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Association *of LEP* G2548A and *LEPR* Gln223Arg Gene Polymorphism with Unexplained Infertility in North Indian Population

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Abstract

To investigate whether *LEP* G2548A and *LEPR* Gln223Arg gene polymorphism associated with the pathogenesis of unexplained infertility in north Indian population. This investigation randomly selected 229 female subjects of age group between 18 to 40 years (120 cases and 109 controls). At first, the members were classified into fertile and infertile. Further, they are separated based on BMI, non-obese (BMI: 18.5 to 24.5) and obese (BMI ≥ 25). The selected gene polymorphisms *LEP* G2548A and *LEPR* Gln223Arg were analyzed by polymerase chain response (PCR) followed by restriction fragment length polymorphism (RFLP). The univariate analysis reveals that Leptin, *LEP* G2548A and *LEPR* Gln223Arg genotypes, the most significant predictor of unexplained infertility (all *p*<0.05). The logistic regression analysis found that these three variables significant in multivariate analysis (all p<0.05) suggesting these as significant and independent predictors of unexplained infertility. The allele frequency of both *LEP* G2548A and *LEPR* Gln223Arg was found significantly different and higher in unexplained infertile than fertile. Moreover, the phenotype frequency of both *LEP* G2548A and *LEPR* Gln223Arg was also found significantly different and higher in unexplained infertile than fertile. G2548A was more frequent than *LEPR* Gln223Arg. This study suggested that high Leptin level and risk genotype increases the risk of infertility. Further investigations in other geographical region of India are to substantiate our finding.

Keywords: Unexplained Infertility; Leptin; Leptin Receptor; Obesity.

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INTRODUCTION

The obesity gene that encodes leptin was initially recognized by Freidman's group in 1994 at Rocke Feller University[1]. Leptin is a 16kDa peptide hormone, found on human chromosome 7q21.3 which encoded by leptin gene (LEP). In the circulation system LEP is discharged by adipose tissue, that ties to leptin receptors that encoded by the leptin receptor gene (LEPR) in the hypothalamus and sign by means of the Jinus kinase begin activator of transcription(JAK-STAT) signal transduction pathway to censored food intake and enhanced energy expenditure[2]. In human LEPR maps to chromosome1p31 and the protein has a five long and short isoform, which have indistinguishable transmembrane and extracellular domains but have different cytoplasmic doman length[3]. Leptin is involved in food intake regulation, immune functions energy balance, and fertility[4]. Leptin can create furthermore by human ovarian follicles-both in cumulus and granulosa cells. It was certified at mRNA and protein levels, human preovulatory follicles express the leptin gene quality[5]. Besides, it has been recognized that leptin may play a significant role in menstrual cycle. Moreover, ladies with anomalous articulation of the *ob* gene product are prone to infertility and menstrual irregularities[6]. Normally infertility is described as year of unprotected coitus along with conception and it is basic issue among reproductive age of men and women[7,8]. Few less study have been carried out in infertile male and female population to investigate the status of serum leptin. Around 30% of infertile couples after the traditional diagnostic evaluation experience unexplained infertility[9]. Negative diagnostic test outcomes would be anticipated if female age were the reason for interruption of fecundity, or when a defect be present in spite of the fact that cannot be found at present available tests. Even if diagnostic test outcomes are normal, the prognosis for live birth is just marginally better than that with different causes of infertility[9].

Leptin may affect on reproduction came from the insight ob/ob mice (lack functional leptin) or db/db mice (means leptin receptor lack) are fertile, and failed to undergo normal sexual development[10]. Recombinant leptin organization to these rodents diminished body weight and reestablished fertility to ob/ob mouse[11,12]. Similar findings have also been reported in human[13,14]. In a reproductive medicine clinic up to 25% of patients who present for investigation are diagnosed to have unexplained infertility[15]. 5 to 37% couples are unexplained infertility of the total proportion [16-19]. A significant difference between unexplained fertility and fertile female in serum leptin levels recommends that Leptin might be associated with pathophysiology of unexplained infertility[20].

SNPs (Single Nucleotide Polymorphisms) is the most broadly recognized hereditary. SNPs are single base substitution of the one nucleotide with another all through the human genome, in both exon (coding region) and entron region (non coding region) variations among people[21]. Researchers also found that the genes *LEP* and *LEPR* have been linked for polymorphisms that could possibly identify with the pathophysiology of obesity and its difficulties[22]. For example Mammes *et al*. (1998) were the first to show that the G2548A variation in the promoter of *LEP* gene was associated with a reduction in BMI in obese women[21].

LEP gene SNP containing substitution of nucleotide G to A at nucleotide - 2548 upstream of the ATG begin site in this gene promoter. The connection among G2548A (rs7799039) and with regards to the *LEPR* polymorphism, the A to G change in exon 6 at 668 nucleotides from the begin codon 223 *LEPR* Q223R (rs1137101) was related with impeded leptin-binding activity[22, 23, 24-28]. The *LEPR* Q223R polymorphism has been related to reduced BMI, blood pressure, leptin levels, and fat mass[29], however, some different examinations are in strife with these findings[30, 31]. There are a couple of studies dealing with the connection between *LEP* G2548A and *LEPR* Gln223Arg polymorphisms, obesity and daily energy intake. Significantly more important, these linked are showing up in a racial dependent design[32-34]. Likewise, the relationship of G2548A and Gln223Arg gene polymorphism with unexplained infertility among Indian has not been examined till date. Leptin plays vital role in reproductive biology of humans, especially in ovulation, and spermatogenesis. An alteration in the leptin flow may demonstrate to be a significant connection between body fat stores and status of fertility among childbearing women and men[35]. Such foundation may give new information into

the cause of infertility and might be prompt better treatment modalities in child bearing females.

