Genetic Profile and Determinants of Homocysteine Levels in Kazakhstan Patients with Breast Cancer

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Abstract. *Aim: To analyze associations between homocysteine level, MTHFR and FTO rs1477196 polymorphisms and folate status in patients with breast cancer (BC) in order to clarify determinants of hyperhomocysteinemia. Patients and Methods: The study included 315 BC cases and 604 controls. Results: The MTHFRC677T genotype was associated with an increased incidence of BC [Odds ratio (OR)=1.71; 95% Confidential interval (CI)=1.21-2.43]. The MTHFR A1298C genotype was associated with a decreased risk of BC [OR=0.68; 95% CI: 0.49-0.95]. The homocysteine level was not associated with either MTHFR C677T or A1298C, nor with FTO rs1477196, but was inversely correlated with folate status in cancer cases ($p<0.0001$) and tended to be higher in patients with the MTHFR 677TT genotype. The folate level ($p<0.0005$) was an independent predictor of hyper-homocysteinemia in patients with BC. Conclusion: These results suggest an important role of homocysteine in breast tumorigenesis. Further studies are warranted to investigate how combined MTHFR genotypes exert their effects on cancer susceptibility.*

Breast cancer (BC) is a complex disease influenced both by the environment and genetics (1), yet most genetic and environmental factors are still poorly understood. Dietary factors may modulate the risk of this malignancy, however the only dietary factor that has been consistently associated with increased breast cancer risk is alcohol consumption (1, 2).

For the past two decades, a mounting body of epidemiological and experimental studies has indicated that low folate intake or status is associated with elevated risk for several types of cancer, including BC (3-5). The biological mechanisms whereby folate deficiency may enhance carcinogenesis may be related to the role of this vitamin in one-carbon metabolism, as folate, in the form of methyltetrahydrofolate, provides the methyl groups required for intracellular methylation reactions and also functions as a co-enzyme in the synthesis of purines and thymidylate for DNA (6). Low folate status may, therefore, alter DNA methylation and influence gene stability and expression (6, 7). Diminished folate status may also result in misincorporation of uracil into DNA, which would lead to chromosome breaks as well as DNA repair disruption, enhanced mutagenesis, and apoptosis (6, 8). Therefore, disturbances in folate metabolism may lead to aberrant DNA synthesis and DNA methylation and may be an adding factor to carcinogenesis.

Folate is also well-known to influence homocysteine metabolism, where it serves as a co-substrate (9). Elevation of homocysteine level is associated with several metabolic disorders, including high body-mass index (BMI), high plasma triglyceride levels, hypertension, and abnormal oxidation of low-density lipoproteins (9-11); this may lead to the development of several types of cancers, including BC (12). Several studies have shown that homocysteine levels are also positively associated with *in vitro* proliferation rates of cells of a variety of tumor types, including breast tumors (13, 14), as well as with oxidative damage to cells (15). Hyperhomocysteinemia has been proposed as a risk factor for the development of estrogen-induced hormonal cancer in

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humans and is believed to cause pathogenic effects through the metabolic accumulation of *S*-adenosyl-L-homocysteine, which is a strong non-competitive inhibitor of the catechol-*O*-methyltransferase-mediated methylation metabolism of various catechol substrates, such as catechol estrogens (15, 16). Methylation of catechol estrogens in target organs is ultimately responsible for decreased formation of 2-methoxyestradiol, a strong anti-angiogenic and anticancer agent, and for the increased accumulation of the pro-carcinogenic 4-hydroxyestradiol, which lead to the development of estrogen-induced hormonal cancer in the target organs (15, 16).

For all these reasons, hyperhomocysteinemia has been regarded as a risk factor for cancer; therefore, the total homocysteine level has been proposed as a new tumor marker since it not only accurately reflects the proliferation rate of tumor cells but also responds to tumor cell death (12). Observational studies undertaken to assess the association between circulating homocysteine and overall BC risk are very limited in number, and findings have been inconsistent. One case control study reported a positive association between homocysteine levels and BC risk (17), whereas a second cohort study did not report on this association (18).

In addition to folate intake, plasma homocysteine levels are regulated mainly by 5,10-methylenetetrahydrofolate reductase (MTHFR), which is involved in the folate-dependent re-methylation of homocysteine (10). MTHFR is a key enzyme in homocysteine metabolism; it catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which directs the folate pool towards re-methylation of homocysteine to methionine, at the expense of DNA and RNA biosynthesis (3). The potential influence of MTHFR on DNA methylation and on the availability of uridylates and thymidylates for DNA synthesis and repair makes *MTHFR* a potential candidate cancer-predisposing gene (5-8).

Previously reported associations between *MTHFR* polymorphisms and various types of cancers (19, 20) have led to a number of studies that examined how these polymorphisms might influence the risk for BC (21-30). Most of these studies have reported on the *MTHFR* C677T and A1298C polymorphisms and risk for BC, with mixed results (13, 14). Accumulating evidence indicates that breast carcinogenesis could be initiated through activation of proto-oncogenes by hypomethylation of their promoter regions, through inactivation of tumor-suppressor genes by hypermethylation (31), or through alteration of estrogen receptor gene methylation patterns (16). Breast tumorigenesis could be triggered by strand break and DNA mutation caused by estrogen metabolism (16). The plausible role of *MTHFR* in DNA methylation, and DNA biosynthesis and repair in actively dividing cells warrants the analysis of BC susceptibility arising from the two most common *MTHFR* polymorphisms.

An association has been found between obesity and BC (32); for example, links with certain types of cancer have been reported for variants within the fat mass and obesity-related gene (*FTO*, OMIM No. 610966), primarily detected as an "obesity-associated" gene, in some, but not all, studies. Initially, an association with obesity and type II diabetes mellitus was found for *FTO* in several genome-wide association studies (33). More specifically, single-nucleotide polymorphisms (SNPs) in intron 1 of FTO, such as rs1477196, have shown significant association with obesity (33). Recently, a clinic-based case-control study by Kaklamani *et al*. revealed that a SNP located in intron 1 of *FTO* (rs1477196) as well as others in a linkage disequilibrium block, were significantly associated with BC risk (34). A recently performed analysis of the DNA methylation profile suggests that common variants within *FTO* are associated with different DNA methylation levels (35). Abundant methylation of regulatory portions of tumor suppressor genes is a common feature of human cancers.

