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Association of MTHFD1 G1958A, MTHFD1 T401C and CBS 844ins68bp with Breast Cancer in Jordan

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MTHFD1 and CBS genes have key roles in folate and homocysteine metabolism. Many studies reported an association between cancer pathogenesis and different functional SNPs of genes involved in the main folate metabolism and the transsulfuration pathway. The current population-based, case-control study examined the association between MTHFD1 G1958A, MTHFD1 T401C, and the CBS 844ins68 insertion with breast cancer (BC) risk in Jordanian women. The studied population included 200 female BC subjects and age-matched female controls. The targeted genotypes MTHFD1 G1958A and MTHFD1 T401C were amplified via PCR followed by subsequent digestion with the proper restriction enzyme (PCR-RFLP), while the insertion/deletion of CBS844ins68bp was visualized and scored directly after gel electrophoresis. Results showed that the examined individual alleles and genotypes of MTHFD 1958A, MTHFD1 401C, and CBS844ins68bp per se were not associated with risk of BC compared with their wild-type genotypes and alleles.

Keywords: Breast cancer; CBS 844ins68; Folic acid; Jordan; MTHFD1 G1958A; MTHFD1 T401C.

Female breast cancer (BC) is the most commonly worldwide diagnosed cancer, with a recorded 2.3 (11.7%) million new cases in 2020 [1]. In Jordan, BC is the most common cancer, accounting for 38.4% of all detected cancers in females (National Cancer Registry (JNCR., 2021). Despite the high prevalence of BC and the identification of various risk factors, the causes of BC are not completely clear. A critical step for interventions and good management of BC is to identify risk factors for its development. There are several known risk factors for breast cancer including genetic factors [2'3] which are highly heterogeneous [3'4]. While only a minor fraction of these genetic factors arise from mutations in established high-penetrance susceptibility genes, the predominant share is believed to stem from common genetic variants, including single nucleotide polymorphisms (SNPs). Identification of the genetic risk factors for any type of cancer in any population is essential for these factors can be changed and adjusted to reduce the risk of cancers. Among those that can allow protective effect against cancers in particular are different common genetic variants, for example, single nucleotide polymorphisms (SNPs) in the genes encoding functional enzymes and coenzymes in the folate/ one-carbon metabolism and lifestyle risk factors [5'6'7'8]. SNPs in genes related to the folate/ one-carbon metabolism alter gene function or regulation by changing the structure and the catalytic activities of the affected enzymes,

and increasing DNA methylation in promoters of many genes and methylation reduction in promoters of other genes. These changes lead to activation of proto-oncogenes and inactivation of tumor suppressor genes [9'10] and alter cell division leading to aberrant chromosomal segregation and tumorigenesis [11'12'13'14]. Different studies reported the characteristics and risk factors for BC in Jordanian women; these included lifestyle risk factors such as alcohol consumption, cigarette smoking [6'15] high-fat nutrient intake pattern, and insufficient exercise [7]. Other risk factors reported for BC in Jordan were age, obesity, body mass index (BMI), high level of estrogen, age at menarche and menopause, reproduction history, exposure to ionizing radiation, and hereditary background [16'17]. Unlike the epidemiological factors, few and limited reports were published on the genetic factors and different gene polymorphisms (SNPs) associated with the risk of BC in Jordan. These included the known germline, high penetrance susceptibility genes, which include the germline *BRCA1* and *BRCA2* mutations [18'19] and few studies examined the association of SNPs in genes related to the folate/ one-carbon metabolism with BC [20'21].

MTHFD1 and *CBS* are genes encoding the enzymes methylenetetrahydrofolate dehydrogenase (*MTHFD1*) and cystathionine β-Synthase (*CBS*) respectively have central roles in folate and homocysteine metabolism through the folate / one-carbon metabolism and are candidate genes for cancer susceptibility [22'23'24'25'26]. MTHFD1 enzyme catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to the primary form of circulatory folate 5-methyltetrahydrofolate [27], and is essential for the synthesis of purine and pyrimidine bases [28] and regeneration of SAM [29]. CBS is a major enzyme in the transsulfuration pathway, at the homocysteine (Hcy) junction of preserving methionine or converting it to cysteine. *CBS* eliminates Hcy by catalyzing the condensation of serine and Hcy to form cystathionine [30], which is then hydrolyzed to cysteine, a precursor of the potent antioxidant glutathione [28'31].

