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Association of Genetic Variants of Enzymes Involved in Folate / One-Carbon Metabolism with Female Breast Cancer in Jordan

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Abstract

Folate/one-carbon metabolism plays key roles in gene expression, and in the synthesis and repair of DNA. Therefore, it differently influences the pathogenesis of different diseases including cancer in different populations. This case-control study examined the association between the risks of female breast cancer with five functional single-nucleotide polymorphisms (SNPs) in the folate/one-carbon metabolism. These were *MTR* A2756G (rs1805087), *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *TYMS* 1494 ins/del 6 (rs151264360) and *MTRR A66G* (rs1801394). This study included two-hundred female breast cancer cases and two-hundred age-matched female controls. The DNA samples were extracted from peripheral blood and were genotyped by PCR-RFLP. Comparisons between frequencies of alleles and genotypes in the different groups were evaluated using Pearson chi square test (P= 0.05), while the associations between each SNP and the risk of breast cancer were estimated by logistic regression analysis and calculation of odds ratios (OR) with 95 % confidence intervals (CI). Results showed that *MTR* 2756GG genotype is directly associated with breast cancer (OR = 4.360; CI= 1.213-15.666; P = 0.011), while its heterozygous genotype *MTR* 2756AG had no such risk, indicating that *MTR* 2756G allele is recessive to the wild type allele *MTR* 2756A in the whole group of cases, but is dominant in the post-menopausal cases. Besides, significant differences appeared in the distributions of both *MTR* 2756G allele (P = 0.022) and its genotypes (P = 0.008) between the pre- and post-menopausal cases respectively, which indicated for the first time, that the post-menopausal status affects the dominance of the polymorphism *MTR* A2756G as a risk factor for breast cancer. The results also showed that *MTHFR* C677T, *TYMS* 1494 ins/del 6 and *MTRR A66G* are strongly associated with breast cancer only when present in double compound heterozygous states (odd ratios between 2.6 and 6.7). In conclusion, folate/one-carbon metabolism contributes to the risk of female breast cancer in Jordan, and this contribution can be modified by the menopausal status of the females.

Keywords: Single nucleotide Polymorphism, *MTR*, *MTHFR*, *TYMS*, *MTRR*, Breast cancer, Jordan.

1. Introduction

Breast cancer constitutes a major concern for public health because it is the most common cancer among women worldwide (Ferlay *et al.*, 2015). In Jordan, breast cancer accounted for 37.3 % of the top five common cancers among Jordanian women (Jordan Cancer Registry, 2016). Hereditary female breast cancer represents about 5 % to 9 % of all breast cancer cases worldwide, while most of the cases are due to sporadic mutations in housekeeping genes that result in abnormal genetic and epigenetic changes (Choi and Mason, 2000).

Folate/one-carbon metabolic pathway is the most essential cellular source of the methyl group that is required for the synthesis and repair of DNA, and is required for the modulation of gene expression (Choi and Mason, 2000; Duthie, 1999; Duthie *et al.*, 2002; Friso *et al.*, 2002; Balaghi and Wagner, 1993), which can influence the pathogenesis of various types of cancers including breast cancer (Gao *et al.*, 2009; Pardini *et al.*, 2011).

Methylene tetrahydrofolate reductase (MTHFR) is a key player enzyme in the folate / one-carbon metabolic pathway that is involved in catalyzing the irreversible reduction of 5,10-methylenetetrahydrofolate (methylene-THF) to 5-methyltetrahydrofolate (5-methyl THF), the major form of circulating folate in the plasma that is essential for the synthesis of purine nucleotides (Friso *et al.*, 2002; Balaghi and Wagner, 1993; Gao *et al.*, 2009). The reduction of 5, 10-methylene THF by MTHFR produces 5-methyltetrahydrofolate (5-methyl THF), the methyl donor for methionine synthesis from homocysteine. Methionine is the precursor of S-adenosylmethionine (SAM), which is the universal methyl group donor in several cellular reactions, especially those essential for DNA synthesis and repair as well as gene expression (Duthie *et al.*, 2002; Zhang *et al.*, 2007).

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A different essential enzyme in this pathway is methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR), which reactivates the enzyme methyltetrahydrofolate-homocysteine methyltransferase (MTR) that uses SAM in the presence of vitamin B12 as a cofactor to remethylate homocysteine to methionine. Both MTRR and MTR are directly involved in methylation of DNA and other cellular components and gene expression. (Choi and Mason, 2000). The same folate form 5, 10 methylene THF is also used as a substrate for the essential enzyme thymidylate synthase (TYMS) in both the regeneration of dihydrofolate and the synthesis of deoxythymidine monophosphate (dTMP) required for DNA synthesis and repair.

In Jordan, deficiencies in folate and Vitamin B12 are associated with colorectal cancer (Waly *et al*., 2012). In addition, MTHFR C677T was reported to be associated with different common disorders such as thalassemia major (Al-Sweedan *et al*., 2009), failure of in-vitro fertilized-embryo transfer implantation (Qublan *et al*., 2006), arterial thrombosis (Eid and Shubeilat, 2005), increased sensitivity to methotrexate (Al-Refai *et al*., 2009), maternal risk for Down syndrome (Sadiq *et al*., 2011), male infertility (Mfady *et al*., 2014) and the risk of breast cancer (Awwad *et al*., 2015).