Recently, we showed that folate deficiency might exist among the Kazakhs, probably due to their traditional diet (36, 37). We screened serum folate and plasma homocysteine in the general population of Kazakh adults, and 50 out of 61 (82.0%) people tested had low concentrations of folate. Supplementation with folic acid is definitely needed in Kazakhstan in order to reduce the risk of hyperhomocysteinemia-related disorders, including BC. However, for the appropriate implementation of this health policy, identification of homocysteine determinants in the Kazakhstan population is needed. To our knowledge, no study has yet investigated the potential association between hyperhomocysteinemia, serum folate level, and *MTHFR*, and *FTO* polymorphisms in Kazakhstan women with BC.

In the present study, we screened biochemical and genetic markers linked to folate and homocysteine metabolism in order to identify the determinants of homocysteine concentration among a group of Kazakhstan patients with BC. The overall aim of this study was to analyze potential associations between total homocysteine levels, *MTHFR* polymorphisms, and rs1477196 SNP in intron 1 of *FTO* and the folate status of patients with BC in order to clarify possible determinants of hyperhomocysteinemia and their role in predicting BC risk.

Materials and Methods

Study participants. The study was performed with the appropriate Institutional Ethics Approvals (No. 2011007) and in accordance with the principles embodied in the Declaration of Helsinki. Prior to the study, informed consent was obtained from all participants.

A total of 315 Kazakhstan women with pathologically-confirmed BC who underwent operations at the Oncological Centers of Semey and Astana, Kazakhstan, were enrolled in this study (cancer cases). An additional 604 healthy women without any diagnosis of cancer, who resided in Semey and Astana regions at the time of enrollment, were included as controls.

Exclusion criteria were the following: no informed consent to the study; acute inflammatory disease; impaired liver (bilirubin level >1.5 mg/dl) or renal (creatinine level >1.5 mg/dl) function; concurrent therapy with anti-folate drugs or medications known to influence homocysteine levels (thus interfering with the genotype/phenotype correlation study), such as current or recent use of a folate or vitamin B12 supplement or of any multivitamin preparation and current or recent use of drugs interfering with homocysteine levels (*e.g.*, anticonvulsants, methotrexate, and penicillamine).

Data collection and laboratory measurements. All participants completed a questionnaire that included questions on diet, demographic characteristics, medical history, family history of cancer, smoking habits, alcohol consumption, physical activities, menstrual and reproductive factors; anthropometric measurements were also obtained. The height and weight of each participant were measured, and the BMI (kg/m^2) was calculated as an index of obesity. The clinical and pathological characteristics such as the age at diagnosis (operation), histological subtype (WHO histological TNM classification), histological grade, T stage, and lymph node involvement were obtained from the medical records.

Fasting blood samples were obtained and after separation of serum and plasma from whole blood, they were kept at –20˚C and –80˚C, respectively, until assayed. Serum folate and vitamin B12 (VB12) were measured using chemiluminescent immunoassay and radioimmunoassay methods (ACS:180 VB12 and Folate kit; Bayer Medical, Tokyo). Serum creatinine and albumin were measured by enzyme (Hitachi 7450; Hitachi, Tokyo) and bromocresol green method (BCG), respectively (38). Plasma total homocysteine was measured using high performance liquid chromatography (39).

The normal ranges of serum folate, VB12, creatinine, and albumin were 3.6-12.9 ng/ml, 233-914 pg/ml, 0.3-1.2 mg/dl, and 3.7-5.5 g/dl, respectively. The normal range of plasma total homocysteine was 6.3-8.9 nmol/ml. Serum concentrations of triglycerides (TG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), calcium, C-reactive protein (CRP) were measured using standard laboratory procedures. Total cholesterol was calculated by the formula TC=HDL-C+LDL-C+(TG/5) (Friedewald equation) (40). The TC/HDL and LDL/HDL ratios were calculated. The TG levels had a skewed distribution, therefore logarithmic transformation was performed for the subsequent statistical analysis. The normal range of TG was 50-149 mg/dl, HDL-C was 40-90 mg/dl, LDL-C 70-139 mg/dl, calcium 8.2-10.0 mg/dl and CRP <0.30 mg/dl. Leptin was measured (ng/ml) using a Human Leptin RIA kit (Cosmic Corporation, Tokyo, Japan). Adiponectin was measured using a Human Adiponectin ELISA kit (Daiichi Pure chemicals Co. Ltd, Tokyo, Japan) using the Latex coagulation method (normal range >4 mg/ml).

DNA isolation and genotyping. DNA was extracted from whole blood lymphocytes using the Master Pure DNA purification Kit (EPICENTRE Biotechnologies, Madison, WI, USA), according to the manufacturer's protocols and was stored at –20˚C until used for genotyping.

Genotyping for the *MTHFR*, 677 C>T (rs1801133 ref dbSNP) and 1298 A>C (rs1801131 ref dbSNP) and *FTO*, (rs1477196 ref dbSNP) was performed by Taqman SNP allelic discrimination by means of an ABI 7900HT (Applied Biosystems, Foster City, CA) using commercial TaqMan SNP Genotyping assays (Applied Biosystems by Life Technologies) C_1202883_20 for *MTHFR*677 C>T and C_850486_20 for *MTHFR*1298 A>C and C_2031262_10 for *FTO* rs 1477196. Primer sets were created using Primer3 (v.0.4.0) software (http://frodo.wi.mit.edu/primer3). Results were ascertained using the SDS software version 2.3 (Applied Biosystems). All results were automatically recorded (*i.e.* the device displays the genotypes automatically with a 95% certainty). A total of 5% of samples were genotyped in duplicate and showed 100% concordance. A total of nine samples – representatives of wild, heterozygous and mutant genotype samples for each polymorphism – were evaluated by TaqMan Assay and then were confirmed by direct sequencing and were further used as internal controls.

