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Homozygosity at the C677T of the *MTHFR* Gene is Associated with Increased Breast Cancer Risk in the Turkish Population

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Abstract. *Background: Folate deficiency is implicated in cancer development. Single nucleotide polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene can modulate the effect of folate. In this case-controlled study, a possible effect of the common MTHFR C677T (ala→val) polymorphism on breast cancer susceptibility in Turkish patients was investigated. Materials and Methods: Polymorphism analysis was performed by melting curve analysis. Results: The variant allele valine (677T) was more frequent among the patients (30.1%) than in controls (23.9%). This difference was weakly significant (p=0.046; OR=1.37) and due to a significantly higher frequency of the valine homozygotes (677TT) among the patients (12.1% vs. 5.4%; p=0.013, OR=2.5). Among the patients diagnosed at more than 40 years of age, a more pronounced association of the valine homozygotes with breast cancer risk was observed (p=0.009; OR=3.3). Conclusion: Homozygosity for the low-activity C677T genotype (TT) may represent a genetic determinant increasing breast cancer risk.*

Breast cancer (BC) is the most common malignancy in women. Several risk factors for BC have been established, most of which are related to reproductive events. The incidence of BC increases with age, doubling every 10 years until the menopause, but the rate of the increase slows substantially after the menopause. Other risk factors associated with reproductive life include the age at menarche and menopause and age at first pregnancy (1). However, these are absent in the majority of women with sporadic BC. Five to 10% of breast cancer are attributed to genetic factors. The fact that age-adjusted incidence and mortality for breast cancer varies by a factor up to five between countries suggests that, on the one hand, environmental factors may be of greater importance than genetic factors or, on the other hand, that there may be ethnic factors associated with BC risk.

Folate deficiency has been implicated in the development of cancer. The mechanism by which folate might protect against cancer is possibly related to its role in DNA methylation and synthesis (2). In BC, a higher intake of folate has been shown to reduce pre- and postmenopausal BC risk (3). The MTHFR enzyme catalyses the irreversible reduction of 5, 10- methylenetetrahydrofolate (5,10-methylene THF) to 5-methyltetrahydrofolate (5-methyl THF). Folate, in the form of 5-methyl THF, provides a methyl group for methionine synthesis, which is further used for synthesis of S-adenosylmethionine, an important compound for DNA methylation reactions. DNA methylation is an epigenetic determinant in gene expression, DNA stability and mutagenesis. 5,10-methylene THF is used for the conversion of deoxyurdylate to thymidylate, needed for DNA synthesis. Consequently, folate deficiency causes an imbalance in DNA precursors leading to the misincorporation of uridylate into DNA. Polymorphisms in the *MTHFR* gene may modulate the utilization and effect of the folate compounds.

Two common polymorphisms of the *MTHFR* gene, C677T (ala→val) and A1298C (glu→ala), affect the activity of the enzyme resulting in a more labile enzyme with decreased activity (4, 5). Of these, the C677T polymorphism is more common, though its frequency varies substantially worldwide, ranging from 10% to over 60% in the normal population (6, 7). Results on the effect of this polymorphism on cancer susceptibility are not consistent. Different types of associations have been reported in solid and haematological malignancies, where both normal and variant genotypes have been found either as protective or as susceptibility factors in different cancer types (7-20).

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Table I. *Characteristics of the BC patients and healthy controls.*

	No.	Median age	Age range
All			
cases	189	50	29-79
controls	223	47	26-70
Age			
<40			
cases	37	37	29-39
controls	55	35	26-29
≥40			
cases	152	52	40-79
controls	168	49	40-70
Premenopausal			
cases	90	42	29-51
controls	134	41	26-50
Postmenopausal			
cases	99	58	48-79
controls	89	56	50-70

Table II. *Genotype frequencies and statistical values in the patients and controls.*

