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ASSOCIATION OF METHYLENETETRAHYDR OF OLATEREDUCTASE GENEPOLYMORPHISMS (C677TRS1801133ANDA1298C RS1801131) WITH BREAST CANCERIN IRAQIPATEINTS

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ABSTRACT

Background

Methylenetetrahydrofolatereductase (MTHFR) is a critical enzyme in folate metabolism. Folate plays an important role in DNA methylation, synthesis and repair.The folate-metabolizing enzymeispolymorphic at nucleotides 677(C→T) and 1298(A→C), resulting in allozymes with decreased activity.Thus, polymorphisms might influence genetic susceptibility to breast cancer.

Aim

To study the association of MTHFR (C677Tand A1298C) gene polymorphisms with breast cancer in Iraqi women.

Methods

Case-control study consisted of 300 breast cancer patients and 170 healthy control. DNA was extracted from whole blood and genotyping was achieved with specific primers to amplify fragments for digestion with restriction enzymes (polymerase chain reaction– restriction fragment length polymorphism (PCR-RFLP)). Followed by electrophoresis on agarose geland UV visualization

Results

The homozygous genotype (TT) of MTHFR C677T in codominant was significantly increased the risk of breast cancer 4.54 folds with respect to those of the wild type (CC). The homozygous genotype (CC) of MTHFR A1298C in codominant was significantly increased the risk of breast cancer 3.05 folds with respect to those of the wild type (AA).

Conclusions

MTHFR (C677T, A1298C) gene polymorphisms were associated with breast cancer in Iraqi women.

KEYWORDS: MTHFR (C677T and A1298C), Gene Polymorphisms, Breast Cancer

INTRODUCTION

In Iraq, breast cancer is the commonest type of female malignancy, accounting for approximately one-third of the registered female cancers according to the Iraqi national cancer research center 2014[(1)].The enzyme which is encoded by MTHFR gene catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5- methyltetrahydrofolate, which is a co-

substrate for homocysteineremethylation to methionine. Genetic variation in this gene influences susceptibility to breast cancer[2, 3]. The mutation of the MTHFR gene which results in the C677T polymorphism is located at exon 4 which causes the conversion of valine to alanine at codon 222, a common polymorphism that reduces the activity of this enzyme. The MTHFR A1298C gene polymorphism results from A to C transition in exon 7 resulting in an amino acid substitution of glutamine to alanine at codon 429 of the protein[4].

PATIENTS AND METHODS

Subjects

Three hundred female patients with primary breast carcinoma were included in this study, their mean age was 49.26 ± 9.86 years,who attended the tumors center at Al-Sader teaching medical city, AL Najaf, Iraq. The control group included 170 healthy females, randomly selected,theirageswas 48.92 ± 12.82 years.

DNA Extraction and PCR Amplifications

Whole blood was collected into EDTA-coated tubes. Genomic DNA was extracted from Whole blood using aReliaPrep™ Blood gDNAMiniprep System (Promega,USA).MTHFR C677T and A1298C mutations were detected after PCR amplification with corresponding primers. The primers for MTHFR C677T gene polymorphism were 5′-TGA AGG AGA AGG TGT CTG CGGGA-3′and 5′-AGG ACG GTG CGG TGA GAG TG-3′.The primers for MTHFRA1298Cgene polymorphism were 5′CTTCTACCTGAAGAGCAAGTC-3′and 5′ CATGTCCACAGCATGGAG-3′.

Data Analysis

Odds ratios (ORs) were used to measure the association of breast cancer risk with the MTHFR polymorphisms.Unconditional logisticregressions were used to obtain maximumlikelihood estimates of the ORs and their 95% confidence intervals.Genotype frequencies of polymorphisms were consistent with Hardy–Weinberg equilibrium.

RESULTS

The baseline characteristics of cases and controls are summarized in table 1.

Table 1: Comparison of Cases and Controls by Selected Characteristics

Parameters	Control Subjects (No.= 170)		Patient Subjects(No.= 300)	P Value
Age (y)	48.92 ± 12.82		49.26 ± 9.86	0.67
BMI (kg/m^2)	27.91 ± 3.39		29.75 ± 4.32	0.000
Residency	**Urban**	120 (70.5%)	198 (66%)	0.3
	Rural	50 (29.5%0	102 (34%)	
Menopausal status	**Premenopausal**	91(39.34±7.68)	158(41.77±5.53)	0.85
	Postmenopausal	79(59.87±7.81)	142(57.59±6.28)	
Histologic types	**Ductal carcinomas**		261 (87 %)	
	Lobular carcinomas		39 (13 %)	

P-value < 0.05 is significant.

