

## Original Article

# Expression of visfatin influences the development of coronary artery disease

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**Abstract:** Excessive intake of fatty substances resulted in an increased occurrence of coronary artery disease (CAD), an affecting people's life disease which has received widespread attentions. We found that patients with CAD had more adipose tissue than non-coronary artery disease in the artery surrounding. So, did increase of this localized fat play an active role? And whether it secretes more fat cells factor? In this study, by using immunohistochemistry, RT-PCR and western blotting methods, the expression of visfatin gene and protein between CAD and Non-CAD's adipose tissue surrounding coronary arteries and around the heart were compared. The purpose of the present study was to investigate the correlations between the expression of visfatin in coronary surrounding adipose tissue and coronary artery disease.

**Keywords:** Visfatin, coronary artery disease, gene expression, adipose tissue

## Introduction

Coronary artery disease (CAD) mainly refers to abnormal lipid metabolism leading to accumulation of lipids arterial intima and forming atherosclerotic lesions, then producing a series of clinical symptoms. With the improvement of people's living standards, the increasing incidences and impact of coronary artery disease might seriously impair human health, even threaten patients' life. Due to abnormal lipid metabolism, blood lipid calm in the smooth artery intima, some similar atherosclerotic lipids deposited by white patches in arterial intima. The elevation of these plaques gradually leads to arterial stenosis and blood flow blocking, which should lead to cardiac ischemia and angina. If the artery wall plaque ruptured or formed ulcers, the formation of blood clots would completely interrupt the whole blood flow, which might acute myocardial infarction, sudden death and even life-threatening. Its prevention, diagnosis and treatment would bring profound effects and influence to medical social burden [1].

Visfatin, known as pre-B cell colony-enhancing factor (PBEF), is identified as a new type of adipokines from visceral fat in humans and mice. It is 473 amino acid residues of polypeptide, and the molecular weight is 52 kD. It is secreted by fat cells and binding to insulin receptor exerted biological effects similar to insulin, which through autocrine, paracrine in regulating the expression of other cytokines and adipokines [2]. Previous studies showed that visfatin might inhibit NO production to impair the function of endothelial cell [3, 4], and vascular endothelial cell injury was considered to the first step of atherosclerosis. On the other hand, visfatin could promote the proliferation and migration of endothelial cells, by reactive oxygen species (ROS) dependent nuclear factor κB (NF-κB) pathway, extracellular signal-regulated kinase (ERK) and phosphatidyl alcohol-3-kinase (PI3K) signaling pathway to promote the expression of metal matrix enzymes (MMP)-2, MMP-9 and vascular endothelial growth factor (VEGF) and other inflammatory mediators, whereas MMP and VEGF were considered to hasten progress and plaque instability of ath-

erosclerosis [5-7]. Dahl found that visfatin was highly expressed in atherosclerotic plaque foam cells and macrophages, as well as in STEMI patients with ruptured plaques. These results suggest that visfatin influences the occurrence and development of coronary atherosclerosis [8].

### Materials and methods

#### *Experimental samples collection*

Experimental subjects were divided into two groups, including CAD and non-CAD. All the subjects were taken from the chest subcutaneous adipose tissue, pericardial adipose tissue, cardiac fat, fat around the coronary artery during surgery. The tissue was divided into two parts: the latter part with sterile phosphate-buffered saline (PBS) to wash bloodstained and quickly into vials, frozen in liquid nitrogen tanks placed for 24 hours, placed in  $-80^{\circ}\text{C}$  refrigerator, was used to detect adipose tissue visfatin mRNA level and adipose tissue visfatin protein level in real-time PCR and western blotting; the other part was fixed into 10% paraformaldehyde solution, paraffin-embedded for immunization staining analysis.

#### *Adipose tissue immunohistochemical staining*

The experimental tissues were removed the endogenous catalase after dewaxing. After washing with PBS, they were added dropwise 5% BSA and anti-secondary antibody into a  $37^{\circ}\text{C}$  incubator. After drying PBS surrounding tissue, it was dropped reagent and incubated at room temperature. Dropping hematoxylin, soaked in distilled water and immersed in a saturated  $\text{Na}_2\text{HPO}_4$  solution. As image acquisition from microscope, a positive result for the cytoplasm stained brown. Visfatin positive expression within fat cells shown brown particles, color intensity varies with the amount of expression, and staining area was according to the number of cells. Application of the United States of Media Cybernetics Image-pro plus immunohistochemical image analysis software for automatic image analysis, were estimated the integrated optical density and the photo area for statistical analysis.

