Kurdistan Journal of Applied Research (KJAR)
Print-ISSN: 2411-7684 | Electronic-ISSN: 2411-7706
Volume 3 | Issue 1 | July 2019 | DOI: 10.24017/science.2019.1.

Received: | Accepted:

The association of codon 392 polymorphism in *ESR2* gene with breast cancer in Iran

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Abstract: ***Epidemiologic studies have revealed that age–incidence patterns of breast cancer in the Middle East differ from those in Caucasians. Genomic data for ESR2 (ER-β) is therefore of value in the clinical setting for that ethnic group and we have investigated whether polymorphisms in the ER-β gene are associated with breast cancer risk among Iranian women.***

Two selected coding regions in ESR2 gene (exons 7) was scanned in Iranian women with breast cancer and PCR single-strand conformation polymorphism technology was performed.

A site of silent single nucleotide polymorphism (SNP) on exon 7 was found. The frequency of allele 1 in codon 392 (CTC → CTG was found only in breast cancer patients (5.7%) (χ2=17.122, P=001). We found that allele 1 in codon 392 (C1176G) had direct association with the occurrence of lymph node metastasis.

Our data suggest that ESR2 polymorphisms in exon 7, codon 392 is correlated with various aspects of breast cancer in Iran. ESR2 gene structure determination, in presurgical evaluation, might be useful as a marker in predicting familial breast cancer and lymph node metastasis

Keywords: breast cancer, estrogen receptor-β (ESR2), polymorphism, metastases, PCR-SSCP

1. INTRODUCTION

Breast cancer is the second leading cause of cancer death among women in most parts of the world, including Iran. The geographical variations in not only in incidence of mortality causes by breast cancer but also in breast cancer development suggest that the environmental factors are of more importance than genetic factors (1). For instance, in Iran the breast cancer patients are 10 years younger than are their Western counterparts (2,3).

However, only 30% of all breast cancers are familial (4), suggesting that might unknown genes act in modification of breast cancer risk. It is still unclear which unknown genes and which biochemical pathways are involve for breast cancer incidence or progression.

2. LITERATURE REVIEW

The epidemiologic evidence suggests that estrogen plays an important role in developing breast cancers. Women with early age at menarche or late age at menopause, even obesity can be associated with breast cancer risk. Therefore, it is suggested that estrogen synthesis in adipose tissue is involved in this increasing risk (5-7). However, ER-α and ER-β genes polymorphisms have been associated with breast cancer risk in Caucasians, with certain clinical features including presence of a family history (8) and lymph node (LN) metastasis (8-12). At the present the literature provide a little information regarding ESR2 gene expression, mutational frequency, and allelic variants in breast cancer. Thus, in this study we tried to identify gene variants in selected region of exon 7 in estrogen receptor β gene (ESR2), and its association with breast cancer risk in order to establish for the first time in Iran as Asian-Caucasian genome, and to test any correlation between gene polymorphism and clinical features of breast cancer in Iranian women.

3. METHODS AND MATERIALS

Study population

A case-control study was conducted from April 2014 to January 2018 in Tehran, Iran. The breast cancer cases (n =150; median age 47.49 ± 11.43 years old) were newly diagnosed at the Imam Khomeini Hospital Complex and were referred to our several clinics of the Cancer Institute, including Women Sections 1 and 3, and Central Clinics of 1 and 2 for breast surgery. The control group (n =147; median age 40.75± 10.54 years) included healthy women neither with any history of breast cancer nor any other neoplastic diseases, and also none of their relatives had a history of breast cancer. By the permission from the hospital ethics committee, all the patients provided with written informed consent to

participate in that protocol before entering into the present study.

Study population

Demographical and risk factor data were collected using a short structured questionnaire, including information on age, weight, height, race, religion, marital status, number of pregnancies and children, age at the first child birth, average lactation term, family history of breast cancer (first-degree relatives), age at menarche, age at marriage, parity, age at first pregnancy, menopausal status, and age at menopause, ABO and Rhesus blood groups, race, age at onset, lymph node metastases, cancer stage at the time of testing and ER expression in breast cancer tissue. An ongoing protocol to collect and store blood samples for future genomic tests had been approved by the institutional review board. Peripheral whole blood was collected and kept in storage at -80°C until genotyping analysis. This information was obtained by interview with patients and family members.

