Association Between Multiple Single Nucleotide Polymorphisms in Folate Metabolism Pathway and Breast Cancer Risk in Georgian Women: A Case-Control Study

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ABSTRACT

BACKGROUND: The folate metabolism pathway plays an integral part in DNA synthesis, methylation, and repair. Methylenetetrahydrofolate reductase (MTHFR) and methylenetetrahydrofolate dehydrogenase (MTHFD1) are both enzymes that are involved in this pathway, and the single nucleotide polymorphisms (SNPs) in genes coding for them have modulatory effects on DNA expression. This study aimed to investigate the relationship between MTHFR C677T (rs1801133) and MTHFD1 G1958A (rs2236225) polymorphisms and the risk of developing breast cancer in Georgian women.

METHODS: A case-control study was performed examining the MTHFR C677T and MTHFD1 G1958A SNP in breast cancer–confirmed cases and healthy matched controls. Real time-polymerase chain reaction (PCR) was used to genotype SNPs. The case individuals' pathology reports were obtained following surgeries for cancer characteristic data. Statistical analysis was performed to investigate the significance of the acquired data.

RESULTS: Statistical analysis of MTHFR C677T SNP revealed that the CT genotype increased the risk of breast cancer by 2.17 folds in the over-dominant model. Statistical analysis of MTHFD1 G1958A SNP showed that the GA genotype increased the risk of breast cancer by 4.12 folds in the codominant model and 2.41 folds in the over-dominant model. No statistically significant link was found between genotypes and lymph node status, however, patients with the CT genotype had higher percentages of proliferative activity.

CONCLUSIONS: Breast cancer seems to have a statistically significant association with the CT genotype in MTHFR C677T and the GA genotype in MTHFD1 G1958A in Georgian women.

KEYWORDS: MTHFR, MTHFD1, single nucleotide polymorphism, breast cancer, RT-PCR, proliferative activity

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Introduction

Cancer is a major public health concern internationally. Breast cancer is the number one diagnosed malignancy globally. In the year 2020, there were 2.3 million new cases worldwide and 44 new cases per 100 000 women in Georgia in the year 2012.[1,2]

The clinical and pathological heterogeneity of breast cancer has been well studied; however, the wide variety of genes that can lead to this wide range of phenotypes has not been well reported. BRCA1 and BRCA2 which are highly associated with breast cancer only account for a small proportion of breast cancer cases. Importantly majority of breast cancer cases are classified as sporadic, but there is a likelihood that low penetrant genetic polymorphisms involved in DNA methylation and repair are involved in the development of cancers.[3] Moreover, the identification of genetic markers that have a role in breast cancer development is essential to guiding the development of new therapeutic approaches.[4]

DNA methylation, an epigenetic process, refers to the covalent binding of a methyl group at 5' cytosine on 5'-CpG-3' regions.[5] A cluster of these regions is called CpG island. Increased methylation of these regions suppresses transcription by 2 main mechanisms: first, these islands can recruit inhibitory proteins and prevent the interaction of transcription factors and DNA, and second, methyl-CpG binding proteins (MBPs) suppress the transcription of DNA when recognizing these methylated regions.[6]

The folate metabolism pathway is essential to DNA methylation and it involves multiple crucial enzymes. One of these key enzymes is methylenetetrahydrofolate reductase (MTHFR) which irreversibly reduces N5, N10-methylene tetrahydrofolate (THF) to 5-methyl THF. 5-methyl THF serves as a methyl group donor in the synthesis of methionine from homocysteine. Consequently, methionine is adenylated to produce S-adenosylmethionine (SAM), a universal methyl donor required in DNA methylation.[5,7-9]

Another vital part of 1-carbon metabolism is methylenetetrahydrofolate dehydrogenase (MTHFD1) which encodes a protein with 3 distinct enzymatic activities: 10-formyltetra hydrofolate synthetase, 5,10-methenyltetrahydrofolate cyclohydrolase, and 5,10-methylenetetrahydrofolate dehydrogenase.[10] These enzymes catalyze 3 sequential reactions in the 1-carbon metabolism pathways and are active in the reversible interconversion of tetrahydrofolate into 5,10-methenyl-THF, 10-formyl-THF, and 5,10-methylene-THF.[11]

