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Research Article

Association of Obesity-Related Genetic Variants (FTO and MC4R) with Breast Cancer Risk: A Population-Based Case–Control Study in Iran

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Background: Heterogeneous breast cancer is the most common cause of cancer-related mortality. Obesity defined by BMI is a known major risk factor for breast cancer.

Objectives: The purpose of this study was to explore the role of obesity related-polymorphisms rs9939609 Fat Mass and Obesity-associated (FTO) and rs17782313 *MC4R* in breast cancer development.

Materials and Methods: Matched peripheral blood serum was obtained from 64 breast cancer patients and 83 normal controls. Height and weight were measured to calculate BMI. All were genotyped for the SNPs rs9939609 and rs17782313 using a Tetra-primer ARMS-PCR method. For statistical analysis, the chi-square test and SPSS software were used.

Results: In subgroup analyses defined by BMI, *FTO* rs9939609 genotypes (TT/AA/AT) were significantly associated with the risk of breast cancer only in non-obese subjects ($p < 0.005$). TT genotypes of *MC4R* rs17782313 in non-obese and genotypes TT/CC in the overweight group were also statistically associated with breast cancer ($p < 0.005$). No significant associations between any variants and breast cancer risk were seen in obese subjects.

Conclusion: Based on the absence of an association between obesity-related SNPs and breast cancer in obese subjects, it is proposed that weight gain in Iranian women will help prevent breast cancer risk. The result help for preparing and designing a safe and versatile recombinant drug in future.

Keywords: Breast cancer risk, *FTO*, *MC4R*, Polymorphism

1. Background

Breast cancer is one of the most frequently diagnosed and a leading cause of cancer-related deaths around the world (1). The incidence of breast cancer has increased alarmingly in Iran over recent years (2). Compared with patients of developed countries, the age of onset of breast cancer is about one decade younger in Iranian women (3). Some different uncertain factors could influence breast cancer risk. Of those, some factors such as age, Body mass index (BMI), weight gain, reproductive history, menopausal status, hormone therapy, etc. have been identified (4). Little is known, however, about modifiable risk factors for this disease. Knowledge of such risk factors may be helpful in the development of preventive and treatment strategies. Because obesity has been found to be a risk factor for many forms of cancer (5), and because it is highly prevalent among Western societies, it seems reasonable to investigate whether obesity could also be a risk factor for developing breast cancer (6).

Estrogen is a well-known factor which is produced more in obese post-menopausal women than in normal-weight ones (7). Another contributing mechanism is the high level of insulin-like growth factor-1 (IGF1) that has a reducing effect on the circulating level of the sex-hormone binding globulin (SHBG). Consequently, with the decreased level of SHBG, the free form of estradiol in the blood will rise, which may lead to breast cancer risk (8).

Single nucleotide polymorphisms (SNPs) such as Fat Mass and Obesity-associated (FTO) and Melanocortin-4 receptor (MC4R) genes have been robustly associated with increased BMI and obesity in multiple study populations (5, 9-11). It has been suggested that this effect is mediated through a reduction in satiety and, consequently, increased food consumption (12). The *FTO* and *MC4R* genes are highly expressed in the hypothalamic nuclei responsible for energy balance (13, 14).

2. Objective

Some evidence has shown that obesity-related traits such as high BMI determine the effect of SNPs on breast cancer development (15, 16). Obviously, a high BMI level doubles the rate of death from breast cancer compared with women with the lowest BMI; however, some studies have reported conflicting results (7).

In the current study, the SNP rs9939609 (T>A) which is located in the first intron of *FTO* and rs17782313 (T>C), laying 188kb downstream of the *MC4*R gene were selected. The study aimed to evaluate whether susceptibility to breast cancer in obese subjects is increased by obesity-related variants. The role of the obesity-related *FTO* gene rs9939609 and *MC4R* gene rs17782313 on breast cancer progression was investigated.

