ORIGINAL ARTICLE

The Effect of Nucleotide Changes in Exon 2 of CYP1B1 (Arg48Gly) on Serum Concentration of Estrogen in Patients with Breast Cancer

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ABSTRACT

Introduction: Cytochrome P450 enzymes play an important role in the removal of carcinogenic compounds from the body. *CYP1B1* is a member of this family that is involved in the metabolism of steroids and acts as a promoter or suppressor of tumor progression through hormonal control. In this study, we investigated the effect of *Arg48Gly* amino acid change in *CYP1B1* (RS 10012) gene on the serum concentration of estrogen and risk of breast cancer. **Materials and Methods:** We selected 128 breast cancer patients and 128 healthy control subjects for this study. After venipuncture, DNA was extracted using the salting out method and genotyping was done through the restriction fragment length polymorphism polymerase chain reaction method. The ELISA method was used to measure the serum concentration of estrogen in the samples. The data were analyzed with the IBM SPSS 23 software using Chi-square and logistic regression. **Results:** The mean age and BMI of the patients and controls had significantly association between two groups. Statistical analysis showed that the CC genotype had a protective role against cancer in carriers ($P = 0.017$, OR: 0.548, CI 95%; 0.334–0.901) while the mutant GG genotype increased the odds of breast cancer by 1.653 times ($P = 0.046$, OR: 1.653, CI 95%; 1.009–2.709). The heterozygous genotype had no significant relationship between the two groups ($P = 0.309$, OR: 2.049, CI 95%; 0.501–8.378). Evaluation of the relationship between the genotypes and other variables showed that the CC and GG genotypes had a relationship with the serum concentration of estrogen. **Conclusions:** The odds ratio of breast cancer in the carriers of the G allele showed that genetic changes related to estrogen metabolism may have a role in the progression of breast cancer. A larger sample size may introduce the genetic changes involved in breast cancer progression with more certainty.

Key words: Breast cancer, CYP1B1 gene, estrogen concentration, restriction fragment length polymorphism polymerase chain reaction, Rs. 10012

INTRODUCTION

Breast cancer is a complex disease in which both environmental and genetic factors play a role.[1] In addition, the serum levels of endogenous estrogens such as estradiol and estrone are related to the risk of breast cancer.[2] Some studies have shown that the effect of sex hormones, especially estrogens, on the risk of breast cancer depends on age and menopausal status. This finding supports the hypothesis that functional single nucleotide polymorphisms (SNPs) in the genes involved in the synthesis of sex hormones can affect signal transduction, metabolism of age related hormones, and menopausal status of the person. However, most studies suggest that the risk of these SNPs has no relationship with age.[1]

The importance of estrogen in the development of cancer probably lies in the polymorphisms that occur in estrogen metabolizing genes. Some cytochrome P450 (*CYPs*) enzymes, including *CYP1B1*, affect oxidative metabolism by estrogens.[3] The large P450 family includes constitutive and inducible enzymes responsible for catalytic hydroxylation in

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many compounds with diverse chemical structures. The P450 enzymes that metabolize xenobiotics include *CYP1*, *CYP2*, and *CYP3* and some types of *CYP4*, while *CYP11, CYP17, CYP19,* and *CYP21* are involved in the synthesis of endogenous substrates such as steroids, fatty acids, and prostaglandins.[4]

Cytochrome P450 1B1 (*CYP*$_1$*B*$_1$) is one of the main enzymes in estrogen hydroxylation. The CYP1B1 human gene is located on the short arm of chromosome 2 (2p21–22) and has 3 exons and 2 introns with a length of about 12kb.[5] This enzymes has a protein sequence of 543 amino acids and is expressed in most tissues, including the prostate,[6] breast,[5,7] ovaries,[8] and kidney and uterus.[9] Polycyclic aromatic compounds[10] and estradiol[11] are the substrates of *CYP1B1* which can be converted into a DNA-binding quinone with carcinogenic potency after 4-hydroxylation.[12]

Several polymorphisms have been identified in relation with the *CYP1B1* gene that can change its enzymatic activity and catalytic characteristic, including Rs 10012 in which cytosine is replaced with guanine at position 142,[13] resulting in the substitution of arginine with glycine at position 48 (*Arg48Gly*).[14] We hypothesized that this nucleotide change in exon 2 of the *CYP1B1* gene might affect the serum concentration of estrogen and increase the risk of breast cancer. To evaluate this relationship, a group of breast cancer patients were compared with healthy controls.

