European Review for Medical and Pharmacological Sciences 2018; 22: 1106-1112

The role of adiponectin gene mediated by NF-κB signaling pathway in the pathogenesis of type 2 diabetes

Y. ZUO

Emergency Department, Third Xiangya Hospital of Central South University, Changsha, Hunan Province, Xiangya, China

Abstract. – OBJECTIVE: To investigate the role of adiponectin (ADPN) gene mediated by NF-κB signaling pathway in the pathogenesis of type 2 diabetes (T2DM).

PATIENTS AND METHODS: The levels of expression of ADPN were measured by fluorescence quantitative PCR technology, enzyme-linked immunosorbent assay, Western-blotting and immunohistochemistry in 78 patients with type 2 diabetes.

RESULTS: Compared with the normal population, the levels of expression of ADPN and NF-κB mRNA in the blood of patients with type 2 diabetes were significantly reduced ($p < 0.05$). The detection of ADPN gene protein expression in normal population and patients with type 2 diabetes by enzyme-linked immunosorbent assay showed that ADPN gene protein expression in type 2 diabetic patients (1.26 ± 0.73) μg/l was significantly lower ($p < 0.05$) than ADPN gene protein expression of the normal population (3.26 ± 1.25) μg/l. At the same time, the detection of the expression of NF-κB gene showed that the protein expression in type 2 diabetic patients (0.58 ± 0.15) μg/l was significantly lower ($p < 0.05$) than that in the normal population (1.67 ± 1.04) μg/l. The results of Western-blotting were consistent with the results of enzyme-linked immunosorbent assay. Immunohistochemical results also showed that the percentage of ADPN positive cells in patients with type 2 diabetes (25.47%) was significantly lower ($p < 0.05$) than that in the normal population (79.47%).

CONCLUSIONS: ADPN gene in the human body can be involved in the pathogenesis of T2DM through the NF-κB signaling pathway.

Key Words:
NF-κB signaling pathway, ADPN gene, Type 2 diabetes, Correlation.

Introduction

Studies[1,2] showed that diabetes is one of the major diseases with high mortality in the world. Its harmfulness has gradually attracted people's attention. Statistics show that by the end of 2014 around the world patients suffering from diabetes mellitus accounted for 6.3% of the total number[3]. With the living standard in our country, the number of people who continue to suffer from diabetes gradually increased. By the end of 2015, the number of diabetes in China was close to 60 million people, ranking second in the world[4]. The numbers of patients with type 2 diabetes accounted for 75.4% of the total number of diabetes[5]. Therefore, strengthening the diagnosis and treatment of diabetes, especially type 2 diabetes, has become an important direction of medical research. In recent years, data from the related research[6,7] showed that type 2 diabetes is mainly due to the lack of insulin and other hypoglycemic substances in the body. Data showed that NF-κB signaling pathway can participate in many body metabolic processes, such as lipid metabolism[9], glycometabolism[10]. With the discovery of the signaling pathway continuing to ascend, the signaling pathways mediate occurrence and development process of many diseases. Additionally, the NF-κB signaling pathway is related to colon cancer, breast cancer and ovarian tumor and many diseases. A relevant research[11] shows that adiponectin (ADPN) as the body's cells, is one of the most important regulatory proteins. Its relevance in the carbohydrates metabolism of the human body has been proven. Studies show that ADPN protein content after injection of insulin in the body increase significantly, which indicates that the ADPN gene may exert certain correlation with insulin. However, there are few reports about the correlation between the ADPN gene and type 2 diabetes (T2DM). Therefore, our research hopes by revealing the correlation between ADPN gene and the pathogenesis of T2DM to deepen

Corresponding Author: Yu Zuo, MD; e-mail: gkjmomgnjjqlpm5@163.com

the study of the pathogenesis of type 2 diabetes to provide certain theoretical and experimental basis for the treatment of type 2 diabetes.

Patients and Methods

Patients

In our hospital, from February 2013 to February 2015, 78 patients with type 2 diabetes were selected as the research subjects. Among them, 42 were males and 36 were females. The average age was (43.6 ± 18.4) years old. 76 normal people of 38 males and 38 females were selected as the control group. The average age was (42.3 ± 17.6) years old. Subjects of both observation and control groups have been detected for diabetes by our hospital according to testing standards. Subjects with incongruent kidney or liver function were excluded from the research.