This study explores the association between the risk of breast cancer in Jordanian females and each of the following functional folate/one-carbon-related single nucleotide polymorphisms (SNPs): MTR A2756G (rs1805087), *MTHFR* C677T (rs1801133), *MTHFR*A1298C (rs1801131), *TYMS* 1494 ins/del 6 (rs151264360), and *MTRR* A66G (rs1801394). These five functional variants were reported to alter the activity of their respective enzymes. Both *MTHFR* C677T (rs1801133) and *MTHFR* A1298C (rs1801131) are associated with the reduction in the MTHFR enzyme activity which is one of the factors responsible for DNA methylation (Weisberg *et al*., 1998; Frosst *et al*., 1995). *TYMS* 1494 ins/del 6 (rs151264360) is associated with the disruption of TYMS enzyme and reduction in its activity (Ulrich *et al*., 2000), leading to the depletion of dTMP and accumulation of dUMP, which consequently causes misincorporation of dUMP in place of dTMP leading to DNA single and double strand breaks as well as genomic instability (DeVos *et al*., 2008). Also, *MTRR* A66G (rs1801395) resulted in the reduction of the MTRR enzyme activity, and leads to reduction in the functional MTR enzyme and consequently the hypomethylation of DNA, and was proved to be associated with the risk of cancers (Wang *et al*., 2017).

In addition, the effect of the menopausal status of breast cancer cases on the risk of breast cancer associated with the studied SNPs was examined in this current study. Furthermore, the possible associations between the double compound genotypes of these SNPs with the risk of breast cancer in all the cases were investigated in this study in order to better understand the molecular bases of breast cancer, which may help in future management of the disease in Jordan.

2. Materials and Methods

2.1. Subjects

This study is a case-control study, which included two-hundred confirmed breast cancer cases and another two-hundred age-matched female Jordanian control women. The average mean ages (± SD) of the case and control female groups were 50.22 ± 10.8 and 49.03± 10.4 years respectively with no significant age difference between them (P= 0.919). All subjects were recruited during the period from March 2009 to January 2012 from two referral hospitals for cancer in the northern and middle regions of Jordan. All participants completed a questionnaire, which included their menopausal status when diagnosed with breast cancer and signed a separate informed consent. This study was approved by the ethics committee at Jordan University of Science and Technology and the Ministry of Health. Each participant signed an informed consent before her inclusion in the study. Respect for the anonymity and confidentiality of information were also strictly complied with the Declaration of Helsinki (World Medical, 2013).

2.2. Blood Samples and DNA Isolation

Peripheral blood samples were collected from each subject in EDTA tubes and genomic DNA was extracted using commercially available kits (Qiagen, Gentra Puregen, Germany) according to the manufacturer's instructions.

2.3. Genotyping

Genotyping was performed by polymerase chain reactions followed by restriction fragment length polymorphism (PCR-RFLP) according to earlier established methods as summarized in Table 1. Amplifications of each SNP was carried out in a total volume of 25µL containing the specific forward and reverse primers, master mix (GOTag® Green Master Mix, Promega, USA or Quick-load® Taq 2X master mix, BioLabs) and 50-100 ng of genomic DNA. Restriction digestions by the proper enzymes (Promega, USA or New England BioLabs) were performed according to the instructions of the manufacturer (Table 1). PCR products and their digestion products were separated on 3 % agarose gels. Results were validated by simultaneously running positive control DNA of known genotypes.

2.4. Statistical Analysis

Comparisons between frequencies of alleles and genotypes in the different groups were evaluated using Pearson Chi square test (P= 0.05). Comparisons between cases and female control groups) and identification of the risk factors associated with breast cancer were achieved using logistic regression analysis and calculation of odds ratios (OR) at 95 % confidence intervals (CI). All statistical analyses were applied using IBM SPSS Statistics software (version 23.0).

Table 1. Primers and conditions used for genotyping analysis of the examined polymorphisms.