Screening for ESR2 variants by single strand conformation polymorphism analysis

To identify any mutation or SNPs in ESR2 gene codon 7 we used the PCR single-strand conformation polymorphism (SSCP) method. The genomic DNA was extracted from whole blood cells using DNGTM-Plus extraction solution kit (Cinnagen Inc, Tehran, Iran) in according to the the manufacturer's instructions. Genomic DNA (50 ng) was used for each run of PCR-based genotyping.

Exons 7 of the ESR2 gene was amplified by PCR methods, set of oligonucleotide primers designed by primer3 (v. 0.8.0) software.

Exon 7 F 5' GATGAGGGGAAATGCGTAGA 3' R 5' GGCCCAGCTGTGTGATTACT 3' 156bp

PCR was performed for 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C and 40 seconds at 72°C. Optimal electrophoretic separation for SSCP was conducted in 12% polyacrylamide gel (29:1 Acrylamide: Bisacrylamide) , in 200 V, 20 hours duration at 16oC. Electrophoresis followed by 0.1% silver nitrate staining. Then the bands with shifting patterns were purified on agarose gel using a DNA Extraction Kit, Fermentas # K0153, Germany, and directly sequenced by big dye Terminator V3.1 Cycle Sequencing kit protocol, (Applied Biosystem Kit, Microgen Co., USA), on a sequencer ABI 3130XL (16capillaries)

We also used the PCR products purification method in order to confirm sequencing by reverse primer. The PCR products were purified using QIAquick PCR purification Kit (50), QIAGEN cat. #28104, USA (through Zistbaran Co. Iran).

Statistical analysis

To assess the influence of polymorphism status on features of breast cancer we performed the $\chi 2$ testing. Unconditional logistic regression analysis was followed using SPSS software (version 16.5 for Windows XP; SPSS Inc., Cary, NC, USA) to calculate odds ratios (ORs) with 95% confidence intervals (CIs) and to examine the predictive effect of each factor on risk for breast cancer. $P < 0.05$ was considered as a statistically significant.

4.RESULTS

The estrogen receptor- β gene is on Chromosome 14, with 8 exons. The encoding region exon 7 of the ESR2 gene was screened for mutation or any variant sites by Single Strand Conformational Polymorphism -PCR followed DNA sequencing. We did not find any novel mutations but it did reveal the presence of a silent single nucleotide polymorphisms (SNP) at codon 392 (Leu 392 Leu) (dbSNP128), rs1256054, in which nucleotide C is converted to G (C 1176 G). Both CTC and CTG are codon which code for Leucine amino acid (13). This SNP in codon 392 was presented only in cases group. The statistically significant frequencies were achieved, 8.7% in for the CG heterozygote genotype, and 1.3%for the CC homozygote genotype in compare with 90.0% with CC normal genotype ($\chi 2=4.769$, P=0.029).

The frequency of allele G in codon 392 was significantly (χ 2= 4.583P = 0.032) higher in cancer patients with the age at menarche </=12 years old (9.2%) than >12 years old (3.3%). The frequency of allele G in codon 392 was significantly very much higher in cancer patients with a first-degree family history (36.8%) than in those without a family history (1.1%), a difference of 35.7%, in compare with the frequency of allele C in codon 392 which is significantly lower in cancer patients with a first-degree family history than in those without a family history, also a difference of 35.7% ($\chi 2 = 78.847$, P = 0.001). The frequency of allele G in codon 392 was significantly very much higher in cancer patients with LN metastases (23.9%) than in those without LN metastases (2.4%) a difference of 21.5%, in compare with the frequency of allele C in codon 392 which was significantly lower in cancer patients with LN metastases (76.1 %) than in those without LN metastases (97.6 %), also a difference of 21.5 % ($\chi 2 = 33.838$, P = 0.001)(Table 1).