RFLP Analysis of MTHFRC677T Gene Polymorphism

The digestion ofPCR productof MTHFR C677Tgene polymorphism by*Hinf1*is shown in figure 1

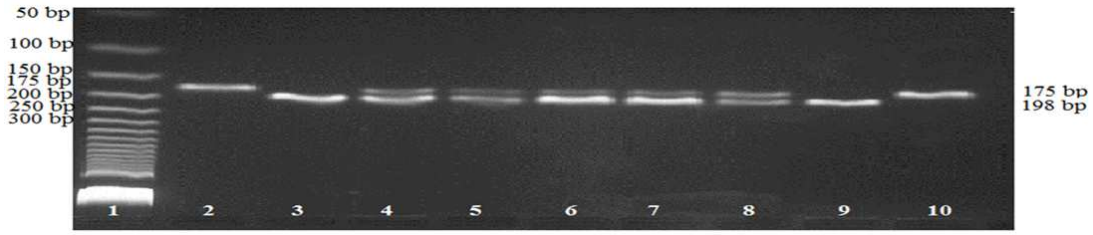

Figure 1: Polymorphism Analysis of MTHFR C677T.The PCR Product Were Digested with Restriction Enzyme *Hinfi*. The digestion products Analyzed by 3 % Agarose Gel Electrophoresis (75 V for 2.5hrs). Line1: DNA Ladder (50-1000 Bp); Lines 3 and 9 for Wild Typecc (198 Bp); Lanes 4, 5, 6, 7and 8 for Heterozygous CT Genotype (198,175 Bp); Lane 2 and 10 for TT Homozygous Genotype (175, 23bp).the Small Fragment of 23 Bp That Formed as a Result of Digestion Is Eluted from the Gel

RFLP Analysis of MTHFRA1298C Gene Polymorphism

The digestion of PCR product of MTHFR A1298C gene polymorphism by MboII was shown in figure 2.

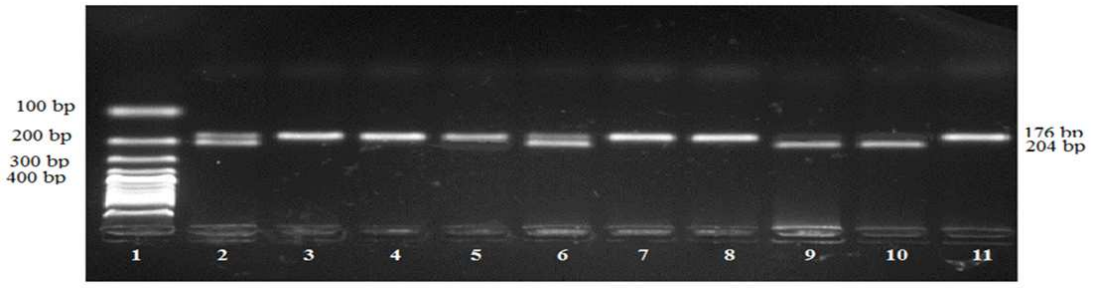

Figure 2.Polymorphism Analysis of MTHFR A1298C the PCR Products Were Digested with Restriction Enzyme *Mboii*. the Product of Digestion Were Analyzed by 3 % Agarose Gel Electrophoresis (75V for 2.5hrs). Line1 :(100-1500 Bp) DNA Ladder; Lines 3, 4, 5, 7, 8, 11: Forcchomozygous Genotype (176bp); Lanes 2, 6: Forca Heterozygous Genotype (204, 176bp); Lane 9, 10: Aa for Wild Type Genotype (204 Bp).

Distributions of Genotypes of Breast Cancer Patients According to Body Mass Index (BMI):

Patients were classified according to WHO classification of obesity.The findings revealed significant difference between patient groups and the highest frequencies were in obese group, table 2.