#### *Real-time quantitative PCR detection of visfatin mRNA expression levels*

The adipose tissue was added 1 mL Trizol to grind until the lysates with no small particles.

Chloroform was added with vigorous shaking, until emulsified solution was allowed to stand at room temperature, and centrifuged supernatant was transferred to a centrifuge tube. Addition of an equal volume of isopropanol and mix thoroughly. After drying at room temperature, it was added 20  $\mu\text{L}$  sterile DEPC to completely dissolved. The resultant cDNA was employed as the template in PCR reaction, containing 5 pmol each of forward and reverse gene-specific primers Primer. Application Premier 5.0 software to design the basic requirements: NAMPT-F 5'-ATCCTGTCCAGGC-TATTCTGT-3; NAMPT-R 5'-CCCCATATTTTCTCACGCAT-3;  $\beta$ -ACTIN-F 5'-CTTCCAGCCTTCCTT-CCTGG-3;  $\beta$ -ACTIN-R 5'-CTGTGTTGGCGTACAG-GTCT-3. Take 1  $\mu\text{L}$  cDNA into PCR tubes and add formulated PCR reaction mixture. PCR reactions were performed on BIOTECH Applied Biosystems 7900 HT real-time PCR instrument. PCR reaction conditions: firstly,  $95^{\circ}\text{C}$ , 30 s; the second step,  $95^{\circ}\text{C}$ , 5 s, temperature of  $60^{\circ}\text{C}$ , 30 s, a total of 40 cycles.

#### *Western blotting measured visfatin gene expression levels*

The appropriate amount of lysis buffer was added phenylmethylsulfonyl fluoride (PMSF), and given a final PMSF concentration of 1 mM. Each 10 mg tissue was added 100  $\mu\text{L}$  lysate at  $4^{\circ}\text{C}$  to shock cracking tissue homogenizer. Preparation of BCA working solution according to the number of samples, mixed thoroughly at  $37^{\circ}\text{C}$  for 30 minutes. Determination in A562, according to the standard curve calculates the protein concentration. Electrophoresis apparatus was using a Bio-Rad SDS-PAGE electrophoresis. Western blotting analysis process to select a clear X-ray image to analyzes system grayscale image. The sample reference gene ratio was used to confirm the relative expression of visfatin genes.

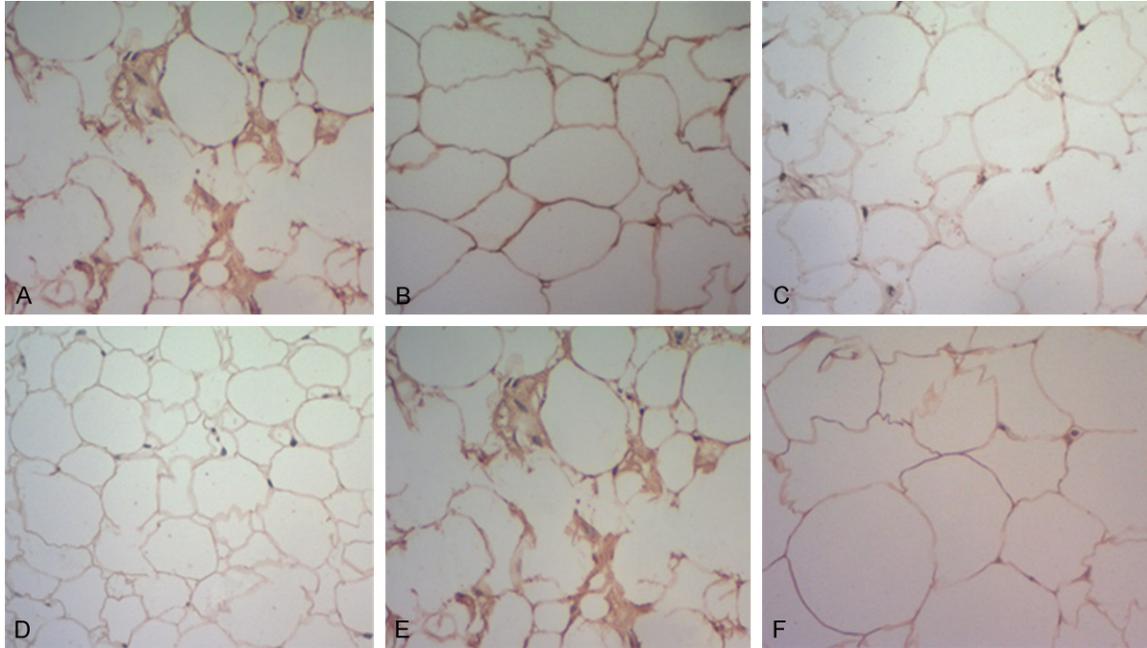
#### *Statistical analysis*

Each set of data are mean  $\pm$  standard deviation, using one-way ANOVA, SPSS13.0 statistical analysis software for data processing,  $P < 0.05$  was considered statistically significant.

