

Common polymorphisms in the visfatin gene (NAMPT/PBEF1) influence visfatin-circulating levels in a Saudi population

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Abstract: The human visfatin (NAMPT/PBEF) gene is located on the long arm of chromosome seven encoding a polypeptide of 491 amino acids with a molecular mass of 52kDa. The aim of this study is to analyze the impact of two common single nucleotide polymorphisms (SNPs) of the visfatin gene on the visfatin-circulating levels in 150 non-obese Saudi subjects. Visfatin levels were analyzed using enzyme-linked immunosorbent assay (ELISA). Genotyping of the promoter variant rs9770242 (T-1001G) and the intron six variant rs4730153 (T+21179C) SNP were performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The two SNPs (rs9770242 and rs4730153) showed significant correlation with visfatin serum level in the entire study cohort ($P=0.002$, $P=0.0001$; respectively). There was a significant tendency toward higher visfatin levels in G-allele carriers of rs9770242 SNP ($4.05\pm 2.64\text{ng/ml}$) compared to the TT genotype carriers ($2.36\pm 1.98\text{ng/ml}$). The C-allele of rs4730153 SNP was found to be associated with increase visfatin serum level ($4.15\pm 2.52\text{ng/ml}$) compared to the TT genotype carriers ($1.90\pm 1.57\text{ng/ml}$). The data suggest that the rs9770242 and the rs4730153 SNPs of the visfatin gene have an effect on visfatin circulating levels. This is the first study to demonstrate that visfatin SNPs at rs9770242 and rs4730153 loci exist in the Saudi population.

[Rowyda Nawwaf Al-Harithy. **Common polymorphisms in the visfatin gene (NAMPT/PBEF1) influence visfatin-circulating levels in a Saudi population.** *Life Sci J* 2014;11(10):205-210]. (ISSN:1097-8135). <http://www.lifesciencesite.com>. 28

Keywords: Visfatin; SNPs; rs9770242; rs4730153; healthy non-obese adult Saudi

1. Introduction

In 2004 an adipokine, visfatin, was identified and named for its high levels of expression in visceral fat cells (Fukuhara *et al.*, 2005). Sequencing revealed that the visfatin gene corresponds to the previously identified cytokine: Pre-B-cell colony-enhancing factor (PBEF). PBEF was described in 1994 as a cytokine produced by lymphocytes (Samal *et al.*, 1994). Visfatin was also previously recognized as Nicotinamide phosphoribosyl transferase (Nampt), the limiting enzyme in nicotinamide adenine dinucleotide (NAD) biosynthesis. Nampt enzymatic activity was originally reported in 1957 and the gene encoding was identified in *Haemophilus ducreyi* in 2001 (Preiss and Handler, 1957; Martin *et al.*, 2001).

Visfatin is not a fat-specific protein. Visfatin is ubiquitously expressed and is associated with a variety of functions in different cells (Ognjanovic *et al.*, 2001; Rongvaux *et al.*, 2002; Ognjanovic and Bryant-Greenwood, 2002; Fukuhara *et al.*, 2005; Ye *et al.*, 2005; Gosset *et al.*, 2008; Garten *et al.*, 2010; Kukla *et al.*, 2010; Lee *et al.*, 2011; Tian *et al.*, 2013). Visfatin can regulate immune action (Luk *et al.*, 2008; sommer *et al.*, 2008) and is involved in the NAD⁺ salvage pathway (Rongvaux *et al.*, 2002; Yoshino *et al.*, 2011). In addition, visfatin helps the regulation of glucose and lipid metabolism (Lai and Chen, 2012). Several early studies indicated that increased visfatin expression has been implicated in life-threatening disorders (Reddy *et*

al., 2008; Patel *et al.*, 2010; Lee *et al.*, 2011; Tian *et al.*, 2013; Ghaemmaghami *et al.*, 2013), in the pathogenesis of atherosclerosis, and in a number of rheumatic diseases (Otero *et al.*, 2006; Laiguillon *et al.*, 2014).