As to the link between *LEP* G2548A and *LEPR* Gln223Arg gene polymorphism with leptin levels, the previous study advice a common gene polymorphism in the promoter *LEP* G2548A influencing secretion of adipose tissue and expression of Leptin[26] might be affecting the unexplained infertility in females. Hence, objective of the present study was to evaluate the link between *LEP* G2548A and *LEPR* Gln223Arg gene polymorphism with unexplained infertility. Moreover, we are also looking at whether *LEP* G2548A and *LEPR* Gln223Arg polymorphism may be associated with the pathogenesis of unexplained infertility in north Indian population.

MATERIALS AND METHODS

For smoothening this research work and earlier ethical approval were obtained from the institutional ethical committee vide letter no-2214/R-Cell-11. Ref. code: 53 ECMIIB/P1 from the King George's Medical University (K.G.M.U.) of Lucknow, India. The present case-control study was performed at the division of Obstetrics and Gynecology, KGMU, Lucknow. The study includes 229 female participants (120 cases and 109 controls females) which were arbitrarily chosen, aged 18 to 40 years. After writing concern, a 5 ml blood samples were collected. At the time of blood collection, information about high and weight (Waist: WC); hip circumference (HC) was recorded (only females) by trained researchers. All volunteers were asked for some information, for example family history of obesity and hereditary diseases. Patients who are on hormonal treatment and pregnant were excluded from this study. The case group (unexplained infertile female) was compared with control group (normal fertile female) for finding the contribution of serum leptin level in the causation of unexplained fertility. Subjects were categorized first into unexplained infertile and fertile group and then further subdivided into subcategories on the basis of BMI, non obese (BMI range 18.5 to 24.5) and obese (BMI more than 25). Leptin level was quantified by Active human leptin ELISA kit unit and BMI was calculated as weight in kg/height in m^2.

Genetic analysis

Blood DNA was isolated and purified by using the classical phenol chloroform Method. Samples were collected in EDTA vials. Approx. 40 ng of the DNA was used for each PCR reaction. DNA was quantified by measuring the optical density at 260nm. 1µl of stock genomic DNA was placed on the plate and the OD was taken at 260nm (Thermo Scientific). DNA concentration of the samples ranged between 100ng to 250ng. The DNA was further diluted to 40ng for using in the PCR reaction. The purity was determined by calculation of absorbance at 260nm to absorbance at 280 nm. DNA samples were aliquoted and one aliquot which was in regular use was kept at 4°C while the rest of the samples were stored at -20°C.

Amplification of LEP G2548A and LEPR Gln223Arg gene

The genetic polymorphism was analyzed by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) method. Isolated genomic DNA was amplified by PCR thermo cycler (Applied Biosystem, Germany) using PCR condition 94 °C for 4 minutes, 35 cycles at 94 °C for 30 second, 58 °C for 40 second, 72 °C for 40 second and lastly 72 °C for 8 minutes. The forward and reversed primers for *LEP* gene were 5'-TTTCCTGTAATTTTCCCGTGAG-3' and 5'-AAAAGCAAAGACAGGCATAAA-3'repectively. For *LEPR* gene 5'-AGT TCA AAT AGA GGT CCA AAT CA-3' and 5' –TTC TGA GGT TGT GTC ACT GGC A-3'respectively. Reaction mixture 100 ng of DNA, 4nM of each primer, 2.5 mM of dNTPs, 2.5 mM of $MgCl_2$, 0.025 U Taq polymerase, and 1xPCR buffer (Invirtogen). Initial melting step of 2 minute at 94 °C, followed by 35 cycle of 30 second at 94 °C, 45 second at 60 °C (for LEP gene), 45 second at 56 °C (for LEPR gene), and 45 second at 72 °C, and final elongation was 6 minute at 72 °C. After PCR, amplified product (242bp for LEP and 367bp for LEPR) was digested with restriction enzyme *Cfo* I for LEP gene and *MspI* for LEPR gene for 2 hours at 37 °C. The digested PCR products (181bp, 61bp and 242bp, 125 bp, respectively) were separated on 3 % Agarose gel.

Statistical analysis

Discrete (categorical) data were summarized in numbers and percentage. Categorical data were compared with the help of Chi square test. The proportions were compared with Z test after corrections for continuity (Z_c).