Different studies showed an association of *MTHFD1* G1958A with different diseases including cardiovascular diseases [27'32] maternal risk for fetal loss [33] and neural tube defects [34'24'35] and psychiatric disorders [36]. Several clinical studies showed over-expression of *CBS* and, increased production of H2S in many cancer types including colon, ovarian, gastric, colorectal, prostate, and gastroesophageal cancer [35'37'38'39'40]. The main CBS-derived metabolites are the anti-inflammatory (H_2S) and Hcy [31]. H2S is associated with signaling and protective effects on antioxidant defenses inhibits the production of hydrogen peroxide (H2O2) and other reactive oxygen species (ROS) and preserves the activity of key antioxidant enzymes including catalase and superoxide dismutase, glutathione peroxidase, and glutathione-S-transferase [41'42'43]. Functional SNPs in *CBS* gene can promote carcinogenesis [11'40'44'45'46].

The association between each of *MTHFD1* and *CBS* genes with BC are still controversial and indefinite. To our knowledge, this is the first study to examine associations of *MTHFD1* and *CBS* genes with breast cancer in Jordanians. Thus in this case-control study, we aimed to examine the possible association of *MTHFD1* G1958A (R653Q), *MTHFD1* T401C (R134K), and the insertion mutation *CBS* 844ins68bp with BC among Jordanian women.

MATERIALS AND METHODS

Subjects

A group of two hundred women with confirmed diagnoses of breast cancer (cases) as well as a group of 200 age-matched unaffected women (controls) were recruited from two major referral hospitals for cancer, King Abdullah Hospital in Irbid, (northern part of Jordan) and Al-Basheer Hospital in Amman (central part of Jordan). Each of the participants in the study provided informed consent to donate samples of their blood and use clinical data for research. All procedures used were in strict compliance with the principles of the Helsinki II Declaration. All procedures used were in strict compliance with the principles of the Helsinki II Declaration. The study was ethically approved by the Yarmouk University (Irbid, Jordan) IRB committee (YU IRB DSR 2023/190).

Blood sampling, DNA isolation and Genotyping

Blood samples (3ml) were withdrawn from subjects into EDTA vacationers and stored at 4°C until DNA extraction. Genomic DNA was extracted from the collected blood samples according to the manufacturer's instructions using

a commercial kit (OMEGA Biokit). Genotyping of both *MTHFD1* G1958A (rs2236225) and *MTHFD1* T401C (rs1950902) was achieved by specific PCR amplification of the genomic DNA, followed by subsequent digestion with the proper restriction enzyme according to [33] and [47] respectively. At the same time, the detection of *CBS*844ins68bp was accomplished according to [48] by direct PCR.

Table 1 summarizes the genotyping conditions including the sequences of the primers, amplification conditions, restriction endonucleases and sizes of the DNA fragments produced. All PCR reactions were carried out in a total volume of 25ìl, containing 1ìl of (5pmole/ìl) of the proper forward and reverse primers specific for each SNP, 12.5ìl of 2X master mix (Promega, USA), and 1ìl of DNA sample in nuclease-free water up to 25 ìl. Amplification products were visualized by electrophoresis on 2% agarose gel (Agarose A; Biobasic) following staining with 0.5ìg/ml ethidium bromide (Sigma, USA).

Statistical analyses

Comparisons between groups and the different allele frequencies were evaluated using Pearson chi-square and the goodness of fit test (P>0.05). The allele and genotype frequencies of the variants in the case and control groups were calculated. Logistic regression analyses were recruited to estimate the associations between the risk of breast cancer and each examined variant, determining odds ratios (ORs) and their corresponding 95% confidence intervals (CIs). The statistical analyses were conducted using SPSS version 22.0 (SPSS, Chicago, IL).

