Influence of thymidylate synthase and methylenetetrahydrofolate reductase gene polymorphisms on the disease-free survival of breast cancer patients receiving adjuvant 5-fluorouracil/methotrexate-based therapy

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This paper considers the influence of thymidylate synthase (TS) and methylenetetrahydrofolate reductase (MTHFR) polymorphisms on the disease-free survival of patients with breast cancer who were treated with adjuvant therapy containing 5-fluorouracil. Relevant clinical data were obtained from the clinical records of 93 patients included in the study. TS and MTHFR genotypes were determined by PCR–agarose gel electrophoresis (TS) and by means of real-time PCR on an ABI PRISM 7000 Sequence Detection System (MTHFR). The median age of 93 patients was 42 years (range 21–76). Fifty patients received CMF, 18 FAC and 25 FEC. The median follow-up of the series was 134 months, with 34 relapses (37%). Sixty patients had a low expression genotype of TS (64.5%) and 33 had a high expression genotype (35.5). No differences in disease-free survival were observed between the two groups ($P=0.42$). The MTHFR genotype of the 50 patients treated with a chemotherapy regime that included methotrexate was as follows: for C677T, 21 C/C, 21 C/T and eight T/T; for A1298C it was 22 A/A, 24 A/C and four C/C. No differences were found in disease-free survival as regards the MTHFR genotypes ($P=0.1$ and $P=0.6$, respectively). Nor were there differences in disease-free survival in the multivariate analyses that included the TS and MTHF genotypes and the relevant clinical variables ($P=0.3$ for TS, $P=0.1$ for C677T and $P=0.6$ for A1298C). This study shows that genotyping the TS or the MTHFR gene is of little value in the individual assessment of the use of adjuvant therapy in breast cancer patients.

Anti-Cancer Drugs 2007, **18**:821–825

Keywords: adjuvant chemotherapy, disease-free survival, MTHFR, polymorphisms

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Received 17 November 2006 **Accepted** 19 January 2007

Introduction

A number of single agents and combination chemotherapy regimens are effective treatments for breast cancer. In metastatic disease the aim of therapy is to improve time to progression, quality of life and possibly overall survival. In the adjuvant setting, whereas the ultimate aim is to improve overall and/or disease-free survival (DFS), combination chemotherapy including 5-fluorouracil (5-FU) has been the standard care for many women for decades [1]. This drug is a fluoropyrimidine which, on activation to the nucleotide form, develops a stable complex with thymidylate synthase (TS), inhibiting the activity of the enzyme [2]. TS catalyses the reductive methylation of dUMP by 5,10-methylenetetrahydrofolate to form 2-deoxythymidine 5 monophosphate, which is a critical reaction for cell proliferation [3].

The TS gene contains a polymorphic tandem repeat of 28 bp in the 5′-untranslated region (variable number of tandem repeats), and although alleles containing two, three, four, five and nine copies of the repeated sequence have been described, alleles with two (TS*2) and three (TS*3) repeats are the most common alleles in all populations studied to date [4–6]. TS mRNA with a three-repeat sequence has greater translational efficiency than that with the two-repeat sequence [7,8]. Two USF family E-box consensus elements have been found within the tandem repeats of the TS*3 allele and one has been detected within the TS*2 allele. Mandola *et al.* [9] identified a common G→C single nucleotide polymorphism (SNP) at the 12th nucleotide of the second repeat in the TS*3 alleles. This polymorphic substitution changes a critical residue in the USF E-box consensus element, abolishes the USF-1 binding and alters transcriptional activity. Kawakami and Watanabe [10] confirmed the presence of the G→C polymorphic change (SNP) and classified each polymorphic allele as *2G, *2C, *3G and *3C in accordance with the combination of the SNP

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and variable number of tandem repeats. The 3G sequence had an efficiency of translation three to four times greater than the other polymorphic sequences.

Although single-agent methotrexate (MTX) is not a common approach to the treatment of BC, one of the oldest and most widely used regimens is cyclophosphamide, MTX and 5-FU (CMF). MTX inhibits dihydrofolate reductase, resulting in partial depletion of reduced folates. The intracellular concentration of 5,10-methylenetetrahydrofolate is controlled by the activity of methylenetetrahydrofolate reductase (MTHFR), an enzyme that plays a key role in the metabolism of folate. The best characterized MTHFR gene polymorphism consists of a 677C→T transition, in exon 4, which results in an alanine to valine substitution in the predicted catalytic domain of MTHFR. This substitution renders the enzyme thermolabile, and homozygotes and heterozygotes have about 70 and 35% reduced enzyme activity, respectively [11]. A second common polymorphism in the MTHFR gene is a 1298 A→C transition in exon 7, which results in a glutamate to alanine substitution within a presumed regulatory domain of the protein [12]. The 1298C allele leads to decreased enzyme activity, although not to the same extent as the 677T allele. Individuals who are compound heterozygous for the 677T and 1298C alleles have a 40–50% reduced MTHFR activity and a biochemical profile similar to the one observed among 677T homozygotes.

