Physiological effects and Molecular characterization of visfatin gene polymorphism in patients with beta-thalassemia major

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Abstract
Beta thalassemia major (βTM) represents the most severe form of beta-thalassemia that is recessively inherited. The study started from January 2021 to October 2021 by collecting blood samples from seventy-five (75) transfusion-dependent β-thalassemia majors. , 38 males and 37 females were registered at the center for thalassemia and inherited blood disorders, Shaheed Hemin teaching hospital, Sulaymaniyah, Kurdistan Iraq. Patients were divided into three groups. Each group included 25 patients whose ages ranged from (2 - 10), (11 - 20), and (21 - 30) years, respectively. And 15 healthy subjects. Assessments of serum levels of visfatin. Restriction Fragment Length Polymorphism (RFLP) polymerase chain reaction (PCR) was employed to detect the single nucleotide polymorphism (SNP) in visfatin. Three genotypes (CC, CT, and TT) were detected in the rs61330082 locus of visfatin, and the differences in the frequency distribution of these genotypes were significant in the two groups ($p \leq 0.001$). The TT genotype was associated with a reduction in visfatin levels
Keywords: Beta thalassemia major, visfatin, polymorphism.

1. Introduction

Beta-thalassemia major (βTM) is a hereditary disorder that is transmitted from disease-carrier parents to their children in an autosomal recessive manner. The major causative factor of this condition is the reduced synthesis of the hemoglobin beta chain due to a genetic defect in the beta-globin gene. This defect makes the body unable to produce sufficient amounts of these chains, thereby an imbalance of hemoglobin chains will lead to ineffective erythropoiesis and microcytic hypochromic anemia. This anemia starts in early childhood and continues throughout the whole life (Arab-Zozani et al., 2021).

Pro-inflammatory adipokine, visfatin, is synthesized and secreted by visceral adipose tissue. It is involved in the upregulation of the production of the pro-inflammatory and anti-inflammatory cytokines IL-1, interleukin-1 receptor antagonist (IL-1Ra), IL-6, IL-10, and tumor necrosis factor by immune cells. These cytokines play an essential role in a wide range of infectious and inflammatory diseases (Ghanem et al., 2020). Previous investigations demonstrated high circulating levels of visfatin in metabolic diseases and in inflammatory disorders (Yamaguchi et al., 2017; 1. Abdulwahed, et al.,2020; 3. Alkanaani et al.,2020). The cytokine, also, has been reported to be elevated in the plasma of patients with βTM (Harbi et al., 2020). The production of visfatin is regulated by glucocorticoids, TNF-α, IL-6, and growth hormones (Francisco et al., 2018).

Visfatin is encoded by the nicotinamide phosphoribosyltransferase (NAMPT) gene located on the long arm of chromosome 7q22 (Vasilache *et al.*, 2020). Many single nucleotide polymorphisms (SNPs) of the visfatin gene have been reported to be associated with low-grade inflammation, T2D, obesity, and coronary artery disease (Yan *et al.*, 2010). The -1535C>T (rs61330082) is a polymorphism located at the promoter region of the visfatin gene. This variant has been widely studied as a disease contributor because it regulates the expression of the visfatin gene (Zhang *et al.*, 2015; Wu *et al.*, 2016).

There are three genotypes of the -1535C>T variant (CC, CT, and TT). It has been shown that the T variant from this SNP resulted in a significant decrease in the transcription rate of visfatin (Wang *et al.*, 2012). In non-small cell lung cancer (NSCLC), the CC genotype was found to be associated with an increased risk for NSCLC patients, while the CT genotype, TT genotype, and T allele may reduce the risk of NSCLC (Zhang *et al.*, 2018).

This study aims to examine the genetic abnormalities in visfatin by Restriction Fragment Length Polymorphism - Polymerase Chain Reaction (RFLP-PCR) Technique.