Table 1. Relationship of LEP G2548A gene polymorphism with clinical parameters of fertile and unexplained infertile group

Variables	Fertile (n=109)				Unexplained infertile (n=120)				Fertile vs. Infertile
	G/G (n=39) (%)	G/A (n=51) (%)	A/A (n=19) (%)	*p* value	G/G (n=22) (%)	G/A (n=59) (%)	A/A (n=39) (%)	*p* value	*p* value
Age (yrs):									
≤ 30	26 (66.7)	39 (76.5)	12 (63.2)	0.439	13 (59.1)	44 (74.6)	26 (66.7)	0.373	0.008*
> 30	13 (33.3)	12 (23.5)	7		9	15 (25.4)	13 (33.3)		0.285
			-36.8		-40.9				
BMI (kg/m^2):									
< 25	27 (69.2)	32 (62.7)	11 (57.9)	0.669	20 (90.9)	39 (66.1)	18 (46.2)	0.002*	0.213
≥ 25	12 (30.8)	19 (37.3)	8		2	20 (33.9)	21 (53.8)		0.002*
			-42.1		-9.1				
WHR:									
< 1	29 (74.4)	38 (74.5)	13 (68.4)	0.864	15 (68.2)	49 (83.1)	27 (69.2)	0.191	0.007*
≥ 1	10 (25.6)	13 (25.5)	6		7	10 (16.9)	12 (30.8)		0.232
			-31.6		-31.8				
TSH (mIU/ml):									
< 3	13 (33.3)	14 (27.5)	5	0.79	15 (68.2)	34 (57.6)	21 (53.8)	0.545	0.089
≥ 3	26 (66.7)	37 (72.5)	-26.3		7	25 (42.4)	18 (46.2)		0.015
			14 (73.7)		-31.8				
LH (mIU/ml):									
< 7	17 (43.6)	19 (37.3)	6	0.656	19 (86.4)	41 (69.5)	27 (69.2)	0.273	0.036
≥ 7	22 (56.4)	32 (62.7)	-31.6		3	18 (30.5)	12 (30.8)		0.021
			13 (68.4)		-13.6				
FSH (mIU/ml):									
< 8	22 (56.4)	32 (62.7)	12 (63.2)	0.804	10 (45.5)	19 (32.2)	15 (38.5)	0.524	0.142
≥ 8	17 (43.6)	19 (37.3)	7		12 (54.5)	40 (67.8)	24 (61.5)		0.01
			-36.8						
PRL (mg/mL):									
<15	25 (64.1)	19 (37.3)	10 (52.6)	0.04	11 (50.0)	34 (57.6)	21 (53.8)	0.816	0.002
≥ 15	14 (35.9)	32 (62.7)	9		11 (50.0)	25 (42.4)	18 (46.2)		0.122
			-47.4						
Leptin (pg/ml):									
< 5	27 (69.2)	30 (58.8)	14 (73.7)	0.408	11 (50.0)	24 (40.7)	5 (12.8)	0.003*	0.196
≥ 5	12 (30.8)	21 (41.2)	5		11 (50.0)	35 (59.3)	34 (87.2)		0.003*
			-26.3						

1st and 2nd p value is the 2x3 chi squire comparisons between lower to higher subgroup within fertile and infertile group separately. Third p value is the 2x3 comparison between lower to lower and higher to higher subgroup between fertile and infertile group of respective parameters. *Asterisk indicates p< 0.05.

Table 2. Relationship of LEPR Gln223Arg gene polymorphism with clinical parameters of fertile and unexplained infertile groups.

Variables	Fertile (n=109)				Unexplained infertile (n=120)				Fertile vs. Infertile
	Gln/Gln (n=67) (%)	Gln/Arg (n=341) (%)	Arg/Arg (n=8) (%)	*p* value	Gln/Gln (n=52)(%)	Gln/Arg (n=46) (%)	Arg/Arg (n=22) (%)	*p* value	*p* value
Age (yrs):									
≤ 30	50 (74.6)	22 (64.7)	5 (62.5)	0.51	37 (71.2)	32 (69.6)	14 (63.6)	0.813	0.020*
> 30	17 (25.4)	12 (35.3)	3 (37.5)		15 (28.8)	14 (30.4)	8 (36.4)		0.333
BMI (kg/m^2):									
< 25	46 (68.7)	17 (50.0)	7 (87.5)	0.065	36 (69.2)	29 (63.0)	12 (54.5)	0.475	0.069
≥ 25	21 (31.3)	17 (50.0)	1 (12.5)		16 (30.8)	17 (37.0)	10 (45.5)		0.020*
WHR:									
< 1	49 (73.1)	25 (73.5)	6 (75.0)	0.993	40 (76.9)	36 (78.3)	15 (68.2)	0.643	0.048
≥1	18 (26.9)	9 (26.5)	2 (25.0)		12 (23.1)	10 (21.7)	7 (31.8)		0.133
TSH (mIU/ml):									
< 3	20 (29.9)	9 (26.5)	3 (37.5)	0.819	31 (59.6)	27 (58.7)	12 (54.5)	0.92	0.219
≥3	47 (70.1)	25 (73.5)	5 (62.5)		21 (40.4)	19 (41.3)	10 (45.5)		0.03
LH (mIU/ml):									
< 7	24 (35.8)	16 (47.1)	2 (25.0)	0.393	38 (73.1)	33 (71.7)	16 (72.2)	0.989	0.089
≥ 7	43 (64.2)	18 (52.9)	6 (75.0)		14 (26.9)	13 (28.3)	6 (27.3)		0.104
FSH (mIU/ml):									
< 8	39 (58.2)	22 (64.7)	5 (62.5)	0.814	17 (32.7)	20 (43.5)	7 (31.8)	0.473	0.088
≥ 8	28 (41.8)	12 (35.3)	3 (37.5)		35 (67.3)	26 (56.5)	15 (68.2)		0.075
PRL (mg/mL):									
<15	33 (49.3)	19 (55.9)	2 (25.0)	0.29	26 (50.0)	29 (63.0)	11 (50.0)	0.377	0.018
≥ 15	34 (50.7)	15 (44.1)	6 (75.0)		26 (50.0)	17 (37.0)	11 (50.0)		0.265
Leptin (pg/ml):									
< 5	40 (59.7)	24 (70.6)	7 (87.5)	0.215	21 (40.4)	12 (26.1)	7 (31.8)	0.321	0.505
≥ 5	27 (40.3)	10 (29.4)	1 (12.5)		31 (59.6)	34 (73.9)	15 (68.2)		0.002*

