



Association of the Human Leptin Receptor Gene (rs1137101; Gln223Arg) Polymorphism and Circulating Leptin in Patients with Metabolic Syndrome in the Indian Population

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Abstract Phenotypic expression of metabolic syndrome is precipitated by environmental variables along with the individual genetic susceptibility to the obesogenic environment and growing body of evidence suggest a paramount role of adipocytokines. Therefore, identifying the genetic influence on circulation leptin levels and clarifying genotype–phenotype correlation of rs1137101 {Leptin receptor gene (LEPR) Gln223Arg (Q223R; A668G)} in metabolic syndrome were the primary objective of this study. A total of 447 adult participants, including 214 metabolic syndrome patients and 233 healthy controls, were genotyped using polymerase chain reaction-restriction fragment length polymorphism method to unravel the effects of genetic risk loci {Leptin receptor gene; Gln223Arg (Q223R; A668G); rs1137101} on the occurrence of metabolic syndrome in consort with circulation leptin levels. Suitable descriptive statistics was used for different variables. The genotype frequencies were found to be in Hardy–Weinberg equilibrium for both cases ($p > 0.2722$) as well as in controls ($p > 0.2331$). However, genotype ($\chi^2: 11.26, 2 \text{ d.f. } p = 0.0036$) and allele distribution ($\chi^2: 10.51, 2 \text{ d.f. } p = 0.0012$) of the LEPR Gln223Arg (Q223R; A668G) differed significantly between cases and controls. Gln/Arg genotype (OR = 1.6099; 95% CI = 1.0847–2.3893; $p \text{ value} = 0.0181$), Arg/Arg genotype (OR = 2.8121; 95% CI = 1.4103–5.6074; $p \text{ value} = 0.0033$) and R allele (OR = 1.5875; 95% CI = 1.1996–2.1008; p

value = 0.0012) were significantly associated with increased risk of metabolic syndrome in univariate analysis. Further a multivariate logistic regression adjusted for potential confounders showed that Arg/Arg genotype (OR = 1.9; 95% CI = 1.271–2.639; $p \text{ value} < 0.05$) and Gln/Arg (OR: 1.3; 95% CI = 0.873–2.034; $p \text{ value} < 0.05$) have a significant risk for the occurrence of the metabolic syndrome. A progressive increase in the serum leptin levels from major homozygous alleles to minor homozygous alleles were observed indicating that rs1137101 modify the serum leptin concentrations in patients with metabolic syndrome. These findings provide enough evidence of a significant association of LEPR Gln223Arg (Q223R; A668G) polymorphism in the LepR gene in Indian patients with increased risk of metabolic syndrome for R allele and Arg/Arg homozygote. Thus, rs1137101 might be a pleiotropic locus for metabolic syndrome and its components in studied population.

Keywords Leptin · Leptin receptor · Gln223Arg · Single nucleotide polymorphism

Introduction

Metabolic syndrome (MetS) is a significant global health concern and its prevalence endures to intensify in India [1]. Moreover, it is not a disease but rather an assemblage of explicit metabolic risk factors viz truncal obesity, hypertriglyceridemia, hyperglycemia, reduced high-density lipoprotein cholesterol (HDL-C) levels and hypertension [2]. Assortment of these altered variables has been associated with a five-fold augmented risk of type 2 diabetes mellitus (T2DM) and a two-fold increased risk of cardiovascular diseases (CVD) [3, 4]. Thus, the foremost objective of MetS management is to reduce the risk of associated morbidity [3]

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and paramount course of action is to identify the individuals at amplified risk of developing metabolic syndrome. A growing body of studies supports that etiopathogenesis of metabolic syndrome involves a complex interplay of genetic, epigenetic and environmental variables, with a dominant focus on the adipocytokines [3, 4].

Among the many potential candidate adipocytokines, leptin (secreted by adipose tissue and regulated by hypothalamus) acts as a principal metabolic regulator; by increasing energy expenditure, plummeting appetite and regulating inflammatory/immune responses [5–7]. Most of its known cellular actions are mediated via the activation of leptin receptor (LepR) [8] and feeble signal transduction through it has been associated with the development of excessive obesity [9, 10]. Therefore, gene encoding for the leptin receptor is one of the imperative contributing factors with both causation or progression of metabolic syndrome and is reasonable to hypothesize that genetic variants in LepR gene modulates receptor function and may modify the risk of developing phenotypic trait.