SNP ID	Variant	[a]Primers sequence (5'→ 3')	PCR conditions	Restriction enzyme, and incubation conditions	Fragment length produced in base pairs	Primers reference
rs1801133	*MTHFR C677T*	F: TGAAGGAGAAGGTGTCTGCGGGA R: AGGACGGTGCGGTGAGAGTG	95°C for 8 min followed by 30 cycles: 94°C for 60s, 63°C for 60s, 72°C for 60s then extension at 72°C for 5 min.	Hinf I, 37°C, 16 hours	C allele: 198 T allele: 175 + 23	Yi *et al*, 2002
rs1801131	*MTHFR A1298C*	F: CAAGGAGGAGCTGCTGAAGA R: CCACTCCAGCATCACTCACT	95°C for 8 min followed by 30 cycles: 94°C for 60s, 63°C for 60s, 72°C for 60s then final extension at 72°C for 5 min.	*Mbo* II, 37°C, 16 hours	A allele: 72+28 C allele: 100	Yi *et al*, 2002
rs 1805087	*MTR* A2756G	F:CATGGAAGAATATGAAGATATTAGA C R: GAACTAGAAGACAGAAATTCTCTA	95°C for 5 min, followed by 35 cycles: 95°C for 45s, 55° C for 35s, 72°C for 72s, then final extension at 72°C for 5 min.	*Hae* III, 37°C, 2 hours.	A allele: 189 G allele: 159 bp + 30 bps	Leclerc *et al* 1996
rs1801394	*MTRR A66G*	F: GCAAAGGCCATCGCAGAAGACAT R: AAACGGTAAAATCCACTGTAACGGC	30 cycles: 94°C for 60s, 35s for 60°C for 60s, 72°C for 60s; then final extension at 72°C for 5 min.	*Nsp* I, 37°C, 16 hours	G allele: 94+24 A allele: 118	Feix *et al*.2004
rs151264360	*TYMS* 1494 ins/del6	F: CAAATCTGAGGGAGCTGAGT R: CAGATAAGTGGCAGTACAGA	95°C for 5 min. followed by 40 cycles: 95°C for 45 seconds, 72°C for 60s; then final extension at 72°C for 5 min.	*Dra* I, 37°C, 2 hours	+6 bp allele: 88 +70 - 6bp allele: 152 -/+ genotype: 158 + 152 + 88 + 70	Ulrich *et al*. 2000

[a] Primers were obtained from Alpha DNA, Canada.

3. Results

3.1. Distribution of the Different Alleles and Genotypes in All Jordanian Cases and Control Females

Genotyping of *MTHFR* C677T, *MTHFR* A1298C and *MTRR* A66G SNPs is presented in Figure 1, while the genotyping of TYMS (1494 ins/del 6) and *MTR* A2756G is presented in Figure 2. The distributions of the different genotypes of each of the five examined SNPs between the cases and the control females were in accordance with Hardy Weinberg equilibrium ($P > 0.05$). The numbers and frequencies of the different alleles and genotypes in the cases and the control female groups with their analyses were verified in odd ratios (ORs) and 95 % confidence intervals (CIs) in Table 2.

Genotypic analysis of all breast cancer cases as one group compared to the control females showed only significant statistical difference between the controls and the cases in the distribution of the homozygous mutant genotype *MTR* 2756GG (OR = 4.36; CI= 1.21-15.7; P = 0.011), while the heterozygous genotype *MTR* 2756 AG showed lack of association with the risk of breast cancer (OR = 0.88; CI=0.88-1-31; P =0.260).

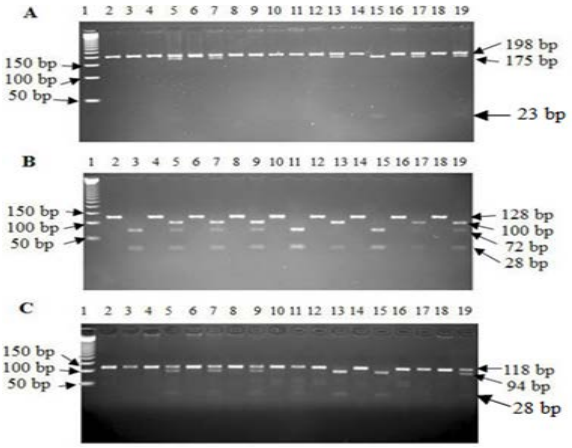

Figure 1. Representative results of PCR-RFLP genotyping of *MTHFR* C677T, MTHFR A1298C and *MTRR* A66G. Lane 1 In all three panels has DNA marker (50 bp ladder). The even numbered lanes of each pair have undigested PCR product, followed by its respective digestion products in the odd numbered lane. **Panel A:** Genotyping analysis of *MTHFR* C677T: lanes 2/3: positive control with previously known wild type genotype CC (single 198 bp band); lanes 4/5: heterozygous CT (198 bp and 175 bp bands). Tested Jordanian Samples: lanes 6/7, 12/13, 16/17 and 18/19: CT genotype; Lanes 8/9 and10/11: CC genotype; lane 14/15: TT genotype. **Panel B:** Genotyping analysis of *MTHFR* A1289C: lanes 2/3: positive control with previously known wild type AA genotype, (72 bp and 28 bp bands); Lanes 4/5: Positive control with previously known genotype: AC (100 bp, 72 bp and 28 bp bands). Tested Jordanian subjects: lanes 6/7, 8/9 and 18/19: AC genotypes; lanes 12/13 and 16/17: CC genotypes; lanes 10/11 and 14/15: AA genotype. **Panel C:** Genotyping analysis of *MTRR* A66G: lanes 2/3: positive control with previously known genotype: AA, wild type genotype (single 118 bp band); lanes 4/5 AG Positive control with previously known genotype: (118 bp and 94 bp). Tested Jordanian subjects: lanes 6/7, 8/9 and 18/19: AG; lanes 12/13 and 14/15: GG.

