Polymorphic Analysis of Leptin Promoter in Obese/diabetic Subjects in Kashmiri Population

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Abstract

Background: The role of common variants in leptin promoter has already been established to play a major role in obesity and diabetes in humans. The study was accordingly focused on leptin promoter variants and their potential association with diabetes and obesity in ethnic population from Kashmir, India. Methods: Allele frequencies of 620 Kashmiri subjects with diabetes (200), obese subjects (200), and ethnically matched healthy controls (200) were tested for the Hardy–Weinberg disequilibrium. Among 200 obese subjects, a total of 50 persons were with diabetes. The genotype and allele frequencies were evaluated using the Chi-square or Fisher’s exact tests. Results: Sequence analysis revealed two reported variations i.e., rs72563764C>T and rs7799039G>A in promoter region. Both variants show homozygous as well as heterozygous genotypes. These variations indicated significant difference with respect to allelic and genotypic frequencies in all groups i.e., persons with diabetes, obese, and obese persons with diabetes (P < 0.05). We also analyzed the association of these variations with biochemical characteristics and found significant association of rs72563764C>T with triglycerides (TG) in obese patients and fasting plasma glucose (FPG) and random blood sugar (RBS) in obese/obese persons with diabetes. Also rs7799039G>A showed association with postprandial plasma sugar (PPPS) in obese patients and FPG and resting plasma glucose (RPG) in obese persons with diabetes. Conclusions: Our results are suggestive of the association of leptin promoter gene variations i.e., rs72563764C>T and rs7799039G>A with both diabetes and obesity.

Keywords: Diabetes, leptin, leptin receptor, obesity, single-nucleotide polymorphisms

Introduction

Leptin (a 16 kDa polypeptide hormone) is predominantly produced by white adipose tissue and plays an essential role in neuroendocrine function, body weight, and energy expenditure homeostasis.[1] Mice deficient in leptin developed marked obesity with diabetes and administration of exogenous leptin to these mice causes dramatic reduction in weight.[2] It was first of all reported by Mamnes et al.[3,4] that G2548A variant in the promoter of leptin is associated with reduction in body mass index (BMI) in overweight women. Leptin receptors were first of all identified in hypothalamic regions and are associated with food intake regulation.[5] Leptin modulates insulin secretion and act via leptin receptors. Few studies have reported that chances of developing obesity are associated with the leptin receptor (LEPR) gene polymorphisms.[6,7] In rodents and humans, mutations in the LEPR gene results in the formation of a truncated receptor, which in turn have been shown to cause obesity and diabetes.[8,9] Although such mutations are very rare, but such events are central in explaining the role played by the leptin in regulating energy balance in rodents as well as in humans. It has been investigated that some polymorphic variations associated with leptin gene (LEP A19G) and leptin receptor gene (LEPR K656N, Q223R, and K109R) are considered as possible factors

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linked with obesity. These authors further reported that the allelic frequencies of these polymorphisms show ethnic variation.

**Materials and Methods**

**Subjects**

A total of 620 ethnic Kashmiri subjects were selected for the study, which included 200 persons with diabetes, 200 obese patients, and 220 healthy controls. Among 200 obese subjects, 50 persons were with diabetes. Inclusion criteria included patients diagnosed with type 2 diabetes mellitus (T2DM), patients were selected for diagnosis with T2DM based on criteria set by the American Diabetes Association (symptoms of diabetes plus a random blood glucose concentration ≥11.1 mmol l⁻¹; fasting plasma glucose (FPG) ≥7.0 mmol l⁻¹; 2-h plasma glucose ≥11.1 mmol l⁻¹; during an oral glucose tolerance test; HbA1c level ≥ 6.5), patients were overweight with a BMI of ≥25, higher lean body mass, people belonging to Kashmir division. Exclusion criteria included people not belonging to Kashmir division, individuals who did not comply with the requisite patient proforma, drug-induced patients, gestational diabetes patients, genetic syndromes associated with diabetes and obesity. Blood samples of the subjects were collected from the Endocrinology Department of the Sher-i-Kashmir Institute of Medical Sciences (SKIMS), Sura, Srinagar, Jammu and Kashmir. Samples of the patients were collected after the complete clinical investigation and all subjects signed written informed consent. Subjects were encouraged to narrate all the details relevant to this study. This included age of the subject, dietary history, gender, history of onset of diabetes/obesity, personal habits, any associated complications, socioeconomic factors, and information regarding close work. Venous 3–4 ml blood samples were collected in ethylenediaminetetraacetic acid (EDTA) for DNA extraction. Samples were kept at −70°C until analyzed.