Genotype	Cases (%)	Controls (%)	OR (95% CI)
All			
CC	98 (51.9)	128 (57.4)	1.0
CT	68 (36)	83 (37.2)	0.9 (0.6-1.4)
CT+TT	91 (48.1)	95 (42.6)	1.3 (0.8-1.8)
TT	23 (12.1)	12 (5.4)	2.5 (1.2-5.3)
Age			
<40			
CC	21 (56.8)	30 (54.6)	1.0
CT	11 (29.7)	20 (36.4)	0.7 (0.3-2)
CT+TT	16 (43.2)	26 (45.4)	0.8 (0.4-2)
TT	5 (13.5)	5 (9)	1.4 (0.4-5.5)
≥40			
CC	76 (50)	97 (57.8)	1.0
CT	58 (38.2)	64 (38)	1.2 (0.7-1.8)
CT+TT	76 (50)	72 (42.2)	1.3 (0.9-2.1)
TT	18 (11.8)	7 (4.2)	3.3 (3-8.2)
Premenopausal			
CC	55 (61.1)	84 (59.6)	1.0
CT	23 (25.6)	50 (35.5)	0.7 (0.4-1.3)
CT+TT	35 (38.9)	57 (40.4)	0.9 (0.5-1.6)
TT	12 (13.3)	7 (4.9)	2.6 (0.9-7.1)
Postmenopausal			
CC	43 (43.4)	43 (52.4)	1.0
CT	45 (45.5)	34 (41.5)	1.3 (0.7-2.4)
CT+TT	55 (66.6)	39 (47.6)	1.4 (0.7-2.5)
TT	11 (11.1)	5 (6.1)	2.2 (0.7-6.8)

With respect to the association of the *MTHFR* C677T polymorphism with breast cancer, discordant results have been reported, depending on the study group or patient characteristics (21-27). It is probable that parameters such as ethnic origin, study design, sample size, or distribution of the cases may have affected the association of the polymorphism with BC. In the present study, the association between the *MTHFR* C677T polymorphism and breast cancer risk in Turkish breast cancer patients was investigated by melting curve analysis.

Materials and Methods

Study population. The study population consisted of patients (n=189) with histologically confirmed breast cancer, admitted to the Istanbul Medical Faculty and the Oncology Institute, Istanbul, Turkey, between 1994 and 2004. The patient group included unselected cases, consisting mostly of sporadic cancers. Ninety patients were premenopausal and 99 postmenopausal. The control group (n=223) consisted of age-matched, healthy women with a similar ethnic background and without any signs of a malign disease. The characteristics of the study groups are given in Table I. The research protocol was approved by the Istanbul Medical Faculty Ethics Committee.

Polymorphism analysis by LightCycler PCR® and melting curve analysis. Genomic DNA was isolated from blood mononuclear cells or tumor tissue by the standard Proteinase K/isopropanol method. LightCycler PCR® (Roche, Mannheim, Germany) and melting curve analyses were performed, as previously described (28). Primers and fluorescence-labelled hybridization probes designed by Nakamura *et al.* (29) were used. The primer sequences were: 5'-TGGCAGGTTACCCCAAAGG-3' (forward) and 5'-TGATGC CCATGTCGGTGC-3' (reverse) (IDT, Coralville, USA) and hybridization probe sequences were: 5'-TGAGGCTGACCT GAAGCACTTGAAGGAGAAGGTGTCT-3'-Flu and 5'-LC-640-CGGGAGCCGATTTCATCAT-3'-PHO (TIB Molbiol, Berlin, Germany). Positive controls were established by PCR-RFLP by digesting the 163-bp product obtained in the LightCycler PCR® using the HinfI restriction enzyme.

Statistical analysis. The Chi-square test was used to determine the association between the allele and genotype frequencies using the SPSS statistical package. A two-sided alpha level of 0.05 was considered statistically significant. Unconditional regression analysis was used to calculate the odds ratios, confidence intervals and risk estimates.

