Adipocyte 4:2, 135–140; April/May/June 2015; © 2015 Taylor & Francis Group, LLC

Genetic variants in leptin: Determinants of obesity and leptin levels in South Indian population

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Keywords: association study, BMI, fat percentage, leptin, leptin resistance, obesity

The revelation of leptin action mechanisms has led to various attempts to establish the association of polymorphisms in the leptin gene with obesity-related phenotypes. But, outcomes have been contradicting, which made the information on the role of the leptin gene in regulating the mechanism of pathophysiology of obesity inexplicable. Moreover, none of the studies are known to have similar implications on the Indian population. To address such contradictions, our study aims to evaluate the association of leptin gene polymorphism with obesity and leptin levels in a South Indian Population. A total of 304 cases (BMI≥27.5) and 309 controls (BMI≤23) from local inhabitants of Mysore, Karnataka were recruited for the study. The leptin gene variants rs7799039, rs2167270 and rs4731426 independently, as well as in 4 haplotype combinations, were found to be significantly associated with the risk of obesity. An increasing trend in BMI and leptin levels was observed with every addition of A and C minor alleles of exonic variant (rs2167270) and intronic variant (rs4731426) respectively. However, only AA genotype of SNP rs7799039 was positively associated with BMI. None of the SNPs were associated with fat percentage and waist to hip ratio. On a whole, this data suggests that the common polymorphisms in the leptin gene are strong predictors of obesity and leptin levels in South Indians.

The escalating rate of obesity throughout the world and its implications for health has obligated us to understand its etiology more clearly. This has led to studies identifying biomarkers for obesity and related disorders and validating their association in various ethnic populations. The exploration of genes encoding biomolecules for physiological systems that regulate energy balance has become the prominent trend in obesity research.

Since then, the emergence of studies that point to adipose tissue as an endocrine system producing various bioactive peptides called adipokines, has become a hot spot in biomedicine research. The basis of this interest, however, lies in a number of metabolic effects demonstrated by these molecules in linking obesity with different chronic disorders. Among them, leptin has invigorated obesity research because of its major role in the control of body-fat stores through co-ordinate regulation of feeding behavior, metabolism, neuroendocrine responses, the autonomic nervous system, and body energy balance.[1,2] Circulating concentrations of leptin have often been positively correlated with indices of adiposity such as weight, BMI, and percentage of fat mass, which further reflects its effect on the size of adipose tissue depot.[3–5]

Hence, it could be anticipated that genetic variations in the leptin gene can modulate its circulating levels and may affect various pathophysiological states, one of which is obesity. Therefore, studying the impact of such genetic variations and replicating studies in different homogeneous populations can provide a better understanding of the role leptin plays in obesity and related pathologies.

Multiple studies have highlighted the leptin gene as a prominent candidate in obesity, but the findings have been inconsistent. Ethnic diversity, phenotype heterogeneity, and sample size limitations could be considerable factors for these inconsistencies. Various polymorphisms in the leptin gene (LEP) have been linked to extreme obesity in a French study[6] and a Pennsylvanian population,[7] but not in Pima Indian sibling pairs.[8] Mammes et al.[9,10] were the first to show that the rs7799039 (G-2548A) variant in the promoter of LEP was associated with BMI reductions in overweight women. However, there are studies that failed to demonstrate this association.[11–13] To address these discordant observations, along with the necessity to conduct additional analyses particularly in countries with diverse populations like India, we proposed to investigate the possible influence of the different variants of leptin gene on obesity traits and leptin levels in a South Indian population.