At gene level, visfatin gene is located on the long arm of chromosome seven between 7q22.1 and 7q31.33, and spans 34.7kb having 11 exons and 10 introns (Jia *et al.*, 2004). It encodes a polypeptide of 491 amino acids with a molecular mass of 52kDa (Samal *et al.*, 1994; National Center for Biotechnology Information accession number AAA17884). Three mRNA transcripts have been identified, comprising 2.0, 2.4, and 4.0kb transcripts with the 2.4kb transcript being more predominant (Samal *et al.*, 1994; Kitani *et al.*, 2003; Yang *et al.*, 2007). Visfatin is highly preserved across animal evolution, closely related sequences were found in prokaryotes, sponges, insects, and mammals (Rongvaux *et al.*, 2002; McGlothlin *et al.*, 2005; Yang *et al.*, 2007; Adegate, 2008). Raising the speculation that it has a vital function and possibly contribute to metabolic interorgan crosslink. Ye and his group reported 11 single nucleotide polymorphisms (SNPs) in the human visfatin gene promoter by direct DNA sequencing from 36 people (Ye *et al.*, 2005). Bailey and his team identified 16 SNPs in 23 unrelated individuals from Quebec. One synonymous polymorphism is in exon 7, nine within the promoter sequence, five are intronic and one within the 3'

untranslated region (Bailey *et al.*, 2006). As of April 9, 2014, dbSNP Build 140 (<http://www.ncbi.nlm.nih.gov/snp>) reported that there occurs 770 SNPs in the human visfatin gene. Many of these SNPs in the dbSNP have not been validated with the population data and the functional consequences of most of these SNPs are currently unknown. Nearly half of references SNPs have been reported in the intron regions. There is an A→T SNP at the position of visfatin mRNA 295 at the 5'untranslated region. There are numbers of SNPs at the 3'untranslated region.

Genotyping of SNPs and SNP data analysis has become extremely important to researchers to understand the relationship between genetic variation and biological functions. To find out all the SNPs that affect visfatin secretion and the molecular mechanisms behind the allelic associations is a challenge. Therefore, the aim of this study is to analyze the impact of two common visfatin SNPs, rs4730153 and rs9770242, on the visfatin-circulating levels in adult Saudi subjects.

2. Materials and Methods

Subjects

Blood samples were obtained from 150 non-obese Saudi middle-age volunteers of body mass index (BMI) < 25kg/m². All the participants underwent physical examinations and routine biochemical analysis of blood. The subjects were excluded if they had high blood sugar level (over 5.9mmol/L), high blood pressure (over 120/80mmHg), BMI ≥ 25kg/m², or if they were under any treatment course. All subjects signed an informed consent for participation in this study. The ethical committees of King Abdul-Aziz University (KAU) had approved the study.

Anthropometric and biochemical measurements

Subjects were weighed to the nearest 0.1kg in standardized light clothes and without shoes on a platform manual scale balance. Height was measured using a fixed stadiometer to the nearest 0.1cm. BMI was calculated as a person's weight in kilograms divided by their height in meters squared. Venous blood samples were obtained from the antecubital vein after a 12-hour overnight fasting. From each subject, seven ml of blood was withdrawn into a plain vacutainer tube for serum preparation and three ml of blood was withdrawn into EDTA vacutainer tube to be used for DNA extraction. Serum visfatin concentration was determined by using a commercial visfatin C-terminal enzyme-linked immunosorbent assay kit (ALPCO Diagnostics, Salem, NH) using the microplate reader (sensitivity 30pg/mL). The intra- and inter-assay coefficient of variation (CV) was 5.04% and 6.67%, respectively. The test kit is effective in the range of 0.073to 4.8ng/ml with observed value of 80-120%.

Duplicate measurements were performed in a single experiment.