RESULTS

The mean ages of the studied BC patients (50.22 ± 10.8 years) and the unaffected controls (49.03± 10.4 years) were statistically not different (P= 0.919). Figures 1 and 2 show representative results of the PCR – RFLP genotyping of *MTHFD1*

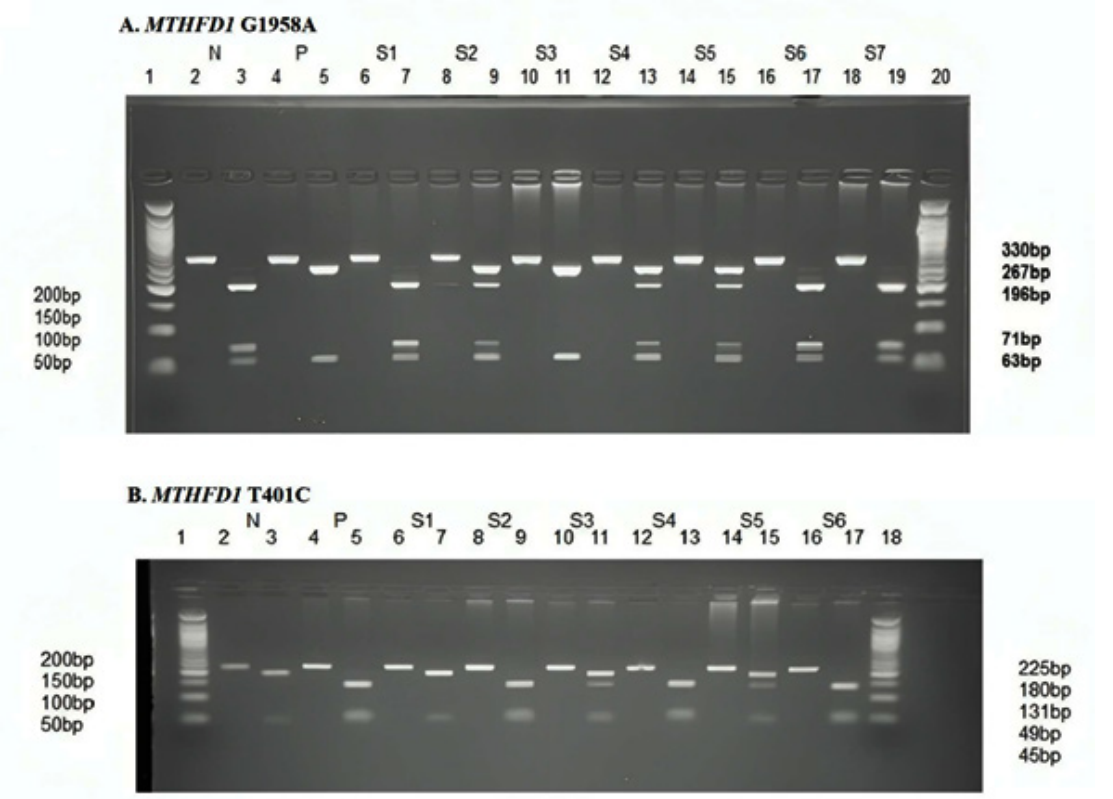

Fig. 1. Gel image shows PCR-RFLP pattern for *MTHFD1* G1958A and *MTHFD1* T401C resolved on 3% agarose gel stained with ethedium bromide. N: negative control. P: homozygous positive control. S: samples results from this study

Table 1. Primers sequences and PCR amplification conditions for the genotyping of the targeted polymorphisms *MTHFD1* G1958A, *MTHFD1* T401C, and *CBS* 844ins 68