### Results

#### *Visfatin protein immunohistochemistry*

The fat cells were round or oval under the observation of light microscope, the nucleus



**Figure 1.** The immunohistochemistry of adipose tissue. A. Coronary surrounding adipose tissue was visfatin-medium positive; B. Subcutaneous adipose tissue was visfatin-positive; C. Cardiac adipose tissue was visfatin-positive; D. Pericardial adipose tissue was visfatin-positive; E. Coronary surrounding adipose tissue was visfatin-medium positive; F. Non-coronary surrounding adipose tissue was visfatin-positive.

was small and biased towards one side of the cell. Comparison the fat cells in different parts found that pericardial fat cells had smaller volume, while other adipocytes groups had no significant difference in fat cell volume. Immunohistochemical staining showed that both in the CAD or Non-CAD patient's subcutaneous fat, pericardial fat, cardiac fat and pericardial adipose tissue had visfatin positive expression. Visfatin protein mainly expressed in the cytoplasm of fat, and had no positive expression in adipocytes nucleus. In the light microscope, both in non-CAD or CAD group, visfatin staining was moderately positive in adipose tissue surrounding coronary arteries, which was deeper stain, stained larger area than other parts (chest subcutaneous fat, pericardial fat, and cardiac fat) group (**Figure 1**).

*Visfatin gene transcription in different parts of adipose tissue*

Amplification and melting curve of visfatin mRNA expression and the internal control  $\beta$ -actin mRNA expression were used real-time PCR assay. The results showed that the chest subcutaneous fat, pericardial fat, myocardial fat and coronary surrounding fat tissues in both

two groups were detected the visfatin gene mRNA transcription. In CAD group, comparing to subcutaneous fat, pericardial fat and cardiac fat, the coronary surrounding fat tissue had higher visfatin mRNA levels ( $P < 0.01$ ); at the same time, those differences also existed in the non-CAD group (**Figure 2**).

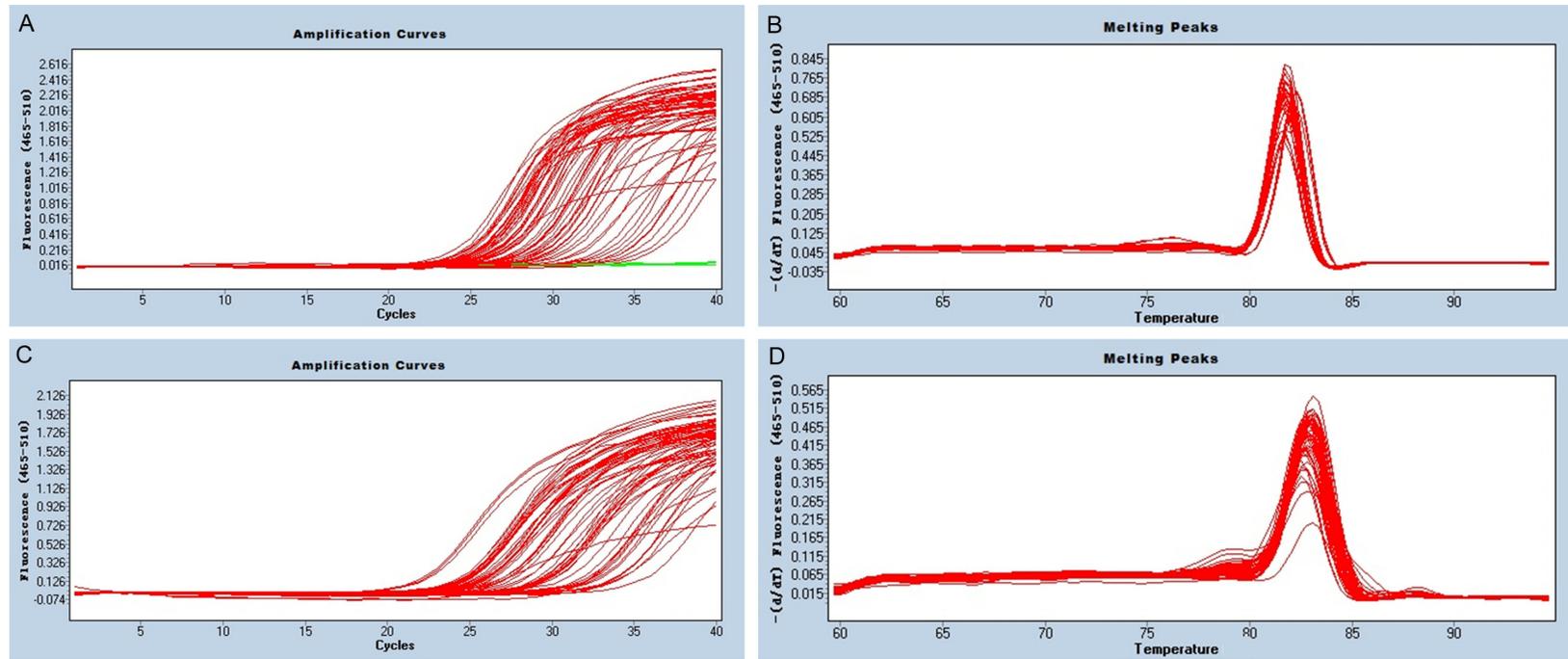
*Visfatin protein expression in different parts of adipose tissue*

