

Effect of leptin receptor Q223R polymorphism on breast cancer risk

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ABSTRACT

Objective(s): Leptin receptor (LEPR) is a member of the class I cytokine receptor super-family that is known implicated in the initiation and progression of breast cancer. We have investigated the effect of Q223R polymorphism on the breast cancer susceptibility in a sample of Iranian subjects.

Materials and Methods: We utilized a polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) method to investigate the association of LEPR Q223R polymorphism with breast cancer risk in a case control study consisting of 100 breast cancer cases and 100 controls without breast cancer. Serum levels of leptin and soluble leptin receptor (sOB-R) were measured by ELISA method.

Results: The genotype (QQ, QR, and RR) distributions were 25, 56, and 19% in breast cancer cases and 54, 40, and 6% in controls, respectively. The frequency of 223 RR genotype was significantly elevated in breast cancer cases as compared to controls ($\chi^2= 20.072, P<0.001$). Similar significance differences were also found in allele frequencies for Q and R between two groups ($\chi^2= 19.027, P<0.001$). Additionally, there were significant association between Q223R genotypes and breast cancer risk; homozygotes for RR genotype (OR= 6.840; 95% confidence interval [CI] = 2.434-19.218), heterozygotes for QR (OR=3.024; 95% CI = 1.620-5.644, $P = 0.001$), and QR+RR genotype (OR= 3.522; 95% CI = 1.934-6.414, $P < 0.001$), respectively.

Conclusion: Our results showed that the LEPR Q223R polymorphism is associated with increased breast cancer risk as well as tumor grade in a sample of Iranian subjects.

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Introduction

Breast cancer is one of the most leading causes of cancer-related deaths for women in the worldwide (1). Though its etiology is still not completely understood, however, overweight and obesity, age at menarche and menopause, diet, reproductive history, steroid hormone administrations, transcription factors, and genetic factors have been suggested as risk factor for breast cancer (2-5).

Leptin, the obese (ob) gene product (6), it seems to play a key role in the food intake and increase energy expenditure (7). Central and peripheral effects of leptin are mediated by specific leptin receptors (LEPR) located on cell surface (8).

The leptin receptor is a single trans membrane protein, belongs to class 1 cytokine receptor family. Due to alternative splicing during transcription of LEPR gene different isoforms of LEPR are formed with varied length of transmembrane domain, but all the isoforms have identical extracellular and trans membrane domains (8, 9). Six isoforms derived from LEPR transcription have been identified, among them a long isoform, LEPR-b, is associated with the signal transduction of leptin (10).

Several single nucleotide polymorphisms (SNPs) in the human LEPR gene have been identified (11). Among them, an A to G substitution at nt 668 from the start codon that converts a glutamine to an

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arginine at codon 223 (Q223R) (12), alters amino acid charge from neutral to positive that could affect the functionality of the receptor and modifies its signaling capacity, which is associated with higher mean circulating levels of leptin (13,14).

This polymorphism is located within the region encoding the extracellular domain of the leptin receptor; the amino acid change affects all forms of the receptor. It has been demonstrated that individuals homozygous for the G (R223R) allele is associated with variation in ligand binding activity than those bearing the A (Q223Q) allele (13). Several studies had shown the association between LEPR Q223R gene polymorphism with BMI, insulin resistance, prostate cancer, postmenopausal breast cancer (15). It can activate ER α and ER α -dependent transcription in a ligand-dependent manner (16). Additionally, Leptin and its receptor (LEPR) are present in human breast tissue and over expressed in breast tumors (17-19). To date, several studies have been reported positively, inversely and not association between single nucleotide polymorphisms (SNPs) in the LEPR gene and breast cancer in different populations (20-25). In addition, breast cancer affects Iranian women at least one decade younger than their counterparts in developed countries (26). Moreover, to our knowledge, effect of leptin receptor gene polymorphism on the risk of breast cancer has not been previously reported in Iranian women. Hence, in the present study we investigated the LEPR Q223R polymorphisms in a sample of Iranian subjects with breast cancer to clarify the effect of Q223R polymorphism on the breast cancer risk.

Materials and Methods

Study participants

The study was performed on two groups. One group was composed of One-hundred unrelated women with confirmed breast cancer. The diagnosis of cancer was confirmed by histopathology 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. Tumor size was measured by the bidimensional product of the horizontal and vertical dimensions. The second group was composed of 100 unrelated age-matched women without any personal or family history of breast cancer or other malignancies to serve as controls. Written informed consent to participate in the study was obtained from all the study subjects. The study protocol was approved by the Clinical Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences. Women with suspected breast cancer without histological confirmation and those that refused sample donation were excluded from the study.