Statistical analysis. Allelic frequencies were calculated by the gene counting method and Hardy–Weinberg equilibrium (HWE) was tested for each SNP. Association of allelic and genotypic counts with BC cases was evaluated by Pearson's Chi-square-test followed by odds ratios (OR) with 95% confidential intervals (CI), using 2×2 contingency tables. The maximum likelihood estimates of haplotype frequencies were calculated from the multisite marker typing data, using the online software SNP Stats (41). Differences between percentages were assessed by Chi-square test. Student's unpaired *t*-test, ANOVA test and Pearson correlation analysis were used for the normally distributed continuous variables. Appropriate non-parametric tests (Mann-Whitney *U*-test, ANOVA and median test and Spearman rank correlation test) were employed for all the other variables. Multiple linear regression analyses were performed to further quantify the relationship between the clinical and biochemical variables. Only two-tailed probabilities were used for testing statistical significance. The data are presented as the mean±standard deviation (SD), or median (25th-75th quartiles). All *p*-values less than 0.05 were regarded as statistically significant. All the calculations were performed using computer software packages (SPSS 19.0, SPSS, Tokyo, Japan).

Results

The study population consisted of 315 BC cases and 604 controls, aged 55.3±9.8 years and 40.5±13.4 years, respectively (Table I). Cases and controls differed significantly by race ($p<0.01$): 48.6% of the cases were Asian and 51.4% were Caucasian, whereas 65.9% of the controls were Asian and 34.9% were Caucasian. Cases, in general, were more likely to have a family history of BC and other cancers, to be older at menarche and menopause, and to have a higher number of pregnancies and live births compared to the controls. Cases had relatively, but not significantly, higher BMI and weight compared to the controls. Significant differences also existed between cases and controls in terms of age at menarche (13.0±1.0 years *vs*. 12.8±0.9 years), parity (4.2±2.6 *vs*. 3.0±2.4), and age at menopause (48.2±4.6 years *vs*. 47.4±5.0 years). No significant differences were found between cases and controls in terms of the proportions of women with regard to alcohol consumption (4.4% *vs*. 5.8%), smoking (8.6% *vs*. 6.8%), and age at first full-term pregnancy (21.8±5.7 years *vs*. 20.4±7.9 years).

The histopathological profile showed that 72.3% of cases had infiltrating ductal carcinoma, 5.1% had intralobular carcinoma, 17.8% had mucinous carcinoma, and 4.8% had ductal carcinoma *in situ*. The majority of the cases were diagnosed at T2 (60.6%) and T1 (16.8%) stages, with fewer

Table I. *Basic characteristics of breast cancer cases and controls.*

	Cases, N=315 n (%)	Controls, N=604 n (%)	*p*-Value
Age (years)	55.3±9.8[a]	40.5±13.4	<0.001
Race			
Asian	153 (48.6)	398 (65.9)	<0.01
Caucasian	162 (51.4)	206 (34.9)	<0.01
Weight (kg)	73.8±13.6	66.7±13.3	0.34
Height (m)	160.7±6.2	162.4±6.8	0.40
BMI (kg/m^2)	28.6±5.2	25.3±5.1	0.94
Smoking			
Yes	27 (8.6)	41 (6.8)	0.397
No	288 (91.4)	563 (93.2)	
Alcohol			
Yes	14 (4.4)	35 (5.8)	0.387
No	301 (95.6)	569 (94.2)	
Family history of any cancer			
Yes	57 (18.1)	71 (11.8)	0.011
No	257 (81.6)	533 (88.2)	
Family history of breast cancer			
Yes	35 (11.1)	43 (7.1)	0.038
No	279 (88.9)	561 (92.9)	
Age at menarche	13.0±1.0	12.8±0.9	0.001
Age at first full-term pregnancy	21.8±5.7	20.4±7.9	0.481
Parity	4.2±2.6	3.0±2.4	0.001
Number of live birth	2.5±1.7	1.9±1.5	0.001
Age at menopause	48.2±4.6	47.4±5.0	0.03
Age at BC manifestation	51.2±9.8	-	
Laboratory variables[b]			
Albumin (g/dl)	4.4	4.6	0.004
Creatinine (mg/dl)	0.8	0.6	0.000
HDL-C (mg/dl)	41.0	54.0	0.000
LDL-C (mg/dl)	81.0	87.0	0.928
TG (mg/dl)	85.0	81.5	0.733
Log TG	1.9	1.9	0.754
CA (mg/dl)	9,4	9,5	0.808
HCY (nmol/ml)	17.2	14.5	0.001
VB12 (pg/ml)	301.0	323.0	0.076
FA (ng/ml)	2.8	2.9	0.019
CRP (mg/dl)	0.2	0.1	0.002
Leptin (ng/ml)	13.1	11.4	0.142
Adiponectin (mg/ml)	11.9	11.6	0.621
TC (mg/dl)	148.2	160.5	0.004
TC/HDL	3.6	3.0	<0.001
LDL/HDL	2.1	1.6	<0.001

[a]Mean±SD (all such values). [b]All values are medians. Differences in mean values between case-control pairs were tested by a paired *t*-test. Differences in median values between case-control pairs were tested by Mann-Whitney *U*-test. Differences in categorical variables between case-control pairs were tested by χ^2 test. HDL-C: High-density lipoprotein-cholesterol; LDL-C: low-density lipoprotein-cholesterol; TG: triglycerides; CA: calcium; HCY: homocysteine; VB12: vitamin B12; FA: folic acid; CRP: C-reactive protein; TC: total cholesterol; TC/HDL: ratio total cholesterol and high-density lipoprotein; LDL/HDL: ratio low density lipoprotein and high-density lipoprotein.

diagnosed at T3 (13.6%) and T4 (8.9%) stages. The frequency of lymph node metastases at diagnosis was 51.1%, and 3.5% of the cases had subsequent metastasis.

