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The *LEP* G-2548A gene polymorphism is associated with age at menarche and breast cancer susceptibility

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ABSTRACT

Leptin is an adipocytokine made by fat cells and plays a key role in proliferation, cell survival, migration and immune response. It has a powerful effect on the initiation of puberty and in determining age at menarche. The current study is the first investigation to examine the effect of G-2548A leptin gene polymorphism on the age at menarche and breast cancer susceptibility. This case–control study was performed on 203 patients with breast cancer and 171 healthy women. The leptin genotypes were determined using the PCR-RFLP method and age at menarche was obtained by questionnaires. There was a significant difference between the leptin G-2548A genotypes between case and control groups ($P < 0.05$). AA genotype is significantly higher in patients compared to the controls. Furthermore, women carrying the AA genotype had a significantly younger age at menarche (12.47 years) than women with the AG (12.94 years) and GG (13.47 years) genotypes. Also, we found that the AA genotype frequency in women with age at menarche <13 years was higher than in women with age at menarche ≥13 years (OR: 3.4, 95% CI: 1.7–6.7, *P*: 0.001). In conclusion, the G-2548A leptin gene polymorphism has an important role in the onset of menarche and breast cancer susceptibility.

1. Introduction

Leptin, a product of the human *LEP* gene, is an adipocytokine secreted by adipocytes in proportion to adipocyte tissue mass (Lorincz and Sukumar, 2006). Several studies showed that leptin and its receptor are overexpressed in breast cancer, especially in high-grade tumors. It has an association with progression and poor survival of breast cancer (Artac and Altundag, 2012). Leptin is important for tumor cell growth and exerts its biologic effects through selective binding to the leptin receptor, which is expressed in many tissues, including the mammary gland (Wang et al., 2012).

Leptin has mitogenetic effects and stimulates the proliferation of breast cancer; these mitogenic effects are directly related to the activation of the extracellular signal regulated kinase and Akt signaling pathways, which are both involved in breast cancer cell proliferation (Vona-Davis and Rose, 2007). Moreover, leptin has an essential role in the female pubertal development (Popovic and Casanueva, 2002). The link between leptin and the timing of puberty has been extensively studied, and leptin levels have been inversely related to age at menarche (Garcia-Mayor et al., 1997; Apter, 2003; Bandini et al., 2008). It is well known that early menarche increases the risk of breast cancer (Kelsey et al., 1993; Petridou et al., 1996; Duarte et al., 2014).

Promoter sequences are potential sources of polymorphism affecting gene expression. To date, there are several sequence variants that have been detected within the 5′ flanking region of the human leptin gene. A leptin G-2548A polymorphism in the promoter region of leptin gene, has been shown to correlate with variations in serum leptin levels, degree of obesity, as well as cancer susceptibility (Mammès et al., 1998; Terrasi et al., 2009).

The aim of this study was to evaluate the impact of leptin G-2548A gene polymorphism on the age at menarche and breast cancer susceptibility in a sample of the Iranian population. To the best of our knowledge, there are no reports regarding the effect of G-2548A leptin gene polymorphism on the age at menarche in breast cancer patients.

2. Materials and methods

2.1. Study population

This case–control study was conducted on 171 healthy women (controls) and 203 breast cancer patients (cases) who were recruited from the chemotherapy department of Nemazi Hospital in Shiraz, south of Iran, from February 2012 to February 2013. In controls, the presence of any malignancy or severe chronic diseases was excluded by

Abbreviations: BMI, body mass index; PCR, polymerase chain reaction; RFLP, restriction length fragment polymorphism; OR, odds ratio; CI, confidence interval.

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standardized complex physical examination and personal history evaluation by a specialist. The case and control groups were matched for age (±5) and sex.

We used the standard questionnaire to collect data, including information on the age, height, weight, body mass index (BMI) and age at menarche. The questionnaire had been completed in all cases at the time of diagnosis, and we used the same questionnaire to collect information for the controls. Ethical approval of this study was obtained from the Research Ethics Committee of Shiraz University of Medical Sciences, Iran.

Blood from breast cancer patients and control women was collected in EDTA anticoagulant containing tube after taking signed consent.

2.2. Genotyping methodology

Genomic DNA was isolated by the salting-out method from peripheral blood samples of the case and control group. Polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) was done to identify the leptin G-2548A gene polymorphism as reported previously (Nieters et al., 2002). The primer sequences for PCR were 5 -TTT CCT GTA ATT TTC CCG TGA G-3 (forward) and 5 -AAA GCA AAG ACA GGC ATA AAA A-3 (reverse) (Bioneer, Korea). The PCR reaction mixture contained 50–100 ng DNA, 0.5 μL dNTP, 10 mm, 0.75 μL $MgCl_2$, 50 mM, 1 μL of each primers (10 pm/μL) and 0.3 U Taq DNA polymerase 5 U/μL (Cinagen, Iran) in a 25 μL mixture.

The PCR conditions included an initial denaturing cycle at 95 °C for 5 min, followed by 35 cycles (denaturing at 94 °C for 1 min, annealing at 51 °C for 1 min and extension at 72 °C for 1 min) and a final extension at 72 °C for 5 min.