Cytogenic location of *LEPR* gene is on chromosome 1p31. A single nucleotide polymorphism (SNPs) with substitution of guanine to adenine in exon 6 of the *LEPR* gene at 668 nucleotide position replacing arginine into glutamine {*LEPR* Gln223Arg (Q223R; A668G)} has been described and is in a genomic sequence that codes for the extracellular subunit of the leptin receptor. *LEPR* Gln223Arg SNP (Q223R; A668G), is confederated with an impaired capacity of leptin to bind with Lep-R; diminished post receptor signalling [9, 11] and has been associated with the pathogenesis of MetS [12, 13], obesity [14, 15], insulin resistance [16] and diabetes mellitus [17]. However, there are lack of consistent findings at all these fronts [18, 19]. Therefore, identifying the genetic influence and clarifying genotype–phenotype correlation of rs1137101 in metabolic syndrome are issues that we pursue with interest in this study.

Materials and Methods

Study Setting and Design

This case–control study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and its subsequent revisions on biomedical research on humans and was approved by the Institutional Human Research Ethical Committee. Written informed consent was obtained from each participant in accordance to related norms and clinical guidelines at the inception of the study after providing a detailed study overview.

The detailed history of each participant including clinical findings and demographic profile was recorded in the proforma. The waist circumference (WC) was assessed at

the superior border of the iliac crest in the horizontal plane using a non-stretchable measuring tape, and the average of the two measurements was taken as the final reading [20]. Total body fat (%) was measured using bioelectrical impedance analyzer (Omron HBF-362). The blood pressure was measured manually in a sphygmomanometer with a mercury column, in a sitting position after 5 min of rest [21].

Sample Size Calculation

LEPR Gln223Arg SNP (Q223R; A668G) status was first assessed in 20 MetS patients, of which 4 patients were found to be positive for the said SNP, accounting a prevalence of 20.0%. Thus, sample size was determined as 210 using the formula $N = (Z^2) (PQ) \div L^2$ (Z = Confidence interval, L = margin of error of 5.0%, P = Prevalence, and $Q = 1 - \text{prevalence}$) [22]. To increase statistical power, 214 and 233 participants were recruited for as cases and controls respectively.

Study Population

This study included a total of 447 participants who were conveniently selected and categorized into two groups: Group I (214 confirmed cases of MetS) and Group II (233 healthy controls), as determined by clinical examination and relevant investigations. MetS was diagnosed using the updated NCEP ATP III criteria (National Cholesterol Education Program, Adult Treatment Panel III) [23]. Patients with any of the following three components out of five were considered cases of MetS: increased fasting blood glucose (FBG) [> 100.0 mg/dL or on regular medical treatment for increased FBG], central obesity (WC in women > 80.0 cm and in men > 90.0 cm), increased blood pressure ($> 135/85$ mmHg or on antihypertensive medication), increased triacylglycerol (TAG) [> 150.0 mg/dL or on regular medical treatment for increased TAG], decreased high-density lipoprotein cholesterol (HDL-C) [≤ 50.0 mg/dL for women and ≤ 40.0 mg/dL for men]. Adult MetS patients of either gender was included in the study. Healthy controls were recruited randomly from the outpatient department, and they were not on any drugs or diagnosed with acute or chronic disorders.

Laboratory Investigations

Venous blood was collected from the peripheral vein via venepuncture after a 12-h overnight fast. The serum fasting plasma glucose (FPG), high density lipoprotein-cholesterols (HDL-C), and triglycerides (TAG) were estimated using commercial kits on fully automated chemistry analyzer. Serum total leptin level was measured by the immunoassay method using a commercially human leptin ELISA kit

according to the manufacturer's instructions (RayBiotech, Inc., Georgia, United States). Serum insulin level was estimated using an electrochemiluminescence-based immunoassay (Roche Diagnostics). The homeostasis model assessment- insulin resistance (HOMA-IR) was used to assess insulin resistance as: Fasting glucose (mM) X Fasting insulin (mU/mL) ÷ 405 [24].

Genotype Analysis

DNA Extraction

EDTA-treated peripheral blood was used to extract genomic DNA from leukocytes by using a commercially available DNA extraction kit. Extracted DNA was stored further at -4°C and amplified within 3 days after the extraction. The integrity and quantity of the DNA were determined by the ratio of absorbance at 260 and 280 nm ($A_{260/280}$) in a spectrophotometer (Systronics).