Genotyping using restriction fragment-length polymorphism

The SNP rs9770242 (T-1001G), the one that showed perfect linkage disequilibrium with rs1319501 in the study by Bailey and his team was selected for the analysis. Furthermore, SNP rs4730153 (T+21179C) was selected from dbSNP (<http://www.ncbi.nlm.nih.gov>). Total genomic DNA was extracted from peripheral blood using a DNA extraction kit (QIAamp DNA Blood Mini Kit; QIAGEN, USA), applying the producer's protocol. Identification of the polymorphisms was performed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Two sets of primers (5.0nmol) from TIB (TIB Molbiol Inc., Germany) were used. For promoter region amplification the forward primer 5'-CCAACTCGTTTCCCAGGATTTAAAG-3' and reverse primer 5'-ACGGGCCAAGCCTTTGA-3' were used. For intron seven variant amplification the forward primer 5'-GGTATGGTTGACCCAGCTAC-3' and reverse primer 5'-CAGATTTACTTAGGCAGACACTTGA-3' were used. The amplification was performed in a volume of 25µL, containing 0.2µg genomic DNA, 1X PCR buffer, 2.5mM MgCl₂, 0.2mM of each dNTP, 0.1µM of each primer, and 0.5U Taq DNA polymerase (Promega). The PCR initial denaturation was done at 96°C for 5min 40 cycles of 96°C for 35s, 58°C for 35s (SNP T-1001G), respectively, 61°C for 35s (SNP T+21179C), 72°C for 35s, and a final elongation step at 72°C for 4 min. 5µL of the PCR products was digested with restriction endonucleases (TspRI for SNP T-1001G and RsaI for SNP T+21179C; respectively) for 2 hours at 37°C. The PCR products of digestion were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide.

Statistical analysis

Numerical variables are presented as the mean ± standard deviation (SD) and differences between the genotype groups were analyzed by one-way ANOVA. The computations were performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) and P<0.05 was considered to indicate a statistically significant difference.

3. Results

PCR-RFLP analysis: The amplification and the RFLP analysis for the regions that contain rs9770242 (T-1001G) SNP in the promoter and rs4730153 (T+21179 C) SNP in intron six were done for all the collected samples. The amplified fragment that contains T-1001G showed a size of 78bp. The wild (TT) genotype showed one band of size78bp. The

heterozygous (TG) genotype produced three fragments of sizes 78, 52, and 26bp and the homozygous (GG) genotype produced two fragments of sizes 52 and 26bp (Figure 1). The other amplified fragment that contains T+21179C showed a size of 157bp. The wild (TT) genotype produced one band of size 157bp. The heterozygous (TC) genotype produced three bands of size 157, 90, and 67bp. The homozygous (CC) genotype produced two bands of size 90 and 67bp (Figure 2).

When the effect of the rs9770242 and rs4730153 SNPs on serum visfatin levels were analyzed, the visfatin level showed a significant difference ($P=0.002$) in serum visfatin levels in TG+GG genotype carriers of rs9770242 SNP ($4.05\pm 2.64\text{ng/ml}$) compare to the TT genotype carriers ($2.36\pm 1.98\text{ng/ml}$). The TC+CC genotype carriers of rs4730153 SNP was found to be associated with increase visfatin serum level ($4.15\pm 2.52\text{ng/ml}$) compared to the TT genotype carriers ($1.90\pm 1.57\text{ng/ml}$).

4. Discussions

The present data confirmed the existence of genotype-specific effect on the visfatin-circulating level. The G-allele of rs9770242 and the C-allele of rs4730153 SNPs on the visfatin gene were found to be associated with the increase of serum visfatin level. This is the first study to demonstrate that the visfatin SNPs at rs9770242 and rs4730153 loci exist in the Saudi population.

Several frequent SNPs, including rs9770242 and rs4730153, in the visfatin gene were reported to be associated with variety of physiological and pathological phenotypes including obesity, dyslipidemia, low-grade inflammation, cardiovascular disease, and type II diabetes (Bailey *et al.*, 2006; Zhang *et al.*, 2006; Körner *et al.*, 2007; Saddi-Rosa *et al.*, 2013). However, such an association was not observed in other studies (Böttcher *et al.*, 2006; Shea *et al.*, 2010; García-Bermúdez *et al.*, 2011). The reason for the discrepancies among these studies is unclear.

The promoter variant rs9770242 was recognized for its linkage disequilibrium group (Böttcher *et al.*, 2006), shown to be associated with higher visceral subcutaneous ratio of visfatin mRNA expression in adipose tissue (Böttcher *et al.*, 2006), and with higher fasting plasma glucose and insulin levels (Bailey *et al.*, 2006). Further studies showed that visfatin T-1001G variant allele is a genetic marker in acute respiratory distress syndrome (ARDS) (Ye *et al.*, 2005; Bajwa *et al.*, 2007). In addition, Ye and his team showed that transfection assay did not reveal a significant change in visfatin gene expression in human microvascular endothelial cells from the lung cells (Ye *et al.*, 2005). In the present study, there was a significant tendency toward higher visfatin levels in G- allele carriers of

rs9770242 SNP compare to the TT genotype carriers in the non-obese Saudi cohort. The change in visfatin level associated with the rs9770242 variant is consistent with Böttcher's result (Böttcher *et al.*, 2006). As the subjects in the present study were all non-obese and healthy, the result supports a significant role of the visfatin rs9770242 on visfatin circulating levels and confirms previous finding (Böttcher *et al.*, 2006).

