The –1535 Promoter Variant of The Visfatin Gene Is Associated with Serum Triglyceride and HDL-cholesterol Levels in Japanese Subjects

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Abstract. Visfatin is a novel adipocytokine that is expressed by the visceral fat cells. We investigated the role of genetic variation in the visfatin gene in the pathophysiology of type 2 diabetes and clinical variables in Japanese subjects. The 11 exons, and the promoter region of the visfatin gene were screened for single nucleotide polymorphisms (SNPs) by PCR-direct sequencing. We found SNPs in the promoter region (SNP – 1535T>C), exon 2 (SNP + 131C>G, Thr44Arg), and exon 7 (SNP + 903G>A). The allele and genotype frequencies of these SNPs showed no significant differences between 200–448 diabetic and 200–333 control subjects. However, the -1535T/T genotype was associated with lower serum triglyceride levels (T/T vs. T/C + C/C (p = 0.015) and T/T vs. C/C (p = 0.043)) and higher HDL-cholesterol levels (T/T vs. C/C, p = 0.0496) in the nondiabetic subjects. Reporter gene assay of 3T3-L1 adipocytes revealed that the promoter activity of –1535T and –1535C was similar, suggesting that the observed association may reflect linkage disequilibrium between –1535T>C and causative variations of the visfatin gene.

Key words: Visfatin, Type 2 diabetes, Genetics, SNP, Metabolism

ADIPOCYTES secrete a variety of proteins known as adipocytokines, which have various important roles in metabolism [1, 2]. Visfatin is a novel adipocytokine strongly expressed by the visceral fat cells of both humans and mice [3]. Visfatin is identical to pre-B cell colony-enhancing factor (PBEF) which has been identified as a growth factor for early stage B cells [4]. Visfatin has an insulin-like effect on cultured cells. It stimulates glucose uptake by adipocytes, myocytes and osteoblasts [3, 5], and inhibits glucose release from liver cells [3]. In addition, administration of visfatin to mice has been shown to decrease the plasma glucose level [3], and mice that are heterozygous (+/−) for visfatin have higher plasma glucose levels than wild-type mice [3]. Furthermore, clinical studies have shown that plasma visfatin levels are about two-fold higher in type 2 diabetic patients than in nondiabetic controls [6, 7]. These findings suggest a role of visfatin in glucose metabolism and in the pathogenesis of type 2 diabetes. It has been reported that variations of the genes for other adipocytokines (e.g. adiponectin) can influence the risk of type 2 diabetes [8–13], but the relation between visfatin gene polymorphism and type 2 diabetes remains unknown.

The visfatin/PBEF gene consists of 11 exons and 10 introns spanning 34.7-kb and is located on chromosome 7q22.2 [14]. In the present study, we systematically investigated polymorphisms of the coding region and promoter region of this gene in Japanese subjects and then performed association studies in type 2 diabetic and non-diabetic subjects. We also examined the
Subjects

The association study enrolled 448 unrelated Japanese subjects with type 2 diabetes (245 men and 203 women) and 333 unrelated nondiabetic Japanese control subjects (191 men and 142 women). Type 2 diabetes was diagnosed in accordance with the World Health Organization criteria. Patients with type 1 diabetes or other types of diabetes (such as maturity onset diabetes of the young) were excluded from the study. The fasting plasma glucose level of all nondiabetic subjects was <110 mg/dl. The demographic characteristics of the type 2 diabetic subjects and the nondiabetic subjects are shown in Table 1. Nondiabetic subjects receiving lipid-lowering therapy, such as statins and/or fibrates, were excluded from the analysis of total cholesterol, triglycerides, and HDL-cholesterol. This study was approved by the ethics committee of Osaka University and written informed consent was obtained from each participant.

Biological measurements

Insulin resistance was investigated by the homeostasis model assessment (HOMA) method, employing the following equation: HOMA = fasting glucose (mg/dl) × fasting insulin (µU/ml)/405 [15]. Plasma adiponectin levels were determined by a sandwich enzyme-linked immunosorbent assay (Adiponectin ELISA Kit, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan), as reported previously [16].