Table 2: Genotypes of Breast Cancer Patients Distributed by Body Mass Index

Genotypes		BMI 18.5 -25 (Kg/ M^2) No. / %	BMI 25 -30 (Kg/ M^2) No. / %	BMI ≥30 (Kg/M^2) No. / %	P Value
MTHFR C677T	CC	31(10.3 %)	43 (14.3 %)	83 (27.7 %)	0.017
	CT	15 (5%)	50 (16.7%)	55 (18.3 %)	
	TT	0 (0 %)	11 (3.7 %)	12 (%4)	
MTHFR A1298C	AA	29 (9.7 %)	34 (11.3 %)	56 (18.7 %)	0.000
	AC	17 (5.7 %)	43 (14.3 %)	68 (22.7 %)	
	CC	0 (0 %)	27 (9 %)	26 (8.7 %)	

Distributions of Genotypes of Breast Cancer Patients According to Family History of Breast Cancer

The results shown statistically significant difference of mutant alleles of MTHFR (C677TTT,A1298C)gene polymorphisms between positive and negative family history of breast cancer ($P < 0.05$).First-degree family history of breast cancer was associated with an increased risk of breast cancer (odd ratio of MTHFR C677T TT:7.12; A1298C:2.22),table 3.

Table 3: Associations between Genotypes and Breast Cancer Patients According to Family History of Breast Cancers

Genotypes		Negative Family History (263) No/%	Positive Family History (37) No/%	OR (95% CI)	P Value
MTHFR C677T	CC	(49 %)147	(3.3 %)10	0.29 (0.13 -0.62)	0.0016
	CT	(34.3 %)103	(5.7 %)17	1.32 (0.66- 2.63)	0.43
	TT	(4.3 %)13	(3.3 %)10	7.12 (2.85-17.78)	0.0001
MTHFR A1298C	AA	(35.7 %)107	(4 %)12	0.69 (0.33- 1.45)	0.33
	AC	(38 %)114	(4.7 %)14	0.79 (0.39- 1.61)	0.52
	CC	(14 %)42	(3.7 %)11	2.22 (1.02- 4.84)	0.043

The Association between Gene Polymorphisms and Grades of Breast Cancer

The patients are classified according to Scarff- Bloom-Richardsonclassification [21]. According to gradesof breast cancer, there were highly significant differencebetween gene polymorphismsand grades,table 4.

Table 4: Relationship between Genotypes and Grades of Tumor in Breast Cancer Patients

Genotypes		Grades			P Value
		Grade I (28)	Grade II (154)	Grade III (118)	
MTHFR C677T	CC	20(6.7 %)	90(30 %)	47(15.7 %)	0.001
	CT	4(1.3 %)	56(18.7 %)	60(20 %)	
	TT	4(1.3 %)	8(2.7 %)	11(3.7 %)	
MTHFR A1298C	AA	12(4 %)	65(21.7 %)	4214 %	0.000
	AC	14(4.7 %)	74(24.7 %)	40(13.3 %)	
	CC	2(0.7 %)	15(5 %)	36(12 %)	

The Association of Gene Polymorphisms with the Tumor Size of the Breast Cancer Patients

Breast cancer patients were classified according to tumor sizes into 4 groups.There was highly significant ($p < 0.05$) differences betweengene polymorphisms and four groups of tumor sizes, table 5.

Table 5: The Association of Gene Polymorphisms with the Tumor Size of the Breast Cancer Patients

Genotypes		Tumor Size				P Value
		T1(34)	T2 (158)	T3(75)	T4(33)	
MTHFR C677T	**CC**	20 (6.7 %)	101 (33.7 %)	23 (7.7 %)	13 (4.3 %)	0.000
	CT	12 (4 %)	47 (15.7 %)	43 (14.3%)	18 (6 %)	
	TT	2 (0.7 %)	10 (3.3 %)	9 (3 %)	2 (0.7 %)	
MTHFR A1298C	**AA**	18 (6 %)	72 (24 %)	15 (5 %)	14 (4.7 %)	0.000
	AC	15 (5 %)	75 (25 %)	30 (10 %)	8 (2.7 %)	
	CC	1 (0.3 %)	11 (3.7 %)	30 (10 %)	11 (3.7 %)	

T2 Tumor ≤ 2 cm, T2 Tumor > 2 cm but ≤ 5 cm,T3 Tumor > 5 cm,T4 Tumor of any size with direct extension to the chest wall and/or to the skin.

Distribution of Gene Polymorphisms of Patients According to Lymph Node Status

The statistical analysis revealed highly significant differences of gene polymorphisms of breast cancer patients and lymph node status, table 6.

