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Visfatin polymorphism may increase tendency to diabetic nephropathy

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Abstract

Visfatin is a novel adipocytokine, which is suggested to play a role in kidney diseases. We hypothesized that diabetics with diabetic nephropathy may have a different gene profile and this study was performed to evaluate the association between visfatin gene promoter region SNPs and diabetic nephropathy. Real-time PCR was used to study SNPs (1001T/G, 423A/G, 1535C/T) of the visfatin gene promoter region in 30 type 2 diabetics with nephropathy, 30 type 2 diabetics without nephropathy, and 30 healthy volunteers who served as control. Routine biochemical parameters, serum insulin, TNF-α, urinary protein and microalbumine were tested in subjects. Insulin resistance was evaluated by the HOMA method. Non-heterozygotes for the SNPs 423 A/G and 1001 T/G had significantly less risk of having nephropathy (for each group odds ratio 0.181 with 95% confidence interval:0.048-0.674). They also had lower serum visfatin levels than subjects with AA and TT genotypes (P=0.035 and P=0.030, respectively). We could not find any relationship between genotype and gender, BMI, HOMA-IR score, HbA1c, proteinuria, serum lipid and TNF-α levels. The two SNPs, 1001 T/G and 423 A/G were in perfect linkage disequilibrium. We, therefore, suggest that visfatin gene polymorphisms may increase the tendency to diabetic nephropathy.

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Key words: diabetes complications, diabetic nephropathies, polymorphism, nicotinamide phosphoribosyltransferase, single nucleotide.

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Contributions: SD, conception and design of the study, analysis and interpretation of data; AÖ, conception and design of the study, data interpretation and analysis; FMİ, analysis and interpretation of the genetic data; KH, NI, analysis of the genetic data; TK analysis of the biochemical data.

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Introduction

Visfatin is a novel adipocytokine identified by Fukuhara *et al.* and abundantly present in the visceral fat cells of both human and mice.[1] This protein had been previously identified as *pre-B cell colony enhancing factor 1*, a cytokine that is expressed by lymphocytes and displays nicotinamide phosphoribosyltransferase activity.[2] The visfatin/PBEF gene is located on chromosome 7q22.2 and consists of 11 exons and 10 introns, spanning 34·7kb of genomic DNA.[3] Visfatin is an adipocytokine and a new marker of inflammation and endothelial damage.[1,4-7] It was suggested to have a role in insulin resistance, dyslipidemia, obesity atherothrombotic disease, pathogenesis of rheumatoid arthritis and infection-induced pre-term birth.[5,8-11] Visfatin genotypes were found to account for insulin resistance and levels of lipid profile.[8] A significant association was found between SNPs 1001T>G, 423A>G and fasting plasma insulin levels and fasting glucose levels.[12] Visfatin was also found to have a role in progression of diabetic nephropathy.[13-15] To the best of our knowledge, no data are available on the relation between visfatin gene polymorphisms and diabetic nephropathy. We hypothesized that diabetics with diabetic nephropathy may have a different gene profile and this study was performed to evaluate if there is any association between visfatin gene SNPs and diabetic nephropathy among type 2 diabetics.

Materials and Methods

The study was approved by Afyon Kocatepe University Ethics Committee and all subjects included in the study gave their written informed consent. The study complied with the principles of the 2008 Declaration of Helsinki. This study included type 2 diabetic patients admitted to our Internal Medicine department between April 1st and June 1st 2009 and healthy volunteers who had been admitted to hospital for routine check-up or dispeptic symptoms and in whom no detectable disease was confirmed. Diabetic subjects were diagnosed as having diabetic nephropathy according to KDOQI guidelines.[16] Among hospitalized diabetics, only those who were hospitalized for blood glucose regulation were included in the study. Exclusion criteria were: patients with any kidney disease other than diabetic nephropathy, acute or chronic inflammatory or infectious disease, any psychiatric or neurological disorder, any malignancy, moderate to severe chronic obstructive lung disease, elevated liver enzymes, blood product transfusion history in the last five years, acute major cardiovascular events in the previous year and alcohol consumption of 40 g/day or over. None of the diabetics who were invited to take part in the study refused to participate. This study included 90 volunteers. Group 1 (n=30) included type 2 diabetic patients with diabetic nephropathy, Group 2 (n=30) consisted of type 2 diabetic patients without nephropathy, and Group 3 (n=30) included healthy volunteers as control.

