Research Article

Specific TaqMan allelic discrimination assay for rs1477196 and rs9939609 single nucleotide polymorphisms of FTO gene demonstrated that there is no association between these SNPs and risk of breast cancer in Iranian women

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Abstract

Background: Breast cancer (BC), is the most common cancer in women, that is the major cause of cancer-related morbidity and mortality in women. Obesity is considered as a major risk factor for BC that increases both the rate and intensity of the disease. Polymorphisms in FTO gene, a known obesity related gene, is shown to be associated with obesity-related traits as well. The aim of this study was to evaluate the association between previously reported single nucleotide polymorphisms (SNPs) of intron 1of FTO gene, rs1477196 and rs9939609 and risk of BC in a subset of Iranian BC patients.

Materials and Methods: We genotyped 99 cases and 100 controls for the two SNPs of rs9939609 and rs1477196 by TaqMan allelic discrimination assay. For each sample in an allelic discrimination assay, a unique pair of fluorescent dye probe is used. One fluorescent dye probe has a perfect match with the wild type allele and the other fluorescent dye probe is perfectly matched to the mutated allele.

Results: Our research has shown that the observed differences between case and control groups in the studied SNPs of FTO gene are not statistically significant ($P > 0.05$).

Conclusions: Our findings suggest that there is no association between rs9939609 and rs1477196 polymorphisms in FTO gene and increase in risk of BC in the studied Iranian population. These results were inconsistent with that of previously reported case–control studies with BC that means presence of these polymorphisms depends on ethnic group.

Key Words: Breast cancer, FTO gene, polymorphism

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INTRODUCTION

Cancer is one of the most serious global health crisis which causes one out of every four deaths in the USA.[1] Several studies revealed that breast cancer (BC) comprises 29% (226,870 patients) of new cases of diagnosed cancers among women of US in 2012, so it is one of the most frequently diagnosed cancers

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in women.[1] Based on the Iranian cancer registry database, incidence of BC in Iran has been growing in the past two decades.[2] In Iran, incidence of BC is 24.4% of all cancer cases that is considered as a relatively high incidence[2,3] also, the patients are diagnosed in advanced stages of the disease. Interestingly the age of onset of BC in Iran is about 10 years younger than the patients in developed countries.[4,5] The alarming rises in the prevalence of obesity worldwide in the last three decades to its epidemic level, has augmented obesity related co-morbidities such as diabetes, cardiovascular diseases and particularly cancer.[6-8] Epidemiological studies of cancer, have shown that in several cancers such as kidney, breast, colon and gallbladder, obesity contributes to the increased incidence and death from cancers as much as 3 times higher.[6,8,9] Genome-wide association studies demonstrated that there are some obesity-associated single nucleotide polymorphisms (SNPs) in the intron 1 of the fat mass and obesity associated (FTO) gene which is reproducibly shown to be associated with body mass index (BMI) and other obesity-related traits in different ethnic groups.[10,11]

Although the exact cellular and physiological function of FTO is ambiguous but based on bioinformatics analyses, FTO double-stranded b-helix fold protein structure has homology with Fe (II)- and 2-oxoglutarate (2OG) -dependent oxygenases.[8,12-14] These enzymes uses non-heme iron as a co-factor and 2OG as a co-substrate and oxidatesa mixture of substrates.[7] Gerken *et al*. verified that the 2OG-dependent nucleic acid demethylase encoded by FTO gene, *in vitro*, removes methyl group from 3-methylthymine and repair deoxyribonucleic acid (DNA) methylation damage by hydroxylating methyl groups on the DNA, in particular single-stranded DNA.[12-17]

A number of studies have revealed that this gene is highly conserved not only in human, vertebrate and other animals but also even in algae. Although this gene is expressed in different tissues but has the highest expression in the hypothalamic arcuate nucleus, one of the main appetite regulating centers.[10]