3. Materials and Methods

3.1. Study Population

A total of 147 unrelated Iranian women (64 BC patients and 83 healthy controls) participated in this study. Patients were recruited from those referring to Shohadaye-7 Tir Hospital (Tehran, Iran) from 2016 to 2017. The mean age of the participants was over 40. Based on BMI levels, cases and healthy volunteers were divided into groups (20>BMI, 25<BMI<30, BMI>30), and (20<BMI<25), respectively. Age, gender, physical activity level, and family history were obtained through interviews. Signed informed consent forms were obtained from all participants in the study.

3.2. Genotype Determination

Approximately three milliliters of peripheral venous blood samples were collected from the study subjects for genotyping rs9939609 of *FTO* and rs17782313 of *MC4R* using the tetra-primer Amplification-Refractory Mutation System (ARMS) Polymerase chain reaction (PCR) method. Genomic DNA was extracted from the blood samples according to standard protocols using DNA extraction kit (GeNet Bio with 846-X-070-141 cat number Company Macrogen Korea). The primer used in the genotyping analysis was initially designed by means of Oligo7 Primer Analysis Software. The sequences and PCR product sizes for each allele are listed in **Table 1**. Negative controls were included in all tests.

The PCR conditions for both polymorphisms of *FTO* and *MC4R* were as follows: Initial denaturation at 95 °C for 5 minutes, 32 cycles constituting the melting step at 95 °C for 30 seconds, annealing at 58 °C (rs9939609 A/T) and 59 °C (rs17782313 T/C) for 30 seconds, elongation at 72 °C for 30 seconds, and a final elongation step at 72 °C for 5 minutes to allow the complete extension of PCR fragments. The PCR products were subjected to electrophoresis on 1.5% agarose gel for rs9939609 of *FTO.* For further confirmation, 10% of the samples were sequenced using an ABI3100 sequencing machine (Applied Biosystems, GenFanavaran, Iran). The provided sequencing outcomes were assessed by Vector NTI software.

Table 1. Details of primers used in tetra-primer ARMS-PCR for genotyping SNPs

Primer position	Sequence of primer (5′-3′ end)	Product size (bp)
rs9939609 (FTO)	Forward inner: CCTTGCGACTGCTGTGAATATA	AA allele=211bp
	Reverse inner: CAGAGACTATCCAAGTGCATCTCA	TT allele=296bp
	Forward outer: GCTGCTATGGTTCTACAGTTCCA	AT Outer =461bp
	Reverse outer: TGTTCAAGTCACACTCAGCCTC	
rs17782313 (MC4R)	Forward inner: GAAGTTTAAAGCAGGAGAGATTGTATACC	TT allele=184bp
	Reverse inner: GCTTTTCTTGTCATTTCCAGCA	CC allele=218bp
	Forward outer: TCCACATGCTATTGGTTTAAGACAA	TC Outer =351bp
	Reverse outer: TGCTGAGACAGGTTCATA AAAAGAG	

3.3. Analytical Methods

Allele and genotype frequencies were evaluated by Hardy-Weinberg equilibrium using the chi-square test. All statistical analyses were performed with SPSS (Statistical Package for the Social Sciences, version 22, windows 10). The association of observed polymorphisms with breast cancer risk was investigated by treating the three-genotype model (homozygous, heterozygous, and mutant). An odds ratio with >1 was reported to show risk. When the *p*-value was lower than 0.05, statistical significance was declared.

4. Results

A total of 147 Iranian people were involved in this research. Participants were adjusted by classes of BMI into four categories: non-obese (BMI < 20), overweight (25<BMI<30), obese (BMI >30), and normal-weight (20<BMI<25). Completed questionnaires were collected from all subjects, including both healthy and patient samples.

4.1. Association Analyses Between FTO and MC4R Polymorphisms and Breast Cancer

According to the analysis, there was a significant relationship between *FTO* and *MC4R* polymorphisms and breast cancer. In the non-obese group, the proportions of homozygous A-A, heterozygous A-T, and wild-type T-T carrier were significantly associated with the risk of breast cancer (odds ratio (OR):>1, $p <$ 0.005). Those with the wild-type carrier T-T had higher odds of developing breast cancer than individuals with alleles (A-A and A-T) (OR= 2.381, $p < 0.005$, 95% CI: 1.189-2.998). In addition, the TT genotype of *MC4R* rs17782313 polymorphism was significantly associated with higher odds than the others (OR = 1.176, $p < 0.005$, 95% CI: 1.083-1.277) (**Table 2)**.