5 ml of blood sample was collected into 10 ml K₃-EDTA vacutainer tube from both breast cancer cases and controls. All the samples were stored at -20°C. Serum leptin concentration was measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available human Leptin ELISA kit (Mediagnost, Reutlingen/ Germany, E07). The inter- and intra-assay coefficients of variation were 6.8 and 2.55%, respectively. Serum soluble leptin receptor (sOB-R) concentration was measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available human sOB-R ELISA kit (Boster Biological technology, LTD C.No. EK0439) composed of two monoclonal antibodies raised against the extracellular domain of sOB-R.

LEPR Q223R polymorphism

Genomic DNA was extracted from peripheral blood leukocytes by a salting out procedure. briefly, 500 μ l of whole blood was mixed with cell lysis buffer (11% sucrose, 10 mM Tris-HCL, 5 mM MgCl₂, 1% Triton X-100, pH=8). Leukocytes were spun down and washed with cell lysis buffer two or three more times according to the samples. The pellet was incubated with nuclei lysis buffer (10 mM Tris-HCL, 1% SDS, 10 mM EDTA, 10 mM sodium citrate, pH=8) and subsequently proteins salted out with a solution of saturated NaCl. The denaturated proteins were precipitated by centrifugation. Next, cold ethanol was added for separation of DNA. The DNA pellet was dissolved in 50-100 μ l TE (Tris EDTA) buffer. Genomic DNA from the cases and controls were analyzed for the presence of the A to G mutation at codon 223 of the LEPR gene by a PCR-based restriction fragment length polymorphism (RFLP) assay. Conditions for amplification were 2 μ l (20 pmol/ μ l) of forward 5'-GGCCTGAAGTGTAGAAAGAT-3' and reverse primer 5'-CTGCTCTCTGAGGTGGGAAC-3, 2 μ l (100-200 ng) of template DNA, 12.5 μ l master mix (200 μ mol/l dNTPs, 1.5 mM MgCl₂, 0.5 unit/ μ l Taq polymerase) and water added to the total volume of 25 μ l. Thermo cycling conditions were initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 58°C for 45 sec, 72°C for 60 sec, followed by final extension at 72°C for 5 min. The PCR products digested with 10 units of *Msp1* restriction enzyme at 37°C for overnight and electrophoresed on 2% agarose gel. A/G substitution at nucleotide 668 in exon 6 of LEPR gene produces a restriction site for *Msp1* restriction enzyme. Digested products were as follow: undigested 642 bp band showed the A allele in the wild type homozygous genotype (QQ), 3 separate bands of 642, 469 and 173 bp in the heterozygous genotype (QR), and 2 separate bands of 469 and 173 bp showed G allele in the mutant homozygote genotype (RR).

Statistical analysis

All the statistical analyses were performed using SPSS software 15.0 (Statistical Package for the Social

Science). 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 (HWE). Chi-square (χ^2) statistics were used to evaluate breast cancer cases and controls differences in the distribution of Q223R genotypes. The relationship between genotypes and breast cancer was also examined by odds ratios (ORs) with 95% confidence intervals (CIs) in logistic regression models. Clinicopathological parameters were dichotomized as follows: SBR (Scarff-Bloom-Richardson) tumor grade (1-2 versus 3) clinical tumor size (T_1 - T_2 versus T_3 - T_4). The means serum level of sOB-R and leptin based three genotypes in each group were compared using a non-parametric Kruskal-Wallis test. All the *P*-values were two sided and the level of significance was taken as *P* < 0.05.

Results

Demographic characteristics

A total of 200 women participants in the study were recruited from Golestan University Hospital, Ahvaz, Iran. One-hundred of them were patients with breast cancer, diagnosed at the department of radiation and oncology of Golestan University Hospital. While the remaining 100 control subjects were selected from the health screening center of the same hospital and being assessed for non-breast cancer and other malignant diseases. Control subjects were matched to breast cancer cases by age (49.0 ± 7.77 vs., 48.16 ± 10.47 years, for controls and cases, respectively), and BMI (28.31 ± 4.71 vs. 27.16 ± 3.96 kg/m², for controls and cases, respectively). Table 1 shows the characteristics of 100 participants with breast cancer and 100 control subjects.