The anthropometric and biochemical characteristics within the study population are summarized by the case-control group in **Table 1**. Both cases and controls were from the same age

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Submitted: 04/11/2014; Revised: 06/24/2014; Accepted: 10/07/2014
http://dx.doi.org/10.4161/21623945.2014.975538

Table 1. Comparison of continuous data among obese and lean subjects

Variables[b]	Lean (N = 309)	Obese (N = 304)	P value[a,c]
Age	46.88 ±16.03	46.37 ±11 .96	0.653
Height (cm)	1.60 ± 0.093	1.58 ± 0.089	**0.044**
Weight (kg)	52.40 ± 8.65	76.30 ±10 .75	**< 0.0001**
Waist (cm)	74.68 ± 9.80	94.34 ±10 .02	**< 0.0001**
Hip (cm)	86.56 ± 6.90	105.30 ± 9.42	**< 0.0001**
BMI (kg/m^2)	20.44 ± 2.18	30.21 ± 2.97	**< 0.0001**
Waist to hip ratio	0.85 ± 0.09	0.91 ± 0.102	**< 0.0001**
Body fat percentage	26.19 ± 7.62	36.98 ± 5.86	**< 0.0001**
Fasting glucose (mg/dl)	93.33 ± 38.93	104.22 ± 42.99	**0.001**
Postprandial glucose (mg/dl)	130.47 ± 72.92	145.31 ± 70.78	**0.011**
Leptin (ng/ml)	11.14 ± 7.82	30.71 ± 15.53	**< 0.0001**

[a]Independent t test P value. [b]Values are represented as mean and SD. [c]Significant p values are in boldface.

group. All variables (enlisted in **Table 1**) were significantly higher in obese than in lean subjects except that leans are comparatively taller. The frequency distributions of categorical variables are shown in **Table 2**. The percentages of females, post-menopausal women, non smokers and non alcoholics were significantly higher among obese and the percentage of people undergoing heavy physical activities are higher among the control group.

Table 3 depicts the minor allele frequencies of all variants in the LEP gene in obese and normal weight subjects. Hardy Weinberg's equilibrium was observed in both cases and controls for the genotypes of the variants except for the SNP rs17151919. Furthermore, the SNP rs17151919 was found to be monomorphic and so was not included in further analysis. Three SNPs in LEP gene viz rs7799039, rs2167270 and rs4731426 showed significantly higher minor allele frequencies in obese rather than in lean individuals.

Binary logistic regression (**Table 4**) was performed to test the association of obesity with genotypes of studied variants, under presumed genetic models after adjusting for the covariates: age, gender, smoking, alcoholism, menopausal stage, occupation,

Table 2. Frequency distribution of categorical variables across the groups

Variables[b]	Category	Lean (N = 309)	Obese(N = 304)	P value[a,c]
Gender	Male	167 (54%)	128 (42.1%)	**0.003**
	Female	142 (46%)	176 (57.9%)	
Smoking	Yes	46 (14.9%)	28 (9.2%)	**0.014**
	No	246 (79.6%)	268 (88.2%)	
	Quit	17 (5.5%)	8 (2.6%)	
Alcoholism	Yes	71 (23.0%)	66 (21.7%)	0.118
	No	226 (73.1%)	234 (77.0%)	
	Quit	12 (3.9%)	4(1.3%)	
Physical activity	Low	147 (48.4%)	180 (58.3%)	**0.014**
	Heavy	157 (51.6%)	129 (41.7%)	
Menopausal stage	Pre	24 (7.8%)	35 (11.5%)	**0.012**
	Peri	7 (2.3%)	20 (6.6%)	
	Post	63 (20.4%)	76 (25%)	
	No	48 (15.5%)	45(14.8%)	

[a]Chi square test P value. [b]Values are represented as frequency and percentage. [c]Significant p values are in boldface.