Table 6: Distribution of Gene Polymorphisms of Patients According to Lymph Node Status

Genotypes		Negative (96) No. / %	Positive (204) No. / %	P Value
MTHFR C677T	**CC**	69(23 %)	88(29.3 %)	0.000
	CT	21(7 %)	99(33 %)	
	TT	6(2 %)	17(5.7 %)	
MTHFR A1298C	**AA**	46 (15.3 %)	73(24.3%)	0.000
	AC	47 (15.7 %)	81 (27 %)	
	CC	3 (1 %)	50 (16.7 %)	

Distribution of Gene Polymorphisms of Patients According to Metastasis of Breast Cancer

The results shown statistically highly significant differenceof gene polymorphisms between metastatic and non-metastatic of breast cancer ($P < 0.05$), table 7.

Table 7: The Association between Gene Polymorphisms and Metastasis of Breast Cancer

Genotypes		Non-Metastatic (238) No. /%	Metastatic (62) No. /%	P Value
MTHFR C677T	CC	145(48.3 %)	12(4 %)	0.000
	CT	78(26 %)	42(14 %)	
	TT	15(5 %)	8(2.7 %)	
MTHFR A1298C	AA	105(35 %)	14(4.7 %)	0.000
	AC	106(35.3 %)	22(7.3 %)	
	CC	27(9 %)	26(8.7 %)	

Relationship between Genotypes and clinical Stages of Tumor of Breast Cancer

Tumor-node-metastasis (TNM) system [5], was employed to classify the stages of patients. The statistical analysis exhibited highly significant variation of gene polymorphisms and the clinical stages, table 8.

Table 8: Relationship between Genotypes and clinical Stages of Tumor of Breast Cancer

Genotypes		Clinical Stages				P Value
		Stage I (26)	Stage II (139)	Stage III (76)	Stage IV (59)	
MTHFR C677T	**CC**	18 (6 %)	91 (30.3 %)	36 (12 %)	12 (4 %)	0.000
	CT	6 (2 %)	38 (12.7 %)	37 (12.3 %)	39 (13 %)	
	TT	2 (o.7 %)	10 (3.3 %)	3 (1 %)	10 (3.3 %)	
MTHFR A1298C	**AA**	15 (5 %)	58 (19.3 %)	36 (12 %)	10 (3.3 %)	0.000
	AC	10 (3.3 %)	73 (24.3 %)	24 (8 %)	21 (7 %)	
	CC	1 (o.3 %)	8 (2.7 %)	16 (5.3 %)	28 (9.3 %)	

Association of Genes Polymorphisms with Types of Breast Cancer

Following the WHO classification of types of breast cancer [6]. Statistical analysis revealed significant impact of MTHFR C677T gene polymorphisms and no significant impact of MTHFR A1298C on the breast cancer tumor types, table 9.

Table 9: The Association of MTHFR C677T and A1298C Gene Polymorphisms with Histopathological Types of Breast Cancer

Tumour Types (NO.)	MTHFR C677T			MTHFR A1298C		
	CC No./%	CT No. /%.	TT No. /%.	AA No./%	AC No./%	CC No./%
Ductal Carcinoma Insitu (10)	**8** 2.7 %	**2** 0.7 %	**0** 0 %	**7** 2.3 %	**3** 1 %	**0** 0 %
Infiltrating ductal carcinoma (215)	**102** 34 %	**93** 31 %	**20** 6.7 %	**78** 26 %	**95** 31.7 %	**42** 14 %
Infiltrating lobular carcinoma (39)	**27** 9 %	**12** 4 %	**0** 0 %	**19** 6.3 %	**16** 5.3 %	**4** 1.3 %
Mucinous carcinomas (16)	**7** 2.3%	**6** 2.0%	**3** 1 %	**7** 2.3 %	**5** 1.7 %	**4** 1.3 %
Medullary carcinomas (11)	**10** 3.3 %	**1** 0.3 %	**0** 0 %	**3** 1 %	**8** 2.7 %	**0** 0 %
IDC with Paget's disease (9)	**3** 1 %	**6** 2 %	**0** 0 %	**5** 1.7%	**1** 0.3 %	**3** 1 %
P. Value	0.008			0.067		

Frequencies and Genotypes of MTHFRC677T Gene Polymorphism

The homozygous genotype (TT) of MTHFR C677T in codominant was significantly (P = 0.006) increased the risk of breast cancer by 4.54 folds with respect to those of the wild type (CC). After adjustment for age and BMI there were significant variation obtained (P = 0.014, OR= 3.99). Similarly the CT genotype significantly (P= 0.000) raised the risk of breast cancer by 2.25 folds in unadjusted and significantly (P= 0.000) raised the risk of breast cancer by 2.28 folds in adjustedodd ratio. Analysis regarding to the dominant, highlighted significant (P= 0.000) association with the risk of breast cancer which raised by 2.45 and 2.43 folds in unadjusted and adjusted (age and BMI) odd ratio, respectively. The results of recessive model showed significant (P= 0.025 and 0.029) raise the risk of breast cancer by 3.44 and 3.02 folds in unadjusted and adjusted odd ratio, respectively, table 10.