Materials and Instruments

Main reagents and materials: RNA Extraction Kit (TaKaRa, Dalian, China); ADPN gene primers (Suzhou Jin Weizhi Biological Co., Ltd. Shanghai, China) ADPN first anti, Goat anti-Rabbit first anti (ABM Company, Canada); Second anti, HRP labeled Rabbit anti-mouse second anti (Keqing Biology, Suzhou, China).

Main instrument: Low temperature high-speed centrifuge (Thermo Scientific, Waltham, MA, USA); Ultra-clean bench (Suzhou Purify, Suzhou, China); PCR instrument (Thermo Scientific, Waltham, MA, USA; Micro quantitative determination of nucleic acid (Thermo Scientific, Waltham, MA, USA); Enzyme standard instrument (Thermo Scientific, Waltham, MA, USA); Film transfer apparatus (Thermo Scientific, Waltham, MA, USA).

RNA Extraction

In the study, RNA was extracted according to the TaKaRa (Dalian, China) RNA Extraction Kit manual operation.

Fluorescence Quantitative PCR

To detect the mRNA expression of ADPN gene in different samples, our study used the SYBRGREEN1 dye method. The specific experimental program referred to the manual. The primers were synthesized by Suzhou Jin Weizhi Biological Engineering (Co., Ltd., Suzhou, China) and its sequence was shown in Table I.

Enzyme-linked Immune Response

The experimental method was carried out according to the specification of ELISA Kit (TaKaRa, Dalian, China) and improved by Haghiac et al[12]. In our study, ELISA standard protein sample was diluted with Assay Buffer in accordance with the ratio of 1:50, and then the standard curve was produced according to the specification of the operation. The samples to be measured were diluted with phosphate buffer saline (PBS) (pH 7.2) according to the scale of 1:100 and 100 ml solution of the liquid to be measured, added into each hole. Then, 50 ml detection solution was added to each hole, was incubated at room temperature for 2 h and TMB chromogenic substrate was added. The light value of absorption was measured at 495 nm. Then, according to the standard curve calculate ADPN content and concentration of the various samples were measured.

Western Blot

ADPN protein expression in different samples was measured by Western blotting assay. Through animal cell, total protein extraction was made according to kit extract "the operation guide to molecular cloning, the third edition". Further, the first antibody, diluted in accordance with 1:800 ratios. The second antibody was used after diluted in accordance with 1:500 ratios.

Immunohistochemistry

The ADPN protein of different samples was determined by immunohistochemistry SP method. With the consent of the patients, family members and the hospital Ethics Committee, and in the process of operation cut kidney tissues and normal renal normal tissue for immunohistochemical experiments, specific experimental procedures referred to "The operation guide to molecular cloning, the third edition", and the relevant literature[10] for operation.

Determination of Blood Glucose

The correlation between blood glucose level and ADPN protein expression in different re-

Table I. Fluorescence quantitative PCR primer.

Primer name	Sequence
ADPN-F	GTCGTAGCTGGATCGATCG
ADPN-R	CGTGATCGGCTAGCTAGCTAGC
GAPDH-F	CGTAGGGCTAGCTAGCTAGATAC
GAPDH-R	CGTAGCTGAGAGTTAGCTAGCATC

search subjects was determined, fasting blood sugar content of normal population and type 2 diabetes was determined in the current study; the operation was according to "the biochemical experiment guide"[13].

Statistical Analysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used for the data analysis. The statistical comparison of data was made by using the X^2-test. Inspection standards a = 0.05, $p < 0.05$ as a significant difference, a = 0.01, $p < 0.01$ as an extremely significant difference.