The estimated risk was much higher for individuals without family history of breast cancer than individuals with family history of breast cancer (94.8% and 5.2% respectively) who were CC normal homozygote. In compare with CG heterozygote, for codon 392 polymorphism, with threefold higher frequency for individuals with family history of breast cancer than individuals without family history of breast cancer (76.9% and 23.1% respectively) (OR 0.016, 95% CI 0.004- 0.073), and with corresponding GG homozygote who were all within individuals with family history of breast cancer.

The estimated risk was nine fold higher for individuals without LN metastases who were CC, normal, in codon 392 (89.6% and 10.4%), but in corresponding CG heterozygote genotype the estimated risk was higher for individuals with LN metastases (53.8%) than for individuals without LN metastases (46.2%) (OR 0.099, 95% CI 0.029-0.337). Although, GG homozygote, in codon 392 individuals, all were only within the patients with LN metastases.

5. DISCUSSION

The SNP of estrogen receptor β was only presented in cancer patients, 8.7% in CG heterozygote genotype and 1.3 %in GG homozygote genotype in exon 7 in Iranian cancer patients. Among German population Five SNPs in ER- β gene have been identified (14), however, one

of them , rs1256049, provided an evidence for association with anorexia nervosa. This sameERS2 SNP had a highly statistically significant association with ovulatory dysfunction in a Chinese and Brazilian population (15-16). In addition, an intragenic CA repeat polymorphism in ESR2 has been associated with bone mineral density in a Japanese research population (17). The Shanghai breast study group reported An ESR2 exon 7 synonymous SNP (rs1256054, L392L) as conferring increased risk of breast cancer (OR, 2.37; 95% CI, 1.18–4.77) in a robust Shanghai breast study (18), and they hypothesized that this SNP may act as an exonic splicing enhancer (19). Although not all polymorphisms in ERs associated with increased risk of breast cancer, such as PvuII and XbaI polymorphisms (21-23).

The frequency of allele G in codon 392 was significantly very much higher in cancer patients with lymph node metastases than in those without LN metastases a difference of 21.5%, in compare with the frequency of allele C in codon 392 which was significantly lower in cancer patients with LN metastases than in those without LN metastases, also a difference of 21.5 % (Table 1).

The estimated risk was very much higher for individuals without family history of breast cancer than individuals with family history of breast cancer (94.8% and 5.2% respectively) who were CC normal homozygote. In compare with CG heterozygote, for codon 392 polymorphism, with threefold higher frequency for individuals with family history of breast cancer than individuals without family history of breast cancer (76.9% and 23.1% respectively) (OR 0.016, 95% CI 0.004- 0.073), and with corresponding GG homozygote who were all within individuals with family history of breast cancer .

In CG heterozygote genotype the estimated risk was higher for individuals with LN metastases (53.8%) than for individuals without LN metastases (46.2%) (OR 0.099, 95% CI 0.029-0.337), in compare with CC normal individuals. Although was less likely in the cases (OR< 1). (Table 2).

Our results revealed that codon 392 polymorphism in ESR2 is significantly different in case and control groups (10.1% and 0.0% respectively) in compare with only one similar study in China population (17), it showed same frequencies in codon 392 polymorphism among case and control groups (93.5% and 94.7% respectively). Taking these results together, codon 392 SNP as conferring increased risk of breast cancer. We also noted that greater the frequency of allele G, the higher the likelihood of LN metastasis in Iranian women population with breast cancer.