Polymorphism	Primer sequence (5"! 3')	PCR conditions: denaturation, annealing, and extension	Restriction enzyme and incubation conditions	Fragment length produced in base pairs (bp)	Primers reference
MTHFD1 G1958A (Arg653Gln)	F:5'CACTCCAGTGTTTGTCCATG-3' R:5'GCATCTTGAGAGCCCTGAC-3'	Total of 35 cycles: denaturation at 94°C for 30 sec, annealing at 58°C for 1 min., and extension at 72°C for 1 min.	**Msp*I incubated at 37°C, 3h	G allele: 196bp, 71bp and 63bp A allele: 267 and 63bp	33
MTHFD1 T401C	F: 5'-GGCGTACAAGGAATGAAAC3' R: 5'-GGATGTGGATGGGTAAGTG3'	35 cycles: 95°C for 30 sec, 48°C for 40 sec, 72°C for 40 sec	**BsmA*I Incubated at 37 °C,16 h	T allele: 180 and 45 bpC allele: 131, 49, and 45 bp	47
CBS 844ins 68	F: 5'-CGCCCTCTGCAGATCATTGG3' R: 5'-CCTTCCACCTCGTAGGTTGTC3'	32 cycles: 95°C for 30 sec, 61°C for 40 sec, 72°C for 40 sec	--	Wild type: 100bp, Homozygous mutant: 168bp Heterozygous: 100bp and 168bp.	48

*Source: (New England Biolabs, Ipswich, MA, USA).

G1958A and *MTHFD1* T401C respectively, while Figure 3 shows representative results of *CBS* 844ins68bp by direct PCR. Table 2 shows the observed frequencies of both alleles and genotypes in BC patients and the unaffected controls. The alleles and genotypes of each of the three examined SNP were in Hardy Weinberg equilibrium (P> 0.05). Table 2 shows that the distribution of the wild type and mutant alleles of the individual polymorphisms *MTHFD1* G1958A, *MTHFD1* T401C and *CBS* 844ins68bp as well as the frequencies of the different genotypes of each examined polymorphism in the BC group were not significantly different from their frequencies in the controls. In addition, the sums of the mutant genotypes of each of these three polymorphisms were not significantly different between the patients and the control groups. The odd ratios of the sums of mutant genotypes *MTHFD1* G1958A (GA+AA), MTHFD1T401C (TC+CC) and CBS 844ins68 (w/ Ins + Ins/Ins) were 0.918 (95% CI=0.601-1.401; p = 0.67), 1.042 (95% CI=0.328-3.306; P=1.00) and 1.197 (95%CI = 0.701-2.044; p=0.498) respectively.

However, the frequencies of the double compound mutant genotypes GA/CC and AA/CC of the two non-synonymous *MTHFD1* polymorphisms G1958A and T401C were higher in BC patients group compared to the unaffected controls. The double compound genotypes *MTHFD* GA/CC and AA/CC seemed to increase the risk for BC by 3.4 and 5.1 respectively (Table 3) but their confidence intervals were wide possibly due to the small and limited numbers of the observed individuals carrying double or triple compound genotypes.

In order to find out the effect of the observed triple compounds of the examined polymorphisms, *MTHFD1*G1958A/ *MTHFD1*T401C/ *CBS* 844ins68 genotypes were examined in the cases and the controls. The results are shown in Table 4, which shows that there are no significant differences between the observed frequencies of the triple compound genotypes between the cases and the controls expect for the triple compound genotype *MTHFD1* 1958AA/*MTHFD1* 40CC/*CBS* 844ins68 (w/ins68) which have increased odd ratio to 5. This suggested that the presence of these three polymorphisms together in the same individual have a synergistic effect on increasing the risk for BC.

DISCUSSION

In this current population-based case-control study, we investigated the association between *MTHFD* 1958A, *MTHFD1* 401C variants and the insertion *CBS*844ins68bp with the risk of BC. *MTHFD1* gene, mapped to chromosome 14 (14q24), codes for the enzyme *MTHFD1*, which is