In regard to intron seven variant rs4730153, extraordinarily limited work has been done. In 2007, Körner and his group have shown that there is no correlation between rs4730153 SNP and lipid phenotype metabolism in children (Körner *et al.*, 2007). Lai and his team suggested that visfatin rs4730153 homozygous CC genotype might effect adjustment of glucose and lipid metabolism in obese Han Chinese children and adolescents (Lai *et al.*, 2013). The rs4730153 polymorphism was not, so far, investigated in the genotype-associated studies with the visfatin-circulating level. In the present study, the C-allele of rs4730153 SNP was found to be associated with the increase of serum level of visfatin compared to the CC genotype carriers in the non-obese Saudi cohort. The result indicates that rs4730153 SNP in the visfatin gene is a significant predictor of circulating visfatin level. Considering that replication is the fundamental tool to validate genetic findings, more studies are warranted to examine the effects of rs9770242 and rs4730153 SNPs on visfatin levels.

Interestingly Liu and his team reported no variation in the T-1001G allele of PBEF in the Han Population of Northeast China. The homozygous TT genotype was the only genotype in the Han cohort (Liu *et al.*, 2012). The result of the present study showed a variation in the T-1001 allele of visfatin in the Saudi population similar to other populations that include Caucasians from Germany (Böttcher *et al.*, 2006), French-Canadian (Bailey *et al.*, 2006), North American and Brazilian (Saddi-Rosa *et al.*, 2013).

Although descriptions of genotype-related effect on serum visfatin levels are still scarce, the present report confirms the importance of the rs9770242 and rs4730153 variants. Also strengthening the notion that SNP data analysis helps to explain the relationship between genetic variation and biological functions. The challenge is to find out all the SNPs that affect visfatin secretion and to understand the molecular mechanisms behind the allelic associations. Additional genetic studies in a larger group as well as functional characterization of these variants are required.

In summary, the study supports a significant role of the visfatin rs9770242 SNPs on visfatin circulating levels and reports novel associations between visfatin rs4730153 SNP and visfatin circulating levels. It also demonstrates that visfatin SNPs at rs9770242 and rs4730153 loci exist in the Saudi population.