Table 2. FTO rs9939609 and MC4R rs17782313 and breast cancer risk stratified by BMI < 20 vs. normal

Genotype	Patients N=2	Patients, %	p-value	Odds ratio
A-A (FTO)	0	0	<0.005	1.163
T-T (FTO)	2	100	<0.005	2.381
A-T (FTO)	0	0	<0.005	1.754
T-T (MC4R)	1	50	<0.005	1.176
C-C (MC4R)	1	50	1.000	1.000
C-T (MC4R)	0	0	1.857	0.032

In the overweight group, the TT/CC genotypes of *MC4R* rs17782313 showed a significant association with breast cancer, but TT had higher odds than CC (OR = 2.667, $p <$ 0/005, 95% CI: 1.336-5.323). However, *FTO* was not associated with breast cancer among overweight women (**Table 3)**.

Table 3. FTO rs9939609 and MC4R rs17782313 and breast cancer risk stratified by 25<BMI < 30 vs. normal

Genotype	Patients N=28	Patients, %	p-value	Odds ratio
A-A (FTO)	3	11	0.521	0.759
T-T (FTO)	12	43	0.886	1.042
A-T (FTO)	13	46	0.669	1.129
T-T (MC4R)	9	32	<0.005	2.667
C-C (MC4R)	6	21	<0.005	0.266
C-T (MC4R)	13	46	0.113	1.582

Finally, no significant association was found between allele and genotype distribution of *FTO* and *MC4R* SNPs and breast cancer risk in the obese group (**Table 4)**.

Table 4. FTO rs9939609 and MC4R rs17782313 and breast cancer risk stratified by 30>BMI vs. normal

Genotype	Patients N=34	Patients, %	p-value	Odds ratio
A-A (FTO)	8	24	0.071	1.940
T-T (FTO)	15	44	0.775	1.085
A-T (FTO)	11	32	0.108	0.624
T-T (MC4R)	14	41	0.054	1.991
C-C (MC4R)	9	26	0.201	0.695
C-T (MC4R)	11	32	0.653	0.874

The results of the PCR products for *FTO* rs9939609 and *MC4R* rs17782313 are shown in (**Fig. 1**.) In the *FTO* polymorphism, the digestion product sizes were 296 bp for wild allele and 211 bp for the polymorphic one (**Fig. 1A**). For rs17782313 of *MC4R*, the PCR product sizes of both alleles were 184 bp and 218 bp, respectively (**Fig. 1B**).

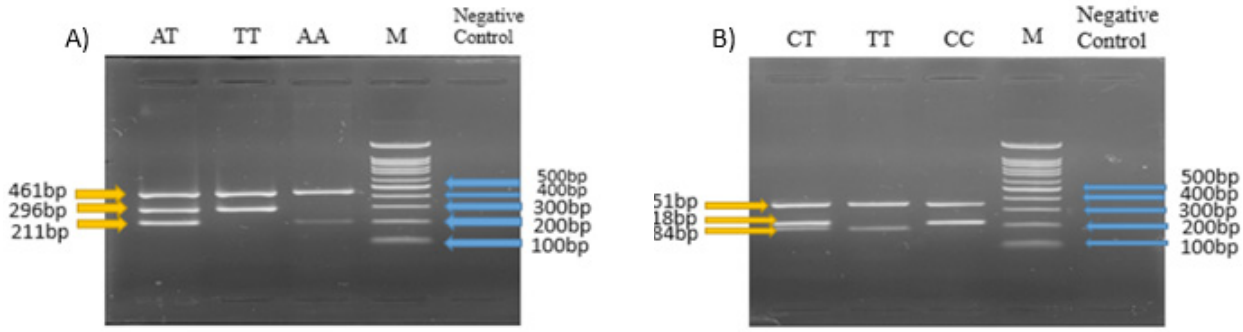

Figure 1. Agarose gel electrophoresis(1.5%) of polymerase chain reaction(PCR) product of tetra-primer Amplification refractory mutation system-PCR (T-ARMS-PCR) for the (A) fat mass and obesity-associated genes and (B) melanocortin-4 receptor.
DNA ladder (100 bp)

