

Polymorphisms in the 3'UTR of the human leptin gene and their role in hypertension

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Abstract. Leptin is a protein hormone, mainly synthesized in adipocytes, that regulates the food intake and energy expenditure of the body. Rare mutations in the leptin gene cause obesity. Common polymorphisms of the leptin gene have been associated with obesity, however their association with arterial blood pressure has not been fully elucidated. The aim of the present study was to examine the effect of variants in the 3' flanking region of the leptin gene on blood pressure in hypertensive subjects with high (35.2±5.12) and low (20.13±1.3) body mass index (BMI). Microsatellite polymorphisms and the C538T SNP in the 3'UTR of the leptin gene were screened in 362 subjects, and different biochemical and anthropometric parameters were measured. The levels of serum urea, creatinine, glucose, cholesterol, triglyceride, leptin and angiotensin II were determined in all subjects. A strong association of microsatellite polymorphisms with essential hypertension was found in subjects with a high BMI, but this association was only slight in subjects with a normal BMI. The C538T variant was not found in this population. The frequency of the Class I/Class I and Class I/Class II genotype for tetranucleotide polymorphisms was also significantly higher in the hypertensive compared to the normotensive group ($p \leq 0.0001$). In addition, a significant correlation was found between serum leptin and Class I/I and Class I/II genotypes. Linear regression analysis showed an independent correlation of leptinemia with BMI ($p = 0.019$), while a notable correlation was found between serum leptin concentration and angiotensin II. The study confirmed that shorter alleles of microsatellites in the 3' flanking region of leptin are significantly associated with

hypertension, however, the underlying mechanism remains unknown.

Introduction

High blood pressure is a major risk factor for cardiovascular disease. The pattern of blood pressure in western populations is such that the majority of the population is at an increasing risk for blood pressure-related cardiovascular diseases (1-5). High blood pressure has been associated with a variety of nutritional abnormalities and an increased prevalence of physical inactivity (6-8). Although some of the early studies argue against the genetic cause of hypertension (9,10), several more recent ones provide evidence in favor of the genetic explanation (11,12). Cloning of leptin gene and characterization of its product leptin was an important advance in the study of hormonal and metabolic alterations associated with this gene (13). Leptin is mainly produced by adipose tissue, and leptin levels are strongly and positively correlated with body mass index (BMI) (14,15). However, the role of leptin deficiency in human obesity has also been considered (16,17).

Previous research has demonstrated that leptin is a pleiotropic hormone with multiple actions that is potentially involved in the control of feeding, as well as in cardiovascular function, insulin secretion, angiogenesis, immune response and haematopoiesis (18). A direct effect of leptin on blood pressure has been reported (19-21), and it has been suggested that this effect is mediated by sympathetic activation (22). Moreover, a direct relationship between plasma leptin and heart rate has been observed in hypertensive patients (20,23). In normotensive men, serum leptin levels were found to be related to serum angiotensin II levels (33), which has a well established role in the regulation of blood pressure. Also, a positive correlation was found between these two parameters in hypertensive subjects (34). This relationship appears to be independent of BMI, plasma insulin and physical activity. The presence of a microsatellite polymorphism in the 3'UTR of the leptin gene was found to be significantly correlated with hypertension, independent of obesity in a Japanese population (24). A similar polymorphism in the leptin gene was found in an Italian population, but no association of this polymorphism with obesity-dependent hypertension was observed (25).

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Table I. Anthropometric and laboratory data of the study subjects.

Variables	Hypertensive lean	Hypertensive obese	Normotensive obese	Normotensive lean	Sign. of difference
Age (years)	53.00±8.35	50.00±8.35	43.20±8.60	42.025±8.60	p=0.0001
BMI (kg/m ²)	22.10±2.80	35.20±5.12	29.50±3.20	21.850±1.01	p=0.0001
Systolic blood pressure (mmHg)	144.51±16.01	145.10±15.21	120.90±2.20	120.500±5.75	p=0.0001
Diastolic blood pressure (mmHg)	92.25±10.61	92.20±12.18	80.50±3.20	80.050±3.12	p=0.0001
Serum creatinine (mg/dl)	0.90±0.23	1.05±0.22	0.90±0.11	0.920±0.25	p=0.4230
Urea (mg/dl)	25.75±9.39	30.00±6.70	31.00±10.50	26.320±8.70	p=0.7780
Cholesterol (mg/dl)	165.00±23.80	175.00±23.80	172.00±19.80	160.770±25.60	P=0.0250 P ² =0.0470
Glucose R (mg/dl)	98.40±22.62	94.00±7.13	102±14	95.500±15.70	p=0.5070
Triglycerides (mg/dl)	165.37±21.90	182.02±15.80	180±23	165±15.20	P=0.3800 P ² =0.7170
Na ⁺	144.00±5.10	141.00±6.20	139±5.30	142±8.23	P=0.2020
K ⁺	4.50±0.46	4.03±0.48	4.14±0.44	3.900±0.56	P=0.4100
Leptin (ng/dl)	11.90±3.77	18.71±15.50	13.82±12.63	9.420±12.20	P=0.0070 P ² =0.0630
Angiotensin II (ng/dl)	1.17±1.36	1.47±1.60	1.34±2.20	1.250±1.23	P=0.9760 P ² =0.1280