2. Materials and Methods

The study was started from January 2021 to November 2021 by a collection of blood samples from a seventy-five (75) transfusion-dependent β-thalassemia major with 15 healthy individuals as control. Among the 75 patients, 38 males and 37 females with age groups ranging from (2-30) were

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registered at the Center for thalassemia and inherited blood disorders, Shaheed Hemin teaching hospital, Sulaymaniyah, Kurdistan Iraq.
Serum level of Visfatin was detected using commercial kits, which were products of SunLong Biotech (China). The kits were based on the principles of the Enzyme-Linked Immunosorbent Assay Technology (ELISA) (sandwich method).

Primer design

Detection of the presence of the single nucleotide polymorphisms (SNPs) in visfatin was performed using primers and restriction enzymes listed in (Table 1). The PCR products of visfatin (-1535C<T: 382 bp) were used for restriction enzyme digestion. The MvaI restriction endonuclease enzyme was used to digest the 1535C/T SNP.

Table 1. Sequences of the primers and restriction enzymes used in the current stud

Gene	SNP	rs number	Primer Sequences (5′ – 3′)	base pair (bp)	Restriction enzymes	Genotype	Reference of primer
Visfatin	1535C>T	rs61330082	F: 5′TGTTTCAAACCTCGTTGCTGA-3′ R: 5′AGTGATGGTGGTGGTGGTA-3′	283 bp	MvaI	CC = 218, 65 CT = 283,218, 65 TT =283	Wu et al.,2016

After PCR amplification, agarose gel electrophoresis 2% was adopted to confirm the presence of amplification products. Further, the first left well of agarose gel was loaded with a DNA marker (100bp ladder). Based on the pattern of migrating bands, the genotypes were determined.
In order to detect visfatin PCR products, PCR conditions shown in (Table 2) were adopted.

Table 2. RFLP- PCR program.

Phase	Temperature (ºC)	Time (minute/second)	No. of cycle
Initial denaturation	95	5 min	1- cycle
Denaturation	95	30 sec	35-cycle
Annealing	59	30 sec	
Extension	72	60 sec	
final extension	72	5 min	1- cycle

The PCR products of visfatin (-1535C<T: 382 bp) were used for restriction enzyme digestion. The *MvaI* restriction endonuclease enzyme was used to digest the -1535C/T SNP. The protocol for the restriction reaction includes the addition of 2 µl from the PCR products to 2 µl of 10 X digestion buffer and 1 µl of each restriction enzyme. The volume then was completed to 20 µl by the addition of 15 µl of distilled water. The reaction mixture was then incubated at 60°C for 1:30 hours (-1535 C/T: 283 bp) and was then incubated at 60°C for 1:30 hours. All the restriction enzyme digested products were analyzed by 2% agarose gel electrophoresis and visualized under a UV transilluminator and photographed

3. Results and Discussion

Level of Visfatin (VF) in Patients and Control Groups

The current results showed that the mean values of Visfatin (ng/ml) levels in male and female subjects in the control group were 1.03±0.13 and 0.99±0.11 respectively. These values were significantly decreased (P ≤ 0.05) in βTM patients of the three age groups to become 0.81±0.06 and 0.82±0.05 (Group 1), 0.75±1.0 and 0.72±0.1 (Group 2), 0.67±0.1 and 0.63±0.09 (Group 3) consecutively, with no significant difference between male and female patients (Figure 1).

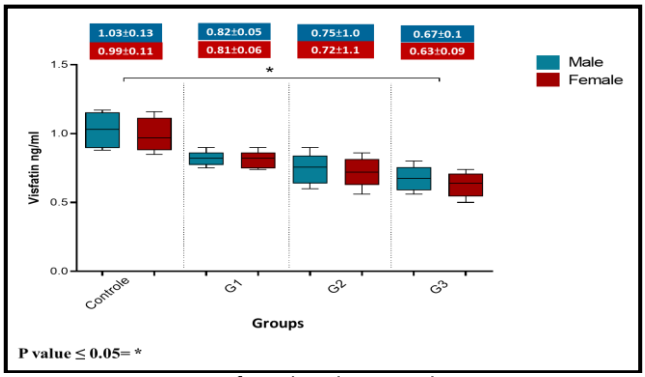

Figure 1. Visfatin levels in study groups.