4.2. Interpreting Sequencing Results

Sequencing was performed to confirm the results of the Tetra Primer ARMS-PCR assay in determining mutation and definitively determining the polymorphisms examined in patients. After loading and ensuring non-negative control bands, all three-normal homozygous, heterozygous, and mutant samples were sent to the GenFanavaran Company for sequencing. Finally, results were analyzed using Vector NTI 10 software. The results of sequencing are presented as graphs (**Fig. 2**).

5. Discussion

Obesity is a major public health problem. According to a national health survey in Iran in 2005, approximately 42.8% men and 57% women of the Iranian population were obese. Obesity and overweight are important risk factors for breast cancer development. The investigation of different genetic variants can lead to the identification of new pathways and, consequently, novel preventive and therapeutic targets to improve early diagnosis and life expectancy for these patients. The role of obesity-related gene polymorphisms in breast cancer development is still uncertain, and there are few studies in this field (17)

The relationship between obesity–susceptibility genes and incidence of breast cancer risk were established, although the biological mechanisms remain essentially obscure (15). Studies have shown that several phenomena can link adiposity to breast cancer risk. High levels of estrogen hormones especially in post-menopausal women, insulin resistance, and hyperinsulinemia contribute to increasing the risk of breast cancer in obese patients (7, 8). Moreover, the role of obesity-related genetic variants in predisposition to breast cancer risk should be noted. SNPs as the simple source of human genetic polymorphisms which causes individual differences in susceptibility to the disease may influence susceptibility to cancer.

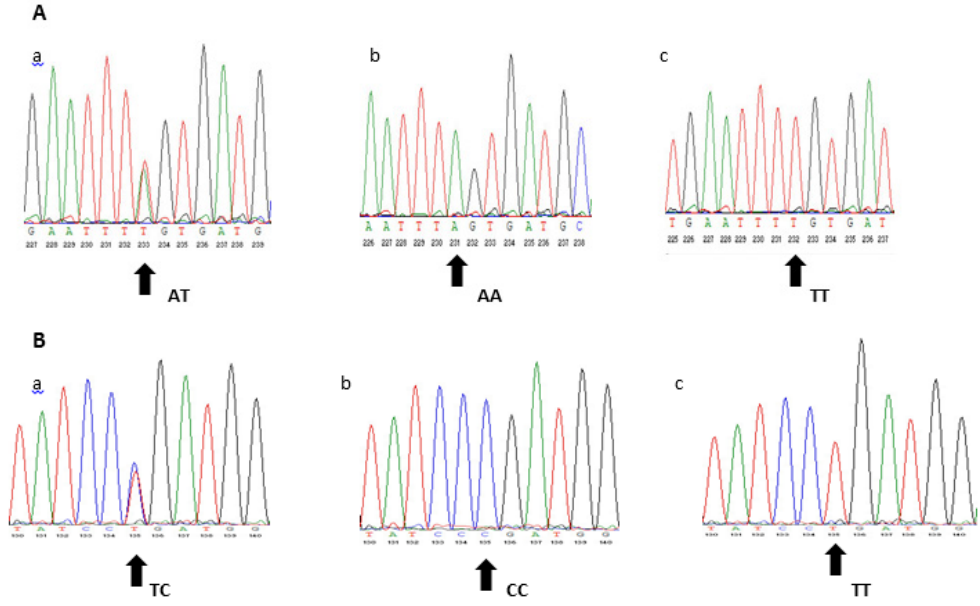

Figure 2: The result of sequencing was consistent with genotypes of the rs9939609 FTO (A) and rs17782313 MC4R (B) variants determined by ARMS-PCR

This case-control study surveyed the association of obesity-related polymorphisms on the development of breast cancer by investigating two common variants, *FTO* and *MC4R***.** The results showed that obesity-associated SNPs, rs9939609 of *FTO* and rs17782313 of *MC4R*, might be protective factors in breast cancer risk. A significant association was seen in non-obese (BMI < 20) and overweight (25<BMI<30) patients compared with the controls. In obese women (BMI >30), no association was seen between obesity-related variations and breast cancer risk. When BMI was under 20 and/or between 25 and 30, the risk was increased; however, the risk was decreased with a BMI over 30.