Results

Genotyping of the *MTHFR* gene (Figure 1) was performed by the LightCycler PCR, and the allele and genotype frequencies were evaluated (Table II). The frequency of the

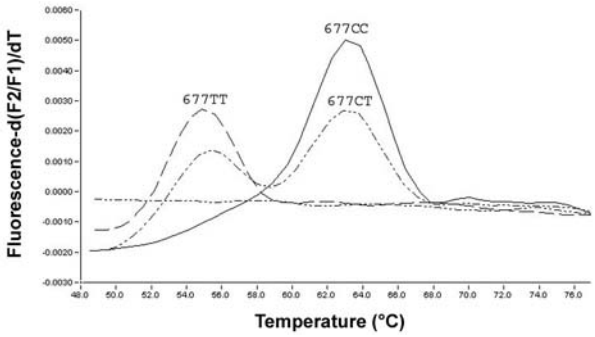

Figure 1. *Genotyping of the MTHFR C677T polymorphism in the LightCycler PCR. Following amplification, melting curves were obtained by increasing the temperature gradually from 40˚C to 85˚C with continuous measurement of the fluorescence. The melting curves were then converted to melting peaks by plotting the negative derivative of the fluorescence with respect to temperature (-dF/dT) against temperature. The homozygote alanine (677CC) and homozygote valine (677TT) genotypes displayed single peaks at approx. 63 and 55˚C, respectively. The heterozygote samples displayed both peaks. Negative control included H_2O instead of DNA and displayed no peak.*

variant valine allele in the control group was in accordance with the Hardy-Weinberg equilibrium. Comparing the allele frequencies between the BC patients and controls revealed that the valine allele (677T) was more frequent in the cases than in controls (30.1% *vs.* 23.9%). This difference resulted in a weakly significant association (p=0.046; OR=1.37). The genotype frequencies were compared between the cases and controls by using the alanine homozygote (677CC) as reference. We found that the difference in the allele frequency was due to the significantly higher frequency of the valine homozygote (677TT) among the cases (12.1% *vs.* 5.4%; p=0.013; OR=2.5; 95% CI=1.2-5.3). In contrast, the valine heterozygote (677CT) displayed a similar distribution in both groups (36% *vs.* 37.2%; p=0.7), while the difference in the frequencies of the combined genotypes (valine heterozygote + valine homozygote) was statistically not significant (48.1% *vs.* 42.6%; p=0.25).

The distribution of the *MTHFR* C677T polymorphism was compared with respect to age at diagnosis and menopausal status. The distribution of the valine homozygote individuals was similar among the patients diagnosed before or after 40 years of age (13.5% *vs.* 11.8%; p=n.s.) or among those in pre- or postmenopausal stage (13.3% *vs.* 11.1%; p=n.s.). These results show that the homozygote valine genotype is homogenously distributed among the BC patients. When the distribution of the homozygote valine genotype was compared to the controls, the difference was not significant for individuals below 40 years of age, although the frequency of the valine homozygotes was higher in the patients (13.5% *vs.* 9%). Among the patients older than 40 years (n=152), a significantly higher frequency of the homozygote valine genotype was observed when compared with the controls in the same age range (p=0.009; OR=3.3; 95% CI=3-8.2). With respect to the menopausal status, the frequency of the valine homozygotes was higher in both the premenopausal (n=90; 13.3% *vs.* 4.9%) and postmenopausal patients (n=99; 11.1% *vs.* 6.1%) than the corresponding control group. However, the differences were statistically not significant (p=0.051 and 0.16, respectively), possibly due to the small number of valine homozygote cases in the subgroups. The heterozygote or combined genotypes were not associated with cancer risk in the subgroups.

Discussion

In this study, a possible effect of the common *MTHFR* C677T polymorphism on breast cancer susceptibility in Turkish patients was investigated. The variant valine allele in the healthy population was found to be in the Hardy-Weinberg equilibrium, indicating that no evolutinary change has occured affecting the distribution of the normal and variant alleles. The frequency of the valine allele in healthy Turkish women (23.9%) appeared to be slightly lower than the reports on other populations, *e.g.* 35% in Greece (30), 39% in Korea (27), 30% Southern England (22) or 27-29% in the USA (11, 31, 32). However, the frequency of the variant allele in the whole population (unselected for sex) displays considerable differences in its distribution pattern worldwide, ranging from 10% in African Americans (6) to 63% in northern China (7). This variation may form the basis of the differences observed regarding the association of the C677T polymorphism with cancer risk in studies from different geographical areas.