DNA Amplification

Following two oligonucleotide primers were used to amplify the DNA by polymerase chain reaction (PCR): forward primer—5'-ACCCTTTAAGCTGGGTGTCCCAAATAG-3' and reverse primer—5'-AGCTAGCAA ATATTTTGTAA GCAATT-3' [25, 26]. PCR was carried out on a final volume of 25 μL , comprising of 200 μM of oligonucleotides, 4.0 μM of each primer, 1X PCR buffer, and 0.3 U Taq polymerase in Thermal Cycler (Applied Biosystems, 2720).

Digestion of PCR Products by Restriction Endonuclease

The restriction fragment length polymorphism (RFLP) analysis of the PCR amplified 80-bp products was performed using the restriction enzyme *MspI* (restriction site: 5'.... C[^]CGG...0.3'). Restriction endonuclease digestion of the 80 bp PCR product was performed by incubating at 37 $^{\circ}\text{C}$ for 16 h with 1x NEB buffer in a total volume of 15.0 μL reaction, followed by heat inactivation at 65 $^{\circ}\text{C}$ for 20 min. Ten μL of restriction fragment was loaded directly onto ethidium bromide-stained 3.0% agarose gel for further separation under UV illumination. The digested product for the Q allele was visualized as band size of 80 bp, while the R allele was visualized as band size of 58 and 22 bp (Fig. 1).

Statistical Analysis

The statistical analysis was performed using the SPSS 16.0 software package. Mean and standard deviations (SD) were calculated for study variables. The Chi-square test was used to compare genotypic, allelic frequencies, and various parameters between two groups. The Hardy–Weinberg

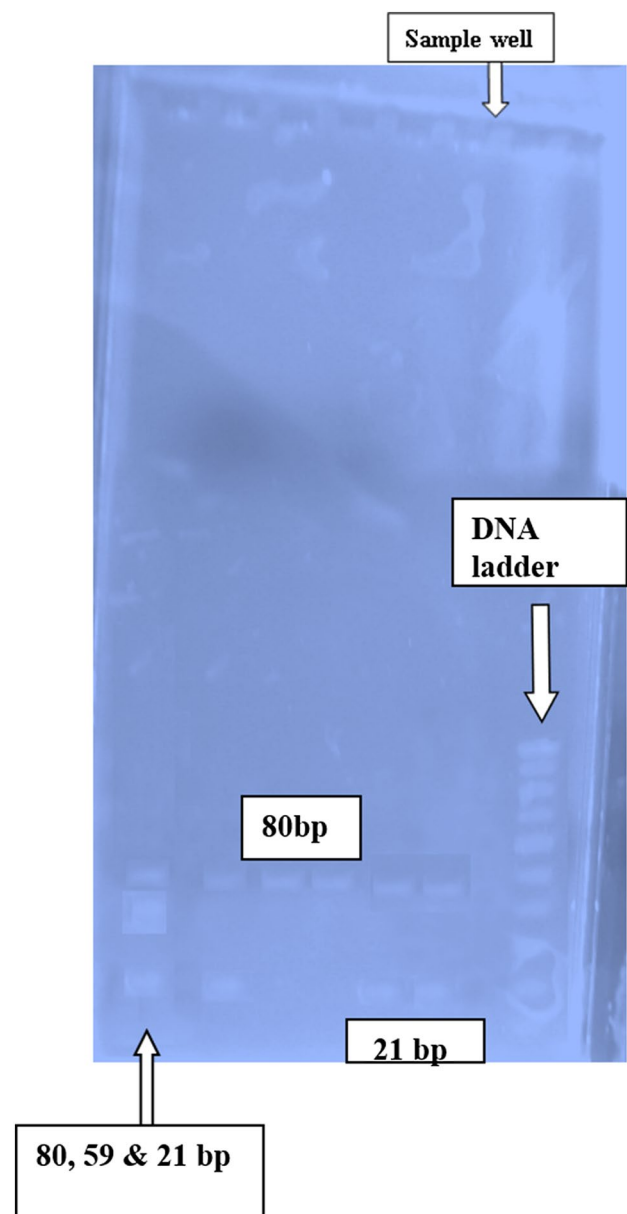


Fig. 1 PCR amplified and digested products

equilibrium was assessed using a goodness-of-fit χ^2 test. The logistic regression was used to calculate the odds ratio (OR) and to evaluate the relationship between MetS as predictor variables and other possible factors. A p-value of less than 0.05 was considered statistically significant.

