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Nucleobindin-2 (NUCB2) gene transcription activity and nesfatin-1 levels in type 2 diabetes mellitus patients in Vietnam Running/short title: NUCB2gene and Nesfatin-1 level in diabetes patients

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ABSTRACT

Background:
Diabetes is mainly caused by obesity and lack of exercise, and is related to genetic inheritance. Therefore, understanding the relationship of resfantin-1 and NUCB2 gene expression is essential to reduce blood sugar and increase insulin in diabetes in the future.

Objective:
In this study, we evaluated nesfatin-1 level and mRNA NUCB2 gene expression in adipose tissue of diabetic patients to contribute to the diagnosis and treatment of diabetes.

Methods:
We conducted a population of 65 patients and 30 controls. Diabetic patients were then divided into two groups, long-term T2DMandnewly diagnosed T2DM. Nesfatin-1 levels and NUCB2 gene expression were analyzed.

Results:
Nesfatin-1was higher in the newly diagnosed T2DM group than in the other groups. Similar results were also detected in the analysis of mRNA NUCB2 gene expression by Realtime-PCr. Meanwhile, no significant difference was found in both analysis of nesfatin-1 and NUCB2 mRNA expression in subjects with l-T2DM compared with the control group. These could explain the effects of long-term treatment with drugs. In the correlation of anthropometric parameters and biochemical indices, nesfatin-1 exhibited significant correlation with BMI, HbA1c, HDL-C, LDL-C, Creatinine and mRNA NUCB2 gene expression in the regression analysis.

Conclusion:
Nesfatin-1 and NUCB2 mRNA gene expression levels may associate with newly diagnosed T2DM. Our study suggeststhe potential of using nesfatin-1 as an effective treatment for type 2 diabetes in Vietnamese patients.

Keyword: Anthropometric parameters, Biochemical indices, mRNA NUCB2 geneexpression,Nesfatin-1,Type 2 diabetes mellitus,Vietnam patients.

I. INTRODUCTION

Diabetes is recognized as the "pandemic" of the 21st century, affecting millions of people around the world. According to statistics of the International Diabetes Federation (2018), the world has about 425 million people with diabetes. Of which over 90% have type 2 diabetes and this trend is increasing [1]. Type 2 diabetes mellitus (T2DM) is a long-term metabolic disorder that is characterized by high blood sugar (glucose) levels, insulin resistance, and relative insulin deficiency [2]. Common symptoms include thirst, frequent urination, and unexplained weight loss, frequent feelings of hunger, fatigue, muscle weakness, and slow healing of wounds or bruises [3]. T2DM is mainly caused by obesity and lack of exercise [4], and some people are more genetically at risk than others [2]. In the past 10 years, the increasing rate of diabetes in Vietnam is 211%, 3 times higher than that of the world (70%). Vietnam is also in the group of 10 countries with the highest rate of increase in diabetes patients in the world with an increase rate of 5.5% per year [5,6]. It is estimated

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that Vietnam currently has 3.53 million people "living" with diabetes and at least 80 people die from related complications every day. The number of infected people will reach 6.3 million by 2045 in Vietnam.

Nucleobindin (NUCB2) is found in plasma membranes and neuroplasma. Several prohormone convertase enzymes of NUCB2 such as PC3/1 and PC2 convert NUCB2 to nesfatin-1 (1-82 aa), nesfatin-2 (85-163 aa) and nesfatin-3 (166-396aa) [7-9]. NUCB2 has a characteristic constitution of functional domains, such as a signal peptide, a Leu/Ile rich region, two Ca^{2+} binding EF-hand domains separated by an acidic amino acid-rich region, and a leucine zipper [10,11]. In humans, the NUCB2 gene length is 55 kb long with 14 exons and 13 introns. The translation region of the NUCB2 gene is described in exon-3. Nesfatin-1 is translated in the region between exon-3 and 5 of the NUCB2 gene [8,12]. However, recent studies have shown that NUCB2 is also expressed in other peripheral tissues, such as stomach, pancreas, reproductive organs, and adipose tissue [7,13,14].