The allelic frequencies and genotype distributions of *MTHFR* C677T (rs1801133), *A1298*C (rs1801131) and *FTO* rs1477196 among cases and controls, as well as their contributions to the risk of breast cancer according to different models, are shown in Table II. The C/C, C/T, and T/T genotype distribution of *MTHFR* C677T in the BC patients (57.5%, 34.6%, and 7.9%, respectively) and controls (47.5%, 44.5%, and 8%, respectively) did not differ significantly from those predicted by the Hardy-Weinberg distribution (HW probability test, p=0.14 for patients and 0.18 for controls).

The distributions of the *MTHFR* A1298C A/A, A/C, and C/C genotypes of the cancer patients and controls (43.8%, 45.1%, and 11.1%; 52.6%, 40.1%, and 7.3%, respectively) did not differ significantly from those predicted by the Hardy-Weinberg distribution (HW probability test p=0.9 for patients and 0.92 for controls). The *FTO* rs1477196 A/A, A/G, and G/G genotype distributions in cases and controls (40%, 49.5%, and 10.5%; 41.7%, 44%, and 14.2%, respectively) were also in conformity with the HWE (HW probability test p=0.17 for patients and 0.25 for controls). The frequency distribution of the *MTHFR* and *FTO* genotypes between cancer patients and healthy participants are depicted in Figure 1.

Taking the *MTHFR* 677CC genotype as the reference, individuals with the 677CT genotype had a 1.3-fold increase in BC risk (OR=1.71, 95% CI=1.21-2.43; p<0.01 co-dominant model), while individuals with the 677CT or TT genotype had a 1.2-fold increase in BC risk (OR=1.55, 95% CI=1.11-2.16; p<0.01) under the dominant model (Table II). For *MTHFR* C677T, the frequency of the T allele was 25.2% in cases and 30.2% in the controls. In the dominant model, using the *MTHFR*1298AA genotype as the reference, individuals with the 1298AC+CC genotype had a 1.2-fold decrease in BC risk (OR=0.68, 95% CI=0.49-0.95, p<0.05). For *MTHFR* A1298C, the frequency of the C allele was 33.7% in the cases and 27.3% in the controls. On the other hand, we found no differences between patients and healthy controls with respect to the *FTO* rs1477196 genotypic frequencies, regardless of which model was used for analysis: dominant (p=0.15), co-dominant (p=0.2), and recessive (p=0.3). Finally, the numbers of alleles were identical in both groups (35.2% in patients and 36.3% in controls for the minor G allele).

Estimated haplotype frequencies among *MTHFR* C677T and A1298C and *FTO* rs1477196 were compared between BC cases and controls. Out of eight possible haplotypes, seven haplotypes were shared by both the cases and the controls. One haplotype (CCA) was associated with a significantly reduced risk of BC (OR=0.62, 95% CI=0.41-0.94; p=0.023) (data not shown). We also analyzed the association of *MTHFR* C677T, A1298C, and *FTO* rs1477196 genotypes with race among cases and controls, and found no significant differences in genotype distributions among cases and controls with

Table II. *Genotypic and allelic frequencies of Methylenetetrahydrofolatereductase (MTHFR) and Fat mass and obesity (FTO) genes among patients with breast cancer and controls and their contributions to the risk of breast cancer by different models.*

SNP	Model	Genotype	Cases N=315 n (%)	Controls N=604 n (%)	OR (95% CI)
MTHFR C677T rs1801133	Co dominant	C/C	181 (57.5)	287 (47.5)	1.0
		C/T	109 (34.6)	269 (44.5)	1.71 (1.21-2.43) **
		T/T	25 (7.9)	48 (8)	0.88 (0.46-1.68)
		C allele	471 (74.8)	843 (69.8)	
		T allele	159 (25.2)	365 (30.2)	
		HWE test[a]	p=0.14	p=0.18	
	Dominant	C/C	181 (57.5)	287 (47.5)	1.00
		C/T-T/T	134 (42.5)	317 (52.5)	1.55 (1.11-2.16) **
	Recessive	C/C-C/T	290 (92.1)	556 (92)	1.00
		T/T	25 (7.9)	48 (8)	0.70 (0.37-1.31)
MTHFR A1298C rs1801131	Co dominant	A/A	138 (43.8)	318 (52.6)	1.00
		A/C	142 (45.1)	242 (40.1)	0.70 (0.49-0.99)
		C/C	35 (11.1)	44 (7.3)	0.61 (0.34-1.09)
		A allele	418 (66.3)	878 (72.7)	
		C allele	212 (33.7)	330 (27.3)	
		HWE test	p=0.9	p=0.92	
	Dominant	A/A	138 (43.8%)	318 (52.6%)	1.00
		A/C-C/C	177 (56.2%)	286 (47.4%)	0.68 (0.49-0.95) *
	Recessive	A/A-A/C	280 (88.9%)	560 (92.7%)	1.00
		C/C	35 (11.1%)	44 (7.3%)	0.72 (0.41-1.26)
FTO rs1477196	Co dominant	A/A	126 (40)	252 (41.7)	1.00
		A/G	156 (49.5)	266 (44)	0.75 (0.53-1.06)
		G/G	33 (10.5)	86 (14.2)	1.13 (0.64-1.98)
		A allele	408 (64.8)	770 (63.7)	
		G allele	222 (35.2)	438 (36.3)	
		HWE test	p=0.17	p=0.25	
	Dominant	A/A	126 (40%)	252 (41.7%)	1.00
		A/G-G/G	189 (60%)	352 (58.3%)	0.81 (0.58-1.13)
	Recessive	A/A-A/G	282 (89.5%)	518 (85.8%)	1.00
		G/G	33 (10.5%)	86 (14.2%)	1.32 (0.78-2.24)

OR, Odds ratios and CI, 95% confidence interval (adjusted by age, race and body mass index). [a]The p-value is for testing the deviation from Hardy-Weinberg equilibrium (HWE) among controls. *p<0.05, **p<0.01, difference between genotypes under the different models.

regard to race, except that the *MTHFR* C677T genotype was associated with BC among Asians (χ^2=9.851, p=0.007) (OR=1.99, 95% CI=1.24-3.17, interaction p-value=0.39).