**DNA analysis**

Genomic DNA was extracted from whole blood samples using the QIAamp DNA Blood Mini Kit (Qiagen). The polymerase chain reactions (PCR) were carried out in a total volume of 50 μl, containing 50–100 ng genomic DNA, 2–6 pmol of each primer, 1× of Taq polymerase buffer, and 0.5 units of taq DNA polymerase (Sigma Aldrich). Following primer sequences were used for amplification:

Forward primer: 5’-TTTCCGTAAATTTCCGATGAG-3’
Reverse primer: 5’-AAAGCAAGACAGGCATAAAAAAG-3’.

Expected PCR products of 242bp were generated successfully. The PCR cycling conditions involved: one cycle of denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 45 s, annealing at 60°C for 45 s, extension at 72°C for 45 s, and one final 7-min elongation cycle at 72°C. PCR products were then purified using purification kit (Sigma).

**Sequence analysis**

Purified PCR products were sequenced to confirm the presence of sequence variations. Sequence results obtained were analyzed using software’s like ClustalX and Chromas Pro for the detailed inspection of individual chromatograms.

**Statistical analysis**

Genotypes were obtained by direct counting with subsequent calculation of allele frequencies. Statistical analysis was undertaken using the Chi-square test and significance value (P). A P value of ≤ 0.05 was considered significant. Adherence to the Hardy–Weinberg equilibrium constant was tested using the Chi-square test with one degree of freedom. Odds ratio (OR) and confidence interval (CI) were also calculated.

**Results**

**Anthropometric and biochemical characteristics of cases and control groups**

The anthropometric and biochemical characteristics of cases and healthy control subjects are presented in Table 1. Affected people were divided into three categories i.e., persons with diabetes, obese subjects, and obese persons with diabetes. The mean age of controls and cases was 50.32 ± 8.83, 51.28 ± 13.05, 49.88 ± 11.21, and 49.08 ± 11.64 years, respectively. Mean BMI of cases and controls was 21.71 ± 1.31 kg/m², 24.02 ± 0.95 kg/m², 34.61 ± 4.99 kg/m², and 35.02 ± 6.11 kg/m², respectively. The cases presented with higher BMI and waist-to-hip ratio (WHR) as compared to controls (P < 0.001). There was no significant difference in parameters like age and high-density lipoproteins (HDLs) in both cases and controls (P ≥ 0.05). Also parameters like resting plasma glucose (RPG) and HbA1c% were not significantly different in controls and obese subjects (P ≥ 0.05). However, parameters like BMI, FPG, total cholesterol (TC), triglycerides (TG), low-density lipoproteins (LDL), and WHR were found significantly different from controls (P ≤ 0.001). Also significant difference was found in HbA1c% and random blood sugar in controls, persons with diabetes, and obese persons with diabetes (P ≤ 0.001).

**Association of leptin promoter polymorphisms with T2DM and obesity**

Two previously reported variations were found in leptin promoter i.e., C > T (rs72563764) and G>A (rs7799039). Both of these variations showed significant difference in allelic and genotypic frequencies between controls and persons with diabetes, controls and obese, and controls and obese persons with diabetes subjects (P ≤ 0.05) [Table 2]. However, frequency of TT genotype in C>A variation was not found different from that of controls and persons with diabetes subjects (P = 0.339). Also variation with rs7799039G>A did not show significant difference between controls and persons with diabetes (P = 0.29) and controls and obese (P = 0.16) with respect to AA genotype. The observed genotype frequencies of these variations were not in the Hardy–Weinberg equilibrium.
Table 1: Anthropometric and biochemical characteristics of controls and cases

