Research Paper

Methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C polymorphisms in breast cancer: a Sardinian preliminary case-control study

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Abstract

Two common polymorphisms in the MTHFR gene, C677T and A1298C, are associated with reduced enzyme activity and may be associated with breast cancer susceptibility.

We performed a case-control study to investigate the association between the two SNPs in the MTHFR gene and risk of breast cancer. In total, 58 breast cancer patients and 58 unaffected controls were enrolled in the study. Polymerase chain reaction/restriction fragment length polymorphism technique (PCR-RFLP) was conducted to determine the genotypes.

No significant differences were found in the genotypes of the two polymorphisms of the MTHFR gene between cases and controls. The OR and 95% CI for the 677CC, 677CT and 677TT genotypes were 1.00, 0.95 (0.39-2.31) and 0.87 (0.27-2.80), respectively; those of the 1298AA, 1298AC and 1298CC genotypes were 1.00, 0.59 (0.26-1.36) and 0.78 (1.32-4.66) respectively. Furthermore, it has been shown in patients with breast cancer a risk of presenting with an aggressive biophenotype about twice or three times higher in the presence of the C677T and A1298C polymorphisms, respectively. Finally, the A1298C polymorphism is significantly associated with increased recurrence risk of lymph node-positive breast cancer.

Our study has not shown a significant association between MTHFR gene polymorphisms and breast cancer risk. However, it highlighted the key role played by the presence of mutant alleles for both polymorphisms in increasing the risk of developing more aggressive phenotypes; moreover, specifically in A1298C, it might also lead to a higher risk of developing lymph node metastasis.

Key words: Methylenetetrahydrofolate reductase (MTHFR), Folate, Polymorphisms, SNPs C677T and A1298C, Breast cancer.

Introduction

The Methylenetetrahydrofolate reductase (MTHFR) gene is located on chromosome 1, it has 11 exons [1] and as shown by quantitative and qualitative studies, its expression is tissue-specific [2]. The MTHFR enzyme plays a central role in intracellular folate homeostasis; it is in fact a key enzyme in the folate metabolic pathway and it catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which is the carbon donor for homocysteine methylation to methionine. Subsequently, methionine is converted into S-adenosyl methionine (SAM) which represents the methyl donor in biological processes, including the methylation of proteins and nucleic acids [3].
Furthermore, MTHFR plays an essential role in de novo synthesis of purines and the pyrimidine nucleoside hence in DNA biosynthesis, repair and maintenance of DNA stability.

Two functional and well characterized SNPs in the MTHFR gene, C677T (rs 1801133) and A1298C (rs 1801131), have been associated with decreased enzyme activity and increased levels of plasma homocysteine [4,5]. In the MTHFR gene, the C677T polymorphism occurs in exon 4, which involves a C to T substitution at position 677, a consequence of transformation from an alanine to a valine at codon 222 in the N-terminal catalytic domain. Individuals with the 677TT homozygous variant have no more than 30% of normal enzyme activity, and heterozygotes CT genotype have 65% of normal enzyme activity and increased thermolability [6,7]. An additional polymorphism, MTHFR A1298C, occurs in exon 7 resulting into the change from a glutamic acid to alanine residue at codon 429 in the C-terminal regulatory domain of the protein and decreases the enzyme activity [8]. The heterozygous (AC) and homozygous (CC) carriers have a reduction of the enzymatic activity of 15% and 30%, respectively. In individuals with reduced MTHFR enzyme activity, DNA hypomethylation in leukocytes is associated with cardiovascular disease, kidney failure, chromosome anomaly, spontaneous abortion and other diseases [9-11].

The present study aims firstly at evaluating the role of C677T and A1298C polymorphisms as risk factors in a small cohort of healthy women and breast cancer patients. In the literature, study findings on such polymorphisms are discordant, due to the fact that they are strongly influenced by differences among specific ethnic groups. For what concerns the Sardinian area, these polymorphisms have never been studied: thus regardless the results found, a higher number of cases will have to be analysed in the future.

Subjects of Study

A total of 116 women were evaluated in this case-control study: 58 breast cancer patients enrolled at the Medical Oncology Unit, Sassari University Hospital, Sassari and 58 healthy women without history of cancer enrolled by the local branch of Lega Italiana Lotta contro i Tumori (LILT), Sassari (Italy). All women taking part to the study were asked to fill in a questionnaire regarding all known risk factors for breast cancer development such as age, body weight, reproductive-hormone profile, cancer familiarity, smoking habits, alcohol, lifestyle and diet. The questionnaire was administered to healthy women at Sassari LILT local office, while women affected by breast cancer were interviewed at the Medical Oncology Unit, Sassari University Hospital.

In addition, venous blood samples were collected from both patient’s groups.

Each part of the questionnaire has been accurately and thoroughly explained during the meetings held to enrol healthy women so as to transform these meetings into learning opportunities aimed at prevention; probably due also to this approach, a study adherence over 90% has been achieved.