Table 2. Alleles and genotypes frequencies of *MTHFR* (C677T), *MTHFR* A1298C, *MTR* A2756G, *MTRR* A66G and *TYMS* (1494 ins/del6) polymorphisms in all breast cancer patients and the control females

Polymorphism Alleles and genotypes	Cases Number and (%)	Control females Number and (%)	OR (95% CI)	*P*-value
MTHFR C 77T				
C	268 (0.67)	264 (0.64)	1.00 (ref.)	
T	132 (0.33)	136 (0.36)	1.04 (0.778-1.391)	0.789
CC	94 (0.47)	92 (0.46)	1.00 (ref.)	
CT	80 (0.40)	82 (0.41)	1.05 (0.687-0.596)	0.830
TT	26 (0.13)	26 (0.13)	1.02 (0.522-1.890)	0.945
CT & TT	106 (0.53)	108 (0.54)	1.04 (0.703-1.542)	0.841
MTHFR A1298C				
A	364 (0.64)	255 (0.638)	1.00 (ref.)	
C	136 (0.36)	145 (0.362)	1.10 (0.826-1.476)	0.505
AA	90 (0.45)	87 (0.436)	1.00 (ref.)	
AC	84 (0.42)	81 (0.405)	0.99 (0.653-1.525)	0.991
CC	26 (0.13)	32 (0.16)	1.27 (0.702-2.310)	0.426
AC & CC	110 (0.55)	113 (0.565)	1.06 (0.716-1.577)	0.763
MTRR A66G				
A	263 (0.658)	239 (0.598)	1.00 (ref.)	
G	137 (0.343)	161 (0.403)	1.27 (0.949-1.685)	0.108
AA	87 (0.435)	72 (0.36)	1.00 (ref.)	
AG	87 (0.435)	95 (0.475)	1.312 (0.861-0.022)	0.203
GG	26 (0.13)	33 (0.165)	1.53 (0.841-2.798)	0.162
AG & GG	113 (0.565)	128 (0.64)	1.39 (0.916-2.046)	0.125
MTR A2756G				
A	335 (0.83)	325 (0.81)	1.00 (ref.)	
G	65 (0.16)	75 (0.18)	1.215	0.352
AA	138 (0.69)	138 (0.69)	1.00 (ref.)	
AG	49 (0.24)	59 (0.29)	0.875 (0.534-1.309)	0.260
GG	13 (0.06)	3 (0.01)	4.360 (1.213-15.66)	0.011
AG & GG	62 (0.31)	62 (0.31)	1.00 (0.655-1.528)	0.543
TYMS (1494 ins/del6)				
+	194 (0.48)	199 (0.49)	1.00 (ref.)	
-	206 (0.51)	201 (0.50)	0.923 (0.695-1.225)	0.724
+/+	48 (0.24)	44 (0.22)	1.00 (ref.)	
+/-	103 (0.51)	106 (0.53)	0.897 (0.546-1.474)	0.764
-/-	49 (0.24)	50 (0.25)	0.938 (0.529-1.665)	0.908
+/- & -/-	156 (0.78)	152 (0.76)	0.893 (0.560-1.423)	0.361

Ref: Reference category (wild type allele or genotype); +: (6bp ins) wild type allele; -: (6bp del) mutant allele; OR: Odds Ratio; CI: confidence interval according to multinomial logistic regression.

3.2. Distribution of the Different Genotypes in the Pre- and Post-Menopausal Breast Cancer Cases

The total number of cancer patients who answered the question related to their menopausal status at the time of diagnoses were 185 subjects. Of these, 113 cases were pre-menopausal cases, while 72 were postmenopausal cases. The analysis of the distributions of the different alleles and genotypes between these two groups showed significant difference only in the distribution of the *MTR* A2756G alleles (P =0.22) and genotypes (P=0.008), which indicates a significant association between the risk of breast cancer and the SNP *MTR* A2756G in the post-menopausal group (Table 3). Such differences indicates a positive dominant association between *MTR* 2756 G mutant allele and the risk of breast cancer in the post-menopausal breast cancer cases. No other significant statistical differences were observed in the distributions of the other studied SNPs between the pre-and post-menopausal cases.

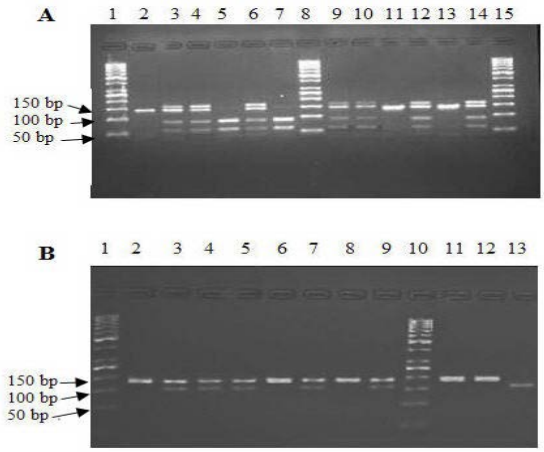

Figure 2. Representative results of PCR-RFLP genotyping of *TYMS* (1494 ins/del 6) and *MTR* A2756G.