| Variables | Control (n=220) | Diabetic (n=200) | Obese (n=150) | Obese with diabetic (n=50) |
|-----------|----------------|-----------------|---------------|---------------------------|
| Gender (M/F) | 100/120 | 75/125 | 51/99 | 9/41 |
| Age (years) | 50.32±8.83 | 51.28±13.05 (P<0.375) | 49.88±11.21 (P<0.673) | 49.08±11.64 (P<0.398) |
| BMI (kg/m²) | 21.71±1.31 | 24.02±0.95 (P<0.001) | 34.61±4.99 (P<0.001) | 35.02±6.11 (P<0.001) |
| FBS (mg/dl) | 92.21±10.71 | 189.14±49.52 (P<0.001) | 95.68±16.73 (P<0.016) | 172.96±34.60 (P<0.001) |
| PPBS (mg/dl) | 109.65±23.73 | 258.95±77.06 (P<0.001) | 117.36±27.79 (P<0.004) | 225.48±67.21 (P<0.001) |
| Random (mg/dl) | 110.41±18.76 | 270.13±70.33 (P<0.001) | 111.91±17.45 (P<0.049) | 237.14±73.67 (P<0.001) |
| HbA1c % | 5.56±1.02 | 8.63±1.56 (P<0.001) | 5.49±0.95 (P<0.491) | 8.81±1.55 (P<0.001) |
| TC (mg/dl) | 151.55±8.48 | 181.78±9.56 (P<0.001) | 191.84±12.85 (P<0.001) | 242.42±15.85 (P<0.001) |
| TG (mg/dl) | 127.21±5.38 | 154.40±6.00 (P<0.001) | 238.39±36.56 (P<0.001) | 273.11±49.06 (P<0.001) |
| LDL (mg/dl) | 87.95±8.80 | 105.08±6.08 (P<0.001) | 181.93±10.49 (P<0.001) | 183.33±7.38 (P<0.001) |
| HDL (mg/dl) | 33.18±5.98 | 32.73±5.38 (P<0.017) | 32.62±3.33 (P<0.357) | 33.05±5.56 (P<0.886) |
| WHR | 0.70±0.095 | 0.82±0.087 (P<0.001) | 1.02±0.079 (P<0.001) | 1.02±0.079 (P<0.001) |

Data represented as mean±SD. BMI: Body mass index, FBS: Fasting blood sugar, PPBS: Postprandial blood sugar. HbA1c: Hemoglobin A1c, TC: Total cholesterol, TG: Triglycerides, LDL: Low-density lipoproteins, HDL: High-density lipoproteins, WHR: Waist-hip ratio. *P-value for inter-group variants measured by the Student’s t-test at 95% confidence interval (P<0.05)

Table 2: Comparison of the distribution of alleles and genotypes of leptin gene polymorphisms in healthy and affected subjects

| Gene/variant | Allele/genotype | Controls (220) | Diabetic (200) | Obese (150) | Obese with diabetic (50) | P | OR (95% CI) | P | OR (95% CI) | P | OR (95% CI) |
|--------------|----------------|---------------|----------------|--------------|-------------------------|---|-------------|---|-------------|---|-------------|
| rs72563764C>T | C | 395 (89.77) | 332 (83.0) | 215 (71.6) | 61 (61.0) | 0.0045 | 1.79 (1.20-2.69) | 0.0001 | 3.38 (2.33-5.16) | 0.0001 | 5.61 (3.89-9.31) |
| | T | 45 (10.22) | 68 (17.0) | 85 (28.3) | 39 (39.0) | - | 1.0* | - | - | - | - |
| rs7799039G>A | G | 405 (92.04) | 346 (86.5) | 253 (84.3) | 70 (70.0) | - | 1.0* | - | - | - | - |
| | A | 35 (7.95) | 54 (13.5) | 47 (15.6) | 30 (30.0) | 0.0098 | 1.80 (1.15-2.82) | 0.0012 | 2.15 (1.35-3.42) | <0.0001 | 4.95 (2.86-8.59) |
| rs72563764C>T | CC | 185 (84.09) | 145 (72.5) | 85 (56.6) | 18 (6.0) | - | 1.0* | - | - | - | - |
| | CT | 25 (11.3) | 42 (1.0) | 45 (30.0) | 25 (50.0) | - | 1.0* | - | - | - | - |
| | TT | 10 (4.5) | 13 (6.5) | 20 (13.3) | 7 (14.0) | 0.339 | 1.66 (0.71-3.89) | <0.001 | 4.35 (1.95-9.70) | 0.0010 | 7.19 (4.42-21.20) |
| rs7799039G>A | GG | 195 (88.63) | 160 (80.0) | 114 (76.0) | 27 (54.0) | 0.039 | 2.11 (1.08-4.13) | 0.003 | 2.85 (1.44-5.63) | <0.001 | 7.70 (3.42-17.34) |
| | GA | 15 (6.81) | 26 (30) | 25 (16.6) | 16 (32.0) | - | 1.0* | - | - | - | - |
| | AA | 10 (4.54) | 14 (7.0) | 11 (7.3) | 7 (14.0) | 0.294 | 1.71 (0.74-4.95) | 0.169 | 1.88 (0.77-4.57) | 0.004 | 5.06 (1.78-14.40) |

