecular mechanisms and the signal pathways involved to need further study.

Chemolin, as a fat factor that has been identified in recent years, is expressed in a variety of tissues. In particular, in adipose tissue, chemerin is highly expressed and plays a role in lipid metabolism [29]. Chemerin has a

proinflammatory effect, and in many diseases is associated with chronic inflammation. The level of chemerin in diabetic patients' serum is significantly elevated [30, 31]. More and more studies have demonstrated that chemerin has both a prophylactic and anti-inflammatory effect. In addition, chemerin also affects the sensitivity of insulin. For example, chemerin can promote an increase in glucose uptake under insulin stimulation [11]. In recent years, chemerin has been increasingly studied in the pathogenesis of diabetes [29]. Although many reports have shown that chemerin is associated with the development of diabetes [32,33], its relationship with DPN is rarely reported. To further explore the possible role of chemerin in the development of DPN, this study measured serum chemerin levels in DPN and healthy patients. However, the results show that there is a difference in the expression of serum in patients with and without DPN, but the difference is not significant. The results show that although chemerin has a certain relevance with the occurrence of diabetes, its role in DPN is not significant. In addition, the results may also be the reason for the small sample size in this study. Therefore, in the follow-up work, we need to expand the sample size for further study and discussion.

CONCLUSION

In this study, the expression of miR-216a, miR-377, ANGPTL4, GAP43, and serum inflammatory response factors PPARG and chemerin in a DPN group and control group were detected by RT-qPCR and ELISA. MiR-216a, miR-377, ANGPTL4, GAP43, and serum inflammatory response factor PPARG were significantly different between the two groups. The correlation analysis showed that miR-216a, miR-377, ANGPTL4, GAP-43, and PPARG were correlated with the clinicopathological factors of DPN. The results further demonstrate that miR-216a, miR-377, ANGPTL4, GAP-43, and PPARG could be potential biomarkers of DPN. Due to the limitations of the article, more research is needed before a concluding statement of the manuscript can be made.

Limitations:

Some of the limitations in this study should be recognized. First, the sample size is relatively small, which may lead to insufficient power for clear results. Second, type 2 diabetes is the most common type of diabetes, accounting for about 90% of all cases of diabetes. In this study, all patients had type 2 diabetes, patients with other types of diabetes were excluded. Therefore, the conclusions have certain limitations.

Third, in this study, all patients with type 2 diabetes were excluded if the disease was related to insulin resistance (such as renal failure, dementia, chronic inflammatory diseases, etc.), but there are still limitations. Fourth, the expression levels of these biomolecules may be affected by multiple factors. In our study, these molecules are associated with some clinical indicators, but we cannot establish causal relationships or provide accurate regulatory mechanisms, which still require further work. Finally, although we demonstrated that there were significant differences in miRNA (miR-216a, miR-377) and their respective target genes (ANGPTL4, GAP-43) in the blood of patients with DPN compared to the control group, the clinical application of these biomolecular changes in the diagnosis and evaluation of DPN remains to be further studied and optimization.

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Declaration of Interest:

The authors declare no conflicts of interest

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