RESEARCH COMMUNICATION

# Mutational Analysis of the MTHFR Gene in Breast Cancer Patients of Pakistani Population

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## Abstract

**Objectives: Since methylenetetrahydrofolate reductase (MTHFR) maintains the balance of circulating folate and methionine and blocks the formation of homocysteine, its regulation in relation to different cancers has extensively been studied in different populations. However, information on Pakistani breast cancer patients is lacking. The MTHFR gene has two most common mutations that are single nucleotide additions which result in change of amino acids C677T to Ala222val and A1298C to Glu429Ala. Methodology: 110 sporadic breast patients with no prior family history of cancer or any other type of genetic disorders along with 110 normal individuals were screened for mutations in exons 1 to exon 9 using single strand conformational polymorphism, RFLP and sequencing analyzer. Results: The p values for the 677CC, 677CT, and 677TT genotypes were 0.223, 0.006, and 0.077, respectively. Those for the 1298AA, 1298AC, and 1298CC genotypes were 0.555, 0.009, and 0.003, respectively. Conclusions: We found an overall a significant, weak inverse association between breast cancer risk and the 677TT genotype and an inverse association with the 1298C variant. These results for MTHFR polymorphism might be population specific in sporadic breast cancer affected patients but many other factors need to be excluded before making final conclusions including folate intake, population and disease heterogeneity.**

**Keywords**: MTHFR - SSCP - breast cancer susceptibility gene - Pakistani breast cancer

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## Introduction

Breast cancer begins in the any part of breast, caused by abnormal cells growth and division. MTHFR association with cancer anomalies also gets a very recent attention. MTHFR gene provides instruction for making an enzyme known as methylenetetrahydrofolate reductase. Methylenetetrahydrofolate reductase (MTHFR) is playing a central role in folate and homocysteine metabolism that catalyzes the conversion of 5-10 methylenetetrahydrofolate to 5-methylenetetrahydrofolate which is circulatory folate which later utilized into homocystein remethylation by methionine (Rosenblatt, 1995). MTHFR gene is located on short arm (p) of the chromosome 1 (1p36.3) and has been identified through fluorescence in situ hybridization (FSH) (Gaughan et al., 2000). MTHFR gene has two promoters and isoform (70 kDa and 77 kDa) (Tran et al., 2002). Human MTHFR gene is composed of eleven (11) exons that encode a protein of 656 amino acids. Analysis of promoter regions of MTHFR gene revealed that it does not have a TATA box that contain CpG islands, which have multiple binding sites for different transcription factors (Rozen,1997). MTHFR enzyme activity is inhibited with the binding of dihydrofolate (DHF) (Matthews & Daubner, 1982) and S-adenosylmethionine (SAM) (Jencks and Mathews, 1987).

MTHFR gene is involved in diseases was firstly published by Mudd (1972) who identified a patient with homocystinuria due to severe deficiency of MTHFR enzyme. Deficiency of MTHFR is also most common inborn error of folate metabolism. In 1988, a MTHFR thermolabile variant was identified through enzymatic assays of lymphocyte extract in cardiovascular disease patients (Kang et al., 1988). Different epidemiological studies showed that folate low level in the development of the cancer in several organs as cervix, colorectal, lungs, brain, pancreas and breast (Kim, 1999).

The variation in MTHFR gene C677T and A1298C have been associated with reduced activity of MTHFR enzyme that increased the availability of folate for thymidilate and purine synthesis. These variants have relation with invasive breast cancer, which was investigated by multiethnic cohort study of man and postmenopausal women of Japan (Goyette et al., 1998). The role of MTHFR C677T gene polymorphism in breast cancer risk is very controversial. Allele T seems to be involved in cancer risk (Henríquez et al., 2010). While the study of treated patients with adjuant therapy show the C/C variant of MTHFR C677T polymorphism suffer a four times higher risk of multifocal lesions in the tumor (Henríquez et al., 2010).

About 9 to 12% of the general population in Taiwan

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population is TT homozygous effected lacking the enzyme responsible for metabolizing folic acid. 44% are CT affected which cannot fully metabolized folic acid to its active form (Molloy, 1997). MTHFR gene has two most common mutations identified in MTHFR that are single nucleotide addition which result in change of amino acids C677T ala222val and A1298C to Glu429Ala (Goyette et al., 1998; Kim, 2000).