The nutritional and metabolic status of the study groups was further analyzed through determination of vitamin B12, folate, total homocysteine, albumin, creatinine, CRP, and calcium levels (Table I). As expected, a significantly lower level of albumin and higher level of creatinine and CRP were found in the patients compared controls, whereas the vitamin B12 and calcium levels were similar in both groups. Both cases and controls had low folate levels; moreover, these were significantly lower in breast cancer patients (2.8 ng/ml *vs*. 2.9 ng/ml, p=0.019). The plasma total homocysteine level was higher in patients compared to the controls (p=0.001). Lipid profiles showed patients with BC to have significantly lower levels of HDL-C, and higher TC/HDL and LDL/HDL ratios, despite having lower levels of total cholesterol than the controls. The cancer cases had a relatively higher level of TGS, but the differences were not statistically significant. No differences were noted in the levels of leptin and adiponectin between cases and healthy controls.

Analysis of the association between the observed genotypes and phenotypes showed no significant association between *MTHFR* C677T, A1298C, and *FTO* rs1477196 genotypes and most of the phenotypic variables for the cases and controls (Table III). Among individuals with the *MTHFR* 677CC genotype, the levels of homocysteine were higher in the cases than in the controls (18.1±6.5 *vs*. 14.3±4.0 nmol/ml, p<0.001), but the differences in total homocysteine levels in *MTHFR* 677TT carriers among cases and controls were not statistically significant (18.0±4.9 *vs*. 19.2±6.3 nmol/ml).

The ANOVA test showed that plasma total homocysteine level was not associated with either *MTHFR* C677T (p=0.67) or A1298C (p=0.36), nor *FTO* rs1477196 (p=0.74), but was inversely correlated with the folate status of the patients (p<0.0001). Further analysis of the plasma total homocysteine

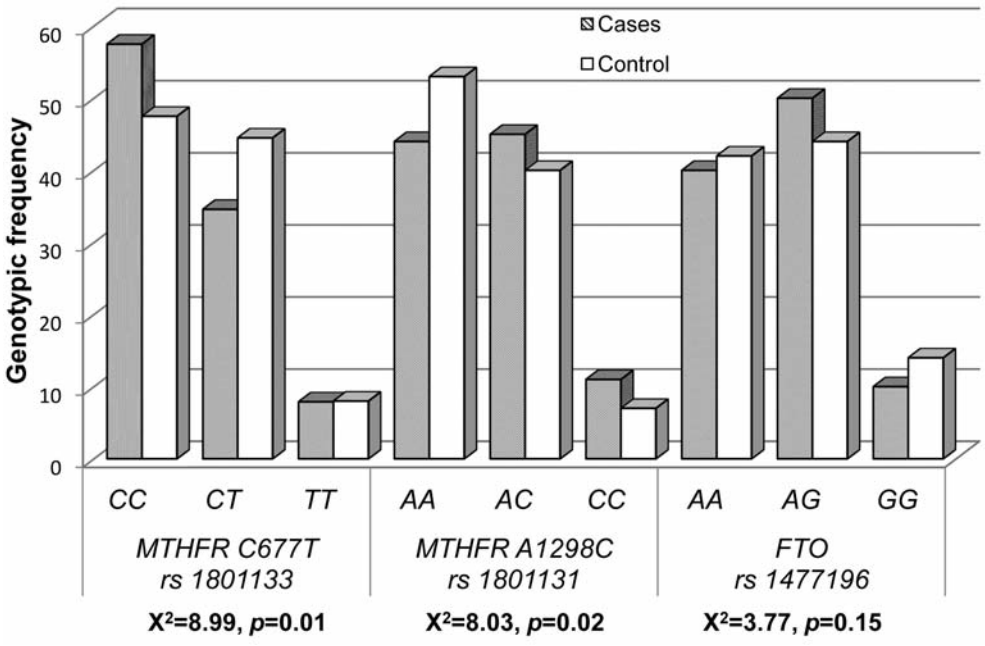

Figure 1. *Frequency distribution of Methylenetetrahydrofolatereductase (MTHFR) C677T and A1298C and Fat mass and obesity (FTO) rs1477196 genotypes between patients with breast cancer and healthy controls.*

distribution in patients with different *MTHFR* C677T genotypes and low or high folate levels (categorized on the basis of the upper quartile of the values observed in all the recruited participants, *i.e.* 4 ng/ml) showed that the highest total homocysteine levels were found in the patients with the TT genotype and low folate levels, although the differences were not significant (F=2.39, p=0.138) (Figure 2).

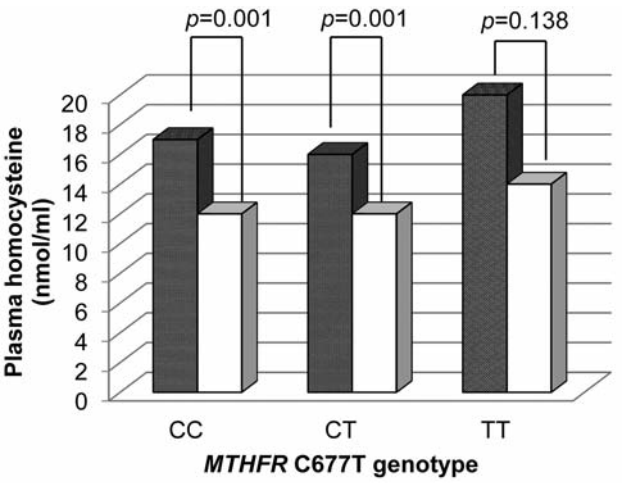

Figure 2. *Plasma homocysteine levels for genotypes of Methylenetetrahydrofolatereductase (MTHFR) C677T, stratified by low (<4 ng/ml, dashed columns) and high (≥4 ng/ml, open columns) serum folate levels, in the breast cancer study population.*

Discussion

Various groups have evaluated the association between the *MTHFR* C677T and A1298C polymorphisms and the risk of breast cancer, but the results have been conflicting (13, 14, 42). The present study identified an association between BC and the *MTHFR* alleles or genotypes. Individuals with the 677CT genotype had a 1.3-fold increase in BC risk (p<0.01) under the co-dominant model, while individuals with the 677CT or TT genotype had a 1.2-fold increase in BC risk (p<0.01) under the dominant model. In contrast, the dominant model using the *MTHFR* 1298AA genotype as the reference showed that individuals with the 1298AC or CC genotype had a 1.2-fold decrease in breast cancer risk (p<0.05).