Western blotting results showed that the relative expression of visfatin protein in different parts of adipose tissue was not all the same ( $P < 0.01$ ). Further analysis showed that in terms of non-CAD and CAD group, the expressions of visfatin protein in adipose tissue surrounding the coronary arteries were higher than the chest subcutaneous fat, pericardial fat and cardiac fat respectively (**Figure 3**).

**Discussion**

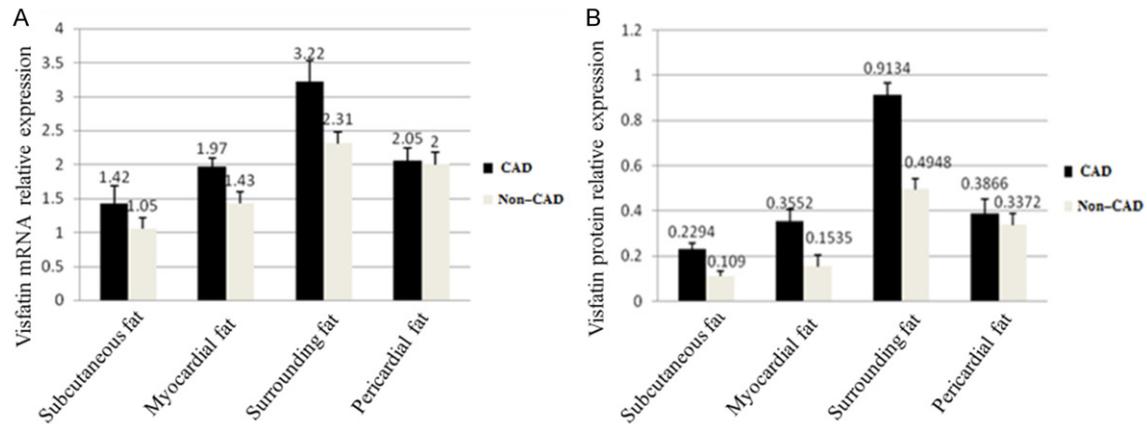
Recent studies have found that fatty tissue has a powerful endocrine function to produce large amounts of fat cells secrete factors, namely "adipokines", action on body organs (including adipose tissue) has an important regulatory

## Visfatin influences CAD



**Figure 2.** Comparison of the visfatin in different parts of the gene transcript levels in adipose tissue. A. Amplification curve of visfatin mRNA; B. Dissolution profile of visfatin mRNA; C. Amplification curve of the internal control  $\beta$ -actin mRNA; D. Dissolution profile of the internal control  $\beta$ -actin mRNA.

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**Figure 3.** The transcript levels of visfatin in fat tissue. A. Comparison of the visfatin mRNA levels in different parts of adipose tissue; B. Comparison of visfatin protein expression in different parts of adipose tissue.

function, such as visfatin, leptin, adiponectin, resistin, omentin, the physiological functions of many factors remain elusive, and the new adjustment factors are constantly being discovered. Marchington found that in animal studies, the level of free fatty acids released from epicardial adipose tissue was twice than those from the pericardial fat and perirenal adipose tissue [9]. And Baker found the adiponectin gene transcription level in hip fat tissue was five times than the epicardial adipose tissue, and adiponectin epicardial adipose tissue gene transcript levels were also significantly lower than the abdominal fat tissue. Tipping that the secretion of adipokines levels in different parts of adipose tissue were not the same, so it had different pathological physiological effects to the adjacent organs and distant organs [10].