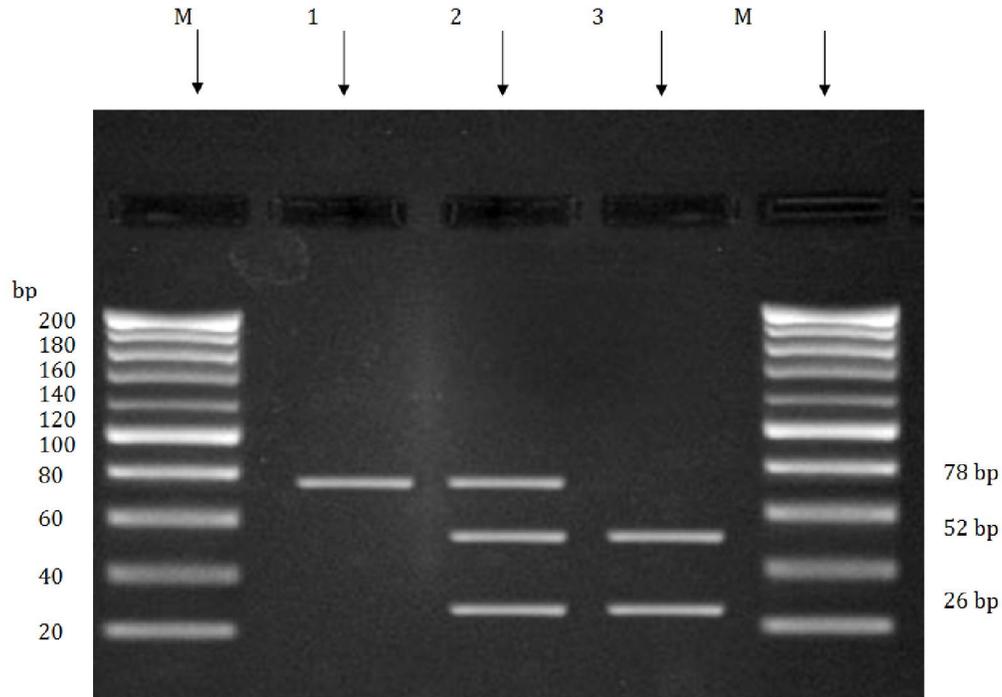


Figure 1. Electrophoretic analysis of rs9770242 (T-1001G) SNP genotyping by PCR-restriction fragment length polymorphism analysis (PCR-RFLP) using TspRI in 2% agarose gel stained with ethidium bromide. Lane M: DNA marker. Lane 1: wild (TT) genotype uncut of size 78bp. Lane 2: Heterozygous (TG) that shows three bands of size 78, 52 and 26bp. Lane 3: Homozygous (GG) that shows two bands of size 52 and 26bp.

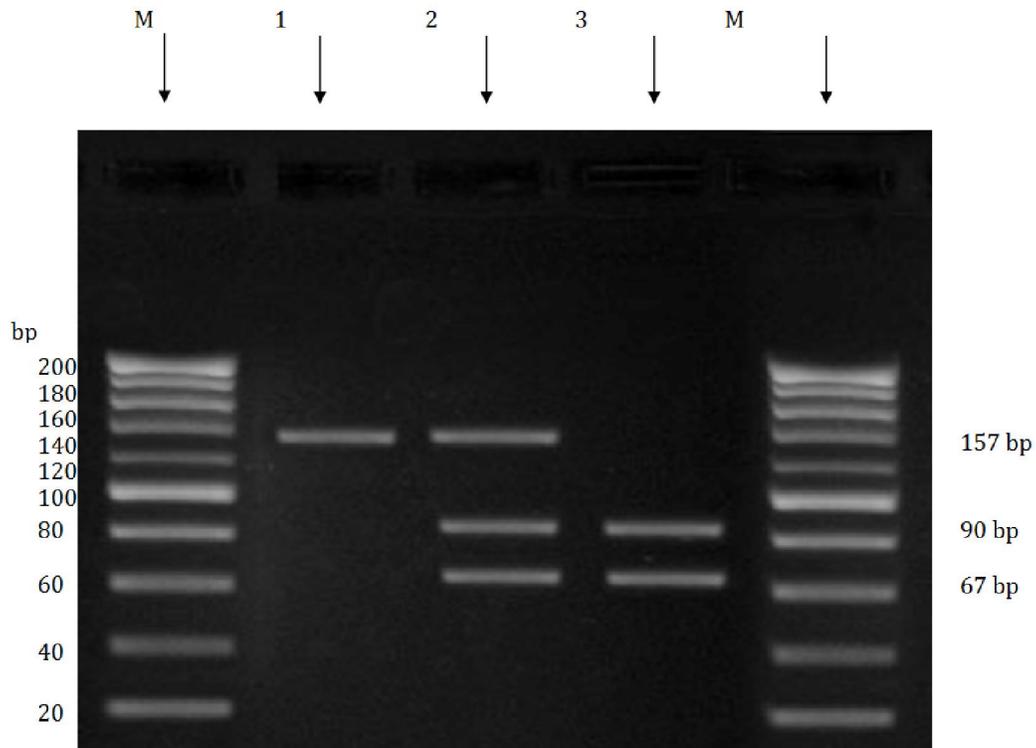


Figure 2. Electrophoretic analysis of rs4730153 (C+21179T) SNP genotyping by PCR-restriction fragment length polymorphism analysis (PCR-RFLP) using RsaI in 2% agarose gel stained with ethidium bromide. Lane M: DNA marker. Lane 1: Wild (TT) genotype uncut of size 157bp. Lane 2: Heterozygous (TC) that shows three bands of size 157, 90 and 67bp. Lane 3: Homozygous (CC) that shows two bands of size 90 and 67bp.

Competing interests:

The author declares that there are no competing interests.

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