Results

Patient Characteristics

Demographic, anthropometric, and blood chemistry characteristics of study participants were described in Table 1.

Table 1 Anthropometric and blood chemistry characteristics of study participants

Parameters	Healthy Controls (n: 233)	Metabolic Syndrome Cases (n: 214)	p value
	Mean (S.D.)	Mean (S.D.)	
Age (years)	45.4 (8.2)	46.8 (7.0)	0.0539*
Male, n (%)	134 (57.51%)	138 (64.48%)	0.1582*
Female, n (%)	99 (42.48)	76 (35.51)	0.1582*
BMI (kg/m ²)	22.6 (1.2)	28.4 (2.4)	<0.0001 [†]
WC (cm)	76.9(6.2)	93.1(8.9)	<0.001 [†]
W/H ratio	0.8 (0.15)	1.0 (0.3)	<0.0001 [†]
SBP (mm Hg)	122.8 (8.2)	138.6 (10.4)	<0.0001 [†]
DBP (mm Hg)	82.2 (4.2)	88.4 (6.6)	<0.0001 [†]
FPG (mg/dl)	90.6 (10.8)	138.2 (22.4)	<0.0001 [†]
TAG (mg/dl)	98.8 (16.2)	178.4 (42.6)	<0.0001 [†]
TC (mg/dl)	164.5(20.4)	208.9 (14.1)	<0.0001 [†]
HDL-C(mg/dl)	53.4 (6.1)	36.5 (5.8)	<0.001 [†]
INSULIN (uU/ml)	7.41 (2.3)	17.17 (5.6)	<0.001 [†]
HOMA-IR	1.78 (1.09)	6.73 (2.2)	<0.0001 [†]
Leptin (µg/L)	10.6 (6.6)	22.8 (5.8)	<0.0001 [†]
Total body fat %	24.7 (5.4)	28.8 (4.2)	<0.0001 [†]
Vegetarian/Non-vegetarian (n)	154 (66.09)/79 (33.9)	127 (59.34)/87 (40.65)	0.1684 [†]

P value is calculated by chi-square test* and student t-test[†]

There was no significant difference in age ($p = 0.539$), gender ($p = 0.1582$), and dietary habits ($p = 0.1684$) between healthy controls and cases. Serum leptin level was significantly high in cases compared to healthy controls (28.8 ± 4.2 versus 24.7 ± 5.4 , $p < 0.0001$). Similarly, BMI, WC, waist to hip (W/H) ratio, blood pressure, FPG, TAG, TC, insulin, HOMA-IR, and total body fat percentage were significantly high in the patient group compared to healthy controls ($p < 0.0001$). However, as expected HDL-C was significantly low in the patient group compared to healthy controls ($p < 0.0001$).

Genotype frequencies of *LEPR* Gln223Arg SNP (Q223R; A668G) in MetS and control participants were described in Table 2. There was no significant difference between observed frequencies and expected Hardy–Weinberg frequencies in cases ($p > 0.2722$) as well as in controls ($p > 0.2331$), substantiating the genotypes of *LEPR*

Gln223Arg SNP (Q223R; A668G) were distributed according to Hardy–Weinberg Equilibrium.

There was a significant difference in the distribution of *LEPR* Gln223Arg SNP (Q223R; A668G) genotype (χ^2 : 11.26, 2 d.f. $p = 0.0036$) and alleles (χ^2 : 10.51, 2 d.f. p : 0.0012) among MetS cases and controls. Gln/Arg (50.93% versus 43.77%) and Arg/Arg (13.08% versus 6.43%) genotypes were significantly high in MetS cases compared to controls. However, Gln/Gln (49.78% versus 35.98%) genotypes were significantly high in controls compared to the MetS group. The Q allele of *LEPR* Gln223Arg SNP (Q223R; A668G) was significantly high in controls (71.67% versus 61.45%), while the R allele of *LEPR* Gln223Arg SNP (Q223R; A668G) was significantly high in MetS cases (38.55% versus 28.33%).