In this study, MTHFR germline and sporadic mutations of breast cancer patients are investigated. So far no single study in relation to MTHFR germline mutations in breast cancer in Pakistan population has been documented yet. We are primarily interested to explore local ethnic sporadically affected breast cancer patients for any polymorphism/mutation. As germline involvements may further impart as an associated risk for this disease.

## Materials and Methods

110 samples of breast cancer patients along with 110 normal individuals without any occurred disease (taken as controls) had been identified. For this purpose, patients with no earlier family history of any type of cancer were involved in this study. Postmenopausal stage and unilateral or multifocal breast cancer affected patients were included. Majority of patients involved in this study were identified from Combined Military Hospital (CMH, Rawalpindi), Nuclear and Oncology Radiation Therapy Institute (NORI, Islamabad) and Allied Hospital (Faisalabad) in Pakistan. Informed consents of these patients with a prior approval of ethical committees of both hospital officials and oncologists were taken prior to sampling. Blood samples (5ml) were drawn from these diagnosed patients and controls. Samples were transported to laboratory for further processing under proper storage conditions.

*DNA Isolation and Estimation*

DNA extraction was carried out from blood samples by using organic method as mentioned in the previous protocol with minor modifications (Helms, 1990). Electrophoresis of these DNA samples was carried out on 1% agarose gel for 45min at 120V. Gel was later immersed in 0.5$\mu$g/ml ethedium bromide solution for DNA staining and quantification.

*Primer Designing and Polymerase chain reaction (PCR)*

MTHFR gene is amplified by different markers that may design by using Primer 3 software. Primers designed in such a way that intron and exon junctions were also screened in this study. The coding exons and exon-intron regions of MTHFR gene were amplified by using polymerase chain reaction (PCR) 20 $\mu$l volume, consist of 0.3 mM deoxyribonucleotide (dNTPS), 1X PCR buffer (10 mM), 2 mM Mgcl2, 0.5 $\mu$M each primers (forward and reverse), 1.5 U Taq Polymerase and 50ng of the genomic DNA. The thermal cycling consisting of an initial denaturation at 95˚C for 4 min, followed by 40 cycles of amplification consisting of denaturation at 95˚C for 45 sec, primer annealing at 55˚C for 1 min and primer extension at 72˚C for 1 min. A final extension step was performed at 72˚C for 10 min. List of primers and their annealing temperature mention in Table 1. Amplified PCR product was electrophoretically separated by using 2% agarose gel with ethidium bromide stained. Bands of PCR were visualized by UV transilluminator and gel doc.

*Mutational Screening of the Amplified products*

Single stranded conformational polymorphism (SSCP) was done following the previously mentioned protocol (Orita et al., 1989) and Restriction fragment length polymorphism (RFLP). Amplified products were screened using SSCP for any mobility shift and alterations and these variants conformed by RFLP technique. Amplified product of desired samples was purified using DNA extraction kit from Fermentas Life Sciences (Burlington, Ontario).

## Results

The genotype frequencies of breast cancer patients and controls are summarized in Table 2. Polymerase chain reaction product of exons 4 and 7 of MTHFR are shown in Figures 1-3. Single strand confirmation polymorphism of amplified product was performed along with their normal samples. The variation of band mobility in patients was identified and genotyped by RFLP and confirmed by sequencing.

Allelic and genotype frequencies for MTHFR C677T polymorphism difference between cancer cases and controls (p values were 0.2230 and 0.0770, respectively) and A1298C polymorphism frequencies in breast cancer cases and controls (p value 0.5545 and 0.0029 respectively). We were finding the evidence for existence of gene dose effect. However in C677T the C/C was slightly overrepresented in patients (p value 0.2230). While in case of A1298C the C/C was slightly overrepresented in patients (p value 0.2230) Furthermore, the heterozygous C/T genotype was 0.0060, pointing to its

