Original Article

The Relationship between -2548 G/A Leptin Gene Polymorphism and Risk of Breast Cancer and Serum Leptin Levels in Ahvazian Women

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Abstract

Background: Potential association of leptin (LEP) gene polymorphisms has been suggested in the processes leading to breast cancer initiation and progression. We investigated whether genetic variations in the LEP -2548G/A gene are associated with risk of breast cancer.

Methods: This case-control study consisted of 100 breast cancer cases and 100 control subjects without breast cancer that matched for age and body mass index (BMI). Genotyping of LEP -2548G/A polymorphism was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Serum leptin level was determined by ELISA in all study subjects.

Results: The genotype distributions (AA, AG, and GG) were 36, 55, and 9% in breast cancer cases and 52, 45, and 3% in control group, respectively. The frequency of LEP -2548 GG genotype was significantly elevated in breast cancer cases as compared to controls ($\chi 2$=6.90, p=0.032). Similar difference was also found in allele frequencies between two groups ($\chi 2$=5.65, p=0.017). A markedly increase risk of breast cancer was associated with the LEP -2548GG genotype when compared to the LEP -2548 AA genotype (OR=4.33, 95% CI=1.09-17.22). In addition, postmenopausal women who bear at least one LEP -2548 G allele were at a markedly increased risk of breast cancer after adjusting for age and BMI confounders (OR=12.24, 95% CI=1.13-131.73).

Conclusion: The LEP -2548 G/A polymorphism is associated with markedly increased risk of breast cancer especially in postmenopausal Ahvazian women and supported the hypothesis that leptin is involved in breast cancer.

Keywords: Brest Cancer; Leptin; polymorphism; PCR-RFLP

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Introduction

Breast cancer is one of the commonest causes of cancer death among women worldwide, but its etiology is still not fully determined [1]. In The recent years, the incidence of breast cancer has increase dramatically in Iran, and consider as of the most frequent malignancies among Iranian women [2]. Although, it has been determined that presence less than 10% of breast cancer is due to dominant mutations in highly penetrate susceptibility genes, including BRCA1 and $BRCA_2$. However, low-penetrate genes, that also increase susceptibility to breast cancer, have been suggested too [3].

Leptin (LEP), an adipocyte-derived satiety hormone, is thought to plays a crucial role in the regulation of energy expenditure and body weight through acting on its receptor expressed mainly in the hypothalamus [4, 5]. Moreover, studies have indicated that the leptin is involved in carcinogenesis of breast tissue and acts to favor the proliferation and angiogenesis of breast cancer cells [6-8]. Interestingly, over expression of leptin in breast cancer appears to be associated with higher tumor grade and size [9-13]. The leptin gene, the human homologue of the rat obese gene (OB), that had been cloned and sequenced by Zhang et al. [14], is located

on chromosome 7q31.3 [15, 16] and the length of its mRNA in adipose tissue is 4.5 kb [14, 17].

In humans, several single nucleotide polymorphisms (SNPs) have been identified in the LEP gene. Among them, G to A substitution at nucleotide (nt) -2548 upstream of the ATG start site in the LEP gene promoter, LEP -2548G/A, has been associated with adipocytes increased leptin production and secretion [18-20]. Increased circulating levels of leptin and over gene expression have been reported in subjects carrying the LEP -2548 A allele [21]. The LEP -2548 A allele has also been associated with a two-fold increase of leptin secretion from adipocytes when compared to secretion by adipocytes bearing only the LEP -2548 G allele [21]. Herein, an association between leptin and breast cancer risk could be proposed. However, only a few studies with inconsistent results have addressed the relationship between leptin and breast cancer [10, 22-26], and to date, no study has addressed the association between polymorphisms within the LEP gene and breast cancer risk in Iranian population. Thus, in this study we investigate the associations of LEP -2548 G/A polymorphism and risk of breast cancer in Ahvazian women, a sample of Iranian population.

Materials and Methods

Study Population

A total of 200 cases were investigated in two groups. One group was comprised of One-hundred Iranian unrelated women with confirmed breast cancer recruited from the department of radiation and oncology of Golestan University Hospital, Ahwaz, Iran as previously described elsewhere (27). The diagnosis of cancer was confirmed by histopathological analyses. Clinical information such as stage of the breast cancer, menopausal status at the time of onset, hormonal receptor status (ER, PR), tumor size, and BMI was obtained from the hospital records. Menopausal status was determined using information provided by the subjects about the date of her last menstrual period. Postmenopausal status was defined as having a last menstrual period more than 6 months before the reference date. The second group was composed of 100 unrelated age and BMI matched women without any personal or family history of cancer and other serious disease to serve as controls. All the breast cancer cases and controls enrolled in the study were Iranians and informed about the study and consent was taken. This study was approved by the Clinical Research Ethics Committee. Women with suspected breast cancer without histological confirmation and those that refused sample donation were excluded from the study.