Values are presented as the means ± SD. P, significance between all hypertensives vs. normotensives; P², significance between all obese vs. all lean subjects.

Another variant, the C538T in the 3'UTR of the leptin gene, was similarly shown to be associated with essential hypertension independent of obesity (26). However, in another study on an African-American group, several genetic markers at the leptin locus, including the one described by Shintani *et al* (24), were not significantly linked to hypertension (27). These contradictory data prompted us to investigate the presence of common tetranucleotide repeat polymorphisms and the rare C538T SNP in the 3' flanking region of the leptin gene in: hypertensive patients independent of obesity, in obese hypertensive patients and in controls. Since several hormones, such as insulin and steroids (35,36), affect angiotensin mRNA, this study was designed to investigate whether leptin upregulates the activity of the gene in adipocytes, thereby contributing to hypertension.

Materials and methods

Subjects. In the present study, 362 patients with hypertension were investigated; 119 subjects were hypertensive with a BMI of 22.10±2.8 and 155 were hypertensive with a BMI of 35.2±5.12.88. Normotensive subjects with different BMIs were also studied as controls. All subjects were either first time diagnosed or had not taken any medication at least 15 days prior to the study. All subjects underwent a physical examination that included measurement of the BMI (evaluated by weight/height²; kg/m²) and levels of serum urea, creatinine, billurubin, cholesterol, glucose and triglyceride. All subjects had normal thyroid function. No patient with renal, hepatic or cardiac disease was included and none of the subjects had diabetes. Therefore, only a specific group of subjects with

essential hypertension was selected. After determining all the biochemical parameters and measuring other anthropometric parameters, blood was obtained from the subjects, collected in EDTA vials and stored at -20°C for genomic studies. The study was approved by the local institutional ethics committee, and informed consent was obtained from all of the subjects.

Genotyping. Genomic DNA was extracted from whole blood using a commercial kit (Zymo Research Corp., Irvine, CA, USA). Genotyping of the tetranucleotide polymorphisms in the 3' flanking region of the leptin gene was detected by polymerase chain reaction using forward primer 5'-AGTTCAAATAGAGGTCCAAATCA-3' and reverse primer 5'-TTCTGAGGTTGTGTCACTGGCA-3' that flanks the microsatellite in the 3' flanking region of the leptin gene. Another primer pair, 5'-CGACCTGGAGAACCTCCG-3' as forward and 5'-GTCCTGGATAAGGGGTGT-3' as reverse, was used for amplification of the 316-bp amplicon in the 3'UTR of the leptin gene for the screening of the C538T variant. PCR contained 100 ng of genomic DNA template, 0.2 μM of each primer, 2 mM of Mg²⁺, 0.2 mM of each dNTP, 1.5 units of *Taq* polymerase and 1X reaction buffer in a total volume of 25 μl. The PCR was performed for 30 cycles of 30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C, with an initial denaturation of 5 min at 94°C and a final extension of 10 min at 72°C. The reaction conditions were the same for both pairs of primers. PCR products were run in 2% agarose gel along with 100-bp ladder as a molecular weight marker. Amplified products were visualized by staining with ethidium bromide. In the case of the tetranucleotide repeat polymorphisms, alleles were distinguished on the basis of amplicon length, which varies for different

Table II. Frequency of the genotypes of the leptin gene polymorphism in hypertensive and normotensive subjects.

Variants/Genotypic distribution	Hypertensive lean	Hypertensive obese	Normotensive	Total
C538T				
C/C	119	155	88	
C/T	Nil	Nil	Nil	362
T/T	Nil	Nil	Nil	
Microsatellite				
I/I	53 (44.5)	71 (45.8)	12 (13.6)	362
I/II	41 (34.4)	60 (38.7)	42 (47.7)	p \leq 0.0001
II/II	25 (21.0)	24 (15.4)	34 (38.6)	

Absolute no. (percentage) of subjects are shown.

Table III. Association of different alleles with serum leptin and serum angiotensin II levels.