MATERIALS AND METHODS

In this case–control study, the blood samples of 128 women with breast cancer and 128 healthy control women who were visited in Shohada-e-Tajrish Hospital were taken after clinical examinations and tests and specialist's confirmation. The control women had a negative history of diseases such as hypertension, diabetes, and cardiovascular disease and there were no cases of cancer in their first-degree relatives. After obtaining informed consent, 3–5 ml blood was drawn from each participant. Part of the drawn blood was mixed with EDTA and frozen at −20°C and part of it was coagulated to separate its serum.

Determine the concentration of estrogen by ELISA

The ELISA method was used to assess the sera using a commercial kit according to the manufacturer's instructions.

Extraction of DNA and determine genotype of samples

The saturated salt method was used for extraction of DNA from the blood samples[15] and the quality of extracted DNA in all of samples was examined by Agarose 1% and Spectrophotometer. Specific primers complementary to the target area were selected to perform the polymerase chain reaction (PCR) in the following order:

Forward: 5'TCT CCA GAG AGT CAG CTC CG 3'Reverse: 5'GTG CCG CGT TTC CGA TCA 3' Each reaction was thermocycled with a total volume of 23 µl, including 10 µl amplicon commercial master mix, 10 µl distilled water, and 1 µl of each primer forward and Reverse (10 pM), and 50 ng of the DNA of each sample. The thermocycler was scheduled for primary denaturation at 94°C for 5 min, followed by 36 cycles of 1 min at 94°C, 30 s at 58°C, 40 s at 72°C, and finally one cycle of 7 min at 72°C for completing the synthesis of DNA strands. Amplified products showed 320bp length on agarose gel 1.5%. To confirm the presence or absence of SNP was used *MspI* enzyme. This enzyme had two cutting sites on the piece that was amplified. One of them was related to the polymorphism. When *MspI* enzyme was added to the PCR products, the mixture was incubated at 37°C for 18 h. Then, the fragmented pieces were loaded on agarose 3% for visualization under ultraviolet light.

When nucleotide of C have changed to G, three pieces 271 bp, 36 bp, and 13 bp in length indicated a mutant CC genotype. If the individual genotype was GG observed two pieces 307 and 13bp. For GC heterozygote genotype was seen four pieces 307, 271.36, and 13bp [Figure 1].

Statistical of analysis

According to Hardy-Weinberg equilibrium were calculated the percentage of each genotype and the frequency of the alleles of each polymorphism. The Chi-square and Fisher's exact test were used to compare the distribution of the genotypes in two groups by IBM SPSS 23 software. The relationship between genotypes and risk of breast cancer was calculated by the odds ratio with a confidence interval of 95% using logistic regression analysis. $P < 0.05$ was considered significant.

RESULTS

Cancer patients and controls aged 32–75 years. The mean age of the cancer patients and participants in the control group was 57.65 years and 51.99 years, respectively. Table 1 shows the biochemical and clinical characteristics of the patients.

Evaluation of the genotypes and calculation of the frequency of the alleles according to the Hardy-Weinberg principle showed that the frequency of the C allele was 0.41 in the cancer and 0.55 in the control group. The frequency of the G allele was 0.59 in cancer patients and 0.45 in control participants. Evaluation of allele frequency distribution in case and control groups showed that the frequency of the G allele, as the risk allele, was higher in patients than controls and the frequency of the C allele was higher in controls. Calculation of the odds ratio showed that the GG genotype increased the odds of breast cancer by 1.653 while the CC genotype had a protective effect against breast cancer. The distribution of genotypes and genetic models and