**Table 1. Primer Sequences for PCR Amplification of MTHFR Exons**

| Exons | | PRIMER Sequence (5'-to-3') | Product size | Tm (°C) |
|---|---|---|---|---|
| Exon 1 | F | TCTGGGACTGAGACCAGGAG | 439bp | 58 |
| | R | TTACACTAATCCCGCGAAGG | | 58 |
| Exon 2 | F | GGCTCCTGCCTTTTAAACCT | 210bp | 58 |
| | R | GTGCTGCCACTCTCTCAAAA | | 58 |
| Exon 3/1 | F | TCACCACACACCCATTCACT | 838bp | 58 |
| | R | AACTTGATGCTGGCCATGTT | | 58 |
| Exon 3/2 | F | GGCTCAGGCAATCCTTCTG | 500bp | 60 |
| | R | CTCCTCAGCTATGGCTGTCC | | 60 |
| Exon 4 | F | TAGCTGTCACCGAGGAGCAT | 700bp | 58 |
| | R | CTCAGGGCTGACAGTTTGCT | | 58 |
| Exon 5 | F | TGAACGCTGATTGGTCTGTC | 465bp | 58 |
| | R | GGCCCAGGAAGAAGTAAAGG | | 58 |
| Exon 6 | F | GGGGCTAGGTAGAGGGAACA | 288bp | 58 |
| | R | CCCTGATCACTGTGTCCTGA | | 58 |
| Exon 7 | F | TCCCTGTGGTCTCTTCATCC | 379bp | 55 |
| | R | CTGGGAAGAACTCAGCGAAC | | 55 |
| Exon 8 | F | ATGATGGAAGGGGGAGAAGT | 470bp | 62 |
| | R | GATGCCCTCTGAGACTGGAG | | 62 |
| Exon 9 | F | TCAGGGGCAGAATTTACAGG | 499 | 55 |
| | R | ACAGGATGGGGAAGTCACAG | | 55 |

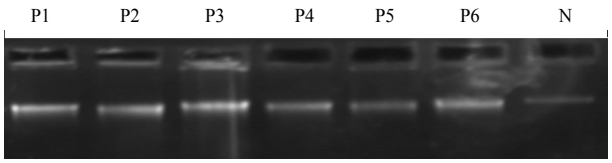


**Figure 1. Electropherogram of the Ethidium Bromide Stained 1% Agarose Gel Showing Extracted DNA of Sample.** The P1 to P6 refer to patients and N refers to standard DNA

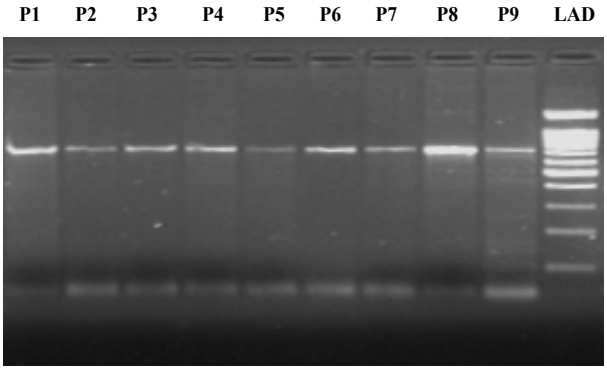


**Figure 2. Electropherogram of the Ethidium Bromide Stained 2% Agarose Gel Showing Amplified PCR Product of Exon 4 Diseased Samples.** The P 1-P9 refers to the Patient involved in present study.

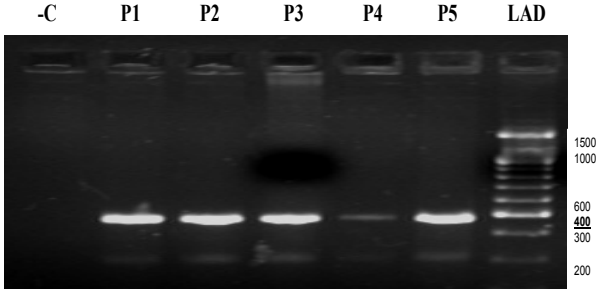


**Figure 3. Electropherogram of the Ethidium Bromide Stained 2% Agarose Gel Showing Amplified PCR Product of Exon 7 Diseased Samples.** The P 1-P5 refers to the patients involved in present study

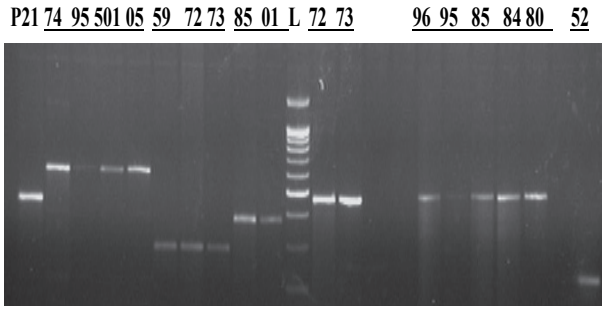


**Figure 4. Electropherogram of the Ethidium Bromide Stained Purified PCR Product on 2 % Agarose Gel Showing Banding Pattern of Product Size Obtained with the Exons 1 2 3 4 5 6 7 8 of MTHFR.** The Numbers refers to the mutant samples involved in present study.

possible protective role and A/C heterozygous genotype was 0.0092.