Nesfatin-1 was first discovered by Oh in 2006. It showed that nesfatin-1 is secreted by peripheral tissues, central and peripheral nervous systems [15]. Nesfatin-1 polypeptides with 82 amino acids derived from the precursor protein NUCB2. The structure of nesfatin-1 has three parts: N-terminal (N23), Middle part (M30), and C-terminal (C29). The middle part was identified as an active part of the physiological effects of nesfatin-1 in the anorexic effect [8,12,16]. In the study of Gonzalez, the homology of nesfatin-1 was up to 85% between humans and mammal species [17]. The role of nesfatin-1 acts as an appetite suppressant and is jointed in the balanced of energy homeostasis related to food and water intake without the leptin gene [18]. The nesfatin-1 enzyme is selected by peripheral adipose tissue, gastric mucosa, pancreatic endocrine beta cells, and testis tissue. It can pass through the blood-brain barrier after secretion [7,17,19-22].

In previous studies, nesfatin-1 was implicated in T2DM pathogenesis by stimulating free acid utilization. Significant reductions in fasting plasma nesfatin-1 levels in T2DM and polycystic ovary syndrome (PCOS) patients have also been confirmed. This can be caused by impaired insulin sensitivity and suggests that nesfatin-1 can be inhibited by insulin resistance, hyperglycemia, and hyperinsulinemia [23]. The plasma level of nesfatin-1 was not increased by acute stress [24,25]. However, serum nesfatin-1 levels were found to be higher in patients with depression than in controls [26].

In Vietnam, to date, no studies have been conducted on diabetic patients on nesfatin-1 levels and the expression of NUCB2 gene. It is difficult to understand whether nesfatin-1 can be used as a supportive tool in the diagnosis and treatment of diabetes in Vietnamese patients. Thus, in the present study, we conducted to assess nesfatin-1 concentrations and mRNA expression of NUCB2 levels in adipose tissue by Realtime-PCr. The correlation between the relative expression of NUCB2 geneand nesfatin-1 levels with several clinicopathological parameters was evaluated as its clinical significance.

II. MATERIALS AND METHODS

2.1. Data Collection and Study Design

A cross-sectional study was conducted on a total of 65 T2DM patients and 30 healthy (control) subjects. Those groups were separated by age and gender (Table **1**). Diabetic patients were divided into two groups based on the duration of diabetes. Long-term type 2 diabetes mellitus (l-T2DM) group included 37 patients over 3 years. The newly diagnosed type 2 diabetes mellitus (n-T2DM) group included 28 patients under 3 years. All patients were randomly selected from those who visited the Endocrinology Department of Hospital, from January to December 2020. The diagnose of T2DM was made according to the American Diabetes Association criteria based on the criteria using a fasting blood glucose (FBG) level $\geq$ 7.0 mmol/L. Clinical exclusion criteria included type 1 diabetes mellitus, history of endocrine disorders, pregnancy, surgery or trauma in recent times, heart failure, and cancer. All people who visited the hospital for regular physical examination were selected as control subjects who had no family history of diabetes or other endocrine disorders. All people in the present study were Vietnamese and consented to join. The present study was conducted based on the principles of the Declaration of Helsinki and approved by the Ethics Committee of Vietnam Military Medical University, Vietnam (Decision number 2883/QĐ-HVQY).

2.2. Experimental Methods

2.2.1. Anthropometry Study

Anthropometric measurements were performed in the morning, before breakfast. Bodyweight and height were calculated using a scale and wall-mounted stadiometer (0.5 kg and 0.5 cm, respectively). Body Mass Index (BMI) is a person's weight in kilograms divided by the square of height in meters. Waist and hip circumferences were calculated using the standard method. The

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waist-to-hip (W/H) ratio was calculated as waist measurement divided by the hip measurement.

2.2.2. Biochemical Study

After overnight fasting, blood samples were collected and centrifuged immediately and frozen at −80°C until assayed. The FBG level was measured using the glucose oxidase technique and glycosylated hemoglobin (HbA1c) with anion-exchange HPLC. The levels of plasma total cholesterol (TC), Serum Creatinine, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were drawn in lithium-heparin vacuum tubes and analyzed enzymatically by an autoanalyzer.