Given the association between obesity and BC is approved, we performed a case control study to evaluate the association between two SNPs in intron1of FTO gene, the region most associated with obesity. Former studies demonstrated that there is controversy about the association of these SNPs with BC.[14,18]

There are several SNP genotyping methods, which can be divided into 4 classes: Hybridization-based methods, polymerase chain reaction (PCR)-based methods, restriction site cleavage methods and mixed methods.[19] Between these methods, real time PCR, due to its actual time detection and specially TaqMan genotyping method because of its accuracy and speed and also because it is less sensitive to the quality and concentration of the DNA is considered as one of the best methods.[20] In this method, probes are designed for each sequence variation, makes this a very specific method which produces more reliable and robust results.

MATERIALS AND METHODS

Study participants

The study was approved by the ethics committee of the Isfahan University of Medical Science. Our case-control study included 99 cases of BC patients and 100 controls from Isfahan province between 2012 and 2013. Only, 62 of the case and controls answered to our questionnaires, but we used both of this population in our studies. Prospective participants were recommended by physicians and the samples were obtained from recruited patients referred to the Omid Hospital, Isfahan, Iran. The controls were selected from apparently healthy people. Cases and controls were matched for sex, age, BMI and all of the participants were of the same ethnicity. Blood samples were obtained from the case and control individuals after obtaining signed informed consent.

DNA extraction

The whole blood Genet Bio DNA Blood Kit was used to extract DNA from 200 μl of blood lymphocytes and extracted DNAs were stored at −20°C until used for genotyping.

SNP genotyping

Two SNPs, rs1477196 and rs9939609 of FTO gene selected for evaluation. Based on previous data, these SNPs which are located in intron 1 of FTO gene, are strongly associated with obesity. All PCRs were prepared in a volume of 10 μl, containing TaqMan Universal PCR Master Mix, specific TaqMan SNP Genotyping Assays (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 99404, USA) and genomic DNA. Thermal cycling conditions were 10 min at 95°C, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. Genotyping of both rs1477196and rs9939609 SNPs, was performed by 5´ nuclease PCR using the TaqMan allelic discrimination system. All results were automatically called by step one software version 2.2. The quality value for all genotype calls (with a 95% certainty) was calculated.

Statistical analysis

Statistical analyses were carried out using SPSS software, version 20. t-test was first used to compare the mean of age, weight, height and BMI between

cases and controls. Genotype frequencies for individual markers were compared in patients and control subjects using Chi-square test. We used logistic regression models to analyze our data simultaneously on each SNP. We first performed our analyses without any co-variates, and then adjusted for age, BMI and menopause status by including them as co-variates.

The odds ratios (OR) was estimated with 95% confidence intervals (95% CI) and $P < 0.05$ was considered statistically significant.

RESULTS

Demographic profile

Demographic properties of the cases and controls are shown in Table 1. Our case control study included 62 cases and 62 controls. Mean age of the patient and control subjects were 50 years (50.16 ± 11.30) and 49 years (49.63 ± 11.88) respectively. Variance of weight, height and BMI in the case and controls were checked to be insignificant (P = 0.863, 0.097, 0.863 respectively). Hence, the matching on these variables were adequate [Table 1].

rs9939609 polymorphism in BC

The genotype of rs9939609 polymorphism in all patients and controls were successfully genotyped. The genotyping by TaqMan genotyping assay was confirmed by sequence analysis of PCR products. The frequency of allelic and genotype distribution of rs9939609 in the case and control subjects is shown in Table 2. The frequencies of the T/T, A/T, and A/A genotypes of rs9939609 T > A were 32%, 52% and 16% in controls and 42%, 40% and 11% of cases, respectively. Frequency of the A allele in cases (38%) and healthy control group (42%) were not statistically significant (P = 0.516.). Therefore, no association was observed between the genotype TT and the T allele of rs6983267 T > A SNP and higher BC risk.

rs1477196 polymorphism in BC

TaqMan geno typing of rs1477196 polymorphism in patients and controls showed that the frequency of A/A, A/G, G/G genotypes of rs1477196 were 53%, 40%, 6%, in cases and 60%, 32%, 8% in control, respectively [Table 2]. Frequencies of A and G alleles were 78% and 22% in controls and 73% and 27% of cases, respectively. These data indicate that there are no statistically differences in the genotype distributions (P > 0.05 or P = 0. 639) and allele frequencies (P = 0.661) of the rs1477196 between the cases and controls.