Genetic analysis

EDTA-blood and serum samples were collected from subjects who had fasted for at least 8 h between 6:45 and 9:45 a.m. and samples were processed within 5-60 min. Two mL of blood was also drawn into tubes containing EDTA for molecular investigations. Genomic

DNA was isolated from the blood samples using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Following DNA isolation, SNPs (1001T/G, 423A/G, 1535C/T) of the visfatin gene promoter region were studied by real time PCR for melting curve analysis on a LightCycler (Roche Diagnostics, Penzberg, Germany). We used 20 μL of reaction mixture containing 5μl DNA sample, 0.5 μlM of each primer (designed and produced by Tib Molbiol, Berlin, Germany), 0.2 μlM of each probe (designed and produced by Tib Molbiol, Berlin, Germany) and 3mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany) in addition to LightCycler® FastStart DNA Master HybProbe (Roche Diagnostics, Mannheim, Germany) for 423A/G, 1535C/T polymorphisms, LightCycler® DNA Master HybProbe (Roche Diagnostics,, Mannheim, Germany) for 1001T/G polymorphism. PCR conditions and melting curve reading parameters were optimized. In the melting curve analysis, the polymorphic sequences demonstrated different melting peaks, representing a distinguishable melting point (Tm), while heterozygotes had both melting peaks. The primer and probe sequences are shown in Table 1.

Biochemical analysis

Routine laboratory measurements were performed at once. Aliquots for visfatin (serum) and TNF-α (serum) measurements were stored immediately at -20°C.

Hemoglobin was measured by Sysmex XT-2000*i* Automated Hematology Analyzer (Sysmex Corporation, Kobe, Japan). HbA1c was assayed using the reagent kit for high performance liquid chromatography (HPLC) analysis from Chromsystems (Munich, Germany). Sedimentation rate was measured by automated systems (Vacuette SRS100/II, Greiner Bio-One, Kremsmünster, Austria).

Biochemical parameters, including glucose, urea, creatinine, calcium, phosphorus, potassium, albumin, triglyceride, total cholesterol, HDL-cholesterol, urinary protein and microalbumine were determined by an automated clinical chemistry analyzer (Cobas c 501 analyzer, Roche, Mannheim, Germany). LDL cholesterol was calculated by the Friedewald equation.

Thyroid stimulating hormone (TSH) and serum insulin levels were measured in a Cobas e 601 automated electrochemiluminescence system by using commercial kits supplied from Roche Diagnostics (Mannheim, Germany).

Serum TNF-α (Biosource, Biosource International Inc., CA, USA) and visfatin (Ray BioTech, Norcross, Georgia, USA) levels were determined using a commercially available enzyme-linked immunoabsorbant assay (ELISA) kit. Insulin resistance was predicted by the insulin resistance score (HOMA-IR) which was computed with the formula:

fasting plasma glucose (mmol/L)
times fasting serum insulin (mU/L)
divided by 22.5.[17]

Low HOMA-IRvalues indicated high insulin sensitivity, whereas high HOMA-IR values indicated low insulin sensitivity (insulin resistance). Body mass index (BMI) was calculated as weight (kg)/height^2(m^2).

Statistical analysis

Statistical analysis was performed using SPSS ver. 17 (SPSS Inc. Chicago, IL, USA) and normality was tested using the Kolmogorov-Smirnov test. Data were tested by χ^2, one-way Anova, Mann-Whitney U test, independent samples t-test and Pearson's correlation analysis. The data were shown as mean±standard deviation and $P<0.05$ were considered statistically significant.

Results

Results of the genetic analysis are listed in Table 2. For 1001 T/G, only one subject had GG, for 423 A/G only one subject had GG, and for 15350 C/T 5 subjects had CC genotype, so they were not included in the statistical analysis and the statistical evaluation was performed among the subjects with TT, AA, TT genotypes, respectively, and the heterozygotes. There was a close relationship between diabetic nephropathy and the SNPs 423 A/G and 1001 T/G (P=0.016 for each). Non-heterozygotes for the SNPs 423 A/G and 1001 T/G had significantly less risk of having nephropathy (for each group odds ratio 0.181 with 95% confidence interval 0.048-0.674).

No relation was found between genotype and gender, BMI, HOMA-IR score, HbA1c, proteinuria, serum lipid and TNF-α levels. However, TNF-α level was significantly higher in diabetics than in the healthy volunteers (P=0.000)

Subjects heterozygote for 1001 T/G and for 423 A/G had lower serum visfatin levels than the subjects with TT and AA genotypes (P=0.030 and P=0.035, respectively) (Table 3). Healthy volunteers and diabetics with nephropathy had significantly lower serum visfatin levels than diabetics without nephropathy (P=0.000 for both goups) (Table 4).