Our study revealed a weak association (OR=1.37) between the variant allele of the *MTHFR* C677T polymorphism and breast cancer risk among Turkish women. This association was due to the higher frequency of the valine homozygotes among the patients (OR=2.5). These results suggest that homozygosity at the C→T (ala→val) transition in the *MTHFR* gene may affect breast cancer risk. However, the homozygote valine genotype occurred in only 12.1% of the cases, demonstrating that in the majority of the cases the BC risk is not associated with the *MTHFR* polymorphism.

The finding that BC risk is increased by the homozygote variant genotype is in line with the fact that the C→T transition impairs enzyme activity and increases its thermolability (4). Thus, homozygosity would lead to accumulation of the 5,10-methylene THF with the consequence of lack of 5-methyl THF for DNA methylation. This condition may impede DNA methylation, increasing breast cancer susceptibility. The importance of the

homozygosity at the C→T conversion for breast cancer was also demonstrated by a recent study (33) indicating that the C677T, but not the A1298C, polymorphism may modify the association between diatary folate intake and BC risk where the effect was more pronounced with the valine homozygotes. Although information on the diatary folate intake of our study participants was not available, the increased risk observed in the valine homozygotes may also be associated with low folate intake.

The finding of our study, namely a weak association between the *MTHFR* polymorphism and breast cancer risk, is in line with previous studies (21-27). In most of these reports the association between the *MTHFR* polymorphism and BC risk appears to vary depending on the study group or patient characteristics. Ergul *et al.* (24), who investigated the *MTHFR* polymorphism in premenopausal Turkish women, reported an increased risk associated with the homozygote variant genotype of the *MTHFR* gene. We also observed a higher frequency of the homozygote valine genotype among the pre- and postmenopausal patients than in the controls, but the difference was still not significant. The reason for this discrepancy may be the small number of valine homozygotes in the subgroups. A positive association in premenopausal, but not in postmenopausal, BC patients (23) or in patients less than 40 years old (22) has also been reported, while others failed to observe an association in unselected Finnish women (25) and among premenopausal BC (26). In our study, a positive association was found among patients with a diagnosis age over 40, but not under 40. A similar inconsistency exists with respect to the association with the bilaterality of the breast tumors (21, 22). The reason for the differential effect of the *MTHFR* C677T polymorphism on BC risk in different studies and populations is not clear, but may be attributable to various parameters such as sample sizes, patient characteristics, folate status or other unknown ethnic factors.

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References

1 McPherson K, Steel CM and Dixon JM: Breast cancer-epidemiology, risk factors, and genetics. BMJ *321*: 624-628, 2000.

2 Duthie SJ: Folic acid deficiency and cancer: mechanisms of DNA instability. Br Med Bull *55*: 578-592, 1999.

3 Eichholzer M, Luthy J, Moser U and Fowler B: Folate and the risk of colorectal, breast and cervix cancer: the epidemiological evidence. Swiss Med Wkly *131*: 539-549, 2001.

4 Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP *et al*: Candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nature Genet *10*: 111-113, 1995.

5 Van der Put NM, Gabreels F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, van den Heuvel LP and Blom HJ: A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet *62*: 1044-1051, 1998.

6 Keku T, Millikan R, Worley K, Winkel S, Eaton A, Biscocho L, Martin C and Sandler R: 5,10-Methylenetetrahydrofolate reductase codon 677 and 1298 polymorphisms and colon cancer in African Americans and whites. Cancer Epidemiol Biomarkers Prev *11*: 1611-1621, 2002.

7 Zhang J, Zotz RB, Li Y, Wang R, Kiel S, Schulz WA, Wen D, Chen Z, Zhang L,Wang S, Gabbert HE and Sarbia M: Methylenetetrahydrofolate reductase C677T polymorphism and predisposition towards esophageal squamous cell carcinoma in a German Caucasian and a northern Chinese population. J Cancer Res Clin Oncol *130*: 574-580, 2004.