Panel A: Genotyping analysis of *TYMS* (1494 ins/del 6) gel: lanes1, 8 and 15: DNA marker (50 bp ladder); lane 2: positive control of mutant homozygote -6bp/-6bp (152 bp band). Tested Jordanian samples: lanes 3, 4, 6, 9, 10, 12 and 14: heterozygotes +6bp/-6bp (158 bp, 152 bp, 88bp and 70 bp); lane 5: wild type homozygote +6bp/+6bp (88 bp and 70 bp bands); lanes 11 and 13: mutant homozygote -6bp/-6bp.

Panel B: Genotyping analysis of *MTR* A2756G: Lanes 1 and 10: DNA marker (50 bp ladder); lane: 2: positive control of wild type AA (189 bp band); lane 3: positive control of a heterozygote AG (189 bp and the 159 bp bands). Tested Jordanian samples: lanes 4, 5, 7 and 9 have AG heterozygotes; lane 13: mutant homozygote GG (159 bp band).

3.3. Frequencies of the Different Observed Compound Genotypes among Breast Cancer Cases and the Control Females.

No significant differences were found between the breast cancer cases and the control women in the observed compound genotypes between *MTR* A2756G with any of the other four studied SNPs (Table 4). However, significant differences were observed between the breast cancer cases and the control females in five out of the eight observed double compound genotypes between *TYMS* ins-del6bp and *MTRR* A66G (Table 5), in addition to four out of the seven observed compound double genotypes between *MTRR* A66G and *MTHFR* 677C as well as with a single double heterozygous genotype between *MTHFR*A1298A and A*MTRR* 66G out of nine observed double genotypes (Table 6).

Table 3. Distribution of the different genotypes in the pre-and postmenopausal breast cancer cases

Polymorphism	Menopausal Status[a]		*P*-value
	Pre-menopause No. (%)	Postmenopause No. (%)	
MTHFR C677T			
C (ref)	151 (40.8)	95 (25.7%)	0.477
T	75 (20.3)	49 (13.2%)	
CC (ref)	55 (29.7%)	31 (16.8%)	0.406
CT	41 (22.2%)	33 (17.8%)	
TT	17 (9.2%)	8 (4.3%)	
MTHFR A1298C			
A (ref)	154 (41.6%)	91 (24.6%)	0.192
C	72 (19.5%)	53 (14.3%)	
AA (ref)	54 (29.2%)	29 (16.8%)	0.599
AC	46 (24.9%)	33 (17.8%)	
CC	13 (7%)	10 (5.4%)	
MTRR A66G			
A (ref.)	145 (39.2%)	98 (26.5%)	0.256
G	81 (21.9%)	46 (12.4%)	
AA (ref)	49 (26.5%)	33 (17.8%)	0.576
AG	47 (25.4%)	32 (17.3%)	
GG	17 (9.2%)	7 (3.8%)	
MTR A2756G			
A (Ref.)	198 (53.5%)	114 (30.8%)	0.022
G	28 (7.6%)	30 (16.2%)	
AA (ref)	87 (47.0 %)	42 (22.7%)	0.008
AG	24 (13 %)	30 (16.2 %)	
GG	2 (1.1 %)	0 (0 %)	
TYMS (1494 ins/del6bp)			
+6bp (ref.)	103 (27.8%)	77 (20.8%)	0.085
-6bp	123 (33.2%)	67 (18.1%)	
(+6bp/+6bp) (ref.)	23 (12.4%)	17 (9.2%)	0.153
(-6bp/-6bp)	33 (17.8%)	12 (6.5%)	
(+6bp/-6bp)	57(30.8%)	43 (23.2%)	

[a]The total number of cases analyzed is185, which included only cases with known menopausal status at their diagnosis with breast cancer; Ref: Reference category, which is the wild type allele or genotype; No: number of subjects; +: (*TYMS* 6bp ins) wild type allele; (-6bp: *TYMS* del6bp) mutant allele; OR: Odds Ratio; CI: confidence interval according to multinomial logistic regression.

Table 4. Frequencies of the observed double compound genotypes of *MTR* A2756G with each of the other studied polymorphisms compared with their wild type double compound genotypes in all BC patients and controls**.**