1st and 2nd p value is the 2x3 chi squire comparisons between lower to higher subgroup within fertile and infertile group separately. Third p value is the 2x3 comparison between lower to lower and higher to higher subgroup between fertile and infertile group of respective parameters. : *Asterisk indicates p< 0.05

Univariate and multivariate logistic regression analysis was done to predict independent predictors of unexplained infertility, considering group as variable (dependent) and clinical characteristics like Age, WHR, BMI and Leptin, and LEP G2548A and LEPR Gln223Arg genotypes the independent variables. The clinical characteristics were sub grouped into low and high on the basis of median value of both fertile and unexplained groups except BMI. For BMI the standard cutoff of normal (BMI: <25 kg/m^2) and overweight (BMI: ≥25 kg/m^2) was used. The control (fertile) genotypes data of both SNPs *LEP* G2548A and *LEPR* Gln223Arg are in HWE (Hardy-Weinberg equilibrium) tested by goodness of fit chi squire. The two tailed p<0.05 was set for the entire experiment. All the statistical analysis was done by Statistical package for social sciences (SPSS, version 16, SPSS inc, Chicago, IL, U.S.A)[36].

RESULTS

Table 1 summarized the clinical characteristics of case (unexplained infertile) and control (fertile) group. Evaluate the frequency (%) distribution of discrete (low and high) variables of two groups, χ^2 test showed Leptin frequency in higher subgroup compared to lower of the case than the fertile group. On the other hand, the distribution of age, BMI & WHR not differed among case and control groups. The association of *LEP* G2548A genotypes with basic clinical characteristics of selected groups is shown in Table 1. Infertile groups, *LEP* G2548A genotypes did not show a significant association with any of the clinical characteristics.

However, in the unexplained infertile group it showed significant association with BMI (p=0.002) as well as Leptin level (*p*=0.003). The higher concentration of TSH and lower PRL is significantly false positive associated with in unexplained infertile in this study.

Further, comparing the genotypes among the case and control group, the genotype showed significant association with lower age (p=0.008), WHR (*p*=0.007), while significant association with higher BMI (*p*=0.002), and Leptin (*p*=0.003). In contrast, LEPR Gln223Arg did not show a significant association with any of the clinical characteristics

Table 3. Frequency distribution of LEP G-2548A and LEPR Gln223Arg polymorphisms between two groups (Wild: G/G or Gln/Gln, Heterozygous: G/A or Gln/Arg, Homogygous mutant: A/A or Arg/Arg)

SNPs	Characteristics	Fertile (n=109) (%)	Unexplained infertile (n=120) (%)	*P* Value
LEP G2548A	*Genotypes:*			
	G/G	39 (35.8)	22 (18.3)	0.003
	G/A	51 (46.8)	59 (49.2)	
	A/A	19 (17.4)	39 (32.5)	
	Allele:			
	G	129 (59.2)	103 (42.9)	<0.001
	A	89 (40.8)	137 (57.1)	
	Phenotype:			
	A+	70 (64.2)	98 (81.7)	0.001
LEPR Gln223Arg	*Genotypes:*			
	Gln/Gln	67 (61.5)	52 (43.3)	0.008
	Gln/Arg	34 (31.2)	46 (38.3)	
	Arg/Arg	8 (7.3)	22 (18.3)	
	Allele:			
	Gln	168 (77.1)	150 (62.5)	<0.001
	Arg	50 (22.9)	90 (37.5)	
	Phenotype:			
	Arg+	42 (38.5)	68 (56.7)	0.016

*Asterisk indicates p< 0.05; A+: sum of G/A and A/A; Arg+= sum of Gln/Arg and Arg/Arg

Table 4. Relationship of LEP G2548A gene polymorphism with clinical parameters of fertile and unexplained infertile group using dominant and recessive model