*Sequencing results*

Seventeen variants were found during SSCP analyses, which are shown in Figure 5 of different exons of MTHFR genes. The sequence results of suspected samples that were identified by SSCP are shown in Figure 6. In breast cancer patients association of MTHFR polymorphism was confirmed by using sequencing analyzer (CEQ, 8800).

**Table 2. Allelic and Genotype Differences for MTHFR C677T and A1298C Polymorphisms for Cancer Cases vs. Controls**

| Genotype | Cases (N %) (N=110) | | Controls (N %) (N=110) | | P Value |
|---|---|---|---|---|---|
| MTHFR C677T | | | | | |
| C/C | 65 | (59.0) | 55 | (50.0) | 0.2230 |
| C/T | 25 | (22.7) | 45 | (40.9) | 0.0060 |
| T/T | 20 | (18.1) | 10 | (9.0) | 0.0770 |
| C allele | 155 | (140.9) | 155 | (140.9) | |
| T allele | 65 | (59.0) | 65 | (59.0) | |
| MHFR A1298C | | | | | |
| A/A | 35 | (31.8) | 30 | (27.3) | 0.5545 |
| A/C | 55 | (50.0) | 75 | (68.2) | 0.0092 |
| C/C | 20 | (20.0) | 5 | (4.5) | 0.0029 |
| A allele | 125 | (113.6) | 135 | (122.7) | |
| C allele | 95 | (86.3) | 85 | (77.3) | |

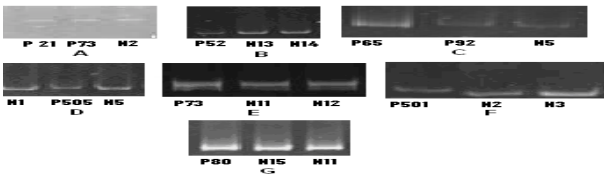


**Figure 5. Variant on SSCP Banding Pattern Obtained with Exons 1 to 9 (A= Exon 1, B = Exon2, C = Exon 3/1, D = Exon4, E = Exon5, F = Exon7 and G= Exon8) of MTHFR.** P refers to the variants occur during screening of gene in diseased samples and N to normal samples

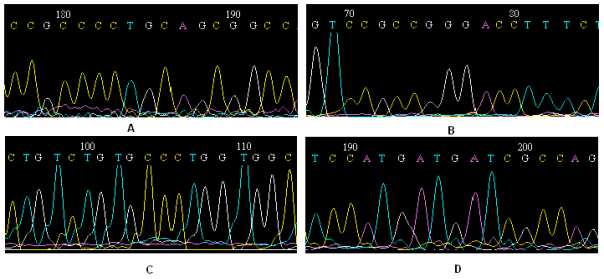


**Figure 6. Sequencing Result Obtained with Samples 90, 52, 30, 73 of Exons 1, 2, 3 and 6 of MTHFR Gene Aligned with Normal**

## Discussion

Breast cancer is most commonly occurring cancer in females of the Pakistan having different causal factors (Mushahida et al., 2005). These factors include hormonal, lifestyle, environmental factors. Genetic make up is another factor that has important functions in the process of carcinogenesis. Different gene mutations have been identified that are particularly linked with the causes of breast cancer including BRCA1, BRCA2, TP53, PTEN, P53 and MTHFR. (Walsh et al., 2006). Different epidemiologists have been suggested that sufficient amount of folate ingestion may be important in the avoidance of breast cancer especially in those women who regularly drink alcohol (Zang et al., 1999; Rohan et al., 2000; Sellers