Our results for the C677T and A1298C SNPs are consistent with those reported in other studies indicating a role of these *MTHFR* polymorphisms in BC risk (21-30). Data are conflicting regarding the role of the *MTHFR*C677T variants in cancer, and an inverse effect (14, 31) or a need for predisposing factors (26-28) has been advocated by some authors. Large case control studies of women showed that White women from the Multiethnic Cohort (27) failed to reveal a BC risk association with *MTHFR* genotypes. In contrast, English-speaking women of the Long Island Study had a significant increase in a BC risk association with the *MTHFR* 677T/T genotype (OR=1.37, 95% CI=1.06-1.78, p=0.03) (29). However, the specific study population is

Table III. *Association between Methylenetetrahydrofolatereductase (MTHFR) C677T, MTHFR A1298C, Fat mass and obesity (FTO) rs1477196 polymorphism genotypes and phenotypes among breast cancer cases and controls.*

	MTHFR C677T			*MTHFR* A1298C			*FTO* rs1477196		
Phenotype	Genotype	Cases	Controls	Genotype	Cases	Controls	Genotype	Cases	Controls
BMI (kg/m^2)	CC	29.0±5.5	25.0±5.0	AA	28.5±5.8	25.6±5.3	AA	28.9±5.1	25.2±4.9
	CT	28.2±4.9	25.8±5.5	AC	28.7±4.8	25.1±4.9	AG	28.5±5.3	25.4±5.3
	TT	27.8±4.6	24.8±4.2	CC	28.9±4.5	24.4±5.0	GG	28.2±5.3	25.4±5.3
Albumin (g/dl)	CC	4.4±0.6	4.5±0.4	AA	4.3±0.6	4.6±0.4	AA	4.3±0.6	4.6±0.5
	CT	4.2±0.7	4.6±0.4	AC	4.3±0.7	4.5±0.4	AG	4.3±0.7	4.5±0.3
	TT	4.4±0.4	4.6±0.5	CC	4.8±0.5	4.6±0.4	GG	4.7±0.7	4.5±0.5
Creatinine (mg/dl)	CC	0.8±0.2	0.7±0.2	AA	0.8±0.3	0.7±0.3	AA	0.8±0.2	0.7±0.3
	CT	0.9±0.3	0.7±0.3	AC	0.8±0.2	0.7±0.2	AG	0.8±0.3	0.7±0.2
	TT	0.8±0.3	0.7±0.2	CC	0.7±0.1	0.7±0.2	GG	0.9±0.2	0.7±0.3
HDL-C (mg/dl)	CC	44.4±10.0	53.9±9.1$^{+\#}$	AA	40.2±10.4$^{*\#}$	56.6±10.7	AA	40.3±9.2$^{*\#}$	54.4±9.2
	CT	43.9±11.6	57.5±11.3	AC	44.9±8.8	55.2±9.8	AG	44.5±10.2	56.6±11.1
	TT	38.1±8.6	57.7±9.0	CC	54.8±13.7	52.3±9.1	GG	51.2±14.0	57.1±10.2
HCY (nmol/ml)	CC	18.1±6.5	14.3±4.0$^{***\#\#\#}$	AA	17.0±6.4	15.2±4.5	AA	18.1±6.9	14.2±4.3
	CT	16.9±7.2	14.6±4.1	AC	18.0±6.9	14.2±4.2	AG	17.3±6.5	14.8±4.2
	TT	18.0±4.9	19.2±6.3	CC	20.4±5.7	15.5±3.1	GG	19.0±6.6	16.4±4.7
VB12 (pg/ml)	CC	330.4±113.2	369.1±186.8	AA	287.2±88.3$^{+\#}$	376.5±184.2	AA	328.2±145.0	370.6±159.1
	CT	321.9±162.9	389.1±204.4	AC	358.2±161.3	374.6±208.2	AG	323.4±129.4	386.9±231.7
	TT	307.4±105.9	315.8±129.9	CC	318.8±75.1	365.7±150.9	GG	319.3±112.5	348.8±134.7
FA (ng/ml)	CC	3.1±1.9	4.3±2.9	AA	3.0±1.1	3.8±3.6	AA	2.8±1.4	4.0±2.8
	CT	2.7±0.9	3.8±3.7	AC	3.2±1.8	4.0±2.9	AG	3.1±1.7	4.1±3.8
	TT	3.2±2.0	2.3±0.6	CC	2.7±2.2	3.9±3.0	GG	3.5±1.3	3.4±2.1
CRP (mg/dl)	CC	0.9±1.6	0.3±0.8	AA	1.1±2.0	0.3±0.4	AA	0.8±1.4	0.3±0.4
	CT	1.8±3.4	0.3±0.7	AC	1.4±2.8	0.3±0.8	AG	1.5±2.9	0.4±1.0
	TT	0.3±0.3	0.2±0.2	CC	0.2±0.6	0.7±1.8	GG	0.3±0.4	0.3±0.4
TC (mg/dl)	CC	151.4±38.9	164.4±35.9	AA	157.0±39.4	168.0±34.7	AA	146.7±36.1	163.1±33.3
	CT	158.2±40.4	165.7±33.6	AC	150.9±35.4	160.7±36.1	AG	156.7±37.7	163.1±34.9
	TT	153.2±23.3	161.6±35.0	CC	157.7±49.0	166.4±22.7	GG	167.3±45.8	175.9±37.3
TC/HDL	CC	3.5±0.8	3.1±0.7	AA	4.1±1.1$^{**+++\#\#\#}$	3.0±0.8	AA	3.8±1.0	3.1±0.7
	CT	3.8±1.1	3.0±0.7	AC	3.4±0.6	3.0±0.7	AG	3.6±0.9	3.0±0.7
	TT	4.2±1.0	2.8±0.5	CC	3.0±1.0	3.2±0.5	GG	3.4±1.0	3.1±0.8
LDL/HDL	CC	2.0±0.7	1.7±0.6	AA	2.4±0.9$^{++\#\#}$	1.6±0.6	AA	2.3±1.0	1.6±0.5
	CT	2.2±0.9	1.6±0.6	AC	2.0±0.5	1.6±0.6	AG	2.1±0.7	1.6±0.6
	TT	2.5±0.9	1.5±0.4	CC	1.8±0.9	1.8±0.4	GG	2.0±0.8	1.8±0.6