Table 10.Frequencies and Risk of Breast Cancer Associated with mthfr C677T Genotypes according to Different Models of Inheritance

MTHFR C677T Rs1801133	Breast Cancer Patients	Control	Unadjusted OR(95% CI)	P Value	Adjusted or (95% CI)	P Value
Codominant						
CC (Reference)	157	124	1.00 1.00			
CT	120	42	2.25(1.47-3.44)	0.000	2.28(1.48-3.51)	0.000
TT (Mutant)	23	4	4.54(1.53 -13.47)	0.006	3.99(1.31-12.09)	0.014
Dominant						
CT + TT	143	46	2.45(1.63 -3.68)	0.000	2.43 (1.60- 3.69)	0.000
Recessive						
CC+ CT (Reference)	277	92	1.00		1.00	
TT	23	4	3.44(1.17-10.13)	0.025	3.02(1.0-9.0)	0.029
C	434 (72.3 %)	290 (85.23 %)	2.218 (1.56-3.14)	0.000	-	
T	166 (27.7 %)	50 (14.7 %)				

OR:odds ratios;CI:95% confidence intervals;significant differences at (P< 0.05).

Frequencies and Genotypes of MTHFRA1298C Gene Polymorphism

The homozygous genotype (CC) of MTHFR A1298C in codominant was significantly (P = 0.001) increased the risk of breast cancer 3.05 folds with respect to those of the wild type (AA). After adjustment for age and BMI there was significant variation (P = 0.002) and increased the risk of breast cancer patients by 2.83folds was obtained. Similarly, the AC genotype significantly (P= 0.009) raised the risk of breast cancer by 1.72 folds in unadjusted and significantly (P= 0.023) raised the risk of breast cancer by 1.62folds in adjusted odd ratio.

Regarding to dominant, showed significant (P= 0.000 and 0.002) association with the risk of breast cancer which raised by 1.97 and 1.85 folds in unadjusted and adjusted odd ratio, respectively. On other hand, results of recessive models showed significantly (P= 0.006 and 0.011) raised the risk of breast cancer by 2.39 and 2.28folds in unadjusted and adjusted odd ratio, respectively, table11.

Table 11: Risk of Breast Cancer Associated with MTHFR A1298C Genotype According to Different Models of Inheritance

MTHFR A1298c Rs1801131	Breast Cancer Patients	Control	Unadjusted Or(95% CI)	P Value	Adjusted Or(95% CI)	P Value
Codominant						
AA (Reference)	119	96	1.00	1.00		
AC	128	60	1.72(1.14-2.58)	0.009	1.62(1.06-2.46)	0.023
CC (Mutant)	53	14	3.05(1.59-5.83)	0.001	2.83(1.46-5.48)	0.002
Dominant						
AC + CC	181	74	1.97(1.34- 2.88)	0.000	1.85(1.25-2.73)	0.002
Recessive						
AA + AC (Reference)	247	156	1.00		1.00	
CC	53	14	2.39(1.28- 4.45)	0.006	2.28 1.21-4.3)	0.011
A	366 (61 %)	252 (74.1 %)	1.83 (1.36-2.45)	0.000	-	
C	234 (39 %)	88 (25.9 %)				

OR: odds ratios; CI: 95% confidence; significant differences at (P< 0.05).

DISCUSSIONS

The study revealed, there were significant differences (P < 0.05) in distributions of MTHFR (C677T, A1298C) genotypes of breast cancer patients according to body mass index,table 2. The increased risk among overweight or obese women is thought to be due to the higher levels of circulating estrogen that arise from aromatization of the androgen precursor androstenedione to estrone in adipose tissue, and this becomes the main source of endogenous estrogens especially after menopause. Obesity is also associated with lower levels of sex hormone binding globulins, which increases bioavailable estradiol in postmenopausal obese women Smith-Warner *et al.*, [7]. This finding agreed withSurekha D*et al.*[8].