Table 3. Allele frequency distribution of LEP and LEPR variants among cases and controls

	Obese	Lean	
LEP SNPs	MAF	MAF	P value[a,b]
rs7799039 (A/G)	0.5395 (328)	0.432 (267)	**0.0001**
rs2167270 (A/G)	0.3734 (227)	0.2298 (142)	**4.251e-008**
rs4731426 (C/G)	0.5197 (316)	0.3447 (213)	**6.08e-010**
rs2071045 (C/T)	0.2056 (125)	0.2298 (142)	0.305
rs17151919 (A/G)	0 (0)	0.0016 (1)	0.3211

[a]Chi square P value for difference in allele frequency. [b]Significant p values are in boldface.

regular physical activity, fasting glucose, and fat percentage. Three variants of Leptin gene, a promoter variant rs7799039 ($P_{recessive} = 0.03775$), an exonic variant rs2167270 $P_{dominant} = 0.03314$, $P_{additive} = 0.00546$,$P_{recessive} = 0.008391$), and an intronic variant rs4731426 ($P_{additive} = 0.0007951$,$P_{recessive} = 4.016e^{-006}$) were significantly associated with risk of obesity. Models predict that only the AA genotype of rs7799039 is at risk of obesity; however, risk of obesity increases with the addition of A and C alleles of rs2167270 and rs4731426 variants respectively.

To assess the effect of LEP variants on traits defining obesity and leptin levels, linear regression analysis was performed assuming genetic models of inheritance (**Table 5**). Variants rs2167270 and rs4731426 were associated with BMI in additive and recessive models implying an increasing trend in BMI with every addition of A allele and C allele of the 2 variants respectively. However, SNP rs7799039 was positively associated with BMI in the recessive model, which predicts that genotype AA of rs7799039 had higher BMI than GA or GG. None of the SNPs were associated with fat percentage and waist to hip ratio.

The SNPs rs2167270, rs4731426, and rs7799039 showed a positive association with leptin levels. Individuals with AA genotype of rs7799039 variant were found to have higher leptin levels than GA and GG. However, there was a linear gradient in leptin levels with every addition of minor allele in the case of rs2167270 and rs4731426 polymorphisms. Variant rs2071045 did not show any significant associations. These results were consistent both before and after covariate adjustments.

The haplotype analysis is shown in **Table 6**. Variant rs17151919 was not included in haplotype analysis because of a significant deviation from Hardy-Weinberg proportions. Since rs7799039, rs2167270 and rs4731426 were in strong LD (i.e. D'≥0.8) they were used as a single block. Variants rs7799039 and rs4731426 were in stronger LD (D'≥ 0.9) with rs2167270 than with each other (LD=0.83). Analysis of observed haplotype in cases and controls revealed that out of 5 discovered haplotypes, 4 haplotypes, H1 (GGG), H2 (AAC), H4 (AGG), and H5 (GGC), were significantly associated with the risk of obesity. Higher frequency of H2 (AAC) (0.337) in cases than in controls (0.220) clearly indicates that minor alleles (A, A and C) of the above mentioned SNPs increases the risk of obesity. Vice versa, H1 (GGG) has a protective function against obesity. The higher

Table 4. Logistic regression analysis to evaluate disease-genotype association[a]

	Dominant		Additive		Recessive	
SNP	OR (95% CI)[b]	P value[c]	OR (95% CI)	P value	OR (95% CI)	P value
rs7799039	1.116(0.647–1.922)	0.6925	1.291 (0.922–1.808)	0.1366	1.837 (1.035–3.261)	**0.03775**
rs2167270	1.699 (1.043–2.766)	**0.03314**	1.682 (1.166–2.427)	**0.00546**	3.243 (1.352–7.78)	**0.008391**
rs4731426	1.34 (0.798–2.248)	0.2679	1.828 (1.285–2.599)	**0.0007951**	5.63 (2.701–11.74)	**4.016e–006**
rs2071045	0.8261(0.498–1.369)	0.4587	0.8979 (0.677–1.19)	0.4532	0.9641(0.455–2.043)	0.924

[a]Adjusted for age, gender, smoking and alcoholism, menopausal stage, regular physical activity, fasting glucose and fat percentage. [b]Values are represented as odds ratio and confidence interval. [c]Significant p values are in boldface.

frequencies of rare haplotypes H4 (0.081) and H5 (0.03) in obese compared to controls implies that there are stronger effects from minor alleles A and C of rs7799039 and rs4731426 for risk of obesity.