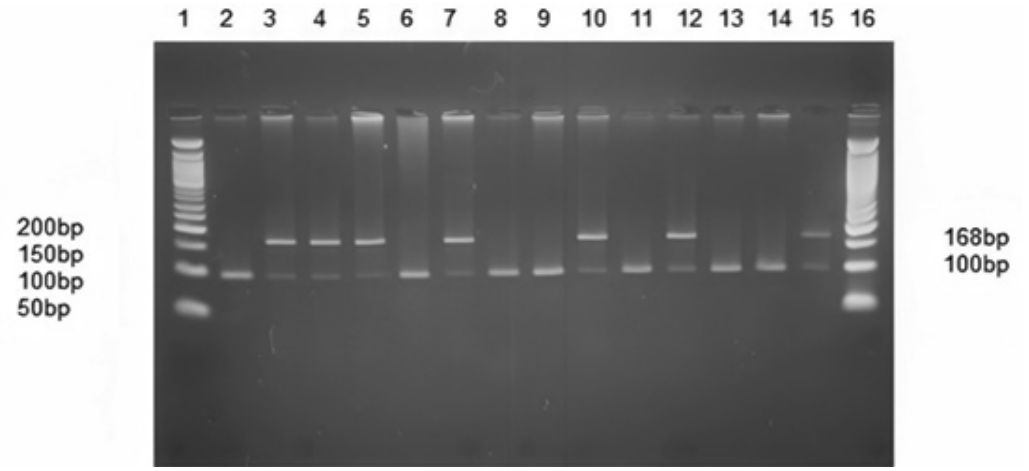

Fig. 2. Gel image shows the PCR analysis of *CBS* 844ins68 resolved on 3% agarose gel stained with ethedium bromide. Lanes 1 and 16: 50 bp DNA ladder; lane 2: normal control; lane 3: heterozygous control; lanes 4 to 15 are samples from breast cancer patients. Lanes 6, 8, 9, 11, 13, 14: normal CBS 844ins68 genotypes with 100bp fragment; Lanes 4, 5, 7, 10, 12 and 15: heterozygous CBS 844ins68genotype with 100bp and 168bp fragments.

a core enzyme in the folate/ one-carbon metabolism [49]. *MTHFD1* is a trifunctional enzyme consists of two major domains, an N-terminal containing the dehydrogenase and cyclohydrolase activities and a synthetase domain in the C terminus [50], and is associated with epigenetic events and DNA methylation that can influence susceptibility to cancers [13]. The SNP *MTHFD1* G1958A is a G to A transition at position 1958 located within the 10-formyl-THF synthetase domain [28], resulting in the substitution of glutamine with arginine residue at position 653 (R653Q). MTHFD1 G1958A causes reduction in the activity and stability of the enzyme [40'51] and facilitates different developmental diseases and different types of cancers, including breast cancer [14], colorectal [52], gastric [47], methotrexate sensitivity in acute lymphoblastic leukemia [53'54]. However, other different studies including a meta-analysis of Asians showed lack of association between *MTHFD1* G1958A and different types of cancers including lung cancer, and head and neck cancer [55'56]. Furthermore, *MTHFD1* G1958A decreased the risk for acute lymphoblastic leukemia [57]. The less common SNP *MTHFD1* T401C in which the arginine at position 134 is substituted by lysine (R134K), lies within the dehydrogenase/ cyclohydrolase domain of the enzyme [50], which may result in disturbance of the folate-mediated homocysteine pathway that is associated with cancer [34'14'40]. The activity of *MTHFD1* G1958A was correlated to reduction in the synthase activity, which is essential for the remethylation of homocysteine (Hcy) to methionine, which is a precursor for the synthesis of SAM [29'40]. Reduced levels of SAM lead to low methyl supply, which can result in global DNA hypomethylation, and very little conversion of dUMP to dTMP, leading to uracil misincorporation into DNA [58], DNA strand breaks, chromosomal instability, alteration of gene expression and consequently promoting carcinogenesis [58'59]. However, current results showed that neither the individual polymorphism

Table 2. Allele and genotype frequencies of *MTHFD1* G1958A, *MTHFD1* T401C, and *CBS* 844ins68 polymorphisms in BC and the control groups