Previous research found that abdominal subcutaneous adipose tissue and visceral adipose tissue all had visfatin expression, and the visfatin expression level of visceral adipose tissue in diabetes, impaired glucose tolerance and impaired fasting glucose was significantly high [11]. Cheng found that the visfatin expression in visceral adipose tissue significantly elevated in patients with coronary artery disease, these results strongly suggested that the increased visfatin expression in abdominal adipose tissue and coronary artery disease had some relevance [12]. So, did the visfatin expression in coronary artery adipose tissue, cardiac, pericardial, and chest subcutaneous adipose tissue correlate with coronary artery disease?

We found that the expression of visfatin detected in CAD and Non-CAD patients fat cells, which

is mainly expressed in the cytoplasm and nucleus of fat cells did not express visfatin. Visfatin was further confirmed that it was secreted by fat cells as a secreted protein. Although the pericardial, cardiac and surrounding coronary arteries adipose tissue were belong to the same visceral fat, but the volume of pericardial fat tissue was significantly lower than the surrounding fat and coronary adipose tissue, suggesting that pericardial fat may have different origin to coronary and myocardial fat tissue.

After comparing the visfatin gene and protein expression of patients with CAD and non-CAD, we found that the visfatin mRNA and protein expression in CAD patients at coronary artery were higher than non-CAD group. This difference is also found in cardiac adipose tissue and subcutaneous fat tissue, and pericardial adipose tissue visfatin mRNA and protein expression was no significant difference in the two groups of patients. It was demonstrated that the secretion of visfatin in surrounding coronary arteries, cardiac and chest subcutaneous adipose tissue associated with coronary atherosclerosis. The secretion of visfatin in pericardial adipose tissue had almost no connection to CAD, which the pericardial fat cells had different origins to the coronary arteries and myocardial fat cells.

We also found that the expression of visfatin in different parts of adipose tissue was different. In immunohistochemical staining, no matter patients of CAD and non-CAD, the expression of visfatin in coronary surrounding adipose

tissues was significantly higher than other parts of fat. Western blotting results showed that the expression of visfatin in visceral adipose tissue was higher than that in the chest subcutaneous tissue; this results found visfatin expression in abdominal subcutaneous adipose tissue was higher than consistent abdominal visceral adipose tissue [13]. In four different parts of the fat tissue, surrounding coronary was the highest expression of adipose tissue, and adipose tissue surrounding coronary arteries > pericardial adipose tissue > myocardial fat > subcutaneous fat. This result was consistent with the findings of Spiroglou that highest visfatin expression was detected in coronary fat, which was significantly higher than the consensus top side and aortic epicardial adipose tissue [14]. Real-time PCR results showed that, at the gene transcription level comparing to subcutaneous adipose tissue, chest visceral adipose tissue had higher visfatin transcript levels, and the highest gene transcription level in coronary surrounding adipose tissue. Coronary surrounding adipose tissue had higher visfatin gene and protein transcription level than other parts of adipose tissue, suggesting its role in the process of coronary atherosclerosis may be greater than other parts of fat tissue.

Mechanism of coronary surrounding adipose tissue adipokines acting on coronary atherosclerosis could be divided into two parts: 1) the secretion of adipokines epicardial in coronary artery adipose tissue by paracrine mechanisms outward diffusion, and through the wall into the coronary artery intravascular; 2) secretion of adipokines were from blood vessels into the coronary artery downstream of the outer wall, which triggered the inflammatory cascade and increased the expression of pro-inflammatory cytokines. Anti-inflammatory cytokine expression cells decreased and increased lipid deposition in intima, which produced a series of damage and led to the formation of atherosclerosis [15]. Gregory vascular research was confirmed at the side of epicardial adipose tissue to derive-leptin affected the coronary endothelial dysfunction: the coronary arteries surrounding adipose tissue-derived leptin by PKC- $\beta$  signaling pathways led to coronary artery endothelial injury, it is due to way of from outside to inside [16]. However, the coronary surrounding tissue-derived visfatin whether through this

“outside-in” approach led to the formation of coronary atherosclerosis remained to be confirmed in future studies.

In summary, the coronary arteries adipose tissue, myocardial fat, chest subcutaneous adipose tissue and pericardial adipose tissue in both had visfatin gene transcription and protein expression, and the highest expression found in the coronary arteries adipose tissue. The increased of visfatin mRNA and protein transcription and expression levels in coronary arteries adipose tissue, heart and chest fat in the subcutaneous adipose tissue was demonstrated to correlate with CAD, wherein high expression of visfatin in coronary surrounding adipose tissue might play more direct roles in coronary atherosclerosis.

### Disclosure of conflict of interest

None.

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