Discussion

Visfatin is a new adipocytokine and a marker of inflammation and endothelial dysfunction which is suggested to have a role in the pathogenesis of insulin resistance, diabetes mellitus, hyperlipidemia and obesity.[4,6] There are only a few studies reporting that visfatin is also related with renal disease.

The synthesis and physiological action of visfatin were studied in cultured mesangial cells (MCs) to investigate the role of visfatin in diabetic nephropathy. It was found that visfatin was also synthesized in mesangial cells and adipocytes, and visfatin synthesis was markedly increased by glucose stimuli rather than by angiotensin II.[13,14] It was suggested that visfatin treatment dramatically increased the synthesis of profibrotic molecules and it plays an important role in the pathogenesis of diabetic nephropathy. Visfatin was also found to have a detrimental effect on diabetic

Table 1. Visfatin gene SNP primer and probe sequences.

Visfatin 1001T/G	
Forward:	5'-GATAATGAGGGGACAAGACCTAA-3'
Reverse:	5'-TGGAATGGTCTGTATTTGGGTGA-3'
Anchor:	5'-GCAACGGGCCAAGCCTTTGAC-FL (Fluorescein)
Sensor:	5'-LC-GGTGCGACACTGACTTTTATC--PH (Phosphate)
Visfatin 423A/G	
Forward:	5'-CCCAGACGCCAGCTCTG-3'
Reverse:	5'-CCTCGTGGCACTGGCAA-3'
Anchor:	5'-CGTGCCGGGAACTCGAACTTA--FL
Sensor:	5'-LC-TAAGCGCCCAGGTCACG--PH
Visfatin 1535C/T	
Forward:	5'-ACTGGAGGCATGGCTGAGA-3'
Reverse:	5'-CCCTCTTGTTTCAAACCTCGT-3'
Anchor:	5'-ACAATACAGGGCAAAGATCATGGAAGTG--FL
Sensor:	5'-LC—AAGGTATCACCAAGCACTCACC--PH

nephropathy through the activation of the intrarenal renin-angiotensin system.[15]

In a recent study performed in a genetic model of type 2 diabetes in rats, higher levels of visfatin synthesis was reported in both glomeruli and tubulointerstitium compared to control rats.[14] Visfatin synthesis was also found to occur in podocytes and proximal tubular cells. In clonal mouse pancreatic beta-cells, it was shown that incubation with visfatin resulted in increased insulin and decreased angiotensin converting enzyme.

The previous studies showed that visfatin 948 g>t polymorphism may be related with fasting insulin.[18] 948g>t polymorphism was also suggested to play a role in determinig type 2 diabetes susceptibility, possibly by modulating chronic, low-grade inflammatory responses. But no relation with BMI, waist circumference, serum glucose levels, or fasting insulin levels was found.[19] Obese carriers of the 948g>t variant allele were found to have significantly higher levels of high-density lipoprotein cholesterol.[20] SNP 4689 g>t was found to be related with insulin resistance and levels of lipid profile.[8] Paschou *et al.* found no major involvement of three tagging visfatin genotypes (rs 2041681, rs 3801272, rs 2098291) in the genetic background of type 2 diabetes mellitus.[21] Blakemore *et al.* studied eight tagged SNPs and the rare rs10487818 SNP and demonstrated strong association between the rare rs10487818 SNP and protection from obesity.[22] Although some studies have examined the relation between visfatin genotype and diabetes and related metabolic disorders, there have been no studies related to the association between diabetic nephropathy and visfatin genotype.

In this study, we found a close relationship between diabetic nephropathy and the SNPs 423 a>g and 1001 t>g. Patients with nephropathy had an increased frequency of heterozygosity for these SNPs and heterozygotes had lower visfatin levels. However, diabetics without nephropathy had significantly higher visfatin levels. It could be thought that high visfatin levels among diabetics without nephropathy may be a compensatory mechanism for amelioration of kidney function. Carrero *et al.* studied these two SNPs and also found that subjects with 1001 TT genotype had higher visfatin levels than those with 1001 TG and GG genotypes, and higher visfatin levels were associated with poor appetite among patients with chronic kidney disease.[23] Kang *et al.* found that visfatin was associated with microalbuminuria and may play a role as an early marker of diabetic nephropathy.[14] Visfatin treatment caused a 3.73-fold decrease in angiotensin converting enzyme level which is important in the pathogenesis of diabetic nephropathy, given that visfatin may be a part of a defense mechanism against nephropathy in early diabetes.[24,25] Axelsson *et al.* studied the same three SNPs as in our study and found no difference in circulating visfatin levels between genotypes.[26] The reason for this discrepancy may be that their study groups were made up of both diabetics and non-diabetics.