Polymorphisms	% Controls (n)	% Cases (n)	OR (95% CI)	P
MTHFD1 G1958A				
G (ref)	0.59% (235)	0.58% (231)	-	-
A	0.41% (165)	0.42% (169)	0.960 (0.725-1.271)	0.774
GG (ref)	0.30 (61)	0.32 (65)	-	-
GA	0.57 (113)	0.51 (101)	0.847 (0.544-1.317)	0.229
AA	0.13 (26)	0.17 (34)	1.270 (0.677-2.375)	0.263
GA and AA	0.70 (139)	0.68 (135)	0.918 (0.601-1.401)	0.667
MTHFD1 T401C				
T (ref)	0.14% (56)	0.13% (52)	0.918 (0. 612-1.377)	-
C	0.86% (344)	0.87% (348)		0.679
TT (ref)	0.03 (6)	0.03 (6)	-	-
TC	0.22 (44)	0.2 (40)	0.955 (0.282-3.233)	0.623
CC	0.75 (150)	0.77 (154)	1.054 (0.329-3.375)	0.623
TC and CC	0.97 (194)	0.97 (194)	1.042 (0.328-3.306)	1.000
CBS 844ins68				
w (ref.)	0.92% (369)	0.91% (365)	-	-
Ins	0.078% (31)	0.086% (35)	0.876 (0.529-1.451)	0.607
w/w (ref.)	0.85 (170)	0.83 (165)	-	-
w/Ins	0.15 (29)	0.18 (35)	1.222 (0.711-2.100)	0.413
Ins /Ins	0.005 (1)	0.0 (0)	0.0	0.0
w/ Ins + Ins/ Ins	0.15 (30)	0.18 (35)	1.197 (0.701-2.044)	0.498

Abbreviations: Ref - Reference category (wild-type allele/genotype); n - number of subjects; OR - Odds Ratio; CI - confidence interval of OR based on multinomial logistic regression; P > 0.05; w - wild-type allele of CBS 844 (100bp); Ins - CBS 844ins68bp."

Table 3. Frequencies and numbers of the observed double compound genotypes among BC patients and the control groups

Compound polymorphisms	Controls (n)	Cases (n)	OR (95% CI)	P
MTHFD1 G1958A/MTHFD1 T401C				
GG/TT (Ref.)	0.02 (4)	0.01 (1)	-	-
GG/TC	0.08 (15)	0.07 (13)	1.467 (0.343 - 35.056)	-
GG/CC	0.21 (42)	0.26 (51)	1.857 (0.523 - 45.127)	0.695
GA/TT	0.01 (2)	0.02 (4)	1.000 (0.500 - 127.900)	0.287
GA/TC	0.13 (26)	0.12 (24)	1.69 (0.385 - 35.400)	0.411
GA/CC	0.43 (85)	0.37 (73)	3.435 (0.376 - 31.425)	0.762
AA/TT	0.00 (0)	0.01 (1)	-	0.220
AA/TC	0.02 (4)	0.03 (5)	1.000 (0.388 - 64.387)	-
AA/CC	0.11 (22)	0.14 (28)	5.091 (0.531- 48.852)	0.736
MTHFD1 G1958A/CBS ins68				
GG/ ww (Ref.)	0.26 (51)	0.26 (51)	-	-
GG/ wIns	0.05 (10)	0.07 (14)	1.400 (0.569 -3.442)	0.400
GA/ ww	0.48 (96)	0.44 (87)	0.906 (0.906 -1.471)	0.366
GA/ wIns	0.09 (17)	0.07 (13)	0.765 (0.337–1.736)	0.448
AA/ ww	0.12 (23)	0.14 (27)	1.174 (0.596-2.313	0.545
MTHFD1 T401C/ CBS ins68				
TT/ w/w (Ref.)	0.02 (4)	0.02 (4)	-	-
TT/ wIns	0.01 (2)	0.01 (2)	1.000 (0.091-11.028)	1.000
TC/ ww	0.18 (35)	0.19 (37)	1.057 (0.245– 4.556)	0.795
TC/ wIns	0.04 (8)	0.02 (3)	0.375 (0.055– 2.555)	0.126
CC/ ww	0.66 (131)	0.62 (124)	0.947 (0.232-3.867)	0.467
CC/ wIns	0.10 (19)	0.15 (30)	1.579 (0.352-7.079)	0.093
CC/ InsIns	0.01 (1)	0 (0)	-	-

OR: odd ratios; CI: confidence interval of OR according to multinomial logistic regression; P > 0.05; Ref: reference ; w: wild type (100 bp); Ins: mutant CBS 844ins68bp (168 bp).