Anthropometric and other Measurements

Anthropometric indices, consisted of height, weight, body mass index (BMI), as well as a full-fasted lipid profile including total cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and triglycerides (TG) were measured as previously described [27].

Polymorphism analysis of the -2548 G/A LEP gene

For DNA extraction, blood samples were collected into K3-EDTA-treated tube from both patients and healthy controls and were stored at -20°C. Genomic DNA was isolated from peripheral blood leukocytes using the salting-out method. The LEP -2548 G/A variants were genotyped by using PCR-RFLP analysis with the forward primer 5'-TTTCCTGTAATTTTCCCATGAG–3′ and reverse primer 5'-AAAGCAAAGACAGGCATAAAA–3′. Conditions for amplification were 12.5 µl master mix, 2.0 µl (10 pmol/µl) forward and reverse primers, 2.0 µl (50 ng/µl) template DNA, and 6.5 µl sterile nuclease free water (18 µl). The PCR conditions were as follows: initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 60 seconds, annealing at 53°C for 45 seconds, and extension at 72°C for 60 seconds, with a final extension of 5 minutes at 72°C. The PCR amplified products were scored in 242 bp in a mixture reaction consisting of: PCR products (10 µl), 10× buffer (2 µl), 10 units *HhaI* restriction enzyme, and sterile nuclease free water (18 µl). The reaction mixture was kept overnight at 37°C for 1-16 hours. The fragments were separated by electrophoresis on 3% agarose gel, stained with ethidium bromide and results were recorded with photographs of gels in UV light. The polymorphism was defined by presence (G) or absence (A) of the *HhaI* restriction site.

Statistical analysis

SPSS software for Windows version 15.0 (SPSS, Inc., Chicago IL, USA) was used for the all statistical analyses. All frequencies were estimated by gene counting. The observed genotype frequencies in the breast cancer cases and controls were tested for the Hardy-Weinberg equilibrium

Table 1. Genotype and allele frequencies of LEP -2548G/A polymorphism in breast cancer cases and controls

Genotype	Breast cancer Cases	controls	$\chi 2$	p value
All Women Genotype frequencies	**(n = 100)**	**(n = 100)**		
AA	36 (36.0)	52 (52.0)		
AG	55 (55.0)	45 (45.0)		
GG	9 (9.0)	3 (3.0)	6.90	0.032
AA	36 (36.0)	52 (52.0)		
AG+GG	64 (64.0)	48 (48.0)	5.19	0.023
Allele frequencies				
A	63.5	74.5		
G	36.5	25.5	5.65	0.017
Pre-menopausal Genotype frequencies	**(n = 55)**	**(n = 60)**		
AA	21 (38.2)	30 (50.0)		
AG	32 (58.2)	28 (46. 7)		
GG	2 (3.6)	2 (3.3)	1.64	0.440
AA	21 (38.2)	30 (50.0)		
AG+GG	34 (61.8)	30 (50.0)	1.62	0.203
Allele frequencies				
A	67.3	73.3		
G	32.7	26.7		
Post-menopausal Genotype frequencies	**(n=45)**	**(n=40)**		
AA	15 (33.3)	22 (55.0)		
AG	23 (51.1)	17 (42.5)		
GG	7 (15.6)	1 (2.5)	6.45	0.040
AA	15 (33.3)	22 (55.0)		
AG+GG	30 (66.7)	18 (45.0)	4.044	0.044
Allele frequencies				
A	58.9	76.3		
G	41.1	23.8		

Data are presented as number of cases with frequency in parentheses. Allele frequencies and genotype distribution of the LEP -2548G/A in breast cancer cases were compared to those in controls by chi-square (χ^2) test.

(HWE). Chi-square (χ^2) statistics were used to evaluate breast cancer cases and controls differences in the distribution of -2548A/G genotypes. The relationship between genotypes and breast cancer was also examined by odds ratios (ORs) with 95% confidence intervals (CIs) in logistic regression models. Stratified analyses according to menopausal status were then carried out. The comparisons of the case-control for serum leptin levels were performed with a non-parametric Mann-Whitney U test. A p value of <0.05 was considered statistically significant.