et al., 2001; Shrubsole et al., 2001, Feigelson et al., 2003). Low folate condition can result in the misincorporation of uracil into DNA that causes chromosomal breaks and DNA repairs disrupted has also been reported in literature (Mason & Levesque, 1996; Blount et al., 1997). Folate deficiency may cause uracil misincorporation in the human DNA that induces chromosomal breakage and is involved in cancer progression (Blount et al., 1997 & Ames, 1999). Three variants regarding MTHFR gene as C677T, G1298C and G1793A MTHFR polymorphisms extensively reported in relation to different populations. SNP (C677T) codes an alanine amino acid into valine as substitution in the N-terminal catalytic domain the second SNP (A1298C) that codes an alanine amino acid to glutamine addition in the C-terminal of regulatory domain. The MTHFR gene at the G1793A polymorphism results an arginine to glutamine change at codon 594 (Rady, 2002) functional significance of this SNP (G1793A) gene has not been reported. These two SNPs are correlated with the enzymes thermolability and reduced invitro enzymatic activity (Frosst, 1995; Weisberg, 1998).

In the present study, association of MTHFR gene polymorphism in sporadic breast cancer patients of local population was evaluated. Both coding as well as non-coding regions were included in this screening to identify the penetrance of any splice site variations as well. Nine exons were selected to be screened for mutational analysis i.e. exons 1 up to 9. The polymorphism (C677T and A1298C) or mutation was found in this study as compared with normal samples. Similar results have been found in earlier studies as well in Korean women (Ford & Bowmwn, 1999; Lee et al., 2004; Marchand et al., 2004; Perry et al., 2004; Justenhoven et al., 2005). Least sporadic mutations in MTHFR gene in context to our population is also in accordance to the previously published study (Langsenlehner et al., 2003; Sharp et al., 2003). In this study low significant association of MTHFR gene polymorphism (677C>T) has been observed while screening 110 breast cancer diagnosed patients along with 110 normal samples. No association among reduced plasma folate level and elevated homocysteine levels were found in relation to the MTHFR polymorphism.

Moreover the polymorphism studies showing the said polymorphism have all been carried out in caucasian population. Only few studies from Asian countries have been published and that has emphasized on the lack of polymorphism involved in breast cancer patients for these variants (Ford and Bowmwn, 1999; Perry et al., 2004). Moreover family history seems to be important for any association of polymorphism/mutations with increased risk of breast cancer. Patients in study showing positive association, for polymorphism for C677T and A1298C SNPs with increased risk of breast cancer had strong family history (Kotsopoulos et al., 2008), whereas all patients recruited in this study had no family history of breast cancer. These findings are also in similar to some earlier report conducted in different populations. As in Jewish population, bilateral breast cancer or combined breast and ovarian cancer causes were higher in individual having 677T allele on MTHFR gene (Gershoni et al., 2000). Diet factors and other genetic polymorphism of MTHFR can influence results. That finding gives useful information to comprehend the molecular basis of breast cancer development, malignancy, progression and outcome (Henríquez et al., 2010).

However, these conflicting results may be attributed to variation in sample size, population involved and folate dietary intake variation. But it is needed to be further explored considering the following possible explanatory reasons for the negative results found in this study.

In conclusions, Pakistani population although may offer potential to explore the contribution to consanguinity of breast cancer, but that may be seems to be specific for hereditary form of the cancer and it might not be the case for sporadic cancer. It is possible that MTHFR gene polymorphism is present in low frequency as compared with that found in many other studies in Caucasian population. Although significant correlation of polymorphism for MTHFR gene (C677, A1298C) has been observed in breast cancer patients, the genotypic data of 677TT 677CC, 677CT, and 677TT genotypes were 0.2230, 0.0060, and 0.0770, respectively. While for the 1298AA, 1298AC, and 1298CC genotypes were 0.5545, 0.0092, and 0.0029, respectively. We also found two unconfirmed single nucleotide polymorphs out of 17 SSCP variants other than the identified polymorphism. These results require a further through analysis to be confirmed and validation via protein expression studies. Since altered regulation of several gene may also be attributed not only to mutation in coding regions but also to variation in expressional levels of transcriptional factors, promoter methylation and loss or gain of binding sites even upstream regulatory regions of the genes. Hence we strongly recommended a further screening of MTHFR protein profile in breast cancer patients along with folate levels in the body with respect to different stages of tumors in a large size cohort study.

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