Relationship between genotypes of LEPR Q223R polymorphism and breast cancer

The genotype distributions and allele frequencies for the LEPR Q223R polymorphism in breast cancer cases and control subjects, and their associated ORs are shown in Table 2. The genotype distributions of this polymorphism in both breast cancer cases and control subjects are in the Hardy-Weinberg equilibrium. In the breast cancer cases 25 (25%) subjects were homozygous for the wild-type

(QQ), 56 (56%) were heterozygous (QR) and 19 (19%) were homozygous for the mutant (RR) genotype. On the other hand in control group 54 (54%) subjects were homozygous for the wild-type (QQ), 40 (40%) were heterozygous (QR) and only 6(6%) of the subjects were homozygous for the mutant (RR) genotype (Table 2). The frequency of LEPR 223 RR genotype was significantly elevated in breast cancer cases as compared to control subjects ($\chi^2 = 20.072$, *P* < 0.001). Similar significance differences were also found in allele frequencies for Q and R of the patients, which were 53% and 47% compared with 74% and 26% of the control subjects ($\chi^2 = 19.027$, *P* < 0.001).

In the breast cancer cases, we also observed an increased risk in the distribution of homozygous for RR, heterozygous QR, and RR+QR, their corresponding ORs being 6.840 (95% CI= 2.434-19.218, *P* < 0.001), 3.024 (95% CI= 1.620-5.644, *P* = 0.001), (95% CI= 1.934-6.414, *P* < 0.001), respectively, (see Table 3). There were significant associations of the main effects of the Q223R polymorphisms and breast cancer risk. Thus, our results suggested that carriers of the RR, QR, and RR+QR genotype in humans have a high risk of breast cancer. In addition, our results indicated that the R allele occurred more frequently than the Q allele in breast cancer cases than those in the control subjects (*P* < 0.05). Subjects with the R allele were at increased risk for breast cancer (OR= 2.524, 95% CI= 1.657-3.844) compared with those having the Q allele. This finding is consistent with data showing that the R allele of LEPR Q223R polymorphism is significantly associated with breast cancer (Table 3).

Prognostic significance of polymorphism in LEPR gene

The genotype distributions for LEPR Q223R polymorphism according to the clinic-pathologic indices of breast cancer severity represented in the Table 4. In order to tumor size breast cancer cases were stratified in two subgroups. In the first group tumor size was less than 5 cm (T_1 - T_2) and the second group consisting of patients with locally advanced breast cancer (T_3 - T_4). In addition, we stratified patients according to hormone receptor status in two

Table 1. Characteristics of the breast cancer cases and control subjects

Variables	Breast cancer cases	Controls
age (year)		
< 40	24	19
40-60	64	74
≥ 60	12	7
pre-menopause	57	60
post-menopause	43	40
body mass index (kg/m ²)		
< 20.0	3	21
20.0-24.9	31	21
25.0-29.9	46	42
≥ 30	20	35

Table 2. Genotype distribution and allele frequencies of LEPR Q223R polymorphism in breast cancer cases and control subjects

	Breast cancer cases	Controls	χ^2	<i>P</i> -value
genotypes				
QQ	25 (25.0)	54 (54.0)		
QR	56 (56.0)	40 (40.0)		
RR	19 (19.0)	6 (6.0)	20.072	<0.001
alleles				
Q	106 (53.0)	148 (74.0)		
R	94 (47.0)	52 (26.0)	19.027	<0.001

The χ^2 test was used to determine whether significant differences (*P*-value) were observed when the breast cancer cases were compared with the controls

Table 3. Distribution of Q223R genotypes in relation to breast cancer risk (Unconditional logistic regression)

Genotype	Breast cancer cases	Controls	OR (95%CI)	P-value
QQ	25 (25.0)	54 (54.0)	1.0	
QR	56 (56.0)	40 (40.0)	3.02 (1.62-5.64)	0.001
RR	19 (19.0)	6 (6.0)	6.84 (2.43-19.21)	<0.001
QQ	25 (25.0)	54 (54.0)	1.0	
QR+RR	75 (75.0)	56 (56.0)	3.52 (1.93-6.41)	<0.001

Data are presented as number of cases with frequency in parentheses

Results were presented for the dominant genetic model (homozygous for LEPR 223 QQ vs. Carriers of R allele). OR: odds ratio; CI: confidence interval

subgroups. The first group includes patients with positive estrogen or progesterone receptor and the second group includes patients with negative estrogen or progesterone receptor. When the data was stratified based on the stage of the disease and genotype frequencies (Table 4). A higher percentage of patients with advance stage breast cancer carrier RR genotype (24.4%) than patients with lower stage cancer (14.5%) (OR=5.500; 95% CI =1.443-20.959, $P = 0.013$) thus the OR was statistically significant. In addition, the frequency of the LEPR 223R allele was significantly higher in patients with higher grade breast cancer. Among breast cancer cases there was no significant association between Q223R polymorphism of LEPR gene and estrogen or progesterone receptor status (Table 4). However the RR genotype frequencies were elevated in both ER-negative (24.24%) and PR-negative (26.67%) compared to ER-positive (16.42%) and PR-positive cases (15.71%), respectively. And no association was found between Q223R polymorphism and the other clinic-pathologic parameter.