Compound genotype	Cases number (%)	Controls number (%)	OR (95% CI)	*P*-value
MTR A2756G/*MTHFR*C677T				
AA/CC	(62) 31.0	(62) 31	1.00 (ref.)	
AG/CC	(30) 15.0	(25) 12.5	1.200 (0.635 - 2.269)	0.575
GG/CC	(2) 1.0	(5) 2.5	0.400 (0.75 - 2.140)	0.284
AA/CT	(55) 27.5	(55) 27.5	1.000 (0.598 - 1.671)	1.000
AG/CT	(24) 12.0	(20) 10	1.200 (0.602 - 2.392)	0.605
GG/CT	(1) 0.5	(7) 3.5	0.143 (0.017 - 1.196)	0.073
AA/TT	(21) 10.5	(21) 10.5	1.000 (0.497 - 2.013)	1.000
AG/TT	(5) 2.5	(4) 2	1.250 (0.320 - 4.876)	0.748
GG/TT	(0)	(1) 0.5		
MTR A2756G/ *MTHFR* A1298C				
AA/AA	(70) 35	(60) 30.0	1.00 (ref.)	
AG/AA	(20) 10.0	(24) 12.0	0.714(0.360 –1.419)	0.337
GG/AA	0 (0)	(3) 1.5	-	-
AA/AC	(53) 26.5	(58) 29.5	0.783 (0.471 - 1.301)	0.345
AG/AC	(29) 14.5	(14) 7.0	1.776 (0.860 - 3.666)	0.121
GG/AC	(2) 1	(9) 4.5	0.190 (0.040 - 0.916)	0.039
AA/CC	(15) 7.5	(20) 10.0	0.643(0.303 - 1.365)	0.250
AG/CC	(10) 5	11 (5.5)	0.779 (0.310 - 1.962)	0.596
GG/CC	(1) 0.5	(1) 0.5	0.857 (0.52 - 14.000)	0.914
MTR A2756G/*MTRR* A66G				
AA/AA	(61) 30.5	(47) 23.5	1.00 (ref.)	
AG/AA	(24) 12	(21) 10.5	0.881 (0.438 - 2.269)	1. 770
GG/AA	(2) 1	(4) 2	0.385 (0.068 - 2.194)	0.282
AA/AG	(60) 30	(69) 34.5	0.670 (0.401 - 1.1.120)	0.127
AG/AG	(26) 13.0	(22) 11.0	0.911 (0.460 – 1.804)	0.788
GG/AG	(1) 0.5	(4) 2	0.193 (0.021 - 1.781)	0.147
AA/GG	(17) 8.5	(22) 11	0.595 (0.285 – 1.246)	0.169
AG/GG	(9) 4.5	(6) 3	1.156 (0.384 – 3.475)	0.797
GG/GG	(0) 0.0	(5) 2.5		
MTR A2756G/ *TYMS* (1494 ins/del 6bp)				
AA/ +/+6bp	(32) 16	(37) 18.5	1.00 (ref.)	
AA/ -/+ 6bp	(72) 36	(67) 33.5	1.243 (.697-2.216)	0.462
AA/ -/- 6bp	(34) 17	(34) 17	1.156 (0.591 - 2.261)	0.671
AG/ -/- 6bp	(16) 8	(13) 6.5	1.423 (0.595 -3.402)	0.427
AG/ -/+ 6bp	(32) 16	(28) 14	1.321 (0.660 -2.644)	0.431
AG/ +/+6bp	(11) 5.5	(8) 4	1.590 (0.570 - 4.437)	0.376
GG/ +/+6bp	(1) 0.5	(3) 1.5	0.385 (0.038 -3.891)	0.419
GG/ +/- 6bp	(2) 1	(8) 4	0.289 (0.057 -1.461)	0.133
GG/ -/- 6bp	(0) 0	(2) 1	-	

Ref.: Reference category(double wild type genotype).

Table 5. Frequencies of the observed double compound genotypes of *TYMS* ins/del 6bp with each of *MTHFR* C677T, *MTHFR* A1298C and *MTRR* A66G compared with their wild type double compound genotypes in breast cancer patients and control females**.**

Compound genotype	No. of Cases (%)	No. of controls (%)	OR (95% CI)	*P*-value
TYMS ins-del6bp / *MTHFR* C677T				
+/+6bp/ CC	21(10.5)	19 (9.5)	1.00 (ref.)	
+/-6bp/ CC	45(22.5)	50 (25)	0.814(0.389-1.706)	0.586
+/+ 6bp/ TT	4(2)	8 (4)	0.452(0.117-1.747)	0.250
+/+6bp/ CT	19(9.5)	21(10.5)	0.819 (0.340-1.969)	0.655
+/- 6bp/ CT	45(22.5)	41(20.5)	0.993(0.469-2.105)	0.985
-/- 6bp/ CT	16(8)	20(10)	0.724(0.293-1.787)	0.483
+/-6bp/ TT	16(8)	12(6)	1.206(0.456-3.19)	0.705
-/- 6bp/ TT	6(3)	6(3)	0.905(0.249-3.289)	0.879
-/- 6bp/ CC	28(14)	23(11.5)	1.101(0.480-2.527)	0.820
TYMS ins-del6bp / *MTHFR* A1298C				
+/+ 6bp/ AA	18(9)	15(7.5)	1.00 (ref.)	
+/- 6bp/ AA	47(23.5)	46(23)	0.851 (0.384-1.889)	0.692
-/-6bp/ AA	25(12.5)	26(13)	0.801 (0.33-1.928)	0.621
-/- 6bp/ AC	17(8.5	18(9)	0.787 (3.303-2.042)	0.622
+/- 6bp/ AC	45(22.5)	40(20)	0.937 (0.418-2.101)	0.875
+/+ 6bp/ AC	22(11)	23(11.5)	0.797 (0.324-1.962)	0.622
-/- 6bp/ CC	8(4)	5(2.5)	1.333(0.360-4.945)	0.667
+/- 6bp/ CC	14(7)	17(8.5)	0.686 (0.256-1.837)	0.454
+/+ 6bp/ CC	4(2)	10(5)	0.333(0.087-1.282)	0.110
TYMS ins-del6bp / *MTRR* A66G				
+/+ 6bp/ AA	22(11)	37(18.5)	1.00 (ref.)	
+/- 6bp/ AA	43(21.5)	67(33.5)	1.079(0.562-2.072)	0.818
-/-6bp/ AA	22(11)	34(17)	1.088(0.513-2.309)	0.826
-/- 6bp/ AG	20(10)	13(6.5)	2.587(1.078-6.208)	0.033
+/- 6bp/ AG	49(24.5)	28(14)	2.943(1.457-5.944)	0.003
+/+ 6bp/ AG	18(9)	8(4)	3.784(1.412-10.966)	0.008
-/- 6bp/ GG	8(4)	2(1)	6.727(1.309-34.572)	0.022
+/- 6bp/ GG	14(7)	8(4)	2.943(1.065-8.132)	0.037
+/+ 6bp/ GG	4(2)	3(1.5)	2.242 (0.459-10.966)	0.319