5.1. Figures and Tables

Table 1. Genotypic and allelic frequencies of estrogen receptor-b exon 7, codon 392 (CTC/CTG) in the study population: breast cancer cases versus control groups and breast cancer cases in the presence versus the absence of major risk factors

Codon 392 / Characteristic		ER-β genotypes			ER-β Alleles	
		CC	CG	GG	C	G
Breast cancer						
Case	(n=150)	135(90.0%)	13(8.7%)	2(1.3%)	283(94.3%)	17(5.7%)
Control	(n=147)	147(100%)	-	-	294(100%)	-
		χ^2=4.769 , *P*=0.029			χ^2=17.122 , *P*=0.001	
Age at menarche(years)						
</=12	(n=60)	50(83.3%)	9(15.0%)	1(1.7%)	109(90.8%)	11(9.2%)
>12	(n=90)	85(94.4%)	4(4.4%)	1(1.2%)	174(96.7%)	6(3.3%)
		χ^2=0.604 , *P*=0.437			χ^2=4.583 , *P*=0.032	
Family history of breast cancer						
First-degree family affected	(n=19)	7(36.8%)	10(52.6%)	2(10.6%)	24(63.2%)	14(36.8%)
Not affected	(n=131)	128(97.7%)	3(2.3%)	-	259(98.9%)	3(1.1%)
		χ^2=27.645 , *P*=0.001			χ^2=78.847 , *P*=0.001	
Lymph node metastases						
Yes	(n=23)	14(60.9%)	7(30.4%)	2(8.7%)	35(76.1%)	11(23.9%)
No	(n=127)	121(95.3%)	6(4.7%)	-	248(97.6%)	6(2.4%)
		χ^2=17.314 , *P*=0.001			χ^2=33.838 , *P*=0.001	
ER expression in breast cancer tissue						
Positive	(n=40)	32(80.0%)	6(15.0%)	2(5.0%)	70(87.5%)	10(12.5%)
Negative	(n=92)	87(94.6%)	5(5.4%)	-	179(97.3%)	5(2.7%)
Not studied	(n=18)	16(88.9%)	2(11.1%)	-	34(94.4%)	2(5.6%)
		χ^2=4.779 , *P*=0.028			χ^2=5.161 , *P*=0.023	

Table 2. Estimated risk for selected demographic characteristic and major risk factors with estrogen receptor-β exon 7, codon 392 in different genotypes

Breast cancer / **Genotype**	**Yes n=150**	**No n=147**	***P* value**	**OR (95% CI)**
Normal CC	135(47.9%)	147(52.1%)	0.029	1.0(reference)
Heterozygote CG	13(100%)	-		-
Homozygote GG	2(100%)	-		-
Age at menarche (years) / **Genotype**	**</=12 n=60**	**>12 n=90**	***P* value**	**OR (95% CI)**
Normal CC	50(37.0%)	85(63%)	0.437	1.0(reference)
Heterozygote CG	9(69.2%)	4(30.8%)		0.261(0.077-0.893)
Homozygote GG	1(50%)	1(50%)		0.588(0.036 - 9.613)
First- degree family history of breast cancer / **Genotype**	**Affected n=19**	**Not affected n=131**	***P* value**	**OR (95% CI)**

Normal CC	7(5.2%)	128(94.8%)		1.0(reference)
Heterozygote CG	10(76.9%)	3(23.1%)	0.001	0.016(0.004-0.073)
Homozygote GG	2(100%)	-		-
Lymph node metastases **Genotype**	**Yes** **n=23**	**No** **n=127**	***P* value**	**OR (95% CI)**
Normal CC	14(10.4%)	121(89.6%)		1.0(reference)
Heterozygote CG	7(53.8%)	6(46.2%)	0.001	0.099(0.029 – 0.337)
Homozygote GG	2(100%)	-		-

4. CONCLUSION

To our knowledge, this was the first study in estrogen receptor gene polymorphism and breast cancer risk. Our data suggest that ESR2 polymorphism is correlated with various aspects of breast cancer in Iran. In ER- β genotype, the more allelic frequency of allele G in 392 codon the more likelihood to develop familial breast cancer and lymph node metastasis in Iranian breast cancer patients. Therefore it represents a candidate marker for predicting for both familial breast cancer and lymph node metastasis.

This research was supported by Tehran University of Medical Sciences & Health services, Research Deputy Grants..

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