On the other hand, the cancer patients enrolled were diagnosed with infiltrating ductal carcinoma and some cases presented with ductal carcinoma in situ, with age between 36 to 85 years. The overall adherence to the study has been approximately 75%.

This experimental study was approved by ASL Sassari Bioethical Committee and written informed consent was obtained from each participant, according to Italian legislation.

MTHFR Genotyping

The MTHFR polymorphisms were analyzed by a polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assay. For each analyzed polymorphism, 10% of the total samples were randomly retested with 100% concordance.

All participants were requested to provide 3 ml peripheral whole blood, which was collected in K3-EDTA, aliquoted and stored at -20°C.

Genomic DNA was isolated from 200 μl of whole blood, using QIAamp DNA Blood Mini Kit (Qiagen, Germany) following manufacturer’s instructions.

MTHFR C677T. PCR was performed with a final volume of 20 μl of reaction mixture containing 100 ng DNA, 1x PCR buffer, 1,5 mM of MgCl2, 0,2 mM of each dNTP, 1,25 units of Taq Gold DNA polymerase (Applied Biosystems, USA) and 0,6 mM of each primer: forward 5I-TGAAGGAGAAGGTCTGC GGGAG-3I and reverse primer 3I-AGGACGG TGGGTGAGAGTG-5I, yielding a 201-bp fragment [7]. PCR conditions consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s with a final extension at 72°C for 5 minutes.

The PCR products were digested with HinfI (New England Biolabs, USA) in a volume of 20μl for 3 hours at 37°C, electrophoresed in a 3% Metaphor
Agarose gel and visualized by ethidium bromide staining. The C→T valine variant creates a HinfI restriction enzyme site and produces fragments of 178-bp and 23-bp (Figure 1A).

*MTHFR A1298C*. This polymorphism consists of a substitution from A to C in codon 429 (gaa→gca) of exon 7 of the gene and converts a glutamic acid into alanine, cancelling the cutting site for the enzyme MboII. PCR was performed with the final volume of 25 μl of reaction mixture containing 150 ng DNA, 5x PCR buffer, 0.2 mM of each dNTP, 2,5 units of Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs, USA) and 0.6 mM of each primer: forward 5I-CTTTGGGGAGCTGAAGGACTACTAC-3I and reverse primer 3I -CACTTTGTGACCATTCCGGTTTG-5I, yielding a 163-bp fragment [12]. PCR conditions consisted of an initial denaturation at 98°C for 30 s, followed by 35 cycles of 98°C for 30 s, 60°C for 30 s, 72°C for 30 s with a final extension at 72°C for 5 minutes.

The PCR products were digested with MboII (New England Biolabs, USA) in a volume of 25 μl for 3h at 37°C, electrophoresed in a 3% metaphor agarose gel and visualized by ethidium bromide staining. The 1298C allele abolishing one of the MboII restriction site, therefore digestion of the 163 bp PCR product of the 1298A allele yields 5 fragment of 56, 31, 30, 28 and 18 bp, whereas the 1298C allele resulting in 4 PCR bands of 84, 31, 30 and 18 bp (Figure 1B).

**Statistical Analysis**

For what concerns the descriptive statistics analysis of continuous quantity variables, the mean and standard deviation were calculated, as well as the median and interquartile range, when appropriate. In relation to quantity variables, the percent distribution was described, while for dichotomous variables the prevalence was calculated. As far as the inferential analysis is concerned, the process of assessing the prevalence and the differences between prevalences observed was conducted by using the Z-test. Differences between mean values of Gaussian distribution variables or whose numerosity was sufficiently consistent, were calculated by a two-tailed Student’s t-test on independent samples. The association strength between determinants and dichotomous outcome has been evaluated through Odds ratio. On the whole, inferential analysis were performed with an error of first type α ≤ 0,05. Where appropriate, the 95% confidence interval estimate was used. The 95% OR confidence intervals were calculated by using the Woolf method for 2x2 tables or through unconditional logistic regression for multivariate analysis.

**Results**

**Demographic characteristics**

The composition of the two analysed samples and data related to the most important variables analysed are shown in Table 1. A statistically significant difference can be noticed between the two groups for what concerns age, Body Mass Index (BMI), nulliparae prevalence and taking oral contraceptives. In fact, it can be seen that the age of cancer patients is higher (55.7 ± 10.4) than healthy controls (49.2 ± 14.7), their BMI is also higher (25.1 ± 4.4 vs 22.3 ± 4.9), a lower number of nulliparae (20.7% vs 37.9%) and a reduced use of oral contraceptives (60.3% vs 82.8%). No statistically significant differences are seen between the two groups for what concerns the onset of menstruation, familiarity of breast cancer and cigarettes and alcohol consumption.
MTHFR polymorphisms and susceptibility to BC