*Reference values for OR; P<0.05 considered statistically significant

Association of leptin gene polymorphisms with biochemical traits

We also analyzed the effect of single-nucleotide polymorphisms (SNPs) on several anthropometric and metabolic traits. SNP rs72563764C>T showed [Table 3] significant association with TG (P<0.021) in obese subjects. Additionally, this variant also showed association with FPG (P=0.015) and RGP (P=0.012) in obese persons with diabetes. However, this variant did not show any association with other parameters. Other SNP with rs7799039G>A showed [Table 4] significant association with postprandial plasma glucose (PPPG) (P<0.0001) and RGP (P<0.0001) in obese subjects and FPG (P=0.05) and RGP (P=0.003) in obese persons with diabetes subjects.

Discussion

This is the first systematic investigation of leptin promoter in persons with diabetes and obese Kashmiri subjects. A total of two known variations in leptin promoter i.e., −2549C>T (rs72563764) and −2548G>A (rs7799039) upon sequencing were reported in current study. Variation with rs72563764C>T showed significant difference in allelic (P=0.004) and genotypic (P=0.008) frequencies between controls and persons with diabetes. Also allelic and genotypic frequencies were significant in controls.
and obese subjects (P ≤ 0.001) and controls and obese persons with diabetes (P ≤ 0.001) in this variation. Additionally, we sought the association of this variant with anthropometric and metabolic characteristics and it showed significant association with TG (P = 0.021) in obese and with FPG (P = 0.015) and RPG (P = 0.012) in obese persons with diabetes subjects. Another variation – 2548G>A with rs7799039G>A showed significant difference in allelic and genotypic frequencies between controls and persons with diabetes subjects, controls and obese subjects, and controls and obese persons with diabetes subjects (P ≤ 0.05). These variations indicated that they have possible bearing with obesity and diabetes. This variation was also analyzed for reporting the association with clinical parameters and it showed significant association with PPPG (P < 0.0001) and RPG (P < 0.0001) in obese and FPG (P = 0.05) and RPG (P = 0.003) in obese persons with diabetes subjects.

Among the variants identified, –2548G/A polymorphism, (rs7799039) is the most studied one in the 5’-untranslated