Ref.: Reference category (double wild type genotype); OR: Odds Ratio; CI: confidence interval according to multinomial logistic regression. No.: number

Table 6. Frequencies of the observed double compound genotypes of *MTHFR* C677T/A, *MTHFR* A1298C and *MTRR* A66G compared with their wild type double compound genotypes in the breast cancer patients and the control females**.**

Compound Genotype	No. of Cases (%)	No. of Control females (%)	OR (95% CI)	*P*-value
MTHFR C677T / *MTHFR* A1298C				
CC/AA	28 (14)	28 (14)	1.00 (ref.)	
CC/AC	41 (20.5)	35 (17.5)	1.17 (0.587-2.338)	0.654
CC/CC	25 (12.5)	29 (14.5)	0.86 (0.408- 1.823)	0.689
CT/AA	40 (20)	38 (19)	1.53 (0.530- 2.092)	0.884
CT/AC	39 (19.5)	41 (20.5)	0.95 (0.480-1.883)	0.886
CT/CC	1 (0.5)	3 (1.5)	0.33 (0.033- 3.402)	0.613
TT/AA	22 (11)	21 (10.5)	1.05 (0.473- 2.320)	0.909
TT/AC	4 (2)	5 (2.5)	0.80 (0.194- 3.294)	0.757
TT/AC	ND (0)	ND (0)		
MTHFR C677T / *MTRRA66G*				
CC/AA	42 (21)	62 (31)	1.00 (ref.)	
CC/AG	40 (20)	25 (12.5)	2.36 (1.252-4.457)	0.008
CC/GG	12 6)	5 (2.5)	3.54 (1.162-10.797)	0.026
CT/AA	34 (17)	55 (77.5)	0.91 (0.511-1.630)	0.757
CT/AG	33 (16.5)	20 (10)	2.44 (1.234-4.806)	0.010
CT/GG	13 (6.5)	7 (3.5)	2.74 (1.010-7.444)	0.048
TT/AA	11 (5.5)	21 (10.5)	0.77 (0.338-1.770)	0.543
TT/AG	14 (7)	4 (2)	5.17 (1.590-16.784)	0.006
TT/GG	1 (0.5)	1 (0.5)		
MTHFR A1298C / *MTRRA66G*				
AA/AA	42 (21)	60 (30)	1.00 (ref.)	
AA/AG	36 (18)	24 (12)	2.14 (1.119-4.104)	0.22
AA/GG	12 (6)	3 (1.5)	5.71 (1.519-21.502)	0.10
AC/AA	34 (17)	58 (29.5)	0.84 (0.470-1.494)	0.548
AC/AG	39 (19.5)	14 (7)	3.98 (1.924-8.232)	0.000
AC/ GG	11 (5.5)	9 (4.5	1.75 (0.665-4.584)	0.258
CC/AA	11 (5.5)	20 (5.5)	0.79 (0.341-1.810)	0.571
CC/AG	12 (6)	11 (5.5	1.56 (0.628-3.865)	0.338
CC/GG	3 (1.5)	1 (0.5)	4.29 (0.31-42.630)	0.214

NO: Number; ref: Reference category (double wild type genotype); OR: Odds Ratio; CI: confidence interval according to multinomial logistic regression; ND: not detected in this study

4. Discussion

In this case-control study, the associations between five SNPs in the folate/one-carbon metabolism and the risk of female breast cancer in Jordan are examined. this study reports for the first time that there is a direct association between the risk of female breast cancer in Jordan and the mutant homozygous genotype GG of the SNP *MTR* A2756G (rs1805087). This is in agreement with previously reported association of this SNP with the susceptibility of different cancers, and is also in agreement with the role of MTR enzyme in activating MTRR that is essential for the synthesis of the universal one carbon donor SAM, that affects DNA methylation and gene expression (Krushkal *et al*., 2016).

The fact that that the homozygous genotype *MTR* 2756GG increased the risk of breast cancer in Jordanian females by more than four folds (GG: OR=4.360; CI=1.213-15.666, p= 0.011), while its heterozygous genotype had no such risk for breast cancer, indicates a recessive role of the mutant *MTR* 2756G towards its wild type allele in being a risk factor for breast cancer.