2.2.3. Serum Nesfatin-1 Assay

The serum nesfatin-1 levels were detected using a commercial enzyme-linked immunosorbent assay kit from Biomatik, Wilmington, USA, with a linear range: 31.25 pg/mL-5000 pg/mL.

2.2.4. Adipose Biopsy Study

Subcutaneous abdominal periumbilical adipose tissue biopsy samples were obtained under sterile conditions and local anesthesia with 1% lidocaine. A biopsy needle with a small incision was used to obtain tissue samples. Samples were treated with liquid nitrogen to flash-freezing tissue the tissue for 1 min. The flash-frozen adipose tissue was cryopulverized 2 times, and stored at −80°C until extraction and analysis.

2.2.5. Expression of mRNA NUCB2

Total RNA was isolated from adipose tissue (approximately 85-100 mg) using Trizol reagent (Invitrogen, USA) according to the manufacturer's guide. It was then treated with DNase I (Invitrogen, USA). First-strand cDNA synthesis was conducted from purified RNA using SuperScript III (Invitrogen, USA). Quantitative Realtime - PCR was run with LC-Fast Start DNA SYBR Green chemistry with LightCycler 2.0 software (Roche, Switzerland). The expressed genes were analyzed by the comparative ct method in relation to GAPDH levels. Relative amounts of mRNA were quantified using the second derivative maximum method of the light-cycler software.

2.3. Data Analysis and Statistical Methods

Statistical analysis was performed with R and R-studio software [27, 28]. Data were expressed as mean ± standard error (SD) or frequency (%). One-way ANOVA with post hoc (least significant difference) analysis was used to assess for differences in body composition, anthropometric, metabolic, and hormonal parameters among l-T2DM patients, n-T2DM patients, and control subjects. The correlation of serum nesfatin-1 with other clinical characteristics including BMI, Serum Creatinine, HDL-C, LDL-C, TC, HbA1c, and Fasting Blood Glucose was determined using Pearson correlation analysis. All analyses were significant at thep< 0.05 level.

III. RESULTS

3.1. Clinical Characteristics of the Patient and Control Subjects

The clinical characteristics and laboratory findings of all subgroups were presented in Table **1**. The highest range of age was given in the l-T2DM group (62.95years). This level was determined to decrease gradually when compared with the n-T2DM and the control groupswith 60.54and 54.83years, respectively. While both the body mass index (BMI) and the Waist hip ratio (WHR) were significantly higher in two patient groups (l-T2DM: 23.25; 0.94 and n-T2DM: 22.61; 0.93) compared with the control group (22.07 and 0.86).The duration of diabetes in the l-T2DM group was approximately 8 years. In n-T2DM patients, under 3- year duration of diabetes in this group accounted for half of the total number of patients, including 7 patients in 1 year, 8 patients in 2 years, and 13 patients in 3 years, with an average of 2.21 years.In the frequency of HbA1c unit, l-T2DM patients also were at the highest level (7.14%) compared to the control group (5.56%) and the n-T2DM group (6.91%). Fasting blood glucose was significantly higher in the l-T2DM group than that in the n-T2DM and control groups (7.44 versus 6.99 and 5.28 mmol/L, respectively,p<0.01). This was an important index for identifying the presence of diabetes (FBG ≥7 mmol/L).The index of cholesterol amount in the body was determined based on the LDL cholesterol, HDL cholesterol, and total cholesterol levels. Total cholesterol levels were significantly increased in the control group, compared with l-T2DM and n-T2DM (4.80 vs 4.69 and 4.61 mmol/L, respectively), but there was no significant difference between the groups.All three study groups achieved an ideal concentration of less than 200mg/dL (equivalent to>5.1 mmol/L), which showed no increase in blood cholesterol and a reduced risk of Coronary Artery Disease (CAD).For HDL-C and LDL-C levels, although in both the l-T2DM and n-T2DM groups, they have not yet decreased to a bad level or need to be cautious (HDL-C <1.0 mmol/L and LDL-C >3.3 mmol/L).However, these indices are being evaluated as inferior to the control group (HDL-C: 1.11 and 1.08 vs 1.55 mmol/L; LDL-C: 2.98 and 3.08 vs 2.52 mmol/L). Especially in the n-T2DM group, lack of early treatment, which can easily lead to worse conditions.The test assessing indices of kidney function such as Creatinine Serum and

Creatinine Urine was also analyzed, however, there were no major effects between the 3 study groups.

