Diabetes & Vascular Disease Research
March-April 2023: 1–5

DOI: 10.1177/14791641231169246
journals.sagepub.com/home/dvr
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High serum Bhlhe40 levels are associated with subclinical atherosclerosis in patients with type 2 diabetes mellitus: A cross-sectional study

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Abstract

Background: Our previous studies have shown that the basic helix-loop-helix family member e40 (Bhlhe40) plays a critical role in regulating calcification and senescence of vascular smooth muscle cells induced by high glucose. In this study, we determined the association between serum Bhlhe40 levels and subclinical atherosclerosis in patients with type 2 diabetes mellitus (T2DM).

Methods: 247 patients with T2DM were included in this cross-sectional study between June 2021 and July 2022. The presence of subclinical atherosclerosis was evaluated by carotid ultrasonography. Serum Bhlhe40 concentrations were measured with an ELISA kit.

Results: Serum Bhlhe40 levels were remarkably higher in the subclinical atherosclerosis group than in the subjects without subclinical atherosclerosis ($p < 0.001$). Correlation analysis showed a positive correlation between serum Bhlhe40 and carotid intima-media thickness (C-IMT) ($r = 0.155$, $p = 0.015$). The optimal threshold of serum Bhlhe40 > 5.67 ng/mL had an area under the ROC curve (AUC) was 0.709 ($p < 0.001$). In addition, serum Bhlhe40 levels were associated with the prevalence of subclinical atherosclerosis (OR: 1.790, 95% CI: 1.414–2.266, $p < 0.001$).

Conclusion: Serum Bhlhe40 levels were significantly higher in T2DM subjects with subclinical atherosclerosis and positively associated with C-IMT.

Keywords

Bhlhe40, subclinical atherosclerosis, carotid intima-media thickness, type 2 diabetes mellitus

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic multisystem disease associated with microvascular and macrovascular complications. Currently, more than 536 million people worldwide are suffering from diabetes, and the number is expected to exceed 780 million by 2045.[1] Cardiovascular disease, especially atherosclerotic cardiovascular disease (ASCVD), is still a major cause of disability and death in patients with diabetes. Identification of diabetic patients at high risk of ASCVD is essential to prevent cardiovascular events and reduce the risk of death. Subclinical atherosclerosis is the early stage of atherosclerosis progression and can be assessed by measuring carotid intima-media thickness (C-IMT) and plaque.[2] Early detection and

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intervention of subclinical atherosclerosis is beneficial to delay the progression of atherosclerosis and improve the prognosis of diabetic patients.

The basic helix-loop-helix transcription factor 40 (Bhlhe40) has been shown to be a key regulator of circadian rhythms, tumorigenesis, and immune response.[3] Our previous studies have found that bhlhe40 was involved in the regulation of high glucose-induced calcification/senescence of vascular smooth muscle cells (VSMCs),[4,5] hinting the involvement of Bhlhe40 in the occurrence and development of atherosclerosis. However, the association between serum Bhlhe40 levels and diabetic macroangiopathy remains unsettled. In this study, we investigated the relationship between serum Bhlhe40 and subclinical atherosclerosis in patients with T2DM.

Methods

Study population

This cross-sectional study included 247 patients with T2DM who were hospitalized in the Department of Geriatrics of The Second Xiangya Hospital of Central South University from June 2021 to July 2022. T2DM was diagnosed according to the American Diabetes Association classification. This study was approved by the Ethics Committee of The Second Xiangya Hospital of Central South University (No. 2022–163). Participants gave informed consent to participate in the study before taking part. The exclusion criteria were as follows: type 1 diabetes mellitus and other special type diabetes, with diabetic acute complications, without carotid ultrasound, history of cardiovascular and cerebrovascular diseases, and other serious diseases, such as autoimmune disease and malignant tumors.

Data collection and measurements

Demographic data, including age, sex, smoking and alcohol intake histories, hypertension history, and diabetes duration, was obtained using a standardized questionnaire, and their weight, height, and blood pressure were measured by a professional caregiver. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Usage of medications was taken from medical records.

Venous blood samples taken after an overnight fast were used to detect biochemical parameters and the remaining serum was stored at −80°C for Bhlhe40 measurement. Biochemical parameters, including fasting blood glucose (FBG), glycated hemoglobin (HbA_{1c}), fasting C-peptide, serum total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum creatinine, uric acid, and urinary albumin-to-creatinine ratio (UACR) well collected. The estimated glomerular filtration rate (eGFR) was calculated using the Chinese-modified Chronic Kidney Disease Epidemiology Collaboration. Serum Bhlhe40 levels were detected using ELISA kits (ZC-55,885, ZCIBIO Technology Co., Ltd.).