| Subject | CC | CT + TT | CC | CT + TT | CC | CT + TT | CC | CT + TT |
|---------|----|---------|----|---------|----|---------|----|---------|
| Age (years) | 50.54±8.9 | 50.01±7.6 | 50.87±13.42 | 53.58±9.88 | 48.13±10.7 | 51.67±11.2 | 46.67±11.6 | 48.79±11.5 |
| BMI (kg/m²) | 21.74±1.30 | 21.70±1.24 | 24.08±0.93 | 23.89±1.07 | 34.57±5.14 | 35.13±5.04 | 34.91±5.35 | 35.58±7.22 |
| FBS (mg/dl) | 39.24±11.0 | 91.21±8.25 | 187.48±44.85 | 198.32±63.97 | 94.95±16.44 | 96.04±16.10 | 184.94±43.79 | 161.5±22.2* | (0.0158) |
| PPBS (mg/dl) | 110.33±24.54 | 107.89±20.96 | 255.03±75.32 | 262.72±79.91 | 118.37±28.96 | 115.61±24.44 | 229.00±70.07 | 211.16±54.9 |
| Random (mg/dl) | 110.59±19.16 | 111.61±18.23 | 266.97±69.87 | 273.03±70.68 | 111.04±18.57 | 112.93±16.99 | 268.33±92.17 | 214.56±55.49* | (0.0129) |
| HbA1c% | 5.61±1.02 | 5.32±1.13 | 8.58±1.62 | 8.86±1.54 | 5.48±1.01 | 5.62±0.84 | 8.39±1.53 | 9.12±1.30 |
| TC (mg/dl) | 151.44±8.49 | 152.31±8.43 | 181.42±9.73 | 182.15±9.33 | 192.01±12.94 | 190.45±11.98 | 242.51±14.01 | 244.45±15.07 |
| TG (mg/dl) | 127.01±5.27 | 128.26±5.58 | 154.33±5.61 | 155.55±6.66 | 233.67±26.26 | 247.60±46.51* | (0.0217) | 287.24±50.59 | 277.65±43.06 |
| LDL (mg/dl) | 87.79±8.86 | 88.33±9.13 | 105.12±6.02 | 104.34±6.75 | 183.35±11.21 | 179.98±9.41 | 184.30±5.70 | 182.32±8.56 |
| HDL (mg/dl) | 33.52±6.02 | 31.06±5.39 | 32.90±5.30 | 32.55±5.68 | 32.92±5.32 | 32.40±5.33 | 34.49±6.84 | 32.82±4.63 |
| WHR | 0.70±0.10 | 0.70±0.07 | 0.82±0.09 | 0.81±0.07 | 1.02±0.08 | 1.025±0.09 | 1.01±0.07 | 1.015±0.08 |

Data represented as mean±SD. BMI: Body mass index, FBS: Fasting blood sugar, PPBS: Postprandial blood sugar, HbA1c: HemoglobinA1c-glucose bound to hemoglobin, TC: Total cholesterol, TG: Triglycerides, LDL: Low-density lipoproteins, HDL: High-density lipoproteins, WHR: Waist-hip ratio. *Statistically significant (P<0.05)
region of the leptin gene. Substantial data indicated that the \( LEP \) –2548 polymorphism was associated with the variations in plasma leptin and BMI in both obese and non-obese individuals.\(^{[11]}\) The mechanism may be that the \( LEP –2548G > A \) polymorphism influences leptin expression, possibly at the transcriptional level, and therefore also adipose secretion levels of the hormone.\(^{[12]}\) However, the direct association between the –2548 G/A polymorphism and obesity remains vague. While most currently published studies have failed to identify a significant association between this genetic variant and obesity in various populations.\(^{[13-18]}\) Three studies had found that the current variant was significantly associated with the risk of obesity in subjects of mixed race\(^{[19]}\) and Caucasians.\(^{[20,21]}\) This is in accordance to our study as the SNP was found to be associated with obesity in our population also. Earlier studies had reported that –2548G/A variant are associated with weight gain although the allelic association was not clear. It should be further noted that some studies had found that allele A is associated with weight gain or increased glucose/lipid measures,\(^{[22-25]}\) while others found significant results with the allele G.\(^{[26]}\) Li et al.,\(^{[27]}\) reported that the G2548A variant is more common in Caucasians than African-Americans within the average weight groups. According to Jiang et al.,\(^{[28]}\) the functional significance of the G2548A polymorphism is uncertain as the polymorphism is not at a conserved region among human, rat, and mouse species. On the other hand, G2548A polymorphism is located at the 5’ end of the promoter region of \( LEP.\)\(^{[19]}\) It has been postulated that this remote region might contain inhibitory elements for transcription in adipocytes.\(^{[29]}\) Although G2548A polymorphism lies in the vicinity to these elements and putative-binding sites, but its effect on leptin expression is still unknown. There is a high variability in the 5’-flanking region in the leptin gene and a large number of studies have been conducted on the association between leptin gene variants and the obesity risk.

**Conclusion**

Our results are suggestive of the association of leptin promoter gene variations i.e., rs72563764C>T and rs7799039G>A with both diabetes and obesity. As per our understanding this is the first report demonstrating the association of the leptin promoter gene with obesity risk and diabetes in the Kashmiri patient population. Our results are interesting but should be replicated in the future in a larger cohort of the same ethnicity to validate the findings of the current analysis to confirm its biological relevance.

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**Conflicts of interest**

There are no conflicts of interest.

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