8 Chen J, Giovannucci E, Kelsey K Rimm EB, Stampfer MJ, Colditz GA, Spiegelman D, Willett WC and Hunter DJ: A methylenetetrahydrofolate reductase polymophism and the risk of colorectal cancer. Cancer Res *56*: 4862-4864, 1996.

9 Esteller M, Garcia A, Martinez-Palones JM, Xercavins J and Reventos J: Germ line polymorphisms in cytochrome-P450 1A1 (C4887 CYP1A1) and methylenetetrahydrofolate reductase (MTHFR) genes and endometrial cancer susceptibility. Carcinogenesis *18*: 2307-2311, 1997.

10 Skibola CF, Smith MT, Kane E, Roman E, Rollinson S, Cartwright RA and Morgan G: Polymorphisms in the methylenetetrahydrofolate reductase gene are associated with susceptibility to acute leukemia in adults. Proc Natl Acad Sci USA *96*: 12810-12815, 1999.

11 Piyathilake CJ, Macaluso M, Johanning, GL, Whiteside M, Heimburger DC and Giuliano A: Methylenetetrahydrofolate reductase (MTHFR) polymorphism increases the risk of cervical intraepithelial neoplasia. Anticancer Res *20*: 1751-1757, 2000.

12 Shen H, Xu Y, Zheng Y, Qian Y, Yu R, Qin Y, Wang X, Spitz MR and Wei Q: Polymorphisms of 5,10-methylenetetrahydrofolate reductase and risk of gastric cancer in a Chinese population: a case-control study. Int J Cancer *95*: 332-336, 2001.

13 Song C, Xing D, Tan W, Wei Q and Lin D: Methylenetetrahydrofolate reductase polymorphisms increase risk of esophageal squamous cell carcinoma in a Chinese population. Cancer Res *61*: 3272-3275, 2001.

14 Wiemels JL, Smith RN, Taylor GM, Eden OB, Alexander FE and Greaves MF: Methylenetetrahydrofolate reductase (MTHFR) polymorphisms and risk of molecularly defined subtypes of childhood acute leukemia. Proc Natl Acad Sci USA *98*: 4004-4009, 2001.

15 Gonzalez Ordonez AJ, Fernandez Carreira JM, Fernandez Alvarez CR, Martin L, Sanchez Garcia J, Medina Rodriguez JM, Alvarez MV and Coto E: Normal frequencies of the C677T genotypes on the methylenetetrahydrofolate reductase (MTHFR) gene among lymphoproliferative disorders but not in multiple myeloma. Leuk Lymphoma *39*: 607-612, 2002.

16 Singal R, Ferdinand L, Das PM, Reis IM and Schlesselman JJ: Polymorphisms in the methylenetetrahydrofolate reductase gene and prostate cancer risk. Int J Oncol *25*: 1465-1471, 2004.