The patient was placed in a supine position with full exposure to the neck. C-IMT was measured by Doppler ultrasound (128XP/10 system; Acuson, Mountain View, CA, USA). Subclinical atherosclerosis was defined as C-IMT >1.0 mm or atherosclerotic plaque formation without clinical symptoms.[6]

Statistical analysis

SPSS 26.0 software (SPSS, Chicago, IL, USA) and GraphPad Prism 9.0 software (GraphPad Prism Inc., San Diego, CA, USA) were used for statistical analysis. Statistical significance was set at $p < 0.05$. The normal distribution data assessed by Kolmogorov-Smirnov test were expressed as mean ± standard deviation (SD) and compared by Student's t-test, whereas the non-normal distribution data were presented as median and percentile (Q1, Q3), and the comparison was conducted by non-parametric Mann-Whitney U test. Categorical variables were expressed as number and percentage (%) and compared by Chi-square test. The area under the receiver operating characteristic (ROC) curve (AUC) of Bhlhe40 was calculated by ROC analysis to assess the diagnostic performance. Logistic regression analysis was performed to evaluate the association between serum Bhlhe40 levels and the prevalence of subclinical atherosclerosis in T2DM patients, adjusting for age, sex, hypertension, diabetes duration, SBP, usage of insulin and statins, fasting C-peptide, TC, serum creatinine, eGFR, UACR, and C-IMT.

Results

Characteristics of the study participants are shown in Supplementary Table 1. T2DM individuals with subclinical atherosclerosis accounted for 60.3% ($n = 149$). Figure 1(a) presented that serum levels of Bhlhe40 were significantly elevated in subjects with subclinical atherosclerosis [4.78 (3.48, 6.07) versus 6.52 (5.21, 8.01), $p < 0.001$]. In addition, correlation analysis presented a positive correlation between serum Bhlhe40 levels and C-IMT ($r = 0.155$, $p = 0.015$) (Supplemental Table 2). Figure 1(b) presented the ROC curve of serum Bhlhe40 for subclinical atherosclerosis. The AUC of serum Bhlhe40 for subclinical atherosclerosis was 0.709 (95% CI: 0.641–0.777, $p < 0.001$). The optimal cut-off value of serum Bhlhe40 was 5.67 ng/mL for distinguishing subclinical atherosclerosis with a high sensitivity (67.1%) and specificity (70.4%). Logistic regression analysis revealed that serum Bhlhe40 levels

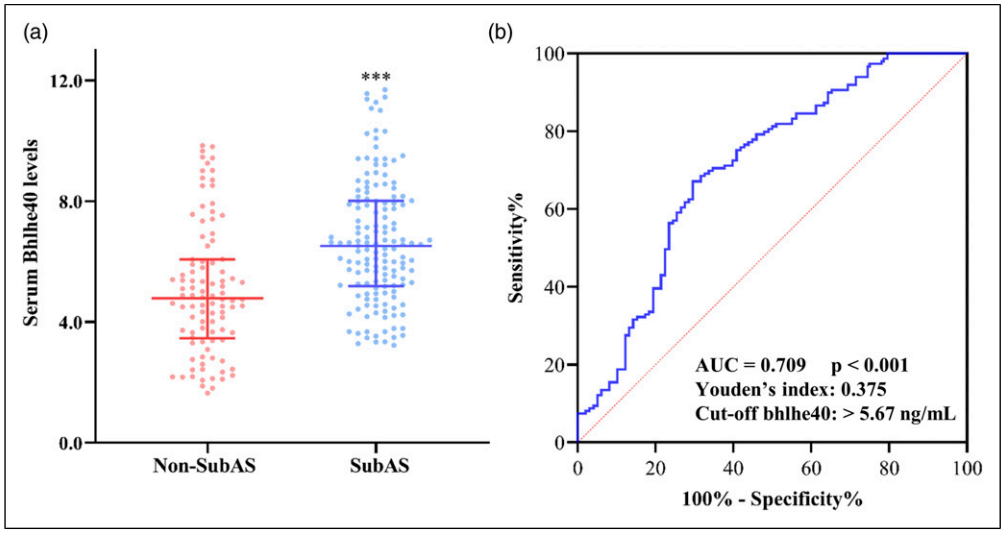

Figure 1. Comparison of the serum Bhlhe40 levels between the subclinical atherosclerosis (SubAS) group and non-subclinical atherosclerosis (Non-SubAS) (a) and receiver operating characteristic (ROC) curve of serum bhlhe40 for SubAS (b). ***$p <$ 0.001 Mann-Whitney U test.

were associated with the prevalence of subclinical atherosclerosis (OR: 1.790, 95% CI: 1.414–2.266, $p < 0.001$) (Supplemental Table 3).

Discussion

The present study showed that significantly higher serum levels of Bhlhe40 were observed in Chinese T2DM patients with subclinical atherosclerosis. Our analyses demonstrated that serum Bhlhe40 levels were associated with the prevalence of subclinical atherosclerosis and positively associated with C-IMT.