For what concerns epidemiological and molecular analysis results, Table 2 shows MTHFR C677T and MTHFR A1298C polymorphism distribution in 58 women for the breast cancer cohort and in 58 controls. Ten cases (17.24%) and eleven (18.97%) controls were homozygous for the C677T variant allele. Taking into consideration together the CT heterozygous and the TT homozygous genotypes, it can be seen a non-significant risk reduction compared to the wild-type CC genotype (OR= 0.93, CI 0.41-2.11). For what concerns the A1298C polymorphism, four cases among the breast cancer patients (6.9%) and four in controls (6.9%) were homozygous for the CC variant allele. Likewise, in genotypes AC and CC together the OR was lower compared to the AA wild-type genotype. Besides, data related to the combined genotypes of the two MTHFR gene polymorphisms are reported.

Association between polymorphisms and clinical data

Among patients enrolled in our study, it can be observed that the most recurrent molecular biophenotype (data not shown) is Luminal A (36.84%), followed by in situ tumour (15.79%) and by Luminal B Her2 negative (15.79%). Less recurrent are those characterized by more aggressive biophenotypes (Luminal B Her2 positive 14.04%, Her2+ non luminal 7.02% and triple negative 10.53%).

Moreover, an association between each polymorphism and variable considered important for prognostic and/or predictive purposes has been analysed. The association with the biophenotype is reported in Table 3: more specifically with MTHFR A1298C in Table 3a and with MTHFR C677T in Table 3b. In general, it can be noticed a tendency towards association between aggressive biotypes and the mutant allele variants in the two MTHFR polymorphisms, leading to a three-fold increased risk of having an aggressive biophenotype in the case of MTHFR A1298C polymorphism and a two-fold risk increase for MTHFR C677T polymorphism.

Cases with metastasis were 46% of the total number of breast cancer. The risk of lymph node metastasis resulted significantly increased of 2.5 times for each mutant allele in A1298C polymorphism (Table 4). No variation has instead been observed in C677T.

In conclusion, a significant increase of overweight (BMI>25) prevalence among patients with a CC homozygous mutant allele of A1298C polymorphism has been observed (Figure 2).

![Figure 2. Patients overweight prevalence distribution in relation to the two analysed polymorphisms.](http://www.medsci.org)
Divergent results have been found [27-34]. It might be a self-selection bias as women with cancer socioeconomic status might access to LILT, but there accessed the LILT local office for preventive women consecutively enrolled who voluntarily control group. The latter was in fact formed by probably related to the method used to enrol the contrast to what should have been expected, is breast cancer familiarity. This result, apparently in menstruation, cigarette and alcohol consumption, and were observed for what concerns age of first oral contraception. Instead, no significant differences in the MTHFR enzyme is influenced by environmental factors such as the proportion of folates introduced with the diet [18]. Several evidences suggest an association between 677T and 1298C alleles and the diet [18]. Several studies have found an association between the C677T and disease with a multifactorial etiology. Several studies have been foreseen, including the collection of a larger number of samples compared to the present study, as well an extra control group enrolled taking into consideration the self-selection bias.

Based on epidemiological and statistical analysis conducted on the different MTHFR polymorphic patterns between the two examined groups, no relevant data emerged to confirm an association of C677T and A1298C with a higher breast cancer risk. With the aim to shed light on the link between the two aforementioned polymorphisms and the breast cancer etiology in our area, a prosecution of the study has been foreseen, including the collection of a larger number of samples compared to the present study, as well an extra control group enrolled taking into consideration the self-selection bias.
In conclusion, although our study has not ultimately confirmed the association between MTHFR gene polymorphism and breast cancer risk, it clearly shows the crucial key-role of mutant allele presence of both polymorphisms in relation to the risk of developing more aggressive biophenotypes and, just in the case of A1298C, an increased risk to develop lymph node metastasis.

The biological plausibility of the association of MTHFR polymorphisms with the risk of developing more aggressive biophenotypes is supported by numerous evidences that the deregulation of DNA methylation, de novo synthesis of purines and pyrimidine nucleoside as well as maintaining DNA integrity by MTFHR polymorphic variants, which determine conformational modifications leading to isoenzymes with altered functional activity, may contribute to malignant transformation [35,36].

However the chance that significant association is really true should be better investigated [37]. Therefore, a higher number of cases analysed might ultimately define the role played by different polymorphisms on cancer risk and might, on one hand, enable to better define the prognosis of observed cases and on the other hand, it might allow the identification of tools for early diagnosis to be stratified on differentiated risk profiles in order to increase the efficacy of screening campaigns.

In conclusion, although with the limitations described, our results suggest a biological role of the MTHFR enzyme and its polymorphisms in the progression of breast cancer, related to the association of MTHFR polymorphisms with aggressive biophenotype and lymph node metastasis. Extensive and independent study should be warranted to evaluate the biological mechanisms of this association.

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Competing Interests

The authors have declared that no competing interest exists.

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