Results

ADPN Gene and NF-PN mRNA Expression in Normal Population and Patients with Type 2 Diabetes Mellitus

The normal population and patients with type 2 diabetes were studied, and the total RNA was extracted. The expression of ADPN gene and NF-κB gene mRNA in different samples were measured by fluorescence quantitative PCR method, and the results are shown in Figure 1. As found, compared with the normal population, the ADPN gene and NF-κB mRNA content in the tissue samples of patients with type 2 diabetes were relatively high. Expression amount of ADPN gene mRNA in normal human tissue was 7.6 times of the expression in tissue samples of patients with T2DM. Expression amount of NF-κB gene in normal human tissue was 5.4 times of the expression

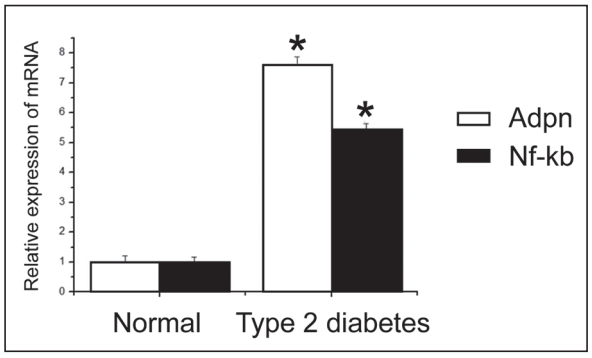

Figure 1. ADPN gene and NF-κB mRNA expression in normal population and patients with type 2 diabetes mellitus: relative expression = (Target gene ct value - Reference gene ct value)/Reference gene ct value. *Represents a significant difference between groups ($p < 0.05$).

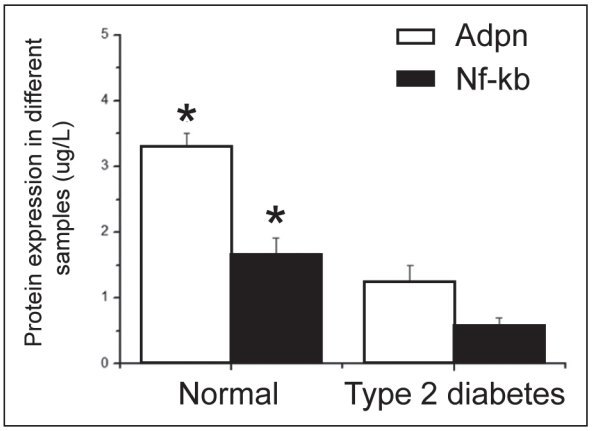

Figure 2. ADPN gene and NF-κB protein expression in normal population and patients with T2DM. *Represents a significant difference between groups ($p < 0.05$).

in tissue samples of patients with T2DM. This showed that the expression of ADPN gene and NF-κB gene in normal population and patients with T2DM was significantly different ($p < 0.05$). There might be a certain correlation between the ADPN gene and the NF-κB gene and T2DM.

ELISA Method for the Determination of ADPN Gene and NF-IS Protein Expression in Normal Population and Patients with Type 2 Diabetes Mellitus

The expression of ADPN and NF-κB gene proteins in the normal population and type 2 diabetic patients were measured by ELISA method. The data are shown in Figure 2 and Table II. ADPN gene protein expression in the normal population (3.26 ± 1.25) mg/l was significantly higher than that in sample tissues in patients with type 2 diabetes mellitus (1.26 ± 0.73) mg/l. At the same time, this study was to determine the expression of NF-κB protein in the normal population and type 2 diabetic patients, results

Table II. ADPN gene and NF-κB protein expression in normal population and patients with T2DM (ug/l ± SD).

Group	ADPN	NF-κB
Normal population	3.26 ± 1.25	1.67 ± 1.04
Type 2 diabetic patients	1.26 ± 0.73*	0.58 ± 0.15*

*$p < 0.05$ represents a significant difference.