The function of this pathway is not only limited to DNA methylation but also to purine and pyrimidine synthesis. Hence, to better understand the effects of these enzymes, it is important to study their genetic variations. Single nucleotide polymorphism (SNP) is an extremely common genome alteration mechanism, in which an individual nucleic acid is substituted by a different one.[12] Single point mutations can be genetic markers for the characteristics of different neoplasms.[13] With the progress of genome-wide association studies (GWASs), there is increasing evidence that cancer is associated with SNPs. A well-studied example is the SNPs of ERCC5 (ERCC excision repair 5 or Xeroderma pigmentosum).[14] A common SNP in the MTHFR gene is MTHFR C677T which is studied in our research and leads to decreased enzymatic activity of MTHFR, and as a result of its involvement in the folate metabolism pathways, decreased DNA methylation.[5] Cytosine substitution with thymine at position 677 causes the substitution of alanine to valine in the enzyme molecule, which leads to decreasing its activity.[15] MTHFD1 G1958A is another SNP studied in this research, which causes a Guanosine substitution with adenine resulting in a change of amino acid arginine to glycine.[11] This substitution has a borderline significant effect on serum folate levels and alters the thermostability of the enzyme. It also leads to a 36% reduction in the enzyme's half-life, hence decreasing its stability.[16]

Here, we aim to investigate the association between MTHFR C677T (rs1801133) and MTHFD1 G1958A (rs2236225) polymorphisms and the risk of developing breast cancer in the Georgian women population. We further investigated the association between the pathological characteristics of patients, like their lymph node status and cancer proliferative activity, with the investigated SNPs.

Materials and Methods

The present case-control study was approved by the Ethics Committee of Tbilisi State Medical University, Georgia. The breast cancer cases were recruited from 2 different oncology hospitals (The First University Clinic and Cancer Research Center) in Tbilisi, who were consecutively admitted to the surgery department. The control group consisted of healthy, age-matched women who were regularly screened for breast cancer in an outpatient clinic in Tbilisi. Both parties were asked to sign an informed consent form, and their clinical information was obtained from their medical charts.

The inclusion criteria for all participants were as follows:

1. The age range of 30 to 80 years.
2. Ability to understand the purpose of the study and provide informed consent.
3. Female sex.
4. Being ethnically Georgian.

The cases were required to have a biopsy confirmed diagnosis of breast carcinoma, with no previous history of breast surgery, preoperative chemotherapy, or radiation therapy. Although minor illnesses (eg, common cold, headaches) were acceptable, having a family history or previously diagnosed cancer was an absolute exclusion criterion for the controls.

As a result of incomplete information, incorrect material, or a non-matching diagnosis, 11 study participants were ultimately excluded. In total, we evaluated MTHFR C677T in 62 confirmed breast cancer cases along with 63 matched, healthy controls and evaluated MTHFD1 G1958A SNP in 59 breast cancer patients and 61 matched, healthy women.

Sample collection and storage

Blood samples were collected in a vacutainer tube containing ethylenediaminetetraacetic acid (EDTA) (lavender top). Genomic DNA was extracted from the whole blood using DNA purification kits (Qiagen, USA). DNA concentration was measured using the fluorometer-based method (Qubit, Thermo Scientific, USA).

Genotyping

TaqMan Assay (Thermo Scientific) was used to perform SNP genotyping. Each TaqMan SNP Genotyping Assay included sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest, as well as 2 TaqMan minor groove binder (MGB) probes with non-fluorescent quenchers (NFQs): 1 VIC-labeled probe to detect Allele 1 sequence and 1 FAM-labeled probe to detect Allele 2. Real-time polymerase chain reaction (PCR) was carried out following standard protocols. DNA polymerase, forward and reverse primers with a final concentration of 200 nM, probes with a final concentration of 250 nM, and 50 ng of genomic DNA were all included in the final volume of the PCR reaction, which was 25 µL. Polymerase activation at 95°C for 10 minutes (hold), denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute were the PCR conditions for amplification (cycle 40). The results of the allelic discrimination (AD) data were plotted by the real-time PCR instrument software as a plot of Allele1(2′-chloro-7′phenyl-1,4-dichloro-6-carboxyfluorescein (VIC) dye) vs Allele 2 (Fluorescein amidites (FAM) dye). Each sample is represented as an individual point on the AD plot. A typical AD plot includes homozygote clusters, heterozygote clusters, and no-template controls. The points in each grouping are closely clustered, and each cluster is isolated from the others.

Table 1. Analysis of MTHFR C677T polymorphism with breast cancer.