Variables	Fertile (n=109)				Unexplained infertile (n=120)				Fertile vs. Infertile
	GG	GA	AA	*p* value	GG	GA	AA	*p*value	*P*- value
Age (yrs):		Dom GG vs GA+AA			Dom GG vs GA+AA				
≤ 30	26 (66.7)	51 (139.7)		0.49	13 (59.1)	70 (141.3)		0.27	0.007*
> 30	13 (33.3)	19 (60.3)			9 (40.9)	28 (58.6)			0.14
		Rec GG+ GA vs AA				Rec GG+ GA vs AA			
≤ 30	65 (143.2)	12 (63.2)		0.43	57 (133.7)	26 (66.7)		0.68	0.01*
> 30	25 (56.8)	7 (36.8)			24 (66.3)	13 (33.3)			0.22
BMI (kg/m^2):		Dom GG vs GA+AA			Dom GG vs GA+AA				
< 25	27 (69.2)	43 (120.6)		0.42	20 (90.9)	57 (112.3)		0.008	0.1
≥ 25	12 (30.8)	27 (79.4)			2 (9.1)	41 (87.7)			NA
		Rec GG+ GA vs AA				Rec GG+ GA vs AA			
< 25	59 (131.9)	11 (57.9)		0.52	59 (157)	18 (46.2)		0.004	0.24
≥ 25	31 (68.1)	8 (42.1)			22 (43)	21 (53.8)			0.007*
WHR:		Dom GG vs GA+AA			Dom GG vs GA+AA				
< 1	29 (74.4)	51 (142.9		0.86	15 (68.2)	76 (152.3)		0.35	0.003*
≥ 1	10 (25.6)	19 (57.1)			7 (31.8)	22 (47.7)			0.38
		Rec GG+ GA vs AA				Rec GG+ GA vs AA			
< 1	67 (148.9)	13 (68.4)		0.59	64 (151.3)	27 (69.2)		0.24	0.05
≥ 1	23 (51.1)	6 (31.6)			17 (48.7)	12 (30.8			0.08
TSH (mIU/ml):		Dom GG vs GA+AA			Dom GG vs GA+AA				
< 3	13 (33.3)	19 (53.8)		0.49	15 (68.2)	55 (111.4)		0.29	0.05
≥ 3	26 (66.7)	51 (146.2)			7 (31.8)	43 (88.6)			0.01*
		Rec GG+ GA vs AA				Rec GG+ GA vs AA			
< 3	27 (60.8)	5 (26.3)		0.75	49 (125.8)	21 (53.8)		0.48	0.122
≥ 3	63 (139.2)	14 (73.7)			32 (74.2)	18 (46.2)			0.23
LH (mIU/ml):		Dom GG vs GA+AA			Dom GG vs GA+AA				
< 7	17 (43.6)	25 (68.9)		0.42	19 (86.4)	68 (138.7)		0.17	0.02*
≥ 7	22 (56.4)	45 (131.1)			3 (13.6)	30 (61.3)			NA
		Rec GG+ GA vs AA				Rec GG+ GA vs AA			
< 7	36 (80.9)	6 (31.6)	0.49	60 (155.9)	27 (69.2)	0.57	0.05		
≥ 7	54 (119.1)	13 (68.4)		21 (44.1)	12 (30.8)		0.06		
FSH (mIU/ml):		Dom GG vs GA+AA			Dom GG vs GA+AA				
< 8	22 (56.4)	44 (125.9)		0.5	10 (45.5)	34 (70.7)		0.34	0.23
≥ 8	17 (43.6)	26 (74.1)			12 (54.5)	64 (129.3)			0.003*
		Rec GG+ GA vs AA				Rec GG+ GA vs AA			
< 8	54 (101.4)	12 (63.2)		0.79	29 (77.7)	15 (38.5)		0.77	0.06
≥ 8	36 (80.9)	7 (36.8)			52 (122.3)	24 (61.5)			0.07
PRL (mg/mL):		Dom GG vs GA+AA	0.02		Dom GG vs GA+AA	0.6			
<15	25 (64.1)	29 (89.9)			11 (50.0)	55 (111.4)			0.004*
≥ 15	14 (35.9)	41 (110.1)			11 (50.0)	43 (88.6)			0.52

FSH (mIU/ml):		Dom GG vs GA+AA		Dom GG vs GA+AA				
< 8	22 (56.4)	44 (125.9)	0.5	10 (45.5)	34 (70.7)	0.34	0.23	
≥ 8	17 (43.6)	26 (74.1)		12 (54.5)	64 (129.3)		0.003*	
		Rec GG+ GA vs AA		Rec GG+ GA vs AA				
< 8	54 (101.4)	12 (63.2)	0.79	29 (77.7)	15 (38.5)	0.77	0.06	
≥ 8	36 (80.9)	7 (36.8)		52 (122.3)	24 (61.5)		0.07	
PRL (mg/mL):		Dom GG vs GA+AA	0.02	Dom GG vs GA+AA	0.6		0.004*	
<15	25 (64.1)	29 (89.9)		11 (50.0)	55 (111.4)		0.52	
≥ 15	14 (35.9)	41 (110.1)		11 (50.0)	43 (88.6)			
		Rec GG+ GA vs AA		Rec GG+ GA vs AA		0.86	0.09	
<15	44 (101.4)	10 (52.6)	0.76	45 (107.6)	21 (53.8)		0.05	
≥ 15	46 (98.6)	9 (47.4)		36 (92.4)	18 (46.2)			
Leptin (pg/ml):		Dom GG vs GA+AA		Dom GG vs GA+AA				
< 5	27 (69.2)	44 (100)	0.002	11 (50)	29 (53.5)	0.06	0.26	
≥ 5	12 (30.8)	26 (67.5)		11 (50)	69 (146.5)		0.02*	
		Rec GG+ GA vs AA		Rec GG+ GA vs AA				
< 5	57 (128)	14 (73.7)	0.12	35 (90.7)	5 (12.8)	0.001	0.33	
≥ 5	53 (72)	5 (26.3)		46 (109.3)	34 (87.2)		0.001*	

1st and 2nd p value is the 2x2chi squire comparisons between lower to higher subgroup within fertile and infertile group separately. Third p value is the 2x2comparison between lower to lower and higher to higher subgroup between fertile and infertile group of respective parameters. : Asterisk indicates p< 0.05; Dom: Dominant; Rec: Recessive; (*p<0.05); NA: not applicable

in both the groups (Table 2). However, between the two groups, it showed significant association with lower age (p=0.020), WHR (p=0.048) and while a significant link with higher BMI (p=0.020), and Leptin (p=0.002).