Amplified PCR products were separated on 2% agarose gel, visualized with ethidium bromide stain. The amplified PCR products were subjected to RFLP using the *HhaI* (Fermentas, Germany) restriction enzyme for 37 °C overnight for enzyme digestion, and visualized on 2% agarose gel stained with ethidium bromide. Three different combinations of fragment lengths were obtained in RFLP, the presence of *HhaI* restriction sites which results in 61 bp and 181 bp corresponds to GG homozygous mutants, 242 bp representing AA homozygous wild types and a combination of three bands 242, 181 and 61 bp represent heterozygous AG genotypes (Fig. 1).

2.3. Statistical analysis

Data processing and statistical analysis were performed by using the SPSS 19. Hardy–Weinberg analysis was performed to compare the observed and expected genotype frequencies using χ^2 test. The association of leptin G-2548A genotypes and breast cancer risk were estimated by odds ratio (OR) and 95% confidence intervals (CIs) calculated by logistic regression analysis. ANOVA was used to compare age at menarche across the genotypes for the G-2548A polymorphism. *P* values were determined by Tukey post hoc test. A *P*-value < 0.05 was considered to be statistically significant.

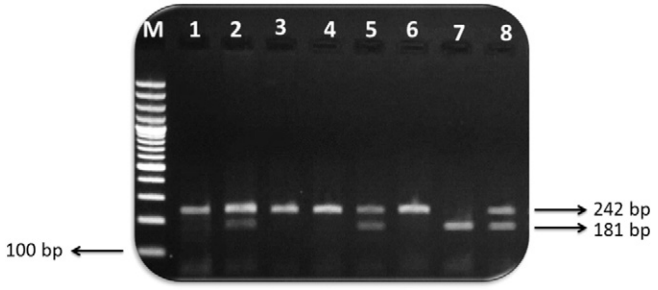

Fig. 1. PCR-RFLP picture of the amplified segment in leptin gene, Lane M: 100 bp DNA ladder, Lanes 1, 3, 4, and 6: AA genotype, Lanes 2, 5, and 8: AG genotype, Lane 7: GG genotype.

3. Results

This study was done on a total of 374 Iranian women, including 203 breast cancer cases with a mean age of 44.8 ± 10.4 and 171 controls with a mean age of 42.2 ± 10.9.

Table 1 shows the anthropometric parameters of the study participants with breast cancer and healthy subjects. Our finding showed that there is no significant difference in height (*P*: 0.8), weight (*P*: 0.74) and body mass index (BMI) (*P*: 0.86) between cases and controls. The mean age at menarche had significant differences between case and control group ($P < 0.001$).

The genotype distribution and allele frequencies of the G-2548A polymorphism in breast cancer patients and controls are presented in Table 2. The genotype frequencies of the control subjects (χ^2: 0.8, df: 1, $P > 0.05$) did not show significant deviation from Hardy–Weinberg equilibrium.

The present study indicated that there are significant differences between the leptin G-2548A polymorphism and risk of breast cancer. Also, significant differences were observed in AA genotype frequencies between case and control (OR: 2.2, 95% CI: 1.5–3.4, $P < 0.01$). Our present findings indicate that the leptin A allele frequency is significantly higher in the patient group compared to the control group (OR: 1.8, 95% CI: 1.3–2.4, $P < 0.001$). On the other hand, the G allele as protective allele, decrease the risk of breast cancer (OR: 0.56, 95% CI: 0.41–76, $P < 0.001$) and has a protective effect against breast cancer.

Furthermore, we found statistically significant differences between cases and controls in the mean age at menarche across genotypes (Table 3). Women carrying the AA genotype had a significantly younger age at menarche (12.47 years) than women with the AG (12.94 years) and GG (13.47 years) genotypes ($P < 0.05$).

To analyze the association between genotype and age at menarche, the women were subdivided into the following two groups based on the mean age at menarche distribution found in our population. One group comprised of women with menarche at younger than 13 years and the second group comprised of women with menarche at 13 years or older. We found that the leptin genotype was significantly associated with the age at menarche (Table 4). The AA genotype frequency in women with age at menarche at younger than 13 years was significantly higher than in women with age at menarche 13 years or older (OR: 3.4, 95% CI: 1.7–6.7, *P*: 0.001). A nonsignificant difference was observed in AG genotype frequencies between age at menarche groups.

4. Discussion

Reproductive and menstrual factors consider the most important risk factors for breast cancer. Leptin has a powerful effect on the age at menarche and pathogenesis of breast cancer in addition to its effect on obesity. Several studies showed that leptin levels have been inversely related to age at menarche. Shalitin and Phillip (2003) indicated that obesity is associated with early puberty, and report that elevated leptin levels have a permissive effect on the pubertal process and pubertal growth. A few studies have demonstrated associations between adiposity-related genes, such as leptin and the age of menarche; but they reported no association with leptin variants (rs13228377, rs2167270, rs791602, −2459 *LEP*) for age at menarche (Gajdos et al., 2008; Rothenbuhler et al., 2009; Kim et al., 2012). We found, for the first time, that the AA genotype in leptin G-2548A (rs7799039) polymorphism was associated

Table 1
Anthropometric characteristics of breast cancer patients and normal women.

	Cases mean ± SD	Controls mean ± SD	T	P
Height	160.8 ± 6.8	161.04 ± 7	0.26	0.8
Weight (cm)	68.5 ± 12	68.1 ± 12/3	0.33	0.74
BMI (kg/m^2)	26.4 ± 4.2	26.3 ± 4/6	0.17	0.86
Age at menarche	12.6 ± 1.4	13.08 ± 1.4	3.55	<0.001