This study aimed to validate the associative inferences of frequently studied variants in the leptin gene with obesity risk. Since the leptin gene sequence is highly conserved, very few variations have been reported. A substantial number of investigations have been done on said polymorphisms for their effects on adiposity and related diseases, but very few studies have been executed in an Asian Indian population.

Among the common variants of the leptin gene, SNPs near the 5' untranslated region such as rs7799039 (G-2548A) and rs2167270 (also known as LEP 19G >A) in the 5' untranslated region of the first exon) has been most consistently associated with obesity. In the Family Heart Study, a common haplotype (49%) including SNP rs2167270, was associated with BMI and predicted to modify transcription-factor binding sites, and hence conclusively proposed to influence the transcription of the LEP gene.[14] Among Caucasians, the same SNP (rs2167270) showed a consistent association with all 3 anthropometric measures in a prospective study.[15] Similarly, we also observed an increase in BMI with the addition of A allele of rs2167270 variant. Despite its strong association with obesity, it is implausible to suggest the direct influence of such a variant on leptin expression and its function in pathophysiology of obesity as it lies within the first untranslated exon of the gene. However, it can be suggested that, being in linkage disequilibrium with the promoter polymorphism (rs7799039), as is the consensus with previous reports[16] and our own findings, rs2167270 may have an effect on gene transcription.

Our study also projected a sturdy association of the lep-2548AA genotype with the risk of obesity. In contrast to the studies in overweight Europeans, we found an inverse trend in a sample of Taiwanese Aborigines with extreme obesity,[17] in Brazilian women,[18] and in Finnish men,[19] where G allele displays as a minor allele and is associated with BMI. Our findings were in concordance with Tunisians,[20] Mexican women,[21] Whites[22] and a population in France.[10] Contrastingly, some of the studies showed a lack of association.[11–13] The discrepancies between the studies may be due to different ethnicity, characteristics of subjects or consideration of varied covariate for adjustment during analysis.

A prospective study reported an independent effect of rs2071045 and rs4731426 on weight regain on subjects following a dietary intervention.[23] The intronic SNP rs4731426 has also been moderately associated with median weight gain and is significantly associated with extreme weight gain in North Indian subjects in an olanzapine treatment group,[24] and was also reported to alter the binding of a transcription factor, zinc finger 5. Previous studies thus implied the influential role of these variants on the body's physiological response to diet. We, however, couldn't find an association of rs2071045 with any of the traits, but SNP rs4731426 could be strongly related to all adiposity parameters in the present study, which is in accordance with previous studies on Caucasians and African Americans.[15]

An increase in circulating leptin levels is directly proportional to size and number of adipocytes.[25] Thus, in obesity, increasing

Table 5. Linear regression analysis to evaluate the association of variants with obesity traits[a, b] and leptin[a,c]

	BMI		WHR		Fat percentage		Leptin	
SNP ID	Beta	P value[c]	Beta	P value[c]	Beta	P value[b]	Beta	P value[c]
Additive								
rs2167270	0.547	0.014					1.815	0.032
rs4731426	0.503	0.0165					1.718	0.017
Recessive								
rs7799039	0.604	0.0501					2.67	0.025
rs2167270	1.304	0.0068						
rs4731426	1.46	0.0001					3.736	0.004

[a]Only significant associations are depicted. [b]Adjusted for age, gender, smoking, and alcoholism, menopausal stage, regular physical activity, fasting glucose, and BMI. [c]Adjusted for age, gender, smoking, and alcoholism, menopausal stage, regular physical activity, fasting glucose, and fat percentage. [d]Values are represented as Beta values.