MODEL	GENOTYPE	CASES (%)	CONTROLS (%)	OR (95% CI)	*P*	χ^2	*P*[a]
Codominant	CC	29 (46.77)	35 (55.55)	1.00 Ref			
	CT	30 (48.38)	19 (30.15)	1.90 (0.89-4.06)	.0948		
	TT	3 (4.83)	9 (14.28)	0.40 (0.09-1.62)	.2012	6.024	**.0492**
	C allele	88 (70.96)	89 (70.63)	1.00 Ref			>.9999
	T allele	36 (29.03)	37 (29.36)	0.98 (0.57-1.69)	.9539		
Dominant	CC	29 (46.77)	35 (55.55)	1.00 Ref			
	CT + TT	33 (53.22)	28 (44.44)	1.42 (0.70-2.93)	.3267		.373
Recessive	CC + CT	59 (95.16)	54 (85.71)	1.00 Ref			
	TT	3 (4.83)	9 (14.28)	0.30 (0.07-1.18)	.0866		.1264
Over-dominant	CC + TT	32 (51.61)	44 (69.84)	1.00 Ref			
	CT	30 (48.38)	19 (30.15)	2.17 (1.02-4.50)	**.0382**		.0712

Abbreviations: CI, confidence interval; OR, odds ratio.
[a]*P* is obtained from the chi-square and the Fisher exact test.
A *P* value of less than 0.05 was considered statistically significant (shown in bold).

Statistics

The study and control groups were analyzed separately. Statistical significance for differences in genotype frequencies was determined by the chi-square and the Fisher exact test, and the level of significance was put at $P < .05$ and was performed by GraphPad Prism 9.3.1 for macOS (San Diego, California, USA). To evaluate associations between the SNPs and the risk of cancer progression odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by MedCalc Software Ltd. Odds ratio calculator (Version 20.123). All statistical tests are planned to be 2-sided.

Results

From the recruited breast cancer patients (n = 64) and controls (n = 64), 62 cases and 63 controls were successfully genotyped for the MTHFR C677T SNP, and 59 cases and 61 controls were successfully genotyped for the MTHFD1 G1958A SNP. In the analysis of MTHFR C677T SNP, we observed that the CT genotype increased the risk of breast cancer 2.17 folds only in the over-dominant model (CT-OR = 2.17; 95% CI = 1.02-4.50; *P* = .0382). For the MTHFR C677T, the frequency of C was 70.96% in cases and 70.63% in controls which is not significantly different at all ($P > .9999$). The genotype distribution of MTHFR C677T and breast cancer risk are presented in Table 1.

In the analysis of MTHFD1 G1958A SNP, we observed that the GA genotype increased the risk of breast cancer 4.12 folds in the codominant model taking MTHFD1 1958 GG as the reference (GA-OR = 4.12; 95% CI = 1.54-11.00; *P* = .0046), 2.41 folds in the over-dominant model (GA-OR = 2.41; 95% CI = 1.15-5.05; *P* = .0189), and 3.34 folds in the dominant model (GA + AA-OR = 3.34; 95% CI = 1.34-8.34; *P* = .0095). The allele frequency in MTHFD1 1958 SNP was not significantly different between cases and controls, with 41.52% and 51.63% for the G allele in cases and controls, respectively. The genotype distribution of MTHD1 G1958A and breast cancer risk are presented in Table 2.

In this study, we also studied the relationship between the genotype of patients in the 2 SNPs with 2 of their cancer characteristics, proliferative activity and lymph node positivity. We observed higher proliferative activity among patients with the CT genotype compared with the CC genotype in the MTHFR C677T SNP (CT-OR = 4.53; 95% CI = 1.23-16.58; *P* = .0224) (Table 3).

Discussion

Folate is an essential part of 1-carbon transfer and is a major player in DNA synthesis, methylation, and repair.[17] Low folate intake and biomarkers for low folate states may be associated with cancers like colorectal cancer, esophageal cancer, and breast cancer.[18-21]

Methylenetetrahydrofolate reductase and MTHFD1 play an important role in folate metabolism, and certain SNPs can affect their activity. For example, mutation at nucleotide 677 of the MTHFR gene can result in a diminished enzymatic activity that can decrease genomic DNA methylation which is even more prominent when the folate intake is low.[17,22] And the mutation at nucleotide 1958 of the MTHFD1 gene leads to changes at the metabolic level like decreased serum folate levels and decreased thermostability and half-life of the enzyme.[16] This study investigated the association between breast cancer and MTHFR C677T and MTHFD1 G1958A. And we found that there is a statistically significant association between breast

Table 2. Analysis of MTFD1 G1958A polymorphism with breast cancer.