These results revealed a decrease in visfatin levels in ßTM patients compared with the control. These results are opposite to most recent studies that demonstrated that serum visfatin is higher in ßTM than in the control group and an association between serum visfatin and the degree of severity in ßTM disease exists. Moreover, serum visfatin and serum ferritin were higher in the ß-TM group.
In ßTM, the disease is characterized by a chronic inflammatory state with an increased level of pro-inflammatory cytokines. Visfatin is a pro-inflammatory adipokine which found or expressed in visceral adipose tissue (Kanavaki et al., 2009).
Its role in inflammatory processes and chronic inflammatory diseases has been demonstrated by many experimental studies (Moschen et al., 2007; Al-Suhaimi et al., 2013). Interestingly, it can upregulate the production of many proinflammatory and anti-inflammatory cytokines such as interleukin (IL)-1, IL-1Ra, IL-6, IL-10, and TNF (Deeb et al., 2019).
Patients with βTM showed a reduced BMI and hypogonadism suggesting a reduced level of visfatin. Also, it has been demonstrated that a positive correlation exists between BMI and visfatin gene expression in visceral adipose tissue and a reverse correlation between BMI and visfatin gene expression in subcutaneous adipose tissue (Pagano et al., 2006; Varma, et al., 2002; Hosseinzadeh-Attar et al., 2013; Taher et al., 2021; Al-Obaedi et al.,2022).
From current study findings, we suggested that the

low levels of visfatin in patients with βTM could be due to reduced adipose tissue, indicated by low BMI and %body fat, in these patients or due to a decreased visfatin mRNA expression as a result of visfatin gene mutations (Taher et al., 2021).

Distribution of Visfatin (-1535 C/T) Genotypes and Alleles in Control and Thalassemic Group [N (%)]

Visfatin is a pro-inflammatory that stimulates the epithelial expression of cytokines such as (TNF-α), (IL-l), and (IL-6) (Moschen et al., 2007; Carbon et al., 2017). On the other hand, the expression of the visfatin gene is upregulated by a pro-inflammatory cytokine IL-1β (Jia et al., 2004).

We detected the visfatin gene polymorphism through amplification by PCR technique and then visualized it by gel electrophoresis. The undigested 283bp visfatin gene was found in all cases (Figure 2). Visfatin gene -1535C/T polymorphism includes three genotypes (CC, CT, and TT). After digestion with restriction enzymes, the results showed that the CC genotype revealed a size of (218 bp, 65 bp, the CT genotype showed a size of (283bp, 218bp, 65bp), and the TT genotype was a size of (283 bp) (Figure 3).

Results of the current study revealed that the frequency of C and T alleles in the control group was 67.6 % and 32.4% respectively (Figure 4). These frequencies became 42.6% and 57.4% in the patient's group with a significant difference (P ≤ 0.007) from the control and OR and CI of 4.41 (1.76 to 11.3). The genotypes (CC, CT, and TT) frequencies in subjects of the control group, showed frequencies of 60 %, 33.3 %, and 6.7 % respectively. In the thalassemic patient group, these frequencies became 17.7%, 48.0%, and 33.3% respectively. The OR between the CT and CC genotypes was 4.63 (CI; 1.416 to 14.96) with a significant difference (P< 0.013) from the control. The OR of the TT vs. CT genotypes was 16.0 (CI; 2.416 to 182.0) with a significant difference of P ≤ 0.002 when compared with the control (Figure 5).

In line with our findings, it has been reported that the CT and TT genotypes are correlated with significantly lower levels of serum visfatin compared with genotype CC in patients with traumatic brain injury (Weng et al., 2013; Wu et al., 2016). Moreover, the T allele of the -1535C/T SNP has been reported to induce a cardioprotective effect on patients with coronary artery calcification (Jin et al., 2016).

The increased frequency of the T allele is associated with low levels of visfatin in patients with βTM in this study. This may indicate a protective effect of this polymorphism in these patients. This explanation is supported by the finding that visfatin gene polymorphisms of the -1535C/T SNP might influence the structure or function of the visfatin protein and the visfatin expression in humans (Wu et al., 2016).