Microsatellite genotypes	Serum leptin (ng/dl)	Serum angiotensin II (ng/dl)	Significance
Genotype I/I (n=26)	19.2 \pm 6.12	1.2 \pm 1.35	P ^a =0.022 P ^{a1} =0.737
Genotype I/II (n=34)	18.7 \pm 15.2	1.40 \pm 1.55	P ^b =0.080 P ^{a2} =0.640
Genotype II/II (n=24)	16.2 \pm 12.6	1.28 \pm 1.26	P ^c =0.298 P ^{a3} =0.336

P^a and P^{a1} show the significance between Class I/I vs. Class II/II of serum leptin and angiotensin II levels, respectively. P^b and P^{a2} show the significance between Class I/I vs. Class I/II of serum leptin and angiotensin II levels, respectively. P^c and P^{a3} show the significance between Class I/II vs. Class II/II of serum leptin and angiotensin II levels, respectively.

alleles due to the variable number of tetranucleotide repeats. For the screening of the C538T variant, a 316-bp fragment was digested with the HypCH4IV enzyme. The digested products were run on 8% PAGE along with pBR322MspI digest as a molecular weight marker. Ten percent of the digested samples were sequenced directly as well.

Analysis of serum leptin concentration. To study the relationship between the leptin gene polymorphism and the serum leptin concentrations, serum leptin levels were determined with the human leptin immunoassay kit (DRG International Inc., Mountainside, NJ, USA). Serum was collected from each patient in the morning. Frozen samples were kept at -20°C until analysis.

Analysis of serum angiotensin II concentration. To study whether leptin upregulates the expression of angiotensin II levels, which is the main regulator of blood pressure, the serum angiotensin II concentration was determined with the angiotensin II ELISA kit (DRG International Inc.).

Statistical analysis. Data are expressed as the means \pm SD. The means were compared by the independent sample t-test. Fisher's exact test or χ^2 test was used to compare the frequencies. Regression analysis was used to describe the relationship between serum leptin as a dependent variable, and the BMI and serum angiotensin II levels as independent variables.

Statistical analysis was carried out using the statistical package SPSS.

Results

Table I shows the characteristics of the population that was genotyped for the microsatellite polymorphisms and the C538T variant in the 3' flanking region of the leptin gene. The age of the hypertensive group ranged from 45 to 65 years (mean \pm SD, 53 \pm 8.35), while the age of the control group ranged from 35 to 50 years (42.025 \pm 8.6). In the hypertensive group, 168 subjects were female and 106 were male, and in the normotensive group 30 were female and 58 male. There was no statistically significant difference in the serum urea, creatinine, glucose, bilirubin, triglyceride and angiotensin II, when compared between the hypertensive vs. normotensive class, or between the obese vs. lean class (p>0.05). A significant correlation was found between serum leptin when compared between the hypertensive vs. normotensive class, and also between the obese and lean class (p \leq 0.05). Linear regression analysis showed an independent correlation of leptinemia with BMI (p=0.019), but no significant correlation was found between the serum leptin concentration and angiotensin II (Table IV). Alleles of the microsatellite were composed of two groups with different size distribution; a shorter one, <190 bp in size (termed as Class I), and a longer one >210 bp in size (termed as Class II; Fig. 2). The frequency of Class I/

Table IV. Linear regression analysis for all subjects (HL, HO, NL and NO), obese subjects (HO and NO), and lean subjects (HL and NL).

Independent variables	Regression coefficients	95% confidence interval of regression coefficient	p-value	R ²
BMI (HO, HL, NO, NL)	0.255	0.02384 to 0.26150	0.019	0.065
ANG II (HO, HL, NO, NL)	0.171	-0.01370 to 0.04150	0.120	0.029
BMI (HO, NO)	0.482	-0.06166±0.01653	0.001	0.232
ANG II (HO, NO)	0.148	-0.05043±0.05405	0.357	0.025
BMI (HL, NL)	0.167	0.02759±0.02397	0.256	0.028
ANG II (HL, NL)	0.203	-0.01253 to 0.04931	0.235	0.041

Serum leptin was used as a dependent variable and body mass index (BMI), genotypic variant Class I/I and angiotensin II (ANG II) as independent variables. H, hypertensive; N, normotensive; L, lean; O, obese. BMI, body mass index; ANG II, angiotensin II.