Table 6. Association of LEP gene haplotypes with obesity[a]

BLOCK	Haplotypes	Total frequency	Case frequencies	Control frequencies	OR	P value[b]
H1	GGG	0.471	0.391	0.549	30.958	**2.6363e-8**
H2	AAC	0.278	0.337	0.220	20.674	**5.4441e-6**
H3	AGC	0.117	0.128	0.107	1.253	0.2631
H4	AGG	0.081	0.062	0.100	6.098	0.0135
H5	GGC	0.030	0.047	0.014	11.439	**7.0 e-4**

[a]Haplotype frequencies were compared using Chi squared test. Haplotypes are defined by selected SNPs in LEP in the order – rs7799039 rs2167270 and rs4731426. [b]Significant p values are in boldface.

fat mass leads to an increase in leptin levels, which exacerbate many of the negative effects of weight gain, such as a local inflammatory response[26] and creating a positive feedback loop for feeding behavior through leptin resistance.[27] Our data shows elevated levels of leptin in obese compared to normal weight individuals perpetuating the idea that obesity is a leptin resistant state. Moreover, a positive association of the same variants with BMI and leptin levels in the present study substantiates the postulation.

Body weight is regulated by a set point mechanism[28] and by the production of leptin from adipocytes with its sensitization at the hypothalamus determining the set point equilibrium.[29,30] Thus, determination of genetic factors in the leptin gene are possibly involved in the regulation of leptin production could be important in defining this set point in obese individuals, and therefore, it may influence therapeutic interventions. The consistency of this idea resulted in several studies reporting an association of SNPs at 5' regulatory region (rs7799039 and rs2167270) with leptin levels and obesity.[9,11,13,18,31]

Previous studies have shown both increased[10,32] and decreased[13] levels of circulating leptin in subjects carrying the AA genotype of the leptin gene promoter rs7799039 polymorphism. We found increased levels of leptin only in the homozygous state of A allele. rs7799039 polymorphism is located at the 5' end of the promoter region of LEP, and it has been suggested that this polymorphism is close to a SP-1 transcription factor binding site, as well as 2 repetitive sequences, MER11 and Alu, that may regulate LEP transcription.[33] In this respect, it has been shown that nuclear extracts (nature unknown) derived from both U937 cells, and human adiposities are able to bind a DNA fragment spanning the rs7799039 polymorphic site. With the presence of nucleotide A at the -2548 position, DNA binding affinity was increased.[32] We also observed association of rs2167270 variant with hyperleptinemia with an additive effect of A allele as reported previously.[34] The same study predicted (using TESS) that, substitution of G allele by A allele of rs2167270 variant modifies a Transcription Factor Binding Site finally affecting leptin expression. Thus, it is conceivable that these polymorphisms could affect leptin transcription and synthesis but the mechanism behind the effect of the G to A substitution at both positions in leptin expression remains unclear.

One of the weaknesses of this study was low sample size, as a result of which we couldn't assess the effect of SNP (rs17151919) with rare frequency. Another was the lack of dietary or calorific intake data, which can be a potent confounder. Even though genotypic associations with leptin levels were adjusted for gender, fat percentage, and menopausal stage, the measurement of hormones such as estrogen and insulin, which have been reported to potentially affect leptin production, might have strengthened the result.

To summarize, common leptin gene polymorphisms are anticipated to regulate obesity traits by influencing leptin levels in a South Indian population. Even though speculation of the functional role of the variants rs7799039 and rs2167270 was beyond the scope of the study, but strong association with traits of obesity and leptin levels is evident to their effects in the determination of these phenotypes. The effect of SNP rs4731426 can be attributed to its strong LD with the aforementioned polymorphisms. More functional studies on these polymorphisms and replication in larger ethnic groups may be useful to better understand the mechanisms by which they influence leptin expression and metabolism contributing to adiposity.

A total of 613 subjects were included within the study consisting of 304 obese (BMI $\geq$ 27.5) and 309 lean (BMI$\leq$23)[35] with ages between 30–60 y. All participants inhabited urban areas of Mysore, Karnataka and were enrolled by subsequent health camps in random sectors within the city. Subjects with chronic disorders like diabetes or cardiovascular abnormality, thyroidism, pregnant or lactating women, participants under medication which might have influenced their body weight (e.g. glucocorticoid, pioglitazone, lipid lowering agents, anti depressants or under infertility treatment), and under some weight reduction regimes were excluded. The study was approved by institutional ethical committee, Anthropological Survey of India.