Table 4. Numbers of the observed triple compound genotypes of *MTHFD1* G1958A, *MTHFD1* T401C and *CBS* 844ins68 among the cases and the control groups

Variants of *MHFD1* G1958A/ *MTHFD1* T401C/ *CBS* 844ins68	Number of Controls	Number of Patients	OR	95% C.I	P value
GG/TT/ ++ (Ref.)	2	1			
GG/ TT/+ins	2	0	-	-	-
GG/ TC/ ++	13	11	1.692	0.135- 21.270	0.674
GG/ TC/ +ins	2	2	1.000	0.090- 44.350	1.000
GG/CC/++	36	39	2.167	0.188- 24.929	0.701
GG/CC/+ ins	6	12	2.000	0.299- 3.468	0.148
GA/TT/++	2	3	2.000	0.150 - 59.890	0.653
GA/TC/++	20	22	2.000	0.185- 26.157	0.744
GA/TC/+ins	6	1	0.333	0.014- 8.182	0.057
GA/CC/++	74	63	1.703	0.151- 19.222	0.246
GA/CC/+ins	11	11	1.000	0.157- 25.404	1.000
AA/TT/ ins,ins	0	1	-	-	-
AA/TC/++	3	5	1.333	0.204- 54.532	0.475
AA/CC/++	20	21	1.100	0.176- 25.010	0.869
AA/CC/+ins	2	7	5.000	0.397- 25.37	0.092

OR: odd ratios; CI: confidence interval of OR according to multinomial logistic regression; P > 0.05; +: wild type *CBS* (100 bp); ins: mutant *CBS* 844ins68 (168 bp); Ref: reference

MTHFD1 G1958A nor MTHFD1 T401C per se were associated with BC, which supported the reported lack of association between MTHFD1 401C and human cancers [34] as well as the lack of association between MTHFD 1958A and BC in west Siberian region of Russia [60]. However, our results were contrary to those obtained from studying a mix of White, African American, Hispanic, Asian, and unknown postmenopausal females, which reported association of MTHFD1 T401C with risk of BC [14]. In addition, results of our current study were also different from those reported association of *MTHFD1* gene with other different types of cancer including gastric, colon and head and neck cancers [47'47]. Different studies also showed that the homozygous mutant genotype *MTHFD1* 1958AA induced significant reduction in the overall cancer risk, and the risk of primary liver and colon cancers [61]. In addition, homozygous patients with *MTHFD1* 1958AA genotype had significantly higher frequency of tumor CpG island hypermethylation compared to wild-type MTHFD1 1958GG homozygotes, which were significantly associated with DNA hypomethylation [13], suggesting that the G allele may exert a protective effect for cancer risk by protecting from DNA hypomethylation [61].

Cystathionine β-synthase (*CBS*) is another core enzyme in the folate/ one-carbon related metabolism, specifically at the reverse transsulfuration pathway, which transfers sulfur from the cytotoxic metabolite homocysteine to cysteine. The CBS gene located in the subtelomeric region q.22.3, of chromosome 21 [62] encodes for the 63-kDa CBS subunits of the tetramer active enzyme [63]. Each subunit of *CBS* consists of N- and C- terminal domains. The N-terminal domain binds to the cofactor heme and is essential for proper folding and assembly of the protein, but is not essential for its catalytic activity [64]. The C-terminal regulatory domain contains the binding sites for the allosteric activator SAM and is responsible for *CBS* subunit tetramerization [63]. The variant CBS ins68, which initially detected in a heterozygous patient with homocystinuria due to *CBS* deficiency [65] had been associated with lowered plasma homocysteine levels, consists of an identical insertion of 68bp DNA repeat within exon 8 of the CBS gene [66].

The observed results demonstrated that *CBS* 488ins68 polymorphism is not a risk factor for BC, which supported previous reports that considered *CBS* 844ins68 as a neutral variant [67], which generates an alternative splice site, which allows elimination of the entire insertion to form a normal *CBS* mRNA transcript [68]. Current results were also in harmony with the lack of association between *CBS* 844ins68 polymorphism with different types of cancer including colorectal cancer [47] carcinomas of the upper gastrointestinal tract [69] and prostatic carcinoma [70]. Furthermore, our results were in harmony with the unaltered expression of *CBS* enzyme in BC and the majority of cancer types where the role of CBS has been examined (with the exception of liver cancer and glioma) [42]. However, our current results were contrary to the reported significant association of *CBS* 844ins68 with risk of BC in Mexicans. In Mexicans, both homozygous and heterozygous genotypes of CBS 844ins68 were associated with the risk of BC [45]. Such controversial results could be due to ethnic differences as well as different lifestyles, diet and uptake levels of folate in the Jordanian and Mexican populations. Furthermore, current results are also different from the reported expression of *CBS* in breast cancer-affected tissue, compared to the normal control unaffected breast tissue in mastectomy samples of BC patients [42' 71].