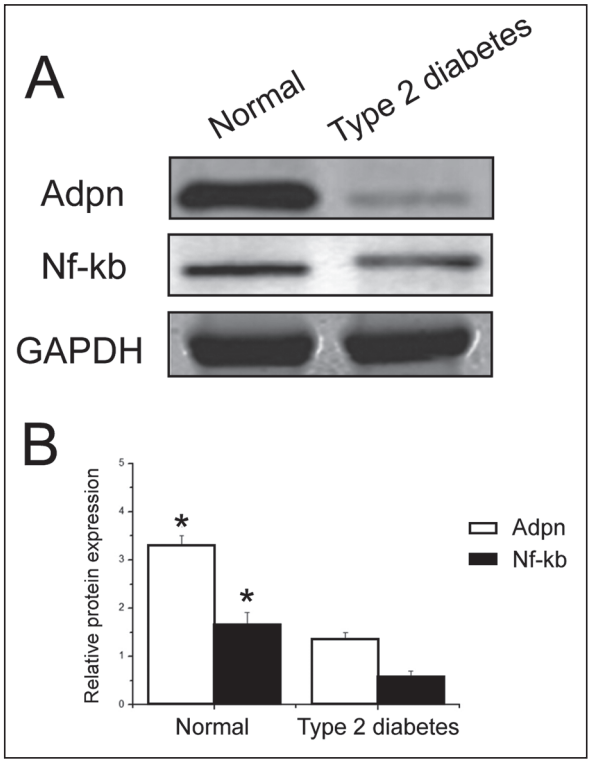

Figure 3. Western-blotting method for the determination of ADPN gene and NF-κB protein expression in normal population and patients with T2DM. ***A,*** Western blotting qualitative data. ***B,*** Western-blotting quantitative data ("*" represents a significant difference between groups; $p < 0.05$).

showed that NF-κB gene protein expression in the normal population (1.67 ± 1.04) mg/l was significantly higher ($p < 0.05$) than that in sample tissues in patients with type 2 diabetes mellitus (0.58 ± 0.15) mg/l.

Western-blotting Method for the Determination of ADPN Gene and NF-th Protein Expression in Normal Population and Patients with Type 2 Diabetes Mellitus

The expression of ADPN and NF-κB gene proteins in the normal population and type 2 diabetic patients were measured by the Western-blotting method (Figure 3). In normal human tissue, both ADPN and NF-κB protein genes content were significantly higher ($p < 0.05$) than those of tissue samples of type 2 diabetic patients. This result was consistent with the results of ELISA and Western-blotting (Figure 4).

Immunohistochemical Determination of ADPN Protein in Different Samples of Type 2 Diabetes Mellitus

Immunohistochemical detection of normal tissue and lesions of type 2 diabetic patients showed that in normal tissue there were more positive ADPN cells, but in lesions, ADPN mRNA positive cells significantly decreased compared with normal tissue (Figure 4) and between the two existed significant differences. Through the comparison of positive cells in normal tissue and lesions, ADPN positive cell rate (92.5%) in normal tissues was significantly higher ($p < 0.05$) than that of the lesions (16.5%) (Table III).

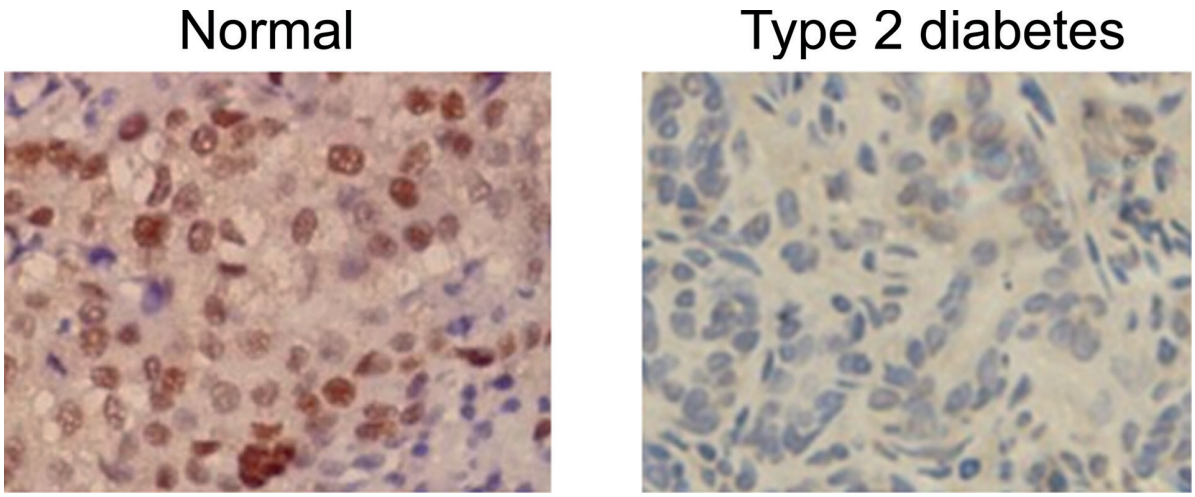

Figure 4. Immunohistochemical determination of ADPN protein in normal population and type 2 diabetes mellitus: brown represents ADPN positive cells, blue indicates ADPN negative cells

Table III. Statistical results of ADPN positive cells in normal tissues and lesions in patients with type 2 diabetes mellitus.