3.2. Serum Nesfatin-1 Concentration in Long-term Type 2 Diabetes Mellitus and Newly Type 2 Diabetes Mellitus Patients.

The level of serum nefatin-1 was significantly higher in the n-T2DM group than in l-T2DM and control groups ($p < 0.05$ vs Control) (Fig. **1**). In l-T2DM patients, this value was slightly lower than in controls, however, no significant difference among them ($p = 0.068$). The serum nesfatin-1 concentrations observed in each group were as follows 0.967 ng/mL (Control), 0.881 ng/mL (l-T2DM) and 1.232 ng/mL (n-T2DM).

3.3. Correlation of Serum Nesfatin-1 with Other Biochemical Characteristics

The linear regression analysis was used to evaluate the correlation of serum nesfatin-1 and other metabolic parameters related to diabetes. Our results showed that in both l-T2DM and n-T2DM groups, nesfatin-1 was positively correlated with BMI, WHR, duration of diabetes, total cholesterol, HCL-C, Serum Creatinine, and Urine Creatinine. Only the correlation with duration of diabetes and total cholesterol was not significantly different in both groups ($p = 0.102$ and 0.764, respectively). For other parameters such as HbA1c, FBG, and LDL-C, all of which were confirmed to be negatively correlated with nesfatin-1, p-value <0.05 for HbA1c and $p<0.001$ for LDL-C, no significant difference in the case of FBG ($p=0.216$ and 0.688) (Table **2**).Based on the correlation between nesfatin-1 index and the parameters with significant differences ($p<0.05$, or <0.001), we conducted a multiple stepwise regression analysis with 6 units including HDL-C, LDL-C, creatinine serum, creatinine urine, BMI, and HbA1c for all 65 patients in l-T2DM and n-T2DM groups (Fig.**2**).

3.4. NUCB2 mRNA Gene Expression in Adipose Tissues

In this study, all of the patient consented to join the subcutaneous adipose tissue experiment, including 30 people in the control group, 37 patients in the l-T2DM group, and 28 patients in the n-T2DM group. The results showed that mRNA NUCB2 geneexpressions were lower in adipose tissue from n-T2DM patients than those from controls and l-T2DM (by ~1.2-fold and ~1.5-fold respectively, $p<0.01$ vs control) (Fig.**3**). The expression of these genes was consistent with nesfatin-1 levels in serum, which was also markedly increased in n-T2DM patients compared with controls and l-T2DM.

IV. DISCUSSION

Previous studies identified NUCB2/nesfatin-1 in several regions of the hypothalamus, and subsequently in peripheral tissues including adipocytes, gastric mucosa, pituitary gland, heart, and medulla oblongata of humans and rats [22]. Recent studies have reported that nesfatin-1 is a potential candidate for the treatment ofT2DM with hypoglycemic effects in conditions of impaired glucose metabolism [29]. It may also act in the brain to regulate insulin sensitivity [30]and increase insulin release in beta cells in response to hyperglycemia [31].In our study, the level of nesfatin-1 in the n-T2DM group was higher than in the healthy group. This result was consistent with previous studies such as the study of Zhangand Guo [30,32]. This could be explained as a physiological response or a compensatory mechanism for the impaired insulin action of n-T2DM[32-34].However, in the study by Li, the fasting nesfatin-1 concentration (after 24 hours of fasting) of T2DM was significantly lower than that of the healthy group [23]. This result has also been shown to be similar in later studies [35-37]. It was different with our data when the nesfatin-1 level of the subjects with long disease duration (8 - 20 years) was not significantly different from the control group. To account for these different results, there may be variations in the selection of patients in the experimental design, as well as in the experimental conditions or duration of the test time. For example, in our experiment, the majority of patients participating in the study were sampled in the morning, after fasting overnight (12 hours). Therefore, the interval after the last meal may not be sufficient to see a difference in nesfatin-1 levels between the 2 study groups, the long-term diabetic patients, and the control group.Besides, to further explain our results, the nesfatin-1 level of the l-T2DM group may be affected by medicine and diabetes treatment. With the goal of reducing blood sugar, increasing insulin sensitivity, and controlling food intake, there are currently many classes of drugs commonly used in the treatment of diabetes, such as Metformin, Sulfonylureas, Meglitinides, Thiazolidinediones, DPP-4 inhibitors, GLP-1 receptor agonists, SGLT2 inhibitors or Insulin. Thus, the effects of these drugs on each patient's concentration of nesfatin-1 were unavoidable.To clarify the correlation between serum nesfatin-1 levels and some related parameters, we conducted a simple regression analysis on the study subjects. With the 3 anthropometric parameters studied including Age, BMI, and WHR, only the BMI parameter showed a significant difference in positive correlation with nesfatin-1 level ($p<0.001$).