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, *vs*. homozygote mutant; $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ *vs*. heterozygote; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, cases *vs*. controls. Data shown are the mean±SD and were evaluated using analysis of variance. BMI: Body-mass index; HDL-C: high-density lipoprotein-cholesterol; HCY: homocysteine; VB12: vitamin B12; FA: folic acid; CRP: C-reactive protein; TC: total cholesterol; TC/HDL: ratio total cholesterol and high-density lipoprotein; LDL/HDL: ratio low density lipoprotein and high-density lipoprotein.

reported to be of mixed ethnicity, and Long Island has the highest BC incidence in the United States; this has been suspected to be potentially linked to environmental pollution with polycyclic aromatic hydrocarbons (43). We may infer that these and yet other unknown environmental confounders may have contributed to this risk association.

Evidence also supports a dependence of the direction and magnitude of the BC risk modification associated with polymorphisms on the folate status and other factors. This view corresponds with observations from the Shanghai Breast Cancer study, where a significant BC risk was observed as an interactive effect between *MTHFR* 677 genotypes and low folate intake, with the highest risk occurring for *MTHFR* 677TT (OR=2.51, 95% CI=1.37-4.60, p=0.003) (26). The Breast Cancer Study from Korea, showed a similar interactive effect for low intake of green vegetables (28). Dietary deficiency in folate by itself is an established risk factor for BC (3, 4); while alcohol intake (1, 2, 44) and estrogen (45) cause folate deficiency, thus promoting the risk of developing BC.

We previously showed that Kazakhstan is a folate-deficient area (36, 37). The traditional food of the Kazakh people consists mainly of meat, such as beef and mutton and the intake of vegetables tends to be deficient, especially in rural areas, owing to their insufficient distribution. We have

screened folate concentrations in the normal Kazakh population and have found that 82% had low folate concentrations, whereas none of an age-matched Japanese population showed low concentrations (36). Indeed, in our present study, low levels of serum folate (<3.6 ng/ml) were recorded not only in BC cases but also in healthy participants (median 2.8 ng/ml and 2.9 ng/ml, in cases and controls, respectively, p=0.019, Table I). In areas such as Kazakhstan, food fortification should definitely be implemented to reduce the incidence of congenital anomalies such as neural tube defects and for prevention of BC.

We found no significant differences in genotypic distributions among cases and controls by race, except for the *MTHFR* 677CT genotype, which was significantly associated with BC among Asians (p=0.007; interaction p-value=0.39). This may reflect the genetic heterogeneity of the ethnic groups in our study. Kazakhstan is a unique country located in Central Asia, lying on the ancient Great Silk roads. Kazakh populations have been strongly influenced by the nomadic lifestyle, and a long history of migration has led to an admixture of Western and Asian populations, which has molded the genetic architecture. It is interesting that studies on the same ethnic groups might reveal opposite findings. A small study with hospital cases and non–aged matched controls from Turkey showed an increased BC risk with both *MTHFR* polymorphisms (24), while another study by Hekim *et al*. found no association between *MTHFR* C677T polymorphism and breast cancer in Turkey (46).

Although these findings seem contradictory, direct comparisons should be made with caution due to the different aspects addressed in these studies. Conflicting reports on an association of *MTHFR* C677T and *MTHFR* A1298C genotypes with BC risk may be attributed to variations in the study size and design, particularly ethnicity and non-sporadic/sporadic BC. Previous studies showing a positive association have primarily included women with a family history (21), or were associated with study design limitations. On the other hand, recent studies on sporadic BC cases showed associations between *MTHFR* polymorphisms and BC risk in Chinese (47), Moroccan (48), Syrian (49), and Iranian (50) women. Lack of an association of *MTHFR* polymorphisms with BC risk was observed in African-American, Latino, and Hawaiian women from the aforementioned Multiethnic Cohort (27) and in smaller studies from Scotland (23), Orange County, CA, USA (25), Korea (26), and Germany (30).

SNPs in intron 1 of *FTO*, such as rs1477196, have been significantly associated with obesity (33). Kaklamani *et al*., in their clinic-based case control study, found that SNPs located in intron 1 of *FTO*, specifically rs1477196 and other SNPs in one linkage disequilibrium block (*i.e*., rs9939609, rs7206790, and rs8047395), were significantly associated with BC risk, with rs1477196 showing the strongest association (34). We could not confirm this finding, as we found no differences between the patients and healthy controls with respect to *FTO* rs1477196 genotypic frequencies, regardless of the analysis model used. The numbers of alleles were identical in both groups (35.2% in patients and 36.3% in controls for the minor G allele).

Haplotype frequencies among *MTHFR* C677T, A1298C, and *FTO* rs1477196 were compared between BC cases and controls. Out of eight possible haplotypes, seven haplotypes were shared by both the cases and the controls. One haplotype (CCA) was associated with a significantly reduced risk of BC (p=0.023). The *MTHFR* 1298CC genotype possibly has a protective effect.