Currently, diabetes is affecting the health and living conditions of hundreds of millions of people, approximately 10.5% of the world's population suffers from diabetes.[1] Although the overall prevalence of cardiovascular diseases in patients with diabetes is decreasing globally, cardiovascular disease remains the leading cause of death in patients with T2DM. Circadian clock was strongly linked to cardiovascular disease. Among the clock genes, Bhlhe40 has proven to be extremely critical for the regulation of metabolic disorders and cardiovascular disease. Our previous studies have shown that the expression of Bhlhe40 was significantly reduced in high glucose-treated VSMCs. Overexpression of Bhlhe40 exhibited a role in alleviating high glucose-induced VSMCs senescence and calcification.[4,5]

This was the first report on the predictive value of serum Bhlhe40 for the prevalence of subclinical atherosclerosis in Chinese adults with T2DM. Unexpectedly, we found that serum Bhlhe40 levels were positively correlated with C-IMT and patients with higher serum Bhlhe40 levels were more prone to subclinical atherosclerosis. In addition, we also determined the optimal threshold point of serum Bhlhe40 for the diagnosis of subclinical atherosclerosis was 5.67 ng/mL. However, when we evaluated the possible association between Bhlhe40 and indicators of glucose and lipid metabolism, no significant relationship was found. Antidiabetic therapy may responsible for this ambiguous or non-significant relationship between Bhlhe40 and indicators of glucose and lipid metabolism in patients with T2DM. These findings indicated that serum Bhlhe40 may be a useful marker for the detection of subclinical atherosclerosis in patients with T2DM. Multivariate logistic regression analyses further validated the predictive value of Bhlhe40 for subclinical atherosclerosis prevalence.

The underlying mechanism by which Bhlhe40 may lead to subclinical atherosclerosis in patients with diabetes has not yet been elaborated. Bhlhe40, a key regulator of immunity during inflammatory responses and autoimmune, is involved in a variety of fundamental biological processes, such as cellular proliferation, differentiation, senescence, apoptosis, and metabolism.[7] Bhlhe40 and insulin resistance (IR) may be co-pathogenic pathways of atherosclerosis, or they may interact with each other. It has been reported that Bhlhe40 was a potential candidate modifier for diabetes and obesity through negatively modulating myocyte fatty acid oxidation.[8] Bhlhe40 transcriptionally inhibited the expression of peroxisome proliferation-activated receptor gama (PPARγ), a key mediator in the regulation of insulin sensitivity.[9] A recent study demonstrated that the high expression of Bhlhe40 was relevant to IR in patients with obstructive sleep apnea and can be utilized for predicting the presence of metabolic syndrome in obstructive sleep

apnea individuals.[10] Moreover, chronic inflammation and oxidative stress are two major hallmarks of atherosclerosis.[11] At the cellular level, overexpression of Bhlhe40 upregulated the generation of reactive oxygen species and the expression of pro-inflammatory cytokines, such as interleukin 6 (IL-6), IL-1β, tumour necrosis factor alpha, and monocyte chemoattractant protein-1 in pulmonary arterial smooth muscle cells.[9] At the population level, the expression of Bhlhe40 was positively correlated with IL-6 and total oxidant status levels and negatively related to total antioxidant status levels.[10] *In vitro* observation showed an elevated proliferation and a declined apoptosis of pulmonary arterial endothelial cells under hypoxia conditions by the upregulation of Bhlhe40.[12] Conversely, our previous studies found a low expression in high glucose-treated VSMCs.[5] The reasons for this discrepancy might be due to the differences in cell types, handling of samples, and study design. Therefore, further studies are urgently needed to explore the mechanism of bhlhe40 in the occurrence and development of atherosclerosis and to verify the relationship between serum Bhlhe40 and cardiovascular disease risk.

To our knowledge, this was the first study to report the predictive value of serum Bhlhe40 levels for subclinical atherosclerosis prevalence in T2DM patients. However, our study has several limitations. Firstly, this study was a cross-sectional and observational study that cannot ensure complete control for all potential confounding factors and would limit the determination of a causal relationship between Bhlhe40 and subclinical atherosclerosis. The results need to be confirmed by subsequent large-scale and prospective studies. Secondly, as our study population only enrolled people with T2DM, our findings may not be directly applicable to the general population due to sampling bias.

Conclusion

The results of the current study showed that serum Bhlhe40 levels were significantly higher in T2DM participants with subclinical atherosclerosis and positively associated with C-IMT. However, more in-depth mechanistic studies are required to verify whether and how Bhlhe40 participates in the pathogenesis of atherosclerosis.

Author contributions

XH obtained the data, performed the analyses, and drafted the manuscript. XQY acquired data and did the interpretation of data. LS contributed to the conception and design of this study, revised the manuscript, and obtained study funding. LYS obtained study funding and provided professional methods. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the National Natural Science Foundation of China (No. 82,071,593 and 82,101,663); National Key R&D (or Research and Development) Program of China (No. 2020YFC2009000 and 2020YFC2009001).

Data availability

Due to the personal privacy of the patients included in this study, the data sets used and analyzed in this study are not publicly available but are available from the corresponding authors upon reasonable request.

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Supplemental Material

Supplemental material for this article is available online.

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