Screening for visfatin gene polymorphism

The exon-intron boundaries were determined based on the human genomic DNA sequence of visfatin/PBEF1 on chromosome 7 (AC007032) and the human PBEF1 cDNA sequence (NM_005746, NM_182790). We initially screened 20 nondiabetic subjects to find relatively common polymorphisms in Japanese population. A 3.2-kb region (including 11 exons, the flanking introns, and the promoter region) of the visfatin gene was screened for polymorphisms by polymerase chain reaction (PCR) amplification and direct sequencing, as described previously [17]. The primers used for PCR and sequencing are shown in Table 2. The 1.7-kb promoter region was amplified in 5 segments, as displayed in Fig. 1. The promoter region (−0.9−0.1-kb) was amplified using TaKaRa LA Taq (Takara Bio Inc., Otsu, Japan), while the other regions were amplified with AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA). Sequencing was performed using a Big Dye Terminator v1.1 Cycle Sequencing Kit and reaction products were analyzed with an ABI PRISM 310 automated sequencer (both from Applied Biosystems, Foster City, CA).

Table 1. Clinical characteristics of the study subjects

<table>
<thead>
<tr>
<th></th>
<th>Type 2 diabetic subjects</th>
<th>nondiabetic subjects</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>448</td>
<td>333</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>65.4 ± 9.9</td>
<td>66.6 ± 10.4</td>
<td>N.S.</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>245/203</td>
<td>191/142</td>
<td>N.S.</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.5 ± 4.1</td>
<td>23.2 ± 3.5 (331)</td>
<td>N.S.</td>
</tr>
<tr>
<td>FPG (mg/dl)</td>
<td>159.3 ± 58.3</td>
<td>93.3 ± 8.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.5 ± 1.5</td>
<td>5.0 ± 0.4 (301)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T-chol (mg/dl)</td>
<td>195.9 ± 37.3</td>
<td>198.0 ± 35.9 (268)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>132.6 ± 9.14</td>
<td>119.7 ± 72.3 (261)</td>
<td>N.S.</td>
</tr>
<tr>
<td>HDL-chol (mg/dl)</td>
<td>52.6 ± 14.8</td>
<td>53.3 ± 15.3 (229)</td>
<td>N.S.</td>
</tr>
<tr>
<td>F-IRI (µU/ml)</td>
<td>9.0 ± 6.0 (125)</td>
<td>6.2 ± 2.6 (167)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA</td>
<td>2.3 ± 1.5 (111)</td>
<td>1.3 ± 0.6 (167)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>6.7 ± 5.3 (151)</td>
<td>7.5 ± 4.1 (135)</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Data are n or mean ± SD. HOMA = FPG (mg/dl) × F-IRI (µU/ml)/405.
Genotyping of visfatin gene polymorphisms

Genotyping of two polymorphisms (SNP + 131C>G and SNP + 903G>A) was performed by using the TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) in 200 diabetic and 200 nondiabetic subjects, while SNP – 1535T>C was genotyped in a total of 448 diabetic and 333 control subjects. The PCR primer and oligonucleotide probe sequences that were used are listed in Table 3. The PCR cycling parameters were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C. Then the allelic nature of the PCR product was determined with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). When the regions with these three SNPs were sequenced in 50 random ly selected subjects, the results were compatible with the genotyping data obtained by using TaqMan.

Plasmids

The human visfatin promoter was subcloned from an Escherichia coli-based bacterial artificial chromosome (RPCI-11 HS BAC CLONE, Clone ID: 22N19, Invitrogen). The promoter region (~1637~ –1) relative to the
translation start codon when A is numbered +1) was amplified by PCR using Pfu Turbo DNA Polymerase (Stratagene, CA, USA) and a pair of primers (5’-ACACAGGGAAAGATCAACCAA-3’ and 5’-CTCGGGCCGGAGGACAGGGGGCCGC-3’). PCR was initiated with 2 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 55°C, and 2.5 min at 72°C, with a final 10 min at 72°C. The PCR product was ligated to the pGL4.10 vector (Promega, Madison, WI, USA). The −1535T>C polymorphism was introduced into the pGL4.10 reporter vector by mutagenesis, as described previously [18]. The sequences of all constructs were confirmed.