We evaluated the association between the genotypes of rs1477196 and rs9939609with menopausal status (pre and post menopause). For both the studied polymorphisms the observed differences between pre and post-menopausal status were not statistically significance (P = 0. 052 for cases, and P = 0. 083 for controls). Association between the polymorphism and individual's menopause status for rs9939609, also was investigated. The observed differences between the pre and post-menopausal women with BC were not statistically significant in the case group (P = 0. 793) and in healthy individuals (P = 0. 543).

When taking into account age, BMI and menopause status [Table 3], the estimated ORs indicated that

Table 1: Demographic characteristics of breast cancer patients and normal controls

Characteristics	Controls	Cases	*P* value
Age (year)	49.63±11.88	50.16±11.30	0.799
Weight (kg)	66.56±10.70	67.89±11.82	0.515
Height (cm)	158.63±14.09	161.95±6.69	0.097
BMI) kg/m^2)	26.01±4.08	25.89±4.16	0.863
Menopause (%)			
Pre	35 (56.45)	35 (56.45)	
Post	27 (43.55)	27 (43.55)	

Data presented as mean±SD and *n* (%), *t*-test; BMI: Body mass index; SD: Standard deviation

Table 2: Allele and genotype characteristics of rs9939609 and rs1477196 in cases and controls

Frequency	Group		*P* value
	Case *n* (%)	Control *n* (%)	
Allele (rs9939609)			
T	77 (62)	72 (58)	0.516
A	47 (38)	52 (42)	
Genotype (rs9939609)			
TT	26 (42)	20 (32)	0.430
AT	25 (40)	32 (52)	
AA	11 (18)	10 (16)	
Allele (rs9939609)			
A	91 (73)	94 (78)	0.661
G	33 (27)	30 (22)	
Genotype (rs9939609)			
AA	33 (53)	37 (60)	0.639
AG	25 (40)	20 (32)	
GG	4 (6)	5 (8)	

Chi-square test

Table 3: Results of association between breast cancer and studied variables in multiple logistic regression model

Variables	Coefficienct *b*	SE	*P* value	OR (95% CI)
Age	−0.037	0.031	0.235	0.964 (0.907-1.024)
Weight	−0.038	0.054	0.481	0.963 (0.867-1.070)
Height	−0.029	0.036	0.412	0.971 (0.906-1.041)
BMI	0.105	0.143	0.462	1.111 (0.839-1.471)
rs1477196	−0.117	0.332	0.725	0.890 (0.464-1.707)
rs9939609	0.195	0.294	0.507	1.215 (0.683-2.161)
Menopause	0.638	0.692	0.357	1.892 (0.488-7.343)
Constant	5.238	5.923	0.377	188.212

Variable entered: age; BMI, rs1477196; rs9939609and menopause; BMI: Body mass index; SE: Standard error; CI: Confidence interval; OR: Odds ratio

although menopause status has the highest effect (OR = 1. 892, 95% CI: 0.488-7.343), but none of these quantities are statistically significant.

When the association of rs9939609 and rs1477196 with BC were studied in 99 patients and 100 control subjects, that were not matched, our results indicated that there was not any association between these SNPs and risk of BC in Iranian women (P = 0. 389 for rs1477196 and P = 0. 385 for rs9939609).