Table 1: Biochemical and clinical characteristics of two groups

Variable	Range	Mean±STD error difference		*P* value
		Case	Control	
	50≥	34 (26.56%)	60 (46.87%)	
	50<	94 (73.44%)	68 (53.13%)	
	Mean± STD error difference	57.65±0.89	51.99±0.91	5.7×10^{-5}
BMI (kg/m2)	25≥	64 (50%)	82 (64.06%)	
	>25	64 (50%)	46 (35.94%)	
	Mean±STD error difference	25.97±0.28	25.18±0.28	0.013
Smoker	Smoker	26 (20.31%)	20 (15.63%)	0.329
	Non-smoker	102 (79.69%)	108 (84.37%)	
Serum E2(pg/ml)	Follicular phase	26 (20.31%)	38 (29.69%)	0.937
	Mean±SD	80.27±23.15	83.12±24.81	
	Ovulatory peak	17 (13.28%)	24 (18.75%)	0.003
	Mean±SD	203.65±30.08	779.29±151.13	
	Luteinic phase	22 (17.19%)	25 (19.53%)	0.646
	Mean±SD	68.65±5.75	72.65±6.34	
	Menopause	63 (49.22%	41 (32.03%)	0.505
	Mean±SD	7.39±2.90	11.11±5.25	
	Total mean±STD error difference	54.92±7.59	97.96±13.09	0.005
Estrogen receptor	Positive	109 (85.16%)		
	Negative	19 (14.84%)		
Progesterone receptor	Positive	81 (63.28)		
	Negative	47 (36.72)		
Type of cancer	Invasive lobular carcinoma	58 (45.31%)		
	Invasive ductal carcinoma	40 (31.25%)		
	Ductal carcinoma *in situ*	16 (12.50%)		
	Lobular carcinoma *in situ*	14 (10.94%)		
Grade of cancer	Grade I	4 (3.13%)		
	Grade II	34 (26.56%)		
	Grade II/III	31(24.22%)		
	Grade III	19 (14.84%)		
	Metastasis	40 (31.25%)		

association with the risk of breast cancer is summarized in Table 2.

The concentration of estrogen was measured in both groups using the ELISA method. The mean serum concentration of estrogen was 54.92±7.59 pg/ml in cancer patients and 97.96±13.09 pg/ml in the control group with a significant difference. The relationship between the CC and GG genotypes and the mean serum concentration of estrogen was significant between two groups. Evaluations showed that the CC and mutant GG genotypes had an effect on the serum concentration of estrogen. The results are shown in Figure 2.

DISCUSSION

Androgen sex hormones, including estrogens, have an important role in the development and progression of breast cancer as a carcinogen. A very close association has been observed between increased serum concentrations of these hormones and the risk of breast cancer.[16] Moreover, some studies have shown a relationship between increased levels of estrogen in the blood (and consequently an increase in the duration of its effect on the breast cancer) and increased risk of breast cancer.[17] The evidence confirms the key role of this hormone in breast cancer.

Table 2: Statistical results of distribution of genotypes and genetic models with the risk of breast cancer

	Genotype	Case	Control	*P Value*	Odds ratio	CI 95%
		n (%)	*n* (%)			
Rs. 10012	CC	50 (39.06)	69 (53.91)		1 (Reference)	
	GG	72 (56.25)	56 (43.75)	0.026	1.774	1.07–2.94
	CG	6 (4.69)	3 (2.34)	0.165	2.76	0.66–11.57
Genetic models						
Dominant	CC	50 (39.06)	69 (53.91)		1(Reference)	
	GG+CG	78 (60.94)	59 (46.09)	0.018	1.824	1.11-2.99
Recessive	CC+CG	56 (43.75)	72 (56.25)		1 (Reference)	
	GG	72 (56.25)	56 (43.75)	0.046	1.653	1.009–2.709
Additive	CG	6 (4.69)	3 (2.34)		1 (Reference)	
	CC+GG	122 (95.31)	125 (97.66)	0.318	0.488	0.119–1.99
Codominant	CC	50 (39.06)	69 (53.91)		1 (Reference)	
	GG	72 (56.25)	56 (43.75)	0.545	0.643	0.153–2.68

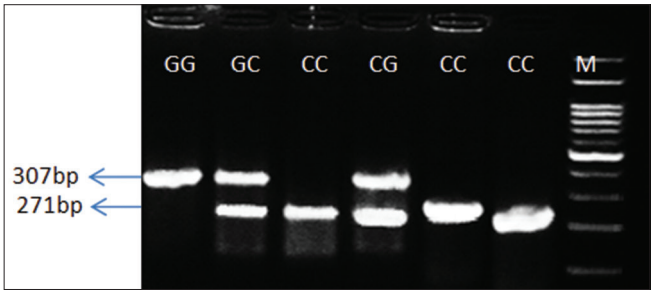

Figure 1: Result of digest samples (from left to right): First well is GG genotype, Wells 2, 4 GC genotype and wells 3, 5, and 6 are CC genotype