Results

Genotype and Allele Frequencies in All Women

The genotypes distributions and allele frequencies for the -2548G/A LEP gene variants in breast cancer cases and controls, and their corresponding ORs are presented in Table 1. The Genotypes distributions were in Hardy-Weinberg equilibrium in both breast cancer cases and controls at this locus. In breast cancer cases the genotype

Table 2. Distribution of LEP -2548G/A genotypes in relation breast cancer risk (unconditional logistic regression).

Genotype	Cases	Controls	p value OR (95%CI)		OR (95%CI)*	p value
All Women Genotype frequencies	**(n=100)**	(**n=100**)				
AA	36 (36.0)	52 (52.0)	1.0			
AG	55 (55.0)	45 (45.0)	1.76 (0.98-3.15)	0.055	1.72 (0.95-3.09)	0.07
GG	9 (9.0)	3 (3.0)	4.33 (1.09-17.22)	0.036	4.23 (1.06-18.83)	0.041
AA	36 (36.0)	52 (52.0)	1.0			
AG+GG	64 (64.0)	48 (48.0)	1.92(1.09-3.39)	0.023	1.87 (1.05-3.33)	0.031
Pre-menopausal Genotype frequencies	**(n=55**)	(**n=60**)				
AA	21 (38.2)	30 (50.0)	1.0			
AG	32 (58.2)	28 (46. 7)	1.63 (0.76-3.46)	0.202	1.50 (0.68-3.28)	0.307
GG	2 (3.6)	2 (3.3)	1.42 (0.18-10.96)	0.732	1.31 (0.16-10.28)	0.796
AA	21 (38.2)	30 (50.0)	1.0			
AG+GG	34 (61.8)	30 (50.0)	1.61 (0.77-3.40)	0.204	1.49 (0.69-3.21)	0.310
Post-menopausal Genotype frequencies	(**n=45**)	**(n=40)**				
AA	15 (33.3)	22 (55.0)	1.0			
AG	23 (51.1)	17 (42.5)	1.98 (0.80-4.91)	0.139	1.96 (0.78-4.95)	0.151
GG	7 (15.6)	1 (2.5)	10.26 (1.14-92.25)	0.038	12.24 (1.13-131.73)	0.039
AA	15 (33.3)	22 (55.0)	1.0			
AG+GG	30 (66.7)	18 (45.0)	2.44 (1.01-5.88)	0.046	2.34 (0.95-5.77)	0.064

Data are presented as number of cases with frequency in parentheses. Results were presented for the dominant genetic model (homozygous for LEP -2548 AA vs. Carriers of G allele); OR=odds ratio. *Adjusted for age and BMI.

frequencies were 36% for AA, 55% for AG and 9% for GG, and were 52% for AA, 45% for AG and 3% for GG in controls (Table 2). The frequency of GG genotype was significantly elevated in breast cancer cases as compared to controls (χ^2=6.90, p=0.032). Similar significance differences were also found in allele frequencies for A and G of the breast cancer cases, which were 63.5% and 36.5% compared with 74.5% and 25.5% of the controls (χ^2=5.65, p=0.017) (Table 1).

Genotype and Allele Frequencies in Premenopausal Women

The LEP -2548 G allele was less frequent in premenopausal controls compared to those with breast cancer cases with allele frequencies of 26.7 and 32.7, respectively. The LEP -2548 AA genotype was less frequent in the women with breast cancer cases compared to controls, with genotype frequencies of 38.2 and 50, respectively (Table 1).

Genotype and Allele Frequencies in Postmenopausal Women

There was a significant difference in the distribution of the LEP -2548G/A genotype in postmenopausal breast cancer cases compared to the controls (χ^2=6.45, p=0.04) (Table 1).