Another study by Ye et al. (2004) performed an experiment for transfecting a C variant or a T variant pGL3 basic vector into human lung microvascular endothelial cells and found that the T allele resulted in a significant decrease in the transcription rate (1.8-fold; P < 0.01) of visfatin. However, the cause of visfatin SNPs is still unclear but we suggested that it could be a consequence of iron overload n patients with βTM. This explanation is strengthened by previous reports demonstrating that intracellular iron overload can cause DNA damage to lymphocytes and immune.

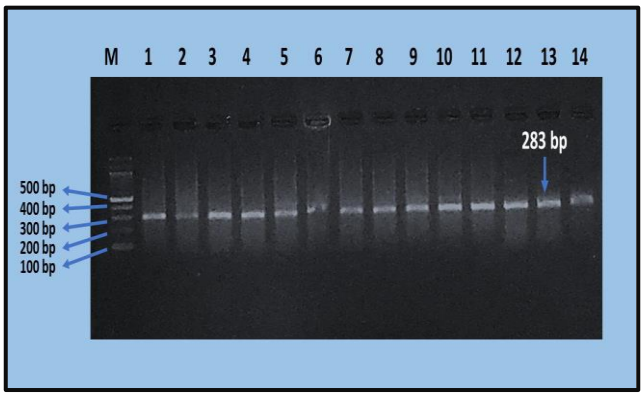

Figure 2. PCR amplification products of visfatin gene on 2% agarose gel

Lane M: DNA ladder (100bp). Lane (1 to 14): PCR amplification products 283bp for visfatin gene for study groups samples.

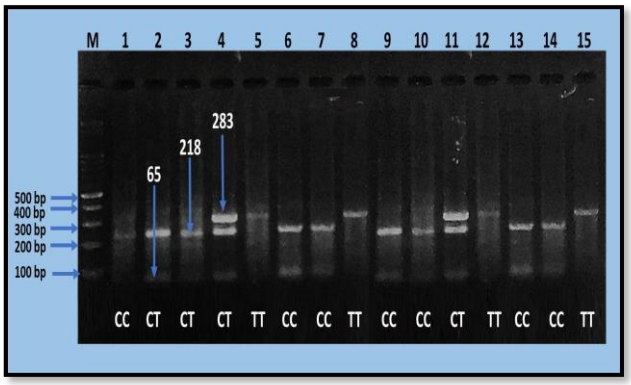

Figure 3. PCR-RFLP and sequencing assay for analyzing the -1535C>T polymorphisms. Agarose gel (2%) showing the visfatin gene (after digestion of the 283 bp with Mva1 enzyme. A PCR-RFLP assay for -1535C>T in the patient's group. M: 100bp DNA ladder. Lane 5, 8, 12, 15: TT (283bp); Lane 2, 3, 4, 11: CT (283bp, 218bp, 65bp). Lane 1, 6, 7, 9, 10, 13, 14: CC (218bp, 65bp).

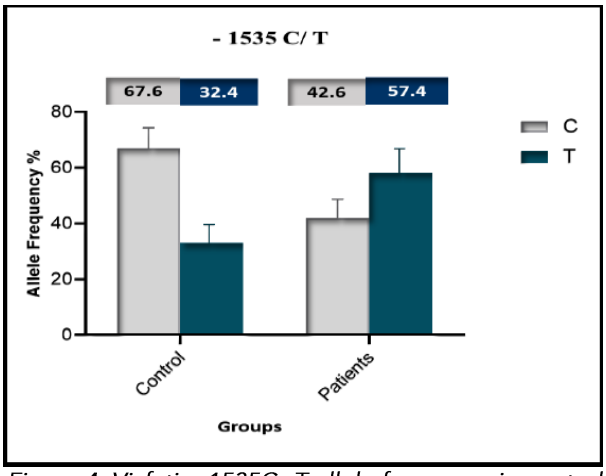

Figure 4. Visfatin -1535C>T allele frequency in control and βTM patients.