In order to determine the effect of LERP Q223R polymorphism on the serum levels of soluble leptin receptor (sOB-R) and leptin in breast cancer cases and control subjects, we compared the means of sOB-R and leptin in each group among LERP Q223R genotypes. Results indicated that there were no significant difference in the means of sOB-R and leptin among LERP Q223R genotypes in both groups (Table 5).

Discussion

A to G substitution at nucleotide 668 from the start codon 223 in exon 6 (Q223R) of the LEPR gene is known to result in several functional consequences due to altered charge distribution from neutral to positive. Because of the Q223R polymorphism take place within the first of two assumed that leptin-binding regions in the extracellular domain of receptor, therefore, the amino acid change affects all isoforms of the receptor and may be associated with impaired LEPR signaling capacity (14). This study investigated whether genetic variations in the LEPR

gene affect susceptibility to breast cancer in a sample of Iranian women.

The present case-control study revealed that the LEPR Q223R variant is highly polymorphic in a sample of Iranian subjects with breast cancer. The LEPR 223R allele frequency of 0.47 in our study subjects is higher than those reported in Caucasians (0.45), Pima Indians (0.32) but much lower than those in Asian populations (0.85) (27). In a study conducted by Chung *et al* the R allele frequency of LEPR polymorphism was low different based on racially groups (11). It is speculated that the small sample size and racial admixture in the U.S. population might be responsible for their results. The 223R allele frequency of the LEPR gene polymorphism reported by international and in Hapmap Project, among Yoruba's in Ibadan, Nigeria African Americans, was 0.61 and 0.53, respectively (28). In addition, our results showed an increased risk of breast cancer in a dose dependent manner, with the inheritance of the LEPR 223R allele. Patients with homozygous for LEPR 223R have more than 6-fold risk to develop breast cancer (OR= 6.840, $P < 0.001$) compared with patients with homozygous for LEPR 223Q allele. The patients with LEPR 223 QR homozygous have a moderate risk for the breast cancer (OR = 3.024, $P = 0.001$). Moreover, the prognostic evaluation of the LEPR genetic markers in breast cancer indicated that LEPR 223R allele is associated with a higher grade of breast cancer at diagnosis.

Although, several studies evaluate the LEPR Q223R polymorphism in relation to breast cancer risk, however, the results are conflicting. Results from a study (15) conducted by Snoussi *et al* in the Tunisian population showed a significant increase risk in both premenopausal and postmenopausal women in a dose dependent manner (OR = 1.68, 95% CI = 1.12-2.5 and OR = 2.26, 95% CI = 1.31-3.90) for the Q223R and R223R genotypes, respectively. Moreover, the authors pointed out that the presence of the LEPR 223R allele associated with poorer overall survival. Results from the other study conducted by Woo *et al* showed no relationship between the polymorphism and breast cancer risk in both premenopausal and postmenopausal in Korean population (OR= 0.54, 95% CI= 0.19-1.81) (25). In

Table 4. Genotype distribution of Q223R polymorphism in relation to pathological indices of breast cancer severity at disease onset

Genotype			OR (95%CI)	P-value
tumor size	T ₁ -T ₂	T ₃ -T ₄		
LEPR (QQ)	17 (29.82)	5 (22.7)	1.0	
LEPR (QR)	29 (50.88)	11 (50.0)	1.290 (0.383-4.346)	0.682
LEPR (RR)	11 (19.30)	6 (27.3)	1.855 (0.453-7.584)	0.390
histological grade	I-II	III		
LEPR (QQ)	20 (36.4)	5 (11.1)	1.0	
LEPR (QR)	27 (49.1)	29 (64.5)	4.296 (1.414-13.054)	0.010
LEPR (RR)	8 (14.5)	11 (24.4)	5.500 (1.443-20.959)	0.013
estrogen receptor	positive	negative		
LEPR (QQ)	21(31.34)	4 (12.12)	1.0	
LEPR (QR)	35 (52.24)	21 (63.64)	3.150 (0.950-10.441)	0.061
LEPR (RR)	11 (16.42)	8 (24.24)	3.818 (0.937-15.554)	0.062
progesterone receptor	positive	negative		
LEPR (QQ)	21 (30.0)	4 (13.33)	1.0	
LEPR (QR)	38 (54.29)	18 (60.0)	2.487 (0.744-8.318)	0.139
LEPR (RR)	11 (15.71)	8 (26.67)	3.818 (0.937-15.554)	0.062