In univariate analysis, Gln/Arg genotype (OR = 1.6099; 95% CI = 1.0847–2.3893; p value = 0.0181), Arg/Arg genotype (OR = 2.8121; 95% CI = 1.4103–5.6074;

Table 2 Genotype frequencies of rs1137101 Leptin Receptor Gln223Arg (Q223R) Gene Polymorphisms in case and control participants

Genotype	Cases (n: 214)		Controls (n: 233)	
	Expected H W frequency	observed	Expected H W frequency	Observed
Gln/Gln	80.8 (37.75%)	77 (35.98%)	119.69 (51.36%)	116 (49.78%)
Gln/Arg	101.39 (47.37%)	109 (50.93%)	94.6 (40.6%)	102 (43.77%)
Arg/Arg	31.8 (14.85%)	28 (13.08%)	18.69 (8.02%)	15 (6.43%)
χ^2	1.2055		1.4218	
P	0.2722		0.2331	

p-value is calculated by chi-square test

Table 3 Association of rs1137101 {Leptin Receptor Gln223Arg (Q223R)} genotype and allele frequency in the case and control participants

Genotype	MetS Cases	Control	CI	p
Gln/Gln	77	116	Reference	
Gln/Arg	109	102	OR: 1.6099; 1.0847–2.3893	0.0181
Arg/Arg	28	15	OR: 2.8121; 1.4103–5.6074	0.0033
Gln/Arg + Arg/Arg	137	117	OR: 1.764; 1.2072–2.577	0.0034
Allele				
Q	263	334	Reference	
R	165	132	OR: 1.5875; 1.1996–2.1008	0.0012

OR is calculated by logistic regression

Table 4 Association of rs1137101 {Leptin Receptor Gln223Arg (Q223R)} genotypes with serum leptin levels

	Upper Quartile Q1 Tertile 1 [Value: > 20.4 (µg/L)] n (%)	Inter-quartile range Q2 Tertile 2 [Value: 17.8–20.4 (µg/L)] n (%)	Lower Quartile Q3 Tertile3 [Value < 17.8 (µg/L)] n (%)	p value
Arg/Arg	18 (64.28%)	8 (28.57%)	2 (7.14%)	< 0.00001
Gln/Arg	25 (22.93%)	54 (49.54%)	30 (27.52%)	
Gln/Gln	13 (16.88%)	23 (29.87%)	41 (53.24%)	

*p-value is calculated by chi-square test

p value = 0.0033) and R allele (OR = 1.5875; 95% CI = 1.1996–2.1008; p value = 0.0012) were significantly associated with increased risk of MetS (Table 3).

Further, in a multivariate analysis that controlled for potential confounders such as age, gender, and HOMA-IR, the Arg/Arg genotype (OR = 1.9; 95% CI = 1.271–2.639; p-value < 0.05) and Gln/Arg (OR: 1.3; 95% CI = 0.873–2.034; p value < 0.05) were found to have a significant risk for the occurrence of the MetS.

Association of rs1137101 {Leptin Receptor Gln223Arg (Q223R; A668G)} genotypes with serum leptin levels is described in Table 4. Most of the patients with Arg/Arg genotype (64.28%) were in the upper tertile group of serum leptin and most of the patients with Gln/Gln genotypes were in the lower tertile group of serum leptin levels. Hence, it implies that the homozygous Arg/Arg genotype of *LEPR* Gln223Arg (Q223R; A668G) was significantly associated with increased serum leptin levels in patients of MetS (Table 4) and is further substantiated by a progressive increase in the serum leptin levels from major homozygous alleles to minor homozygous alleles (Table 5).

Discussion

Recent experimental and clinical studies along with new improved molecular/biological tools have extended

Table 5 Serum leptin levels according to rs1137101 {Leptin Receptor Gln223Arg (Q223R)} genotypes

Genotype	Serum leptin level (Mean ± SD)		P-value
	MetS cases (n: 214)	Controls (n: 233)	
Gln/Gln	10.1 (4.9) (n: 77)	8.9 (3.8) (n: 116)	0.0575
Gln/Arg	22.2 (5.4) (n: 109)	11.6 (6.8) (n: 102)	< 0.0001
Arg/Arg	24.2 (3.6) (n: 28)	14.8 (4.2) (n: 15)	< 0.0001

*p-value is calculated by a one-way ANOVA test

supplementary acquaintance around hereditary variables of metabolic syndrome. The revelation of the genetic component of the MetS and the major susceptibility locus may assist to comprehend the pathogenesis of MetS. Thus, this study explored the genotype–phenotype correlation of rs1137101 polymorphism with respect to the condition, since as per PubMed database no study has investigated *LEPR* Gln223Arg SNP (Q223R; A668G) and its association with serum leptin level in patients with metabolic syndrome in the Indian population. Primary finding of the study reveals a significant association of the locus with the occurrence of MetS, together with an augmented serum leptin level.