MODEL	GENOTYPE	CASES (%)	CONTROLS (%)	OR (95% CI)	*P*	χ^2	*P*
Codominant	GG	8 (13.55)	21 (34.42)	1.00 Ref			
	GA	33(55.93)	21 (34.42)	4.125 (1.54-11.00)	**.0046**		
	AA	18 (30.50)	19 (31.14)	2.48 (0.88-7.02)	.0856	8.490	**.0143**
	G allele	49 (41.52)	63 (51.63)	1.00 Ref			.1226
	A allele	69 (58.47)	59 (48.36)	1.50 (0.90-2.50)	.1170		
Dominant	GG	8 (13.55)	21 (34.42)	1.00 Ref			
	GA + AA	51 (86.44)	40(65.57)	3.34 (1.34-8.34)	**.0095**		**.0101**
Recessive	GG + GA	41 (69.49)	42 (68.85)	1.00 Ref			
	AA	18 (30.50)	19 (31.14)	0.97 (0.44-2.10)	.9396		>.9999
Over-dominant	GG + AA	26 (44.06)	40 (65.57)	1.00 Ref			
	GA	33 (55.93)	21 (34.42)	2.41 (1.15-5.05)	**.0189**		**.0272**

Abbreviations: CI, confidence interval; OR, odds ratio.
P is obtained from the chi-square and the Fisher exact test.
A *P* value of less than 0.05 was considered statistically significant (shown in bold).

cancer and the CT genotype in MTHFR C677T and the GA genotype in MTHFD1 G1958A.

Babyshkina et al conducted a stratified analysis of MTHFR C677T polymorphism and breast cancer in premenopausal and postmenopausal cohorts. They found a significant association between the 677CT genotype and tumor size and ER positivity in the postmenopausal sample. Moreover, homozygous patients with the 677TT genotype had better progression-free survival. They also reported a poorer prognosis in MTHFD1 1958AA patients when compared with wild-type homozygotes GG.[23] A meta-analysis involving 57 case-control studies performed by Li et al[24] found a significant association between MTHFR 677C > T and breast cancer risk, especially in Asian populations. A study done in the Kazakh population showed an increased risk of breast cancer in patients with the MTHFR 677CT genotype in the codominant model, which is consistent with our results.[25] Another study done on women living in Long Island, New York, showed an increased breast cancer risk in women with the 677TT genotype. And the risk was even stronger in women with low dietary folate consumption.[26] In contrast, a study done on the Brazilian population by Batschauer et al showed no significant difference in the frequency of MTHFR C677T in women with breast cancer and controls. However, the study showed an association between MTHFR C677T and lymph node involvement.[27] Chou et al[28] observed a conflicting decreased risk of breast cancer in women with 677 CT and TT genotypes in Taiwan.

Regarding MTHFD1, a study by Krajinovic et al[29] showed that patients with acute lymphoblastic leukemia (ALL) who were treated with methotrexate had a lower probability of event-free survival (EFS) if they had MTHFR 677T or MTHFD1 1958A allele. The G > A substitution at position 1958 leads to an arginine to glutamate substitution that may have functional consequences.[16] Methylenetetrahydrofolate dehydrogenase G1958A SNP has mostly been studied in neural tube defects (NTDs), showing an increasing influence on a mother's risk of having a child with NTD.[30,31] Wang et al[32] observed an increased risk of gastric cancer in the 1958AA genotype compared with 1958GA and GG. In contrast, Moruzzi et al observed that the MTHFD1 1958AA genotype is associated with a reduction in cancer risk, especially in colon cancer. Moreover, higher DNA methylation levels in Peripheral blood mononuclear cells (PBMCs) were observed in the presence of the A allele, suggesting a possible protective effect of this allele against DNA hypomethylation.[9] In our study, we found that GA increased the risk of breast cancer in codominant and over-dominant models.

This study has limitations in size, lack of full data on phenotypic status like metastasis, and lack of data on potential confounders like diet and smoking status. However, GWASs like this are one of the few tools that can determine the casualty of disease. Many GWAS studies lead to downstream analyses that have been helpful in the discovery of novel biological mechanisms and can have diverse clinical applications, establishing the importance of conducting primary studies that can show association and lead to bigger studies and advances. In addition, many risk loci show considerable differences in frequency and/or effect size in different ethnic groups, highlighting the importance of conducting GWAS in different ethnicities.[33,34]

Conclusions

Breast cancer seems to have a statistically significant association with the CT genotype in MTHFR C677T and the GA

Table 3. MTHFR 677 and MTHFD1 1958 gene polymorphisms and breast cancer progression.