Transfection studies of 3T3-L1 cells

3T3-L1 cells were maintained in DMEM containing 10% FBS. On the 5–7th day after the cells reached confluence in 6-well plates, the medium was changed to 10% FBS-supplemented DMEM containing 5 µg/ml insulin, 0.5 mmol/L 1-methyl-3-isobutyl-xanthine, and 1 µmol/L dexamethasone. The medium was changed again to DMEM containing 10% FBS after 2 days and the cells were incubated for a further 2 days to achieve differentiation into adipocytes [19]. On day 4 of incubation for differentiation, the medium was changed to OPTI-MEM (Invitrogen), and the cells were transfected with the pGL4.10 luciferase reporter vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Transfection was performed with 2 µg of pGL4.10 plasmid containing the human visfatin promoter or an empty pGL4.10 plasmid along with 1 µg of pCMX-β-galactosidase. Five hours after transfection, the medium was changed to DMEM containing 10% FBS. After 48 hours of incubation, the luciferase reporter assay was performed using a Luciferase Assay System (Promega, Madison, WI, USA). The measured activity values were adjusted for that of β-galactosidase (internal control) and were expressed as the relative luciferase activity.

Statistical analysis

Results are shown as the mean ± SD. The demographic characteristics of the subjects were compared by Student’s t-test or χ² analysis. The frequencies of alleles or genotypes were compared by χ² analysis. Differences of continuous variables, such as BMI or other clinical parameters, among genotypes were evaluated by one-way ANOVA. All statistical analyses were performed with StatView Ver. 5.0 software (SAS Institute Inc., Cary, NC). Statistical significance was defined as P<0.05. Pair-wise linkage disequilibrium (Lewontin’s D’) was measured in the 200 unrelated nondiabetic subjects using SNPAlalyze Ver. 5.0 software (Dynacom, Chiba, Japan).

Results

Detection of visfatin SNPs

Direct PCR sequencing of the ~1.6-kb promoter region, as well as the 11 exons and flanking introns, revealed one SNP in the promoter region (SNP – 1535T>C, described as C-1543T in reference [14]), one SNP in exon 2 (SNP + 131C>G) and one SNP in exon 7 (SNP + 903G>A) (Fig. 1). SNP + 131C>G in exon 2 resulted in the replacement of Thr in codon 44 by Arg (Thr44Arg). SNP + 903G>A was a silent mutation (rs2302559). Other SNPs reported in Caucasian subjects [14, 24], including SNP – 1458T>C, SNP – 1001G>T, SNP – 948G>T, SNP – 520G>A, SNP – 423G>A, SNP – 422G>T, SNP – 194C>T, and SNP – 129C>T, were not detected in the present study.

Association between visfatin SNPs and type 2 diabetes

To examine the influence of these SNPs on the risk of type 2 diabetes, we genotyped the SNPs in Japanese subjects. There were no significant deviations of the Hardy-Weinberg equilibrium with respect to these SNPs. The allele frequency of the Thr44Arg mutation (SNP + 131C>G) was quite low (0.5%). The allele frequencies of SNP – 1535T>C and SNP + 903G>A in the diabetic group were 0.55/0.45 and 0.91/0.09, respectively, while the frequencies in the non-diabetic group were 0.56/0.44 and 0.92/0.08, respectively (Table 4). SNP – 1535T>C and SNP + 903G>A showed linkage disequilibrium (D’ = 0.833). The allele and genotype frequencies of these SNPs showed no significant differences between the diabetic and non-diabetic subjects (Table 4).

Associations between SNP – 1535T>C and clinical parameters

We next examined the effect of SNP – 1535T>C on
the clinical parameters of our nondiabetic subjects. Since SNP + 131C>G and SNP + 903G>A were uncommon (0.5% and 8.2%, respectively), we excluded these variants from further assessment. No differences were observed with respect to the levels of fasting plasma glucose, HbA1c, BMI, fasting insulin, homeostasis model assessment of insulin resistance (HOMA-IR), total cholesterol, and adiponectin (Table 5). However, we found a significant association between SNP – 1535T>C and the triglyceride level. This promoter variant was associated with a lower serum triglyceride concentration (104.0 ± 44.9 mg/dl for T/T vs. 126.8 ± 75.1 mg/dl for T/C + C/C (p = 0.0148) and 128.4 ± 94.4 mg/dl for C/C (p = 0.0425)). We also found a significant association between SNP – 1535T>C and the serum HDL-cholesterol concentration (56.2 ± 17.2 mg/dl for T/T vs. 50.4 ± 13.9 mg/dl for C/C, p = 0.0496). Although not significant, there was a trend for an association between SNP – 1535T>C and the adiponectin level (8.5 ± 4.7 µg/ml for T/T vs. 7.1 ± 3.7 µg/ml for T/C + C/C, p = 0.079). No significant association was observed between SNP – 1535T>C and the levels of triglycerides, HDL-cholesterol, and adiponectin in the patients with type 2 diabetes (data not shown).