The *FTO* gene is localized in chromosome 16q12.2. The protein 2-oxoglutarate-dependent enzyme encoded by FTO can be found in the nucleus (18). This gene was first identified by Frayling *et al*. as a risk factor for type 2 diabetes and obesity (9). Rs9939609 (A/T) polymorphism in the *FTO* gene was known to influence the consumption of fat and fiber and modify susceptibility for obesity (16). Thereafter, the association of *FTO* rs9939609 with breast cancer was broadly replicated in different populations. In 2011, Kaklamani *et al*. reported for the first time the association between *FTO* SNPs and breast cancer risk in North-American women. They found that *FTO* SNPs are significant classifiers in predicting breast cancer risk (18).

In recent years, another common genetic variant (*MC4R*) has been reported to contribute to common obesity susceptibility. *MC4R* encodes a seven domain-trans membrane G protein coupled receptor. Mutations in this gene are the most common cause of the monogenic form of obesity (19).

A study of European subjects in 2008 was the first report of SNP rs17782313 associated with increased BMI in adults and children. This association has been replicated in many studies in different populations (20-22).

An analysis of *FTO* rs9939609 in Iranian people and a meta-analysis in Asian, Caucasian, and African populations failed to find an association between this variant and breast cancer risk (2, 23).

Hung *et al*. examined the relation of *FTO* rs9939609 with endometrial, pancreatic, and breast cancers; only breast cancer type did not reveal an association. Another study analyzed *FTO, MC4R,* and Neurexin 3 (NRX3) genes in Polish women. Similarly, no significant association was found between *FTO* rs9939609 and *MC4R* rs17782313 and breast cancer risk except for rs10146997 of *NRXN3* (17)

These results are in line with the current findings in which obesity SNPs showed no significant association with breast cancer risk in obese subjects.

Da Cunha *et al*. found a notable association with breast cancer risk when analyzing *FTO* SNPs rs1121980 and rs9939609 in combination with SNP rs17782313 of *MC4R* in a Brazilian population. They also presented that this association indicated a possible direct role of this *MC4R* polymorphism in the development of breast cancer, and this role seemed to be independent of its effect on BMI (24).

It is well known that both obesity and breast cancer are multifactorial and have multi-genic traits; therefore, due to the different genetic backgrounds of the populations, the study of different polymorphisms in different parts of the world can have different results. Differences in ethnic backgrounds and the different forms of breast cancer may somehow explain the contradictory results observed in different studies, which may be due to limitations in the assessment of phenotypes, racial differences in genotype frequency of *FTO* genes, uncontrolled interfering factors in some studies, and differences in demographic and environmental risk factors for breast cancer. Overall, the results of this study indicated that SNPs in the *FTO* gene have no role in breast cancer risk in the Iranian population.

The current study further demonstrated that the role of obesity in the development of cancer is very complicated and the role of an individual' genetic background polymorphisms in this pathway has remained even more elusive. To clarify the role of SNPs on this route, which may play a role in carcinogenesis, further studies in different populations which consider the influence of environmental factors are required.

Due to a lack of association in the obese subjects in the current study, it is suggested that in the process of gaining weight, some biological mechanisms might have an effective role in breast cancer prevention. More studies are important to identify novel susceptibility loci for breast cancer, which might contribute to the development of new prevention strategies, early detection, and more specific treatment approaches.

6. Conclusion

The current study found that in obese women, specific obesity-related SNPs may prevent the prediction of breast cancer risk when adjusted by BMI. It would not imply that one should be obese to restrain the breast cancer risk; however, if the valid mechanisms in obese populations against cancer were conclusively assessed, new treatment approaches may be considered. Obviously, more studies are needed to clarify the effect of obesity-related polymorphisms in the prevention of breast cancer. Further investigation is also required to genotype other known SNPs in these regions, which could help our understanding of the genetic basis of breast cancer.

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