DISCUSSION

SNPs as the simplest and most common source of human genetic polymorphisms, which causes individual differences in susceptibility to the disease, may influence the susceptibility to cancer. Several studies have shown that some polymorphisms in non-coding areas, such as intron, may be located in the regulatory sequence of the gene and may affect the expression and function of proteins and have an association with the cancer risk. A study conducted in 2010 by Berulava and Horsthemke clearly showed that the FTO transcripts containing risk allele (A) of rs9939609, are abundant compare to the transcript containing T allele. Transcript high levels may be associated with high levels of the protein product.[11] Obesity is an important risk factor for BC that increases 3-fold (OR = 3.21) BC risk in obese Iranian women, compared with women with normal BMI.[21] It is shown that obesity related hormones such as Adiponectin and Adipokine, have a role in BC risk.[22,23] Given that, influence of stated SNPs on obesity development are well-assessed and the association of these SNPs with BC, has been demonstrated by Kaklamani *et al*.[14] we evaluated the association between BC and two FTO polymorphisms among Iranian patients. In our study, one of the evaluated polymorphisms that its effect on risk of BC was investigated, was rs9939609 SNP on chromosome16 q22.2:53800954. A study carried out by Kaklamani *et al*. in the USA, is shown that this polymorphism has significant association with increased risk of BC. It is reported that individuals carrying the A allele have a higher risk of developing BC[14]. Interestingly, in another study conducted in the Netherlands by Renata Kusinska, Allele A of rs9939609 showed no association with increased risk of developing BC.[18] We carried out this study to evaluate the correlation between this polymorphism and BC in Iranians. Table 2 shows the results of allele and genotype related to rs9939609 SNP for BC susceptibility. Our findings are not consistent with those of a previous study in the USA population which showed that individuals carrying the A allele have a significantly higher risk of developing BC.

For the other SNP, the rs1477196 SNPs, genotype distribution and allele frequency between the control and case groups were not statistically different. In this study, no association was detected between G allele and GG genotype with BC. Only, in a study conducted by Kaklamani *et al*., association of rs1477196 with BC was examined Their results demonstrated that rs1477196 SNP showed the strongest association with BC, compared with other SNPs located in intron 1 of the FTO gene.[14]

Studies conducted on the association of obesity with risk of BC in Western women, have shown conflicting results. Studies on postmenopausal women, has demonstrated that there are association between obesity and BC risk, whereas this relationship between pre-menopausal women, is negative. In our study, although there was a link between the high BMI and menopausal status and hence that postmenopausal individuals had high BMI. However when participants were divided into two groups according to their menopause status; and rs9939609, rs1477196 with their menopausal status was examined, the results indicated that the allelic frequency and genotype distributions of these two SNPs, in menopause and non-menopause healthy and patient women did not show any significant difference.

When patients and controls were stratified on the basis of age, BMI and menopausal status, no association was found between these variables and genotype distribution. These findings suggest that both SNPs studied in this survey, rs9939609 and rs1477196 might work in an ethnic-specific mode rather than in a general mode.

Due to the different genetic background of the populations, the study of different polymorphisms in different parts of the world can have different results. For example, allele frequency of the risk factor (A) of rs1477196 in Asia, Europe, Africa and America, are 26%, 35%, 44% and 8% respectively.[24] Unfortunately, the allele frequencies of these polymorphisms in Iran, is not known.

The differences in ethnic background and the different forms of BC may somehow explain the contradictory observed in different studies. These conflicting results may be due to limitations in the assessment of phenotypes, racial differences in genotype frequency of FTO gene, uncontrolled interfering factors in some studies, and differences in demographic and environmental risk factors for BC.

Overall, results of this study indicate that SNPs in the FTO gene, has no role in BC risk in Iranian population.

Our study further demonstrated that the role of adipose tissue in the development of cancer is very complicated and the role of the individual's genetic background polymorphisms in this pathway, has remained more elusive. In order to clarify the role of SNPs on this route, which may play a role in carcinogenesis, we require further studies in different populations, as well as considering the influence of environmental factors.

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