### Discussion

Visfatin is a novel adipocytokine that is strongly ex-
pressed by the visceral fat cells of both humans and mice [3]. Visfatin exerts various insulin-like effects both in vitro and in vivo. It has also been reported that plasma visfatin levels are higher in type 2 diabetic patients than in non-diabetic controls [6, 7, 28]. Although the precise influence of visfatin on glucose metabolism remains to be determined, it has been suggested to play a role in the pathogenesis of type 2 diabetes.

To determine whether variations of the visfatin gene were associated with type 2 diabetes in Japanese subjects, we screened this gene extensively for SNPs and found one SNP in the promoter region (SNP –1535T>C), one missense mutation in exon 2 (SNP +131C>G), and one silent mutation in exon 7 (SNP +903G>A). However, the frequencies of these SNPs were not significantly different between the diabetic and control groups, indicating that there was no evidence for a major influence of visfatin gene polymorphism on susceptibility to type 2 diabetes in the present study population.

Recent genetic studies performed in non-diabetic Mexican-Americans have obtained evidence of a linkage between triglyceride and HDL-cholesterol levels and chromosome 7q22.2 [25, 26], where the visfatin gene is located. Interestingly, we found a significant association between the SNP –1535T>C genotype and serum triglyceride and HDL-cholesterol levels. Non-diabetic subjects who had the SNP –1535T/T genotype showed significantly lower serum triglyceride levels compared with those who had the T/C + C/C genotype or the C/C genotype (104.0 ± 44.9 mg/dl for T/T vs. 127.3 ± 81.3 mg/dl for T/C + C/C (p = 0.015) and 128.4 ± 94.4 mg/dl for C/C (p = 0.043)). In addition, SNP –1535 was significantly associated with serum HDL-cholesterol levels (Table 5). We have no clear explanation as to why the SNP –1535 was associated with serum triglyceride and HDL-cholesterol levels. However, a similar association between the haplotype containing SNP –1535 and triglyceride levels has been reported in Chinese subjects [27]. Visfatin has an adipogenic effect that enhances the synthesis of triglycerides from glucose by primary cultured preadipocytes [3]. Since we did not obtain informed consent from participants to measure serum visfatin levels, we could not directly test the visfatin levels in the present study. It would be of interest to investigate the influence of SNP –1535 on the circulating visfatin concentration.

Ye et al. recently reported that SNP –1535 (described as –1543 in their study) influences the promoter activity of the human visfatin gene [20]. The –1535T allele was associated with a 1.8-fold decrease of promoter activity compared with the –1535C allele, indicating that this SNP is functional. Therefore, we also examined the effect of this SNP in 3T3-L1 adipocytes. In sharp contrast to the previous result, we could not detect any difference of promoter activity between –1535T and –1535C in 3T3-L1 adipocytes expressing the visfatin gene [19]. Thus, this variation itself is not likely to be the cause of the association. The reason for the different results with respect to promoter activity is unknown, but it may be related to differences of the experimental conditions (a 1.6K-bp reporter in 3T3-L1 mouse adipocytes vs. a reporter gene with 147-bp fragment in human lung microvascular endothelial (HMVEC-L) cells [20]). Our results suggest that the observed association may reflect linkage disequilibrium between –1535T>C and an unknown causative variation of the visfatin gene. Therefore, identification of this causative functional variation of the gene will be required to elucidate the molecular mechanism in the future.

Although not significant, there was also a trend for an association between SNP –1535 and plasma adi-
Adiponectin concentrations in this study, and it has been reported that higher adiponectin levels are associated with higher HDL-cholesterol and lower triglyceride levels [21]. Further studies will be needed to clarify whether the adiponectin and visfatin levels show any association.

Nicotinamide adenine dinucleotide (NAD) is an essential coenzyme regulating many metabolic pathways in the cells. Interestingly, it has been reported that PBEF/visfatin is the rate limiting component in the mammalian NAD biosynthesis pathway [22, 23]. NAD biosynthesis mediated by visfatin might also be involved in the regulation of the lipid metabolism.

In conclusion, we found no association between visfatin SNPs and type 2 diabetes in Japanese subjects, but the –1535T/T genotype of visfatin was associated with lower serum triglyceride levels and higher HDL-cholesterol levels in nondiabetic subjects. These findings suggest that visfatin may play a significant role in lipid metabolism.

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References


**note added in proof**

After the acceptance of this paper, a paper by Fukuhara [3] was retracted.