We found no relation between BMI, insulin resistance, proteinuria, serum lipid and TNF-α levels for three SNPs. Böttcher *et al.* studied seven SNPs (two of these were also examined in our study: -1001 T>G and -423 A>G) and found no association with type 2 diabetes, BMI, waist-hip ratio and percentage of body fat.[18] Another SNP -1535C>T that examined in our study was a frequently studied polymorphism which was reported to be associated with the regulation of serum lipid level and reduced risk of coronary artery disease.[27] Tokunaga *et al.* found there was no significant difference in the frequencies of three SNPs (-1535T>C, +131C>G, +903G>A) in the visfatin gene between the diabetic and control groups. This indicates that visfatin gene polymorphism did not increase susceptibility to type 2 diabetes mellitus.[3] Jian *et al.* studied three SNPs; one of these was -1535 C/T. These authors found no difference in type 2 diabetes mellitus, impaired glucose regulation and normal glucose tolerance.[28]

Table 2. Genetic analysis results of the subjects.

Polymorphism	Genotype	Diabetes mellitus with nephropathy (n)	Diabetes mellitus without nephropathy (n)	Healthy volunteers
1001T/G	TT	17	26	17
	Heterozygote	12	4	13
	GG	1	0	0
15350 C/T	TT	21	20	17
	Heterozygote	6	8	13
	CC	3	2	0
423 A/G	AA	17	26	17
	Heterozygote	12	4	13
	GG	1	0	0

n, number of subjects.

Table 3. Mean serum visfatin levels in different visfatin genotypes.

	Mean serum levels of visfatin (ng/mL)		
Genotypes	1001 T/G (n)	1535 T/C (n)	423 A/G (n)
TT/TT/AA	447±82 (54)	434±81 (48)	446±83 (53)
Heterozygote	406±55 (23)	436±74 (26)	406±55 (23)
P	0.030	0.928	0.035

n, number of subjects.

Table 4. Demographic and biochemical analysis of serum specimens among all subjects.

	Diabetics with nephropathy	Diabetics without nephropathy	Healthy volunteers	P
Age	64±9.9	57.4±12.7	46.3±13.9	0.000
Gender (F/M)	13/20	8/28	10/21	0.829
Serum visfatin level	409±54	484±74	397±65	0.000
BMI (kg/m^2)	29.6±5.9	28.9±4.8	28.4±4.3	0.330
Insulin (μu/mL)	9.2±8	10.4±10	7.2±3	0.637
TNF (pg/mL)	14.1±6.8	11.6±11.5	5.8±3.1	0.002
HbA1c (%)	7.3±1.7	7.4±1.9	-	0.886
Proteinuria (g/day)	1,89 ± 2,08	0,08 ± 0,06	0,09 ± 0,04	0.000
Triglyceride (mg/dL)	153±70	230±212	144±79	0.024
Total cholesterol (mg/dL)	174±61	205±55	190±46	0.097
LDL (mg/dL)	111±46	114±43	107±44	0.806
HDL (mg/dl)	34±14	41±12	47±13	0.001
HOMA-IR score	6.2±9.3	5.3±4.9	4.9±9.6	0.848

In our study, the two SNPs, 1001 T/G and 423 A/G were in perfect linkage disequilibrium (LD), as previously reported.[12,18]

To the best of our knowledge, this is the first study suggesting that visfatin gene polymorphisms may increase tendency to diabetic nephropathy. However, there are some limitations to this study. First, this is a single center study and the study groups are small. Second, although serum visfatin level does not seem to be related with age,[29] and age cannot affect genotype, mismatched mean age between 3 groups was a further limitation. Third, dietary intake, which was suggested to be related with serum visfatin level,[30] was not evaluated.

In conclusion, we suggest that visfatin polymorphisms may increase tendency to diabetic nephropathy. Further studies to evaluate these polymorphisms and their relationship with visfatin expression and end-organ damage may provide new insights into the treatment of diabetes and its complications.

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