Table 3 summarized the genotype frequency distribution of *the LEP* G2548A *gene* (Wild: G/G, Heterozygous: G/A and Homozygous mutant: A/A) and gene LEPR Gln223 Arg (Wild: Gln/Gln, Heterozygous: Gln/Arg and Homozygous mutant: Arg/Arg). The χ^2 test showed significantly different and higher homozygous mutant frequency of both *LEP* G2548A (A/A: 17.4% vs. 32.5%, p=0.003) and *LEPR* Gln223Arg (Arg/Arg: 7.3% vs. 18.3%, p=0.008) in unexplained infertile than the fertile group. Further, the allele frequency of both *LEP* G2548A (A: 40.8% vs. 57.1%, p<0.001) and *LEPR* Gln223Arg (Arg: 22.9% vs. 37.5%, p<0.001) was also found significantly different and higher in unexplained infertile than fertile. Moreover, the phenotype frequency of both *LEP* G2548A (A+: 64.2% vs. 81.7%, p=0.001) and LEPR Gln223Arg (Arg+: 38.5% vs. 56.7%, p=0.016) was also found significantly different and higher in unexplained infertile than fertile. Further , using dominant model, genotype was also observed tobe associated with unexplained infertility as compaired to their wild type genotype for both polymorphism (table 4,and 5). Unfortunately, some calculation such as BMI>25;LH<7 in LEPG2548A gene polymorphism in dominant model and age >30;BMI>25;WHR>1;TSH<3;LH< and >7; FSH, PRL and Leptin in recessive model was restricted by small number of participants (table 4 and 5)

The univariate and multivariate regression analysis was done in evaluating out independent predictors of unexplained infertility the clinical characteristics and genotypes of both the SNPs were regressed against fertile and unexplained infertile groups which are summarized in Table 6. The univariate analysis found that the level of leptin, gene LEP G2548A as well as *LEPR* Gln223Arg genotypes the significant (p<0.05 or p<0.01 or p<0.001) predictors of unexplained infertility. This univariate statistical analysis further found these variables significant (p<0.05 or p< 0.01 or p<0.001) in the further multivariate analysis suggesting these as significant and independent predictors of unexplained infertility.

Table 5. Relationship of LEPR Gln223Arg gene polymorphism with clinical parameters of fertile and unexplained infertile groups using dominant and recessive model

Variables	Fertile (n=109)				Unexplained infertile (n=120)				Fertile vs. Infertile
	Gln/Gln	Gln/Arg	Arg/Arg	*p* value	Gln/Gln	Gln/Arg	Arg/Arg	*p*value	*P*- value
Age (yrs):	Dom Gln/Gln vs Gln/Arg+Arg/Arg				Dom GG vs GA+AA				
≤ 30	50(74.6)	27(127.2)		0.24	37(71.2)	46(133.2		0.68	0.009*
> 30	17 (25.4)	15(72.8)			15(28.8)	22(66.8)			0.29
	Rec Gln/Gln +Gln/Arg Vs Arg/Arg				Rec Gln/Gln +Gln/Arg Vs Arg/Arg				
≤ 30	72(139.3)	5(62.5)		NA	69(140.8)	14(63.6)		0.55	0.04
> 30	29(60.7)	3(37.5)			29(59.2)	8(36.4)			NA
BMI (kg/m^2):	Dom Gln/Gln vs Gln/Arg+Arg/Arg				Dom Gln/Gln vs Gln/Arg+Arg/Arg				
< 25	46(68.7)	24(137.5)		0.22	36(69.2)	46(68.7)		1.0	0.007*
≥ 25	21(31.3)	18(62.5)			16(30.8)	21(31.3)			0.35
	Rec Gln/Gln +Gln/Arg Vs Arg/Arg				Rec Gln/Gln +Gln/Arg Vs Arg/Arg				
< 25	63(118.7)	7(87.5)		NA	65(132.2)	12(54.5)		0.29	0.31
≥ 25	38(81.3)	1(12.5)			33(67.8)	10(45.5)			NA
WHR:	Dom Gln/Gln vs Gln/Arg+Arg/Arg				Dom Gln/Gln vs Gln/Arg+Arg/Arg				
< 1	49(73.1)	31(148.5)		0.92	40(76.9)	51(146.5)		0.81	0.02*
≥ 1	18(26.9)	11(51.5)			12(23.1)	17(53.5)			0.11
	Rec Gln/Gln +Gln/Arg Vs Arg/Arg				Rec Gln/Gln +Gln/Arg Vs Arg/Arg				
< 1	74(146.6)	6(75)		NA	76(155.2)	15(68.2)		0.35	0.07
≥ 1	27(53.4)	2(25)			22(44.8)	7(31.8)			NA
TSH (mIU/ml):	Dom Gln/Gln vs Gln/Arg+Arg/Arg				Dom Gln/Gln vs Gln/Arg+Arg/Arg				
< 3	20(29.9)	12(64)		0.88	31(59.6)	39(113.2)		0.80	0.08
≥ 3	47(70.1)	30(136)			21(40.4)	29(86.8)			0.03
	Rec Gln/Gln +Gln/Arg Vs Arg/Arg				Rec Gln/Gln +Gln/Arg Vs Arg/Arg				
<3	29(56.4)	3(37.5)		NA	58(118.3)	12(54.5)		0.68	NA
≥ 3	72(143.6)	5(62.5)			40(81.7)	10(45.5)			0.02
LH (mIU/ml):	Dom Gln/Gln vs Gln/Arg+Arg/Arg				Dom Gln/Gln vs Gln/Arg+Arg/Arg				
< 7	24(35.8)	18(72.1)		0.46	38(73.1)	49(143.9)		0.88	0.15
≥ 7	43(64.2)	24(127.9)			14(26.9)	19(55.6)			0.03
	Rec Gln/Gln +Gln/Arg Vs Arg/Arg				Rec Gln/Gln +Gln/Arg Vs Arg/Arg				
< 7	40(82.9)	2(25)	NA	71(144.8)	16(72.2)	1.0	NA		
≥ 7	61(117.1)	6(75		27(55.2)	6(27.3)		NA		
FSH (mIU/ml):	Dom Gln/Gln vs Gln/Arg+Arg/Arg				Dom Gln/Gln vs Gln/Arg+Arg/Arg				
< 8	39(58.2)	27(127.2)		0.53	17(32,7)	27(75.3)		0.43	0.035
≥ 8	28(41.8)	15(72.8)			35(67.3)	41(124.7)			0.04
	Rec Gln/Gln +Gln/Arg Vs Arg/Arg				Rec Gln/Gln +Gln/Arg Vs Arg/Arg				
< 8	61 (122.9	5 (62.5)		NA	37 (76.2)	7 (31.8)		0.60	NA
≥ 8	40 (77.1)	3 (35.3)			61 (123.6)	15 (68.2)			NA
PRL (mg/mL):	Dom Gln/Gln vs Gln/Arg+Arg/Arg				Dom Gln/Gln vs Gln/Arg+Arg/Arg				