LEP -2548G/A Genotypes and Breast Cancer Risk in All Women

Compared to women with the LEP -2548 AA genotype, those bearing the heterozygous LEP -2548 AG genotype (OR=1.765, 95% CI=0.98-3.15, p=0.055) had no significant increased risk of breast

Table 3. Mean leptin levels according to LEP -2548 G/A genotypes, menopausal status, estrogen and progesterone receptor status at disease onset

variables	Mean leptin level ±SD (ng/ml)	p value*
Genotypes		
Breast Cancer Cases		
AA	68.20±45.90	
AG+GG	69.33±41.92	0.957
Controls		
AA	34.35±25.28	
AG+GG	30.97±28.80	0.364
Pre-menopausal		
Breast cancer cases	67.39±43.26	
Controls	35.13±28.23	0.001
Post-menopausal		
Breast cancer cases	71.56±43.08	
Controls	28.88±24.80	< 0.001
Pre-menopausal in breast cancer cases		
ER positive	68.45±42.68	
ER negative	59.10±45.39	0.258
PR positive	73.43±40.83	
PR negative	36.00±40.65	0.008

*p values derived by non-parametric test

cancer. However, Compared to women with the LEP -2548 AA genotype, those bearing the homozygous -2548 GG genotype (OR=4.33, 95% CI=0.09-17.22, p=0.036) have significant increased risk of breast cancer. Thus, the presence of at least one LEP -2548 G allele (LEP -2548 AG + LEP -2548 GG genotypes) was associated a significant risk of breast cancer in these women (OR=1.926, 95% CI=1.09-3.39, p=0.023) (Table 2).

LEP -2548G/A Genotypes and Breast Cancer Risk in Premenopausal Women

Among premenopausal women, there was no significant association between the LEP -2548G/A polymorphism and breast cancer risk (Table 2).

LEP -2548G/A Genotypes and Breast Cancer Risk in Postmenopausal Women

Among premenopausal women, compared to women with LEP -2548 AA genotype, those bearing the homozygous LEP -2548 GG (OR=10.26, 95% CI=1.14-92.25, p=0.038) had significant increased risk of breast cancer. Moreover, Adjusting for age and BMI confounders, did markedly and significantly increased risk of breast cancer in these women (OR=12.24, 95% CI=1.13-131.73, p=0.039) as shown in table 3. Therefore, the presence of at least one LEP -2548G allele (LEP -2548AG +LEP-2548GG genotypes) was associated with a significant increased risk of breast cancer in these women (OR=2.44, 95% CI=1.01-5.88, p=0.046) (Table 2).

Relationships between serum leptin level and breast cancer

Because of the opposite effect of obesity on breast cancer risk among pre- and post-menopausal women, we compared the serum levels of leptin between breast cancer cases and controls according to the menopausal status. The mean leptin levels was significantly different between breast cancer cases

and controls according to the pre-menopausal (67.39±43.26 vs. 35.13± 28.23 ng/ml, p=0.001) and post-menopausal (71.56±43.08 vs. 28.88±24.80 ng/ml, p<0.001), respectively. Pre-menopausal breast cancer cases with positive progesterone receptors (PR) on breast cancer tissue indicated significantly higher serum leptin levels than those with negative PR (73.43±40.83 vs. 36.00±40.65 ng/ml, p=0.008). There was no significant difference in leptin levels in pre-menopausal breast cancer cases with positive estrogen receptor (ER) and negative ER (68.45±42.68 vs. 59.10±45.39 ng/ml, p=0.258). Additionally, to determine whether LEP -2548G/A polymorphism is associated with differences in serum leptin levels in breast cancer cases and controls, we compared the mean levels of serum leptin in each group according to the LEP -2548G/A genotypes. Results indicated that there were no significant difference in mean levels of serum leptin in both breast cancer cases and controls according to the LEP -2548G/A genotypes (Table 3).

Discussion

This study showed significant differences in genotypic distribution and allele frequencies of the LEP -2548G/A polymorphism between breast cancer cases and controls. Additionally, women with the LEP -2548G allele have markedly increased risk of breast cancer, an association that was modified by menopausal status. In this way, our results supported the hypothesis that breast cancer might be associated with the serum levels of leptin.

Our results indicated that there was no significant difference regarding the mean serum levels of leptin between case and control groups according to the LEP -2548G/A observed genotypes. Data regarding to the potential action of the LEP -2548G/A genotypes on the serum levels of leptin is still controversial. A study conducted in the Taiwanese population reported that no association was found between this LEP polymorphism and serum levels of the leptin [28]. Conversely, reports from a cohort study in men French showed that there was association between AA genotype and increase serum levels of leptin [29]. This finding was confirmed by a Sweden study, which revealed that the -2548 A allele of this polymorphism was associated with increased leptin mRNA levels and increased rate of leptin secretion from adipose tissue [20]. Inversely, data from a study conducted in a French population, revealed that the -2548G/A LEP polymorphism could potentially alter leptin expression, and female subjects with the AA homozygous had lower mean levels of leptin than girls with other genotypes [19]. The association between the AA genotype of LEP -254G/A and lower serum levels of leptin was also described in healthy subjects from Greece, in French morbidly obese patients and in obese Brazilian women [30-32]. The reason of these different results is not completely understood but suggested due to interaction of LEP -2548G/A polymorphism with other polymorphisms in leptin and/or leptin receptor genes, sample size of population, or from to the model used in statistical analysis.