The conflicting and controversial reports in the literature concerning the association of *MTHFD1* G1958A, *MTHFD1* T401C and CBS 844ins68bp with the risk of BC could be reflection to the complexity of the regulation of *CBS* and *MTHFD1* genes, as well as the complexity of the carcinogenesis process itself.

Both *CBS* and *MTHFD1* are involved in methyl group metabolism [70]. *CBS* catalyzes different but interrelated cellular biochemical pathways, including availability of SAM and DNA methylation, while *MTHFD1* G1958A is associated in breast cancer with hormone receptor content and DNA methylation frequency [13]. BC patients homozygous for the *MTHFD1* 1958AA genotype had a significantly higher frequency of tumor CpG island hypermethylation compared to the wild-type homozygotes *MTHFD1* 1958 GG [13]. *CBS* transcription is regulated by different mechanisms including the methylation status of the CpG islands in its two principal GC-rich promoters [72'73] and by several hormones and transcription factors [57'74'75'76]. *CBS* also regulates

the production of both ROS, which is triggered by glutathione abundance [65] and the intrinsic cellular regulator H2S [77'78]. In addition, the process of carcinogenesis involves altered methylation cycle accompanied with promoter hypermethylation, which leads to inactivation of genes in almost all pathways protective of carcinogenesis such as DNA repair, cell cycle control and apoptosis [31]. All these factors including ethnicity, which is a possible cause for differences in genetic variants in *MTHFD1*, *CBS* and other genes in the folate/ one-carbon metabolism in different ethnic groups, could contribute to differential risks of developing breast cancer between different populations [79]. In addition, other factors specific for different populations such as environmental, lifestyles, nutrition and uptake levels of folate may act upon these SNPs to generate a gradient of intermediates in the folate/ one-carbon metabolism and associated transsulfuration pathway.

Various studies showed that different individual polymorphisms in different genes of the folate/ one-carbon metabolism were not per se associated with breast cancer. These include the polymorphisms *MTHFR* C677T and *MTHFR* A1298C in the methylenetetrahydrofolate reductase, TYMS 1494 ins/del 6 in the thymidylate synthase and *MTRR* A66G in 5-methytetrahydrofolate homocysteine methyltransferase reductase (*MTRR*). However, the presence of mutant alleles for two polymorphisms of these genes increased the risk of BC or were associated with increasing the risk of developing more BC aggressive phenotypes [80'81]. We examined the effect of double compound genotypes of MTHFD1 T401C, MTHFD1 1958A and *CBS* 488ins68 *CBS* 488ins68 on the risk of BC. Results showed that the double compound states of both MTHFD1 T401C and *MTHFD1* 1958A (1958GA/ 401CC) and (1958AA/401CC) seemed to increase the risk of BC by 3.4 and 5.1 folds respectively (Table 3), but such increase was accompanied by large confidence intervals possibly due to the observed small numbers of these compound genotypes. Further investigations are required to explore the effect of double compound genotypes of *MTHFD1* T401C, *MTHFD1* 1958A and *CBS* 488ins68 on the risk of BC. In conclusion, *CBS* 844ins68bp, *MTHFD1* G1958A and MTHFD1 T401C polymorphisms per se are not risk factors of BC in Jordan females. For better BC management, further studies can be considered for understanding the influence of double compound genotypes of these polymorphisms on the risk of BC in Jordan.

CONCLUSION

This study revealed that each of MTHFD1 G1958A, T401C and CBS 844ins68 polymorphisms alone had no direct risk for BC in the Jordanian women, compared to the wild-type genotypes.

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Conflict of interest

The authors have no conflict of interest to declare.

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