< 8	39(58.2)	27(127.2)	0.53	17(32.7)	27(75.3)	0.43	0.035
≥ 8	28(41.8)	15(72.8)		35(67.3)	41(124.7)		0.04
	Rec Gln/Gln +Gln/Arg Vs Arg/Arg			Rec Gln/Gln +Gln/Arg Vs Arg/Arg			
< 8	61 (122.9	5 (62.5)	NA	37 (76.2)	7 (31.8)	0.60	NA
≥ 8	40 (77.1)	3 (35.3)		61 (123.6)	15 (68.2)		NA
PRL (mg/mL):	Dom Gln/Gln vs Gln/Arg+Arg/Arg			Dom Gln/Gln vs Gln/Arg+Arg/Arg			
<15	33 (49.3)	21 (80.9)	0.92	26 (50)	40 (113)	0.33	0.01
≥ 15	34 (50.7)	21 (119.1)		26 (50)	28 (87)		0.17
	Rec Gln/Gln +Gln/Arg Vs Arg/Arg			Rec Gln/Gln +Gln/Arg Vs Arg/Arg			
<15	52 (105)	2 (25)	NA	55 (113)	11 (50)	0.60	NA
≥ 15	49 (94.8)	6 (75)		43 (87)	11 (50)		NA
Leptin (pg/ml):	Dom Gln/Gln vs Gln/Arg+Arg/Arg			Dom Gln/Gln vs Gln/Arg+Arg/Arg			
< 5	40 (59.7)	31 (158.1)	0.13	21 (26.1)	21 (57.9)	0.23	0.5
≥ 5	27 (40.3)	11 (41.9)		31 (73.9)	49 (142.1)		0.01*
	Rec Gln/Gln +Gln/Arg Vs Arg/Arg			Rec Gln/Gln +Gln/Arg Vs Arg/Arg			
< 5	64 (130)	7 (87.5)	NA	33 (66.5)	7 (31.8)	0.86	NA
≥ 5	37 (69.7)	1 (12.5)		65 (133.5)	15 (68.2)		NA

1st and 2nd p value is the 2x2chi squire comparisons between lower to higher subgroup within fertile and infertile group separately. Third p value is the 2x2comparison between lower to lower and higher to higher subgroup between fertile and infertile group of respective parameters. : Asterisk indicates p< 0.05; Dom: Dominant; Rec: Recessive; (* p<0.05); NA: not applicable

DISCUSSION

Apparently, this is the first Indian study to look at the association of polymorphism of LEP G2548A and LEPR Gln223Arg with unexplained infertility among North Indian population. The key findings of this work were that leptin levels were higher in UI (unexplained infertility) group as compared to control, and that the LEP G2548A and LEPR Gln223Arg polymorphism demonstrated a critical relationship between leptin focus and UI among North Indians.

In the present investigation, no difference was found with weight, height and BMI, screened crosswise genotypes in the obese compared with no obese group. Likewise, when the subject was divided into female and male, the difference was found to be null in BMI and others parameters. This result is consistent with two studies, which identified no relationship between Gln223Arg, BMI and leptin levels[29,37].