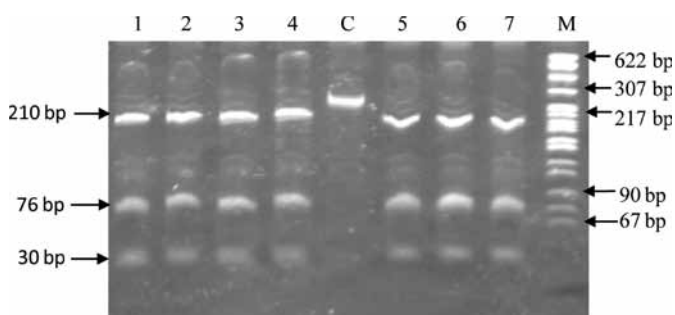


Figure 1. Representative polyacrylamide gel image (8%) showing the results of the HpyCh4IV digested PCR product. Lanes 1-7 show the digestion pattern of different samples under evaluation [homozygous wild-type (C/C) genotypes]. Lane M shows the separation pattern of pBR322MspI digested DNA marker. C represents the undigested PCR product (control).

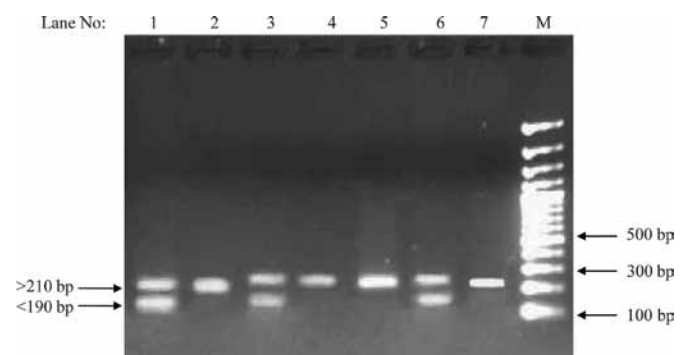


Figure 2. Representative gel image showing the PCR results. Lanes 1, 3 and 6 represent heterozygotes, and lanes 2, 4, 5 and 7 represent homozygotes for the Class II allele of the microsatellite polymorphism of the leptin gene. M, marker (100-bp ladder).

Class I genotype were higher in the hypertensive group than in the normotensive group (Table II). Class I/I and Class I/II was found to be significantly correlated with serum leptin concentration ($p \leq 0.05$), but no such correlation was observed with serum angiotensin II levels (Table III). In the case of the C538T variant, upon digestion with the HpyCH4IV restriction enzyme, the wild-type genotype provided three fragments of size 210, 76 and 30 bp, carriers had four fragments of size 210, 76, 30 and 286 bp, whereas mutant homozygotes gave two bands of size 286 and 30 bp. All 362 subjects studied showed the digestion pattern of wild-type homozygotes (Fig. 1) and direct sequencing of 10% of the subjects confirmed this.

Discussion

Obesity and essential hypertension are considered to be the result of multiple environmental and genetic determinants. These two factors are closely linked in epidemiological studies (28), and essential hypertension is estimated to be up to three times more prevalent among obese populations (29). One of the major mechanisms leading to the development of obesity-induced hypertension appears to be leptin-mediated sympatho-activation. In African-Americans, highly polymorphic markers in leptin locus were not significantly linked to the trait of essential hypertension (27). Later, several studies

on a Japanese population showed a significant association of the polymorphism in the 3'UTR region of the leptin gene with hypertension, while this association was independent of BMI. In another study involving a British population, another rare variant in the 3'UTR of the leptin gene was found to impact pulse pressure considerably, independent of obesity and cortico-intima-mediated thickness (26). The results of our study confirmed that the 3' flanking region polymorphism of the gene previously described by Shintani *et al* (24) is also found in a pure ethnic population of Kashmir. A significant association of the microsatellite polymorphism with hypertension was found. Another variant, C538T, was observed to be very rare in some populations (26,32), but seems to be either absent or even rarer in the Kashmiri population. However, a larger sample size is required to confirm this possibility. Although the microsatellite polymorphism examined is located in non-coding region, similarly located variants in the 3'UTR of other genes are known to play a key role in their expression (30,31). There are generally some cis-acting determinants in the 3'UTR to which proteins bind and either stabilize or destabilize the mRNA. Cis-acting determinants in the 3'UTR may also interact with other sequences within that mRNA. Therefore, the variation in these cis-acting determinants may affect the expression of this particular gene. This case may be similar to the present study, as we found that serum leptin is significantly

associated with Class I/Class I and Class I/Class II genotypes. Thus, the Class I/I and I/II genotypes were shown to be closely associated to hypertensive and serum leptin concentration. To determine whether this relation of leptin is mediated through angiotensin II, whose role in the regulation of blood pressure is well established, the concentration of angiotensin II was also assessed. However, a negative correlation was found between leptin and angiotensin II in all groups. The present data suggest that the common polymorphism in the 3' flanking region of the leptin gene is relative to essential hypertension. Further *in vitro* and animal model research is necessary to elucidate how the variation in the size of microsatellite in the 3' flanking region of the gene is involved in the regulation of blood pressure in obesity-dependent and obesity-independent hypertension.

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