Group	Total cell count	Number of positive cells	Number of negative cells	Positive cell rate (%)
Normal tissue	400	370	30	92.5
Lesion tissue	400	66*	334*	16.5*

*$p < 0.05$ represents a significant difference.

Determination of Fasting Blood Glucose in Normal People and Patients with Type 2 Diabetes Mellitus

The current study was to explore the correlation between the ADPN genes and type 2 diabetes, and to determine the fasting blood glucose level in normal people and patients with T2DM. Fasting blood glucose level of patients with T2DM (10.7 ± 2.83) mmol/l was significantly higher than that of normal population levels (4.3 ± 1.32) mmol/l (Table IV).

Correlation Between ADPN Protein Expression and Blood Glucose Concentration

Related research shows that fasting blood glucose content as an important indicator of diabetes plays an important role in the detection of diabetes. In our study, linear relationship (y = 3,2814 × +0.2816, r = 0.025) was found between fasting blood glucose levels and ADPN protein expression in normal and diabetic subjects. This showed that there was a significant correlation between the ADPN protein content and the fasting blood glucose.

Discussion

Statistics show that[14] at present, the incidence rate of type 2 diabetes mellitus in our country is increasing year by year, and the incidence of the disease tends to be younger. By 2015 in China, type 2 diabetes incidence has more than 35 million people and rapidly grows with the speed of nearly 1.2% per year. Therefore, to strengthen the diagnosis and treatment of T2DM becomes an important direction to improve people's quality of life. However, due to the complex causes of type 2 diabetes, it is affected by environmental factors, genetic factors and dietary habits and so on. Therefore, the research on the pathogenesis of type 2 diabetes is not clear at present[15], so there is no specific drug for the diagnosis and treatment of T2DM.

Research shows that[16] adiponectin (ADPN), a class of collagen secreted by fat cells, in human plasma mainly exists as trimmer and six polies. In recent years, adiponectin in human body has been found to enhance insulin resistance[17]. Relevant researches[18,19] showed that adiponectin in the human body improves the body's metabolism of fatty substances by lowering triglyceride levels in plasma. Because adiponectin can improve the body's resistance to insulin, it was speculated that it might have a certain correlation with diabetes. Further study[20] found that adiponectin secretion of 3T3-L1 adipocyte was regulated by the phosphatidylinositol 3-kinase pathway, which had a close correlation with NF-κB signaling pathway in the human body. For example, in the pathogenesis process of breast cancer, NF-κB can regulate phosphatidylinositol 3 kinase to inhibit the diffusion process of cancer cell[21]. However, the correlation between NF-κB signaling pathway and ADPN gene has not been reported. Because adiponectin gene is associated with insulin resistance, in this study, we examined the expression of ADPN in patients with type 2 diabetes and normal population. We found that the levels of ADPN protein and mRNA in type 2 diabetic patients were significantly lower than those in the normal group, and the ADPN gene and NF-κB gene expression was determined in patients with type 2 diabetes and normal population with a certain correlation that ADPN gene expression increased with the increase of the expression of

Table IV. Fasting blood glucose levels in normal subjects and type 2 diabetic patients.

Group	Fasting blood glucose (mmol/l)
Normal population	4.3 ± 1.32
Type 2 diabetic patients	10.7 ± 2.83

*$p < 0.05$ represents a significant difference.

NF-κB, decreased with the decrease of its expression. However, in this study, we found that there were some differences in the content of ADPN gene protein in patients with T2DM, and it did not have a perfect linear relationship with the change of NF-κB protein content.

Conclusions

Research[22] showed that in human NF-κB signaling pathway may be involved in many physiological and biochemical reactions, cell proliferation, inflammatory reaction and metabolism of lipid material inside the body. Our study shows that the correlation of ADPN gene mediated by NF-κB signaling pathway and T2DM is affected by disease duration, severity of the disease, and many other factors of patients. The main reason may be that type 2 diabetes mellitus with the extension of time may lead to other related diseases such as arthritis, cardiovascular disease, and other diseases. Additionally, NF-κB signaling pathway is proven to be closely related to the disease mentioned above. Thus, correlation of adiponectin gene mediated by NF-κB signaling pathway and type 2 diabetes may be affected by other diseases in the later period of T2DM.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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