In the present study, the χ^2 test showed significantly different and higher homozygous mutant frequency, the allele frequency and phenotype frequency of both *LEP* G2548A and *LEPR* Gln223Arg in unexplained infertile than the fertile group. Infertile groups, *LEP* G2548A genotypes did not show any kind of association with clinical parameters. Moreover, in the unexplained infertile group it showed significant association with BMI and Leptin. In addition, in population, no association was found between the LEP-2548G/A polymorphism and obese and their related variables. On the other hand, higher level of leptin was acknowledged in-2548GG carriers[38]. In other research, workers found that there was no association between LEP-2548G/A polymorphism and obesity in Tunisian obese and their control group[26]. But, same author like Le *et al*. (2000) stated that there was a relationship of this polymorphism with extremely obese subjects in North American, Caucasian ladies, in whom the G allele was more frequent[25].

Sahin Daniz Say *et al*. (2013) reveals that gene LEP 2548 AA or AG genotype are key predictor for increased leptin concentration and BMI in obese subjects and reveals that it might be a useful marker for risk of obesity [32]. However in this present study show, in unexplained infertile group it showed a link with BMI (*p*=0.002) and Leptin (*p*

Table 6. Identification of predictors of unexplained infertility among clinical characteristics and gene polymorphisms using logistic regression analysis

Predictors	Univariate Analysis		Multivariate analysis	
	Odds Ratio (OR), 95% CI	*P* value	Odds Ratio (OR), 95% CI	*P* value
Age (yrs):				
≤ 30	Ref		Ref	
> 30	1.07 (0.61-1.89)	0.808	0.93 (0.43-2.00)	0.848
BMI (kg/m^2):				
< 25	Ref		Ref	
≥ 25	1.00 (0.58-1.72)	0.993	0.43 (0.19-0.96)	0.038*
WHR:				
< 1	Ref		Ref	
≥ 1	0.88 (0.48-1.60)	0.672	1.06 (0.48-2.34)	0.885
Leptin (pg/ml):				
< 5	Ref		Ref	
≥ 5	3.74 (2.16-6.46)	<0.001	5.24 (2.47-11.11)	<0.001*
LEP G2548A:				
G/G	Ref		Ref	
G/A	2.05 (1.08-3.90)	0.029	4.71 (1.86-11.97)	0.001*
A/A	3.64 (1.71-7.76)	0.001	6.00 (2.08-17.31)	0.001*
LEPR Gln 223Arg				
Cln/Gln	Ref		Ref	
Gln/Arg	1.74 (0.98-3.09)	0.057	2.07 (0.88-4.89)	0.097
Arg/Arg	3.54 (1.46-8.60)	0.005	7.21 (2.26-23.00)	0.001*

CI: confidence interval ; *Asterisk indicates p< 0.05

= 0.003) and genotype GA & AA (*p*-value =0.001 both) is more significant.

Present study shows a significant association of Gly223Arg with higher BMI (p=0.020), and Leptin (*p*=0.002) where AA (Arg/Arg) is more significant than GA (Gln/Arg)(*p* value 0.001 vs 0.097). This study is supported by a Boumaiza Imen *et al* which showed Q223R (Gln223Arg) polymorphism is associated with Weight and obesity risk [33]. Boumaiza *et al* (2012) also showed that G2548A has significant association with obesity [33]. Zayani *et al* 2018 were also found a significant association between Q223R and G2548A SNPs and obesity[39].

Suryaprom *et al* 2014 likewise demonstrated that there was a relationship between LEPR Gln223Arg polymorphism and Leptin Level and metabolic disorder[34,35]. Farooq *et al* similarly demonstrated that leptin assumes a basic role in particularly, ovulation and spermatogenesis. A fluctuation in circulating leptin also presumed that obesity is related with infertility in men as well as women. In addition, Sex hormonal irregularity may likewise be related BMI and serum leptin in infertility[35]. From our present study it might conceivable that there is role of Serum Leptin, G2548A and Gln223Arg polymorphism in UI.

CONCLUSION

This present study showed an association between G2548A and Gln223Arg polymorphism and serum leptin with UI. Statistical analysis reveals that these parameters are significant and independent predicators of UI. The allele frequency of both *LEP* G2548A and LEPR Gln223Arg was also found significantly different and higher in unexplained infertile than fertile. Moreover, the phenotype frequency of both *LEP* G2548A and *LEPR* Gln223Arg was also found significantly different and higher in unexplained infertile than fertile. G2548A was more frequent than *LEPR* Gln223Arg. Recommended to conduct, more studies with large sample size may be helpful for

the knowing the association and impact of these Gene in unexplained female infertility in Indian population.

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None.

CONFLICTS OF INTEREST

The authors declares that there is no conflict of interest.

AUTHORS' CONTRIBUTION

PK did design study, review and substantially participated in all other work. SPJ guided Infertility in Assays and Data compilation. PS and SD supervised in sample collection of review and sample. AAM and KA guided in all the Molecular and Biochemical studies. WA helped in statistical analysis.

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DATA AVAILABILITY

The datasets are available from the corresponding author on reasonable request.

ETHICAL STATEMENT

A prior approval was obtained from the King George's Medical University (K.G.M.U.) of Lucknow, India ethics committee vide letter no-2214/R-Cell-11. Ref. code: 53 ECMIIB/P1 to conduct this research.

Participants: human Participant

Consent: Informed consent were taken from all the patients.

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