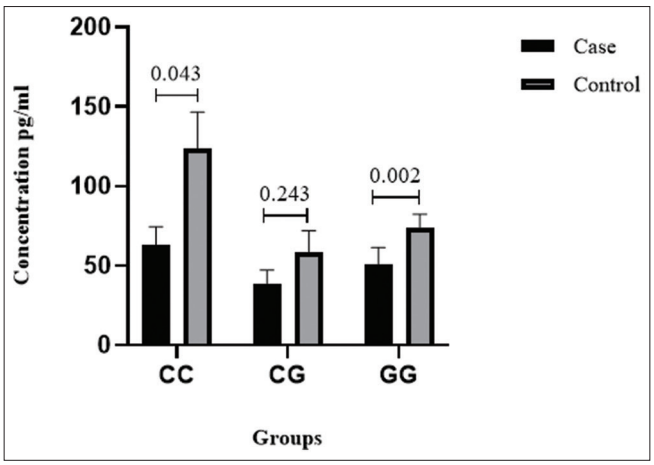

Figure 2: The Mean serum concentration of estrogen in cancer and control groups with GG and CG genotypes was observed less than CC genotype

Estrogen exerts its carcinogenic effect through stimulating cell proliferation, affecting the activity of intermediate receptor, and increasing the production of intermediate metabolites by Cytochrome P450 (due to gene mutations).[17] *CYP1B1* gene is involved in estrogen metabolism.[18] Numerous polymorphisms of this gene have been in association with breast cancer.[19-21] The product of this gene is an enzyme that contributes to the production of carcinogenic metabolites, including E2, from 4-OH-E2.[22] The level of E2 is markedly higher in cancer tissues versus normal tissues.[23] The ratio of 4-OH-E2/2-OH-E2 is also significantly higher in cancer tissues.[22,24] This evidence confirms the important role of *CYP1B1* in the development and progression of breast cancer.

Two polymorphisms 355G>T (m2) and 142-C>G (m1) have been identified in exon 2 of this gene which results in the substitution of *Arg48Gly* and *Ala119Ser*.[12] According to a study by Hanna *et al*., the presence of Gly48 allele in the enzyme increases the catalytic activity of estrogen.[14] Another study showed that although the Gly48 allele did not alter the expression of the *CYP1B1* gene, it caused minor changes in the Vmax and Km of the enzyme in 2,4 hydroxylation of estradiol. Since this reaction has an important role in carcinogenesis, it is likely that *CYP1B1* polymorphisms have an important role in susceptibility to diseases in which this gene is active in target tissues.[25] Zimarin *et al*. found a significant association between the C allele of *Arg48Gly* polymorphism and the risk of breast cancer.[26] Moreover, Lubinski *et al*. also found a close relationship between this polymorphism and breast cancer.[27] However, Gaudet *et al*. found no significant association between this polymorphism and breast cancer[28] and McLellan *et al*. reported that R48G polymorphism caused no change in the folding and stability of *CYP1B1* enzyme, and therefore would not change its activity.[29]

CONCLUSION

In the present study, we evaluated the relationship between the serum concentration of estrogen and rs10012 polymorphism,

and found that the GG mutant genotype was associated with the serum concentration of estrogen. Moreover, the grade of cancer and BMI had a significant association with CC and GG genotypes. Our investigation showed that the risk of breast cancer was higher in the carriers of the GG genotype by a factor of 1.653 while the CC genotype decreased the odd of breast cancer by a factor of 0.548. In line with the results of previous studies, our study showed that genetic changes of *CYP1B1* enzyme had a key role in the development and progression of breast cancer and some genetic changes in the metabolism pathway of estrogen affected the risk of breast cancer.

ACKNOWLEDGMENTS

The author would like to express their gratitude to the medical personnel at Shohadaye Tajrish Hospital in Tehran and all the patients who participated in this study. A special thanks to Dr. Akbariand Dr Nafisi for introducing female patients for the research.

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How to cite this article: Tahmasebi Fard Z. The Effect of Nucleotide Changes in Exon 2 of CYP1B1 (Arg48Gly) on Serum Concentration of Estrogen in Patients with Breast Cancer. J Clin Res Oncol 2020;3(2):1-6.