However, we found a significant difference of serum lptin levels between case and control according to the both premenopausal and postmenopausal status. These results conflict with the reports of several studies [2, 33]. According to another study in northern Sweden, no significant association between leptin levels and risk of post-menopausal breast cancer was observed [24]. The issue regarding leptin levels and breast cancer was controversial and an additional study with a larger number of cases would be helpful to clarify this relationship. Compared with the leptin levels used in several experiments that claimed that leptin caused significant effects on cellular proliferation [12, 21, 25, 34] there was a middle gap between the mean serum leptin levels in experiments, above 100 ng/ml, and in that of our post-menopausal patient group 71.56 ng/ml. Further studies are needed to elucidate to what degree the serum leptin levels reflect tissue levels in the breast and whether or not there would be a threshold of leptin which could stimulate the development of cancer.

Reports in premenopausal breast cancer cases were showed some association between leptin level and increased tissue levels of PR from cancer tissue. In a study conducted by Tessitire et al. in patients with breast cancer, found that, increased tissue levels of PR were associated with increased leptin levels [10]. In consideration of a finding that leptin is a hormone in adipose tissues that are regulated by transcriptional control with sex steroid hormones including progesterone [35], it thought to be there was some relationship between leptin and progesterone. Thus, leptin could stimulate the production of progesterone, or amplified signals through PR could increase the production of leptin.

Leptin, seems to be a growth factor, has several effects on the growth of normal and tumor cell, migration and invasion of tumor cell, development of angiogenesis, and proposed that can promote an aggressive of breast cancer phenotype [36]. Previous studies regarding to the association between LEP -2548 polymorphism and breast cancer risk have been controversial. Data reported in a case-control study conducted in Tunisian population showed that subjects who carried the LEP -2548AA genotype having a threefold increase in risk of breast carcinoma, those carried the heterozygous GA genotype having an intermediate risk compared to women who were homozygous for the LEP -2548 G allele [37]. In a large population-based study of mostly European - American women reported that the LEP -2548AA genotype was associated with a modest (30%) increase in the risk of breast cancer when compared to those with the LEP -2548GG genotype, an association that was not modified by menopausal status. Moreover, they did not observe an increased association with breast cancer risk for subjects bearing a single A allele [38]. Conversely, we observed 4-fold increased association with breast cancer risk for carrying a single G allele. Whether the differences in the magnitude of the association according to the LEP genotype between two studies is due to differences in ethnicity, sample size, or statistical analysis, is not clear. Although, the functional status of the LEP -2548G/A polymorphism is not completely understood, however, previous reports have shown that the A allele is associated with increased mRNA expression and higher circulating levels of leptin [20]. Additionally, the A allele of the LEP -2548 polymorphism has also been linked to other cancers, such as those of the lung and prostate [39, 40]. In spite of evidence that leptin induces proliferation of breast cancer cell in vitro [41] however, epidemiological reports regard to the effects of circulating levels of leptin on the breast cancer development and progression has been limited. A study conducted by Hu et al. showed an inverse association between circulating levels of leptin and breast cancer development in premenopausal women only [21], though in two other studies no such association was found [24, 26]. With regard to breast cancer prognosis, one study found no association between leptin levels and either disease-free or overall survival [42]. Why these studies failed to show evidence that leptin levels are associated with breast cancer is unknown. One probability may be that the leptin levels in these studies were measured in circulation which could vary significantly from tissue concentrations found in the breast.

Conclusion

The results of this study have demonstrated a markedly increased risk of breast cancer in women bearing the LEP -2548G allele, and the association may be markedly profound in the postmenopausal women. According to our knowledge, this is the first study to provide information on the role of LEP -2548G/A polymorphism in breast cancer risk in a sample of Iranian women. These results should be confirmed with additional studies to further evaluate the potential action and interaction between this polymorphism, obesity and breast cancer development both in-vivo and in-vitro.

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Conflicts of Interest

There is no conflict of interest in this article.

Authors' Contribution

All authors participated in the study design, data analysis, interpretation and manuscript writing.

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