A standardized questionnaire was administered that included demographic, clinical such as personal and family history of chronic disorders, medication intake, regular physical activity, addictive behaviors, etc. Anthropometric measurements such as height, weight, waist (WC) and hip circumference (HC) were taken. Height was measured using Holtain Anthropometric rod and weight was taken with lighter clothing and no footwears. Waist circumference was measured from the midpoint of the bottom of the ribcage and the top of the lateral border of the iliac crest during minimal respiration. Body mass index (BMI) was calculated as weight in kilograms by height in meters squared and ratio of waist to hip circumference was also calculated. The Omron fat monitor was used to measure the total body-fat percentage. Physical activity during the day was categorized into high and low. High physical activity was attributed to persons who engage in heavy physical activity (i.e., resulting in sweating

and elevated pulse rate during 20 minutes or more at least 3 times per week) and less than those were grouped into the other category.[36]

Blood (after 12hours fasting) was collected with prior consents. Postprandial plasma glucose (PGLU) was measured after 2 hours of administering 75g of glucose to the subjects (WHO, OGTT). Plasma was immediately frozen until shipped to the laboratory for estimation of analytes. Fasting and postprandial glucose was estimated with the help of automated biochemical analyzer EM360 (Transasia, Germany) and ERBA kits. Leptin concentration in plasma was quantified using multiplex assay kits that utilize fluorescent microbead technology, allowing simultaneous quantification of several target proteins within a single plasma inoculation. These included pre-mixed and fully customized panels that utilize the Luminex xMAP technology platform (Luminex Corp, TX, USA). For Leptin intra assay variations was 3% and inter assay variation was 7% and minimal detectable range was 35pg/ml.

Genomic DNA was extracted from leucocytes using phenol-chloroform extraction. Most frequently studied 5 SNPs of leptin gene were identified from literature viz rs7799039, rs2167270, rs4731426, rs2071045 and rs17151919. SNPs were genotyped using the TaqMan allelic discrimination assay implemented on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).[37]

The characteristics of the study population were expressed as mean±standard deviation or ratios. Difference in the means and proportions were checked using independent t- test and chi-square test respectively. Further, chi-square test was used to check for the Hardy Weinberg equilibrium between the genotypic frequencies of the SNPs in cases, controls and total population and general allelic association with disease. Allelic and genotypic association with the phenotype both qualitative and quantitative traits, presuming various genetic models were assessed through linear and logistic regression analysis respectively. All the above tests were performed using SPSS version 17.0 and plink (http://pngu.mgh.harvard.edu/purcell/plink/) software. Haplotype associations were examined by the program Haploview (Haploview Web site, Whitehead Institute). The D' for all pairs of SNPs was calculated, and the haplotype blocks were estimated using the solid spine of LD method. The default settings were used for these analyses, which invoke extend spine if D'>0 .8.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We are thankful to all subjects in Mysore, Karnataka who voluntarily participated in this study and provided blood samples. We are grateful to Mr S Yaseen Saheb, Dr BV Raviprasad and Dr Jaishankar Rao for their guidance and support throughout the work. We are also thankful to Lions Club Mysore, Dr. Vikas Modi and Dr Ramakrishna of Amritakripa Hospital, Rupanagar, Mysore and paramedical staff for organizing medical camps and extending their valuable help during the collection of samples. Thanks are due to our colleagues Mr Abrar Alam, Mr Ranjith and Dr Pushpa Jogihalli for their sincere involvement at various levels in this project.

Funding

This work is a part of the Anthropological Survey of India's project "People of India: Bio cultural adaptations." We express our gratitude to the Director Anthropological Survey of India, Ministry of Culture, Government of India, Kolkata for supporting the project financially.

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