Although there was no significant difference in the correlation between nesfatin-1 and age, our result was consistent with that reported in the study by Li [23]. There is a further explanation for the results reported on the Vietnamese group of patients, possibly because the habit of periodic health check-ups has not been established in young people, so most of the patients only conduct health check-ups after retirement age (standard supported by the government). Therefore, the patient group identified with T2DM in the study was relatively old, leading to the result that the correlation with the age group in our study was not clear. In addition, BMI and WHR parameters were also different from previous studies, possibly as a different result between groups of people around the world.Positive correlations and significant differences were reported in the two creatinine test indices. This result is equivalent to the study of Li [23]. The reported negative correlation coefficient between nesfatin-1 levels with HbA1c, HDL-C, and LDL-C is similar to some previous studies [23,35]. The above results suggest that these factors may contribute to the uniformity observed in the overall analysis of nesfatin-1 serum concentrations of T2DM patients.To further clarify whether the NUCB2 gene expression level affects the serum nesfatin-1 level in Vietnamese patients, we analyzed the mRNA NUCB2 gene expression level by Realtime-PCr. Previous studies have shown the of mRNA NUCB2 geneexpression in peripheral tissues including heart, spinal cord, pancreas, stomach, muscle, and adipose tissue[17,22]. They have important physiological roles in body weight and contribute to the pathophysiology of insulin resistance and related metabolic problems in obesity and diabetes. Guo found that mRNA NUCB2 and protein levels of muscle and adipose tissue were significantly increased in T2DM patients [32]. Nesfatin-1 has been described to be able to cross the blood-brain barrier across an unsaturated membrane [20,21]. The expression of mRNA NUCB2 genewas established in the gastric mucosa higher 10 times more than in the brain, suggesting that the stomach was the major base of circulating nesfatin-1 [38]. The expression of NUCB2 and nesfatin-1 was also released in adipocytes. This expression is mainly evident in subcutaneous adipose tissue cells[8].In the present study, the expression level of the mRNA NUCB2 gene in Vietnamese patients with l-T2DM was high in n-T2DM group. While the expression level of NUCB2 gene transcription activity in the l-T2DM group was observed to be slightly decreased compared with the control group. This may be explained by the fact that the l-T2DM patients have been treated with different classes of drugs, which compensate for the lack of insulin. Thus, the mRNA NUCB2 gene expression level in this group was not different compared with the healthy group.

V. CONCLUSION

The novelty of our study was the first study to evaluate the nesfatin-1 concentration and mRNA NUCB2 gene expression level in a group of Vietnamese patients with type 2 diabetes mellitus. Moreover, the relationship between nesfatin-1 in serum with some anthropometric and biochemical parameters of the long-term disease and newly diagnosed diseases of Vietnamese patients was determined for the first time. Our findings also suggest some potential of using nesfatin-1 as an effective treatment for type 2 diabetes in Vietnamese patients in the future.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All people in the present study were Vietnamese and consented to join. The present study was approved by the Ethics Committee of Vietnam Military Medical University, Vietnam (Decision number 2883/QĐ-HVQY).

HUMAN AND ANIMAL RIGHTS

No animals were used for studies that are the basis of this research. This research was conducted on patients are in accordance with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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