This study is the first to provide genotyping data of *MTHFR* and *FTO* gene polymorphisms within a breast cancer association study. One of the objectives of this study was to correlate the distribution of plasma homocysteine levels with the *MTHFR* genotypes. We found that the total homocysteine level was significantly higher in the patients compared with the controls (p=0.001) (Table I), and also significantly higher in individuals with the *MTHFR* 677CC genotype, but differences were not significant for the *MTHFR* 677TT carriers (Table III). The total homocysteine level was not associated with either *MTHFR* C677T (p=0.67), A1298C (p=0.36), or *FTO* rs1477196 (p=0.74), but were inversely correlated with the folate status of the patients (p<0.0001). After stratification of patients by folate level, the highest total homocysteine levels were found in individuals with the *MTHFR* 677TT genotype and low folate levels, although this was not statistically significant (p=0.138) (Figure 2). Despite the lack of significant association of *MTHFR* and *FTO* genotypes with total homocysteine levels in both groups, the genotypic correlation in the cancer population confirmed that the T-variant at nucleotide 677 of the *MTHFR* gene was a major determinant of plasma total homocysteine level, but only in the patients with low folate status, in agreement with a previous study performed in other subsets of patients (51).

Multivariate linear regression analysis of the cancer patients, adjusted for age and race, demonstrated that folate (β=–0.523, p<0.0005) was the only independent predictor of elevated total homocysteine level. We found no significant associations between observed *MTHFR* and *FTO* genotypes and most of phenotype variables examined in the cases and controls (Table III). One explanation for a lack of significant genotype/phenotype associations might be the relatively low number of individuals in each genotype group.

We recorded higher levels of CRP, creatinine, total cholesterol, TC/HDL and LDL/HDL ratios, and lower levels of albumin and HDL-C in cases than in controls (Table I). Lipid profiles showed significantly lower levels of HDL-C and LDL-C and higher TC/HDL and LDL/HDL ratios, despite lower levels of TC in BC patients than in the controls. Cases

had higher levels of TGS, but the difference was not statistically significant. No differences were noted in the levels of leptin and adiponectin between the cases and healthy subjects, in agreement with previous results from several recent epidemiological studies (51-56), where lipid profiles were investigated in the context of BC. Some studies have indicated possible associations between cholesterol and lipoprotein levels and BC risk, but the data on these associations remain inconclusive. A hospital-based case control study in Italy demonstrated significantly higher total cholesterol and higher LDL among cases (226.4 *vs*. 215.0 mg/dl; and 148.3 *vs*. 138.7 mg/dl , respectively) and no difference in HDL or TG levels (54.5 *vs*. 52.9 ; and 112.7 *vs*. 109.6 , respectively). In contrast, another Italian study (53) demonstrated no significant differences in total cholesterol, HDL, LDL, or triglyceride levels between BC cases and controls.

Our observation that low HDL levels may be associated with an increased risk of BC is in line with the hypothesis that high HDL levels may elicit a protective effect. HDL transports circulating cholesterol within the arteries back to the liver for excretion or re-utilization. Therefore, as the TC level increases, potentially stimulating an increase in HDL level, BC risk may possibly decrease (and *vice versa*) (54). Forsberg *et al*. hypothesized that the aromatization of androgens to estrogens within adipose tissues is the causal mechanism for an inverse association between HDL and BC (55).

We are aware that given the multiple pathophysiological changes known to be associated with cancer, statistical correlation does not necessarily indicate a biological relationship; therefore, we cannot conclusively define the pathophysiological significance of total homocysteine and many other factors in BC. Nonetheless, the present findings showing that the MTHFR polymorphisms did not significantly contribute to the total homocysteine level in BC, but only in cases with low folate levels, are consistent with the hypothesis that hyperhomocysteinemia may be a cancer-related factor that operates through different pathways (*e.g*. aberrant DNA methylation (31); inflammation, possibly involving a relevant pathway (51); or metabolic accumulation of intracellular *S*-adenosyl-L-homocysteine, a strong inhibitor of the catechol-*O*-methyltransferase-mediated methylation metabolism of endogenous and exogenous catechol estrogens (16).

This study has a number of limitations that should be noted. One limitation was the lack of detailed information on folate intake status (dietary, supplement use), intake of other folate co-factors (*i.e*. methionine, vitamins B6 and B12) for further examination of the gene–nutrient interaction. We did not evaluate the interaction of genotypes, homocysteine, and folate by hormonal receptor status of patients with BC nor with other genes involved in one-carbon metabolism, something that has previously been demonstrated (26, 30, 56).

Conclusion

Despite existing limitations, this study provides evidence for an association between the *MTHFR* C677T polymorphism and BC risk, supporting the hypothesis that *MTHFR* C677T polymorphism contributes to overall BC risk. In order to confirm our findings, well-designed studies including different ethnic groups with a careful matching between cases and controls should be considered in future association studies in Kazakhstan. Further evaluation is also necessary to determine the effect of gene–gene and gene–environment – life style interactions on *MTHFR* C677T and A1298C, and *FTO* (rs1477196) polymorphisms and BC risk. We have shown a significant association between elevated plasma homocysteine levels and increased risk for BC, suggesting the possibility that a high plasma homocysteine level could be a metabolic risk factor for breast cancer. Given the growing evidence of the important role of homocysteine metabolism in breast tumorigenesis, we need a better understanding of the modifiable determinants of total homocysteine concentration. Until recently, population studies suggested that a low folate status was the most important determinant of mild-to-moderate hyperhomocysteinemia (10). Consequently, much of the research on determinants of total homocysteine concentration has focused on folate. Despite the recent folic acid fortification of enriched grain production in the United States, which has altered the prevalence of elevated total homocysteine concentrations associated with low folate (10), evaluation of folate levels, as well as other determinants of hyperhomocysteinemia, remains important in the Kazakhstan population. We also need to continue our efforts to identify additional risk factors of hyperhomocysteinemia in patients with cancer. Food fortification with folic acid could possible help reduce the incidence of BC in Kazakhstan.

Conflicts of Interest

All Authors have no conflicts of interests to declare.

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