However, in the post-menopausal cases, *MTR* 2756G mutant allele as well as its homozygous and heterozygous genotypes were positively associated with breast cancer, which indicated that the menopausal status of Jordanian women has a significant modifying effect on their risk of breast cancer. This observed positive association between *MTR* 275G with breast cancer was in agreement with the role of wild type MTR in DNA synthesis, repair, and methylation (Krushkal *et al*., 2016).

The observed lack of associations between the risk of breast cancer and each of the single alleles of the other examined four SNPs in both groups of cases as well as the separated pre- and post-menopausal breast cancer cases, indicated that the menopausal status of Jordanian women had no effect on the association between any of the other four examined SNPs and the risk of breast cancer.

Despite the observed lack of association between the risk of breast cancer with each individual SNPs *TYMS* (1494 ins/del 6) and *MTRR* A66G, the results show a significant association between their compound double heterozygous genotypes with the risk of breast cancer, which could be related to an additive effect of both altered MTRR and TYMS enzymes. MTRR function is to regenerate the active form of MTR that is involved in DNA methylation, while TYMS enzyme is directly involved in DNA synthesis. The alteration of both enzymes in these double heterozygotes seemed to cause perturbation in both DNA synthesis, repair, and gene expression that is expected to lead to pleiotropic cellular effects that may end up with carcinogenesis (Choi and Mason, 2000).

The observed lack of significant association between *MTHFR* C677T and the risk of breast cancer in the Jordanian females was different from the results reported previously in Jordan (Awwad *et al.*, 2015), which suggested a positive association between *MTHFR* C677T and breast cancer. Such difference may be due to the differences in the design of the two studies; unlike this current study, Awwad *et al*., (2015) used 150 female cases and 150 age-matched healthy individuals at Al-Basheer hospital, which is located in the central part of Jordan. Besides, there is still some doubt regarding their control group, which were defined as "150 age-matched healthy individuals". Furthermore, their sample collection was limited to 9 months between the 15th of March till 21st of December 2014, which may not be significantly random and representative of the Jordanian population, as much as this study's 200 samples, which were collected from two different hospitals in the central and the northern parts of Jordan over a period of more than two years.

In this study, the observed lack of association between *MTHFR* C677T and the risk of breast cancer in Jordanians is similar to those reported in Saudi (Alshatwi, 2010), Greek (Kalemi *et al.*, 2005), Japanese (Ma *et al.*, 2009) and Brazilian populations (Batschauer *et al.*, 2011), but it is different from those reported in Turks (Ergul *et al*., 2003; Deligezer *et al*., 2005), Chinese (Gao *et al*., 2009), and the Americans (Chen *et al.*, 2005). Similarly, the observed absence of association between the risk of breast cancer and *MTHFR* A1298C alleles and genotypes in this study is in harmony with those found in Saudis (Alshatwi, 2010), Polish (Lissowska *et al.*, 2007), Chinese (Gao *et al*., 2009) and Russian populations (Vainer *et al*., 2010), but it is contrary to the results reported in the Turkish (Ergul *et al*., 2003) and the American (Chen *et al*., 2005) populations.

There is a lack of association between the risk of breast cancer in the carriers of *MTHFR* C677T and *MTHFR* A1298C as well as their double heterozygous genotype *MTHFR* 677 CT / 1298AC, although these genotypes lead to the reduction in the MTHFR activity compared to homozygous wild-types (Narayanan *et al.*, 2004); however, such unexpected results may be explained by either the genetic backgrounds of our subjects and/or the compensation of folate-rich diet and supplementation by Jordanian women before and during pregnancies, which leads to the compensation of the plasma levels of the central metabolite 5, 10-methylene-THF in the folate/one-carbon metabolism. This is in harmony with the differences in the risk factors for breast cancer in different populations, which are thought to be affected by the different genetic backgrounds of the populations, as well as the difference in their life styles and diets (Alshatwi, 2010; Gao *et al.*, 2009; Ma *et al*., 2009; Ericson *et al*., 2007), in addition to the compensation for the lack of MTHFR catalytic activity through different side pathways within the folate/one-carbon metabolism that utilize MTHFD1 and TYMS enzymes (James and Hobbs, 2002). More studies of the compound genotypes of the folate-related genes, which need a larger number of cases, will allow for a better understanding of the link between folate metabolism and the risk of breast cancer in Jordan.

5. Conclusions

This study shows that *MTR* A2756G (rs1805087) homozygous mutant genotype *MTR* 2756GG is significantly associated with the risk of breast cancer in Jordanian females. Furthermore, the menopausal status of these females influences the risk of breast cancer in the females heterozygous to *MTR* A2756AG genotype. In addition, this study found out that the compound double heterozygous genotypes between *TYMS* (1494 ins/del 6), *MTRR* A66G and *MTHFR* C677T) are significantly associated with the risk of breast cancer in Jordan, which indicates that the folate/one-carbon SNPs constituted potential contributing risk factors for breast cancer in Jordanian females.

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Conflicts of Interest

The authors declare no conflict of interest.

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