Fist degree family history of breast cancer wasreported in 12.3 % of patients.It was associated with an increased risk of breast cancer of homogenous genotypes of MTHFR C677T:OR=7.12(2.85- 17.78) and forMTHFR A1298C:OR= 2.22(1.02- 4.84), table 3. Studying family history of breast cancer can highlight the genetic predisposition to develop the disease, and in this regard, the results clearly established for women who have breast cancer in their families[9].

According to gradesof breast cancer, there were highly significant difference between gene polymorphismsand grades of breast cancer, table 4. It has been found that mutant alleles (MTHFR C677T: TT, MTHFR A1298C: CC) are more frequently associated with moderately and poorly differentiated cancer as compared towell differentiated grade. These results may explain the aggressiveness of breast cancer tumours when they were developed due to gene polymorphisms and might reflect the fact that grade II and III in general carry a bad prognosis. This suggests that this gene polymorphisms are a predisposing genetic factor implicated in the carcinogenesis of breast cancer.

It has been suggested that tumor size is crucial for breast cancer staging to determine the invasiveness of tumor, and it is one of the most important prognostic factors in breast cancer. Accordingly, more than 88% of the patients were at a greater risk of metastasis (table 5), as their tumor size exceeded two centimeters[10].The findings are identical with those of Iraqi [11].In contrast, in a study from a western country, the tumors are predominantly less than 2cm [12], this could be due to the early detection programs prevalent in the western countries and absence of efficient national breast cancerprevention and screening program in our country and high rate of malignant breast tumors in Iraq with poorly differentiated cells.

Regional lymph node status is the most important predictor of disease-free and overall survival in patients with breast cancer. In developed countries, majority of the patients, the lymph nodes were not involved [13]. High positive lymph node results were seen in studies in Iraq 77% [162], 81.6% [193] and 84.8% [11]. Alsodue to the early detection programs prevalent in the western countries and absence of efficient national breast cancer screening program in our country and high rate of malignant breast tumors in Iraq. Significant (p=0.000) differences of gene polymorphisms were evident with lymph node status of breast cancer patients, table 6. The frequency of homozygous genotypes were found to be increased significantly in patients with node-positive status.

The present frequency of 20.7 % distance metastasis worth to pay such factor a pronounced attention in Iraqi patients. The study shown significant difference of studied gene polymorphism MTHFR C677T between metastatic and non-metastatic of breast cancer ($P < 0.05$).The frequency of CC of MTHFR A1298C genotype was found to be increased in breast cancer patients with respect to stage of the disease, table 7. Results could be explained through the time of cancer is diagnosed.

The statistical analysis exhibited highly significant ($P< 0.05$) variation of gene polymorphisms and the clinical stages, table 8.

The current study found that there was significant difference in the frequency of the heterozygous mutant CT genotype of C677T polymorphism as compared to that of control (OR of CT vs. CC =2.257, 95% CI: 1.478-3.445). Also, there was a significant increase in the frequency of the homozygous mutant TT genotype of C677T polymorphism as compared to that of control (OR of TT vs. CC =4.541, 95% CI: 1.531 -13.475).There was a significant difference in the risky T allele of C677T polymorphism in cases as compared to that of control, T allele had a significantly increased risk of breast cancer, with (OR of T vs. C =2.218, 95% CI: (1.564-3.146)),table 10. The present results are in agreement with results in Brazil [14], Sweden [15],Turkey [16], Egypt [17],Australia [18],USA[19],Italy [20], Iran[21], Morocco[22] and China [23, 24]. Meta-analysis with regard to C677T polymorphism, significant association was found with breast cancer risk, in Asian populations [25].

On other hand, the results of the present study do not agree to results in Pakistan [26], India [27], Syria [28] andTaiwan [29], who found no significant association between breast cancer risk and the 677TT genotype. These differences might be due to ethnicity, race or sample size.

This study also found that there was significant difference in the frequency of the heterozygous mutant AC genotype of A1298C polymorphism as compared to that of control (OR of AC vs. AA=1.721, 95% CI: 1.145-2.588). There was significant increase in the frequency of the homozygous mutant CC genotype of A1298C polymorphism as compared to that of control (OR of CC vs. AA=3.054, 95 % CI: 1.598-5.835). C allele had significantly increased risk of breast cancer, with (OR of C vs. A =1.8308, 95% CI: 1.365-2.454),table 11.From the results that there is clear relation betweenA1298C genotype polymorphism and breast cancer. The results agree to results in China [23], Brazil [14], Sweden [15], Turkey [16], Iran[21]and Syria [28].These results are in contrast to results in Pakistan [26] and Taiwan [29].

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