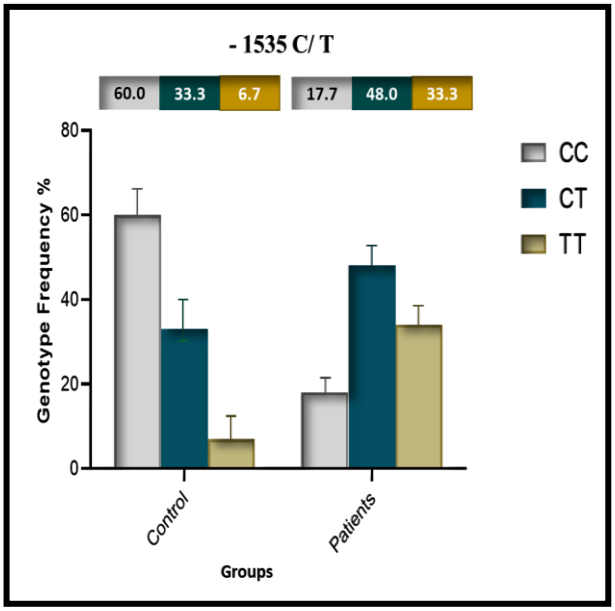

Figure 5. Visfatin -1535C>T genotype frequency in control and βTM patients

The effect of -1535C>T Polymorphisms on visfatin levels in patients with βTM

Our results revealed that there is no significant difference in serum levels of visfatin of the CC genotype carriers in the control and patients groups, where these values were (0.85±0.45 and 0.83±0.2) respectively. These values were significantly decreased (P<0.001) in patients with the CT genotype to become (0.78±0.6 and 0.73±1.4) respectively. More reduction was seen in visfatin levels in patients having the TT genotype to become (0.70±2.4 and 0.65±1.3) respectively (Figure 6). It has been reported that visfatin is increasingly produced at visceral adipose tissue, and it decreases at the subcutaneous level (Stastny et al., 2012; Vasilache et al., 2020). There are no/or limited studies about the link between visfatin gene variants and βTM.

In the present study, we demonstrated that visfatin -1535 C/T is associated with a decrease in plasma visfatin protein. In support of this finding a study that analyzed 243 obese children showed that -1535 C/T (rs61330082) variant allele carriers had lower fasting serum visfatin and lower fasting plasma glucose as compared with wild-type allele carriers (Ooi et al., 2014).

Other previous reports suggested that the −1535C>T polymorphism is associated with decreased plasma levels of inflammatory markers in patients with coronary artery disease (CAD) (wang et al., 2011).

In non-small cell lung cancer (NSCLC), the CC genotype was found to be associated with an increased risk for NSCLC patients, while the CT genotype, TT genotype, and T allele may reduce the risk of NSCLC (Zhang et al., 2018). Dysfunction in a patient with βTM (Shaw et al., 2017).

Moreover, The T allele of the rs61330082 SNP in the visfatin gene had a cardioprotective effect on patients with coronary artery calcification (CAC) (Jin et al., 2016)

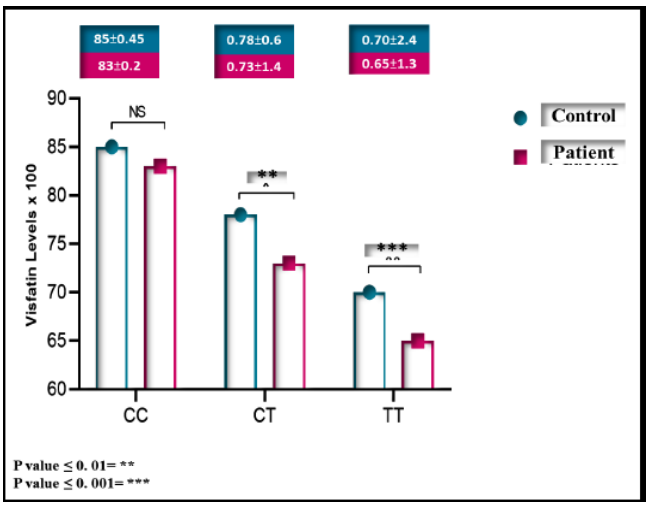

Figure 6. Effects of -1535C>T genotypes on visfatin levels in control and βTM patients.

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