The underlying mechanisms for the onset/progression of MetS is still unidentified and remains mysterious, although alteration in serum leptin level is reported to be associated with obesity/obesity related disorders, signifying an imperative factor for the development of MetS [27]. This study also reported a significant association of serum leptin level with MetS and these findings were consistent with previous studies conducted on East Azerbaijan populations [28], Lebanese participants [29], and Korean populations [30].

Leptin plays a critical role in the regulation of food intake, fatty acid oxidation and glucose uptake. It decreases food intake by inhibiting agouti-related protein (AgRP) and by activating melanocortin which further stimulates α -melanocyte-stimulating hormone (α -MSH) [31]. Hence, the diminished activity of leptin or leptin resistance stimulates food intake and increases the risk of obesity [8]. *LEPR* Gln223Arg SNP (Q223R; A668G) affects the function of the leptin receptor and its signalling

capacity, which might lead to disturbance in hunger sensation and increases the risk of obesity as well as of MetS. In addition, *LEPR* Gln223Arg SNP (Q223R; A668G) has been found to be independently associated with components of metabolic syndrome i.e. impaired glucose tolerance, insulin resistance, and type 2 diabetes mellitus [16]. The result of this study advocates an association between the *LEPR* Gln223Arg SNP (Q223R; A668G) and higher leptin levels in the Indian population, which is consistent with findings in the Romanian [19] and Dutch [32] populations. However, contrary to this study's findings, the *LEPR* Gln223Arg (Q223R; A668G) polymorphism in Turkish children had no effect on leptin levels [18].

There have been conflicting findings regarding the relationship between the *LEPR* Q223R (A668G) and MetS. Arg/Arg and Gln/Arg genotypes of *LEPR* Gln223Arg SNP were significantly associated with the occurrence of MetS in this study. Similar allele and genotype frequencies of *LEPR* Q223R were observed in the Tunisian [33] and Brazilian [34] populations. In contrast to this finding, the R allele was significantly more prevalent in the Japanese population [35]. Similar to the findings of this study in the Indian population, Gottlieb et al. reported a significant association between *LEPR* Gln223Arg SNP and MetS in the Brazilian population [13], and Suriyaprom et al. discovered that *LEPR* Gln223Arg SNP significantly affects MetS susceptibility in the Thai population [25]. In contrast, Heo et al. found no association of the *LEPR* Q223R polymorphism with waist circumference and body mass index [36].

The significant genetic association of *LEPR* Gln223Arg SNP (Q223R; A668G) with the susceptibility of MetS in this study may be justified by increased serum leptin levels and the modified function of the leptin receptor. Further, leptin might affect the function and secretion of insulin by its action on leptin receptors in the beta cell of the pancreas [37]. Finally, there are certain limitations in the current study such as; it is a hospital-based case–control study, thus the recruited participants may not be a representative of the generalized population; secondly, the unavailability of the entire *LEPR* gene analysis or haplotype analysis, hence we could not address gene–gene or gene–environment interactions; thirdly, hormones analysis which affects leptin levels like estrogen were not done. From these respect, additional study with a larger sample size and broad spectrum of leptin receptor gene analysis is needed to fully evaluate the impact of SNP with the phenotypic expression of metabolic syndrome. Nonetheless, we believe that our findings suggest with enough evidence that *LEPR* Gln223Arg SNP (Q223R; A668G) seemed to be a significant genetic element in etiopathogenesis of metabolic syndrome and genetic variants might serve as a predictor for the susceptibility.

Conclusion

Our study revealed a significant association of *LEPR* Gln223Arg (Q223R; A668G) polymorphism in the *LepR* gene in Indian patients with increased risk of metabolic syndrome for R allele and Arg/Arg homozygote. These findings indicate that rs1137101 might be a pleiotropic locus for MetS and its components in studied population. In the future, well-designed studies with large sample sizes and broad genetic spectrum of adipocytokines are warranted.

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