17 Siemianowicz K, Gminski J, Garczorz W, Slabiak N, Goss M, Machalski M and Magiera-Molendowska H: Methylenetetrahydrofolate reductase gene C677T and A1298C polymorphisms in patients with small cell and non-small cell lung cancer. Oncol Rep *10*: 1341-1344, 2003.
18 Gemmati D, Ongaro A, Scapoli GL, Della Porta M, Tognazzo S, Serino ML, Di Bona E, Rodeghiero F, Gilli G, Reverberi R, Caruso A, Pasello M, Pellati A and De Mattei M: Common gene polymorphisms in the metabolic folate and methylation pathway and the risk of acute lymphoblastic leukemia and non-Hodgkin's lymphoma in adults. Cancer Epidemiol Biomarkers Prev *13*: 787-794, 2004.
19 Matsuo K, Hamajima N, Suzuki R, Ogura M, Kagami Y, Taji H, Yasue T, Mueller NE, Nakamura S, Seto M, Morishima Y and Tajima K: Methylenetetrahydrofolate reductase gene (MTHFR) polymorphisms and reduced risk of malignant lymphoma. Am J Hematol *77*: 351-357, 2004.
20 Skibola CF, Forrest MS, Coppede F, Agana L, Hubbard A, Smith MT, Bracci PM and Holly EA: Polymorphisms and haplotypes in folate-metabolizing genes and risk of non-Hodgkin lymphoma. Blood *104*: 2155-2162, 2004.
21 Gershoni-Baruch R, Dagan E, Israeli D, Kasinetz L, Kadouri E and Friedman E: Association of the C677T polymorphism in the MTHFR gene with breast and/or ovarian cancer risk in Jewish women. Eur J Cancer *36*: 2313-2316, 2000.
22 Campbell IG, Baxter SW, Eccles DM and Choong DYH: Methylenetetrahydrofolate reductase polymorphism and susceptibility to breast cancer. Breast Cancer Res *4*: R14, 2002.
23 Semenza JC, Delfinom RJ, Ziogas A and Anton-Culver H: Breast cancer risk and methylenetetrahydrofolate reductase polymorphism. Breast Cancer Res Treat *77*: 217-223, 2003.
24 Ergul E, Sazci A, Utkan Z and Canturk NZ: Polymorphisms in the MTHFR gene are associated with breast cancer. Tumour Biol *24*: 286-290, 2003.
25 Forsti S, Angelini F, Festa S, Sanyal S, Zhang Z, Grzybowska E, Pamula J, Pekala W, Zientek H, Hemminki K and Kumar R: Single nucleotide polymorphisms in breast cancer. Oncol Rep *11*: 917-922, 2004.
26 Langsenlehner U, Krippl P, Renner W Yazdani-Biuki B, Wolf G, Wascher TC, Paulweber B, Weitzer W and Samonigg H: The common 677C>T gene polymorphism of methylenetetrahydrofolate reductase gene is not associated with breast cancer risk. Breast Cancer Res Treat *81*: 169-172, 2003.
27 Lee SA, Kang D, Nishio H, Lee MJ, Kim DH, Han W, Yoo KY, Ahn SH, Choe KJ, Hirvonen A and Noh DY: Methylenetetrahydrofolate reductase polymorphism, diet, and breast cancer in Korean women. Exp Mol Med *36*: 116-121, 2004.
28 Deligezer D, Akisik E and Dalay N: Genotyping of the MTHFR gene polymorphism, C677T in patients with leukemia by melting curve analysis. Mol Diagn *7*: 181-185, 2003.
29 Nakamura S, Aoshima T, Ikeda M, Sekido Y, Shimokata K and Niwa T: Simultaneous detection of methylenetetrahydrofolate reductase gene polymorphisms, C677T and A1298C, by melting curve analysis with LightCycler. Anal Biochem *306*: 40-43, 2002.
30 Antoniadi T, Hatzis T, Kroupis C, Economou-Petersen E and Petersen MB: Prevalence of factor V leiden, prothrombin G20210A, and MTHFR C677T mutations in a Greek population of blood donors. Am J Hematol *61*: 265-267, 1999.
31 Goodman MT, Mc Duffie K, Hernandez B, Wilkens LR, Bertram CC, Killeen J, Le Marchand L, Selhub J, Murphy S and Donlon TA: Association of methylenetetrahydrofolate reductase polymorphism C677T and dietary folate with the risk of cervical dysplasia. Cancer Epidemiol Biomarkers Prev *10*: 1275-1280, 2001.
32 Gerhard DS, Nguyen LT, Zhang ZY, Borecki IB, Coleman BI and Rader JS: A relationship between methylenetetrahydrofolate reductase variants and the development of invasive cervical cancer. Gynecol Oncol *90*: 560-565, 2003.
33 Shrubsole MJ, Gao YT, Cai Q, Shu XO, Dai Q, Hebert JR, Jin F and Zheng W: MTHFR polymorphisms, dietary folate intake, and breast cancer risk: results from the Shanghai Breast Cancer Study. Cancer Epidemiol Biomarkers Prev *13*: 190-196, 2004.

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