Data are presented as number of cases with frequency in parentheses. Results were presented for the dominant genetic model (homozygous for LEPR 223 QQ vs. Carriers of R allele). OR, odds ratio; CI, confidence interval; LEPR: leptin receptor

addition, they pointed rarity of 223Q allele of the LEPR polymorphism in the Korean population, and the low study population (45 cases and controls) could be partly corresponding of their findings. Our results indicated that there were no associations among genotypes of LEPR Q223R polymorphism, serum levels of sOB-R and leptin in both groups. Although, previous studies showed that -2548A allele of leptin polymorphism was significantly associated with higher serum levels of leptin (29, 30). However, the association among genotypes of the LEPR Q223R polymorphism, serum levels of leptin and sOB-R are controversial. Findings from a study conducted by Quinton *et al* showed LEPR 223R allele associated with lower serum levels of leptin (13). Inversely, Yiannakouris *et al* have demonstrated that carriers of the 223R allele of LEPR Q223R polymorphism had a significant higher level of leptin than those no carrier (14).

Although, the functional role of leptin in the breast cancer risk have not been completely understood, however, the substantially biological effects of leptin, such as modulating several neuroendocrine

pathways, which play a key role in the pathogenesis of breast cancer, have been supported by several evidence (31). Moreover, tumor markers that are elevated in breast cancer can upregulate leptin and leptin receptor production. For example, studies reported that some pro-inflammatory factors such as TNF- α , IL-1 α , IL-1 β , VEGF, and fibroblast growth factor 2 (FGF-2), increased serum levels of leptin and promote cell differentiation and tumor growth (32, 33). Additionally, leptin has effect on the expression of several genes involved in the angiogenesis such as matrix metalloproteinase (MMP-2 and MMP-9) (34, 35). It was reported that the levels of expression of leptin and leptin receptor correlate with distant metastasis (18). In addition, it has been demonstrated that patients with LEPR negative and low expressing-leptin tumors have shown an exceedingly good outcome, and the survival rate trend to be lower in patients with LEPR positive or high expressing-leptin-tumors (18). Previous reports suggested that a single amino acid changed in the LEPR Q223R polymorphism, substitution of glutamine by arginine with a change from neutral to

Table 5. The mean serum levels of sOB-R and leptin according to the Q223R genotypes in two groups

Genotype	Mean sOB-R \pm SD (ng/ml)	P- values*	Mean leptin level \pm SD (ng/ml)	P- values*
Breast cancer cases				
QQ	10.61 \pm 34.13		61.35 \pm 39.11	
QR	4.07 \pm 14.37		72.62 \pm 44.44	
RR	3.68 \pm 10.74	0.307	68.28 \pm 45.17	0.534
Controls				
QQ	27.22 \pm 50.33		35.87 \pm 29.96	
QR	23.23 \pm 47.72		30.81 \pm 24.61	
RR	7.73 \pm 12.32	0.457	22.88 \pm 19.69	0.535

* P- values derived by non-parametric test. sOB-R: Soluble leptin receptor

positive, might affect the signaling capacity and functionality of the receptor (11, 34, 37). The finding of higher leptin binding activity levels in homozygous carriers of the G allele (LEPR R223R) and higher levels of leptin in the LEPR R223R homozygote and our finding of increased breast cancer risk in women carrying the LEPR R allele provides supportive evidence for this proposition. There was a little epidemiological study about the casualty role of LEPR gene polymorphisms on the risk of breast cancer in the most populations. However, much study has been done on the mechanisms of leptin-induced carcinogenesis in animal models and association serum levels of leptin with breast cancer risk (29, 30, 38, 39). To date, a number of studies have demonstrated the association of LEPR Q223R polymorphism with breast cancer risk, but reports from one population to the other population due to the study ethnic population, design, and significant differences in allele frequency are inconsistency. In addition, results from the studies on the interaction of Q223R genotypes, menopausal status and obesity from various populations might also explain the existence differences. Due to much higher prevalence of postmenopausal breast cancer and a higher proportion of obese women in the industrialized countries, suggest that Q223R polymorphism of LEPR gene have more effect on the susceptibility of the breast cancer than the other population with a lower prevalence of obesity.

Conclusion

This study has demonstrated an increased risk of breast cancer in a sample of Iranian women bearing 223R allele of the LEPR Q223R polymorphism. To the best of our knowledge, ours is the first study to provide information about the role of Q223R polymorphism of the LEPR gene on the risk of breast cancer in a sample of Iranian patients.

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