	TOTAL PATIENTS (N=64)		OR (95% CI)	*P*			OR (95% CI)	*P*
	TUMOR SIZE				CANCER PROLIFERATIVE ACTIVITY			
	N+ (N=30) NUMBER (%)	N– (N=32) NUMBER (%)			PROLIFERATIVE ACTIVITY > 30 NUMBER (%)	PROLIFERATIVE ACTIVITY < 30 NUMBER (%)		
MTHFR 677 gene polymorphism and breast cancer progression								
CC	12 (40)	17 (53.1)	1.00 Ref		4 (18.1)	16 (48.4)	1.00 Ref	
CT	16 (53.3)	14 (43.7)	1.61 (0.57-453)	.3591	17 (77.2)	15 (45.4)	4.53 (1.23-16.58)	**.0224**
TT	2 (6.6)	1 (3.1)	2.83 (0.22-34.92)	.4164	1 (4.5)	2 (6.0)	2.00 (0.14-27.99)	.6067
C allele	40 (66.6)	48 (75)	1.00 Ref		25 (56.8)	47 (71.2)	1.00 Ref	
T allele	20 (33.3)	16 (25)	1.50 (0.68-3.27)	.3082	19 (43.1)	19 (28.7)	1.88 (0.84-4.18)	.1219
MTHFD1 1958 gene polymorphism and breast cancer progression								
GG	2 (7.4)	6 (18.7)	1.00 Ref		2 (14.2)	3 (7.8)	1.00 Ref	
GA	18 (66.6)	15 (46.8)	3.60 (0.6-20.52)	1.442	7 (50)	26 (68.4)	0.40 (0.05-2.90)	.3680
AA	7 (25.9)	11 (34.3)	1.90 (0.29-12.26)	.681	5 (35.7)	9 (23.6)	0.83 (0.10-6.78)	.8647
G allele	32 (50)	27 (42.1)	1.00 Ref		13 (43.3)	32 (42.1)	1.00 Ref	
A allele	32 (50)	37 (57.8)	0.72 (0.36-1.46)	.3758	17 (56.6)	44 (57.8)	0.95 (0.40-2.23)	0.9083

Abbreviations: CI, confidence interval; OR, odds ratio.

genotype in MTHFD1 G1958A in Georgian women in certain inheritance patterns. The difference in results of different studies can be due to differences in ethnicities and selection bias as some studies recruited controls from hospital patients and some from healthy communities. Therefore, much larger studies, including different ethnicities, that can combine gene expression (like global DNA methylation) and genotype are required to illuminate the full role that these SNPs can play in cancer development and progression. This study has established a primary association, but the full understanding of these SNPs role in cancer is important in developing new therapeutic approaches along with markers that can help in diagnosis and prognosis.

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Author Contributions
S.A. contributed to conceptualization, data curation, formal analysis, investigation, methodology, writing original draft, and editing. SS contributed to formal analysis, investigation, methodology, writing original draft, and editing. DK contributed to formal analysis, investigation, methodology, writing original draft, and editing. AG contributed to formal analysis, investigation, methodology, visualization, writing original draft, and editing. EK contributed to formal analysis, investigation, methodology, supervision, writing original draft, and editing. EA contributed to formal analysis, investigation, methodology, supervision, writing original draft, and editing. KK contributed to formal analysis, investigation, methodology, funding acquisition, supervision, writing original draft, and editing. All authors approved the manuscript.

Availability of Data and Materials
In case you want to access our PCR raw data, you can contact the fifth author by email at: e.kvaratskhelia@tsmu.edu.

Consent to participate
Signed informed consent was collected from every study participant.

Consent for publication
Not applicable.

Ethical approval
The study is approved by the Ethics Committee of Tbilisi State Medical University, Georgia under approval number #44/3 with the following members: (1) Givi Javashvili, Chair of the Ethics Committee, (2) Guram Kiknadze, (3) Tamar Kurtanidze, (4) Davit Gelovani, (5) Irma Manjavidze, (6) Ramaz Shengelia, (7) Temur Silagadze, (8) Zaza Chafichadze, (9) Zaza Berishvili, (10) Magda Bethaneli, (11) Akaki Barkalaya, and (12) Gocha Shatirishvili.

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