Despite the fact that antifolates are still an important component of breast cancer treatment, pharmacogenetic studies in this type of cancer have received little attention to date. This study considers the influence of TS and MTHFR gene polymorphisms on the DFS of patients with breast cancer who were treated with adjuvant therapy containing 5-FU/Methotrexate.

Patients and methods

Patients

In this retrospective study, 93 patients undergoing 5-FU-containing adjuvant chemotherapy were studied. All patients were required to have normal bone marrow and organ function before administration of the adjuvant chemotherapy. Written informed consent was obtained from all patients, and the study was approved by the Institutional Ethics Committee.

Chemotherapy regimen description

The two different regimens administered in this group of patients were:

- Regimen CMF: Includes cyclophosphamide 600 mg/m^2, MTX 40 mg/m^2 and 5-FU 600 mg/m^2 (days 1 and 8 each 4 weeks; 6 cycles).
- Regimen FEC: includes 5-FU 600 mg/m^2, epirubicin 75 mg/m^2 and cyclophosphamide 600 mg/m^2 (every 3 weeks, 6 cycles).

Clinical parameters and prognostic factors

Relevant clinical data (age, menopausal status, histological type and grade, tumour size, lymph node involvement, hormone receptor status) were obtained from clinical records. DFS was calculated from the beginning of the therapy to the time of relapse or death [13].

Genotyping

After receiving informed consent, we obtained ethylenediaminetetraacetic acid whole blood from 93 patients and DNA was isolated by the salting out procedure [14].

TS

For the analysis of the 28-repeat polymorphism, a fragment containing the repeats was amplified using the primers and the PCR conditions described by Horie *et al.* [15]. DNA fragments of 242 bp (*3/*3), 214 bp (*2/*2) or both (*2/*3) were observed. Half of the PCR products of all cases except those with a *2/*2 genotype were digested with *Hae*III followed by electrophoresis in 3.5% agarose gel containing ethidium bromide. The G→C change in the second repeat of the *3 alleles removes a *Hae*III site, enabling the identification of the different alleles: *2 (66, 47, 45, 44 and 12 bp), *3C (94, 47, 45, 44 and 12 bp) and *3G (66, 47, 45, 44, 28 and 12 bp). Following Kawakami and Watanabe [10], TS genotypes of the patients were classified into two groups: high expression type (*2/*3G, *3C/*3G and *3G/*3G) and low expression type (*2/*2, 2/*3C and *3C/*3C).

MTHFR

The SNPs MTHFR C677T and MTHFR A1298C were additionally analysed in the group of patients receiving the CMF scheme by means of real-time PCR on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, California, USA). Primers and TaqMan probes were as described previously [16]. Each reaction contained template DNA and a final concentration of 1 × TaqMan PCR Master Mix (Applied Biosystems), 300 nmol/l of each primer, 100 nmol/l wild-type probe (Applied Biosystems) and 100 nmol/l variant probe (Applied Biosystems). Thermocycling was performed with an initial 50°C incubation for 2 min followed by a 10-min incubation at 95°C. A two-step cycling reaction was performed for 40 cycles, with denaturation at 95°C for 15 s and annealing/extension at 55°C for 1 min. Analysis of the amplification reaction was performed using the Sequence Detector software, version 2.0 (Applied Biosystems).

Statistical analysis

Kaplan–Meier estimates and the log-rank test were employed in univariate analysis of DFS. A Cox regression model was used for DFS multivariate analysis. The results were considered to be statistically significant when bilateral P values were less than 0.05.

Results

The clinical characteristics and the clinical prognostic factors of the 93 patients included in the study are summarized in Table 1. The mean and median follow-up of the entire series was 147 and 134 months, respectively. Thirty-four patients (37%) relapsed during this period of follow-up.

Table 2 shows the distribution of TS and MTHFR genotypes along with the univariate analysis of DFS in relation to these genetic patterns. We found no statistically significant differences in DFS between the different groups of patients whether classified according to their TS genotype ($P = 0.24$) or according to the TS expression-related genotypes ($P = 0.42$) (Table 2a; Fig. 1a)

In the group of women treated with CMF (the regimen that contains MTX) no differences were found between groups according to the C677T ($P = 0.1$) and A1298C ($P = 0.6$) substitutions of the MTHFR gene. In an additional univariate analysis, we compared the DFS of these patients with MTHFR genotypes associated with low (homozygous and compound heterozygous patients) or with high (heterozygous and wild-type patients) enzymatic activity. No relationship was observed ($P = 0.5$) (Table 2b; Fig. 1b).

Table 1 Clinicopathological characteristics of the 93 patients

Median age (range, year)	42 (21–76)
Menopausal status	
Premenopausal	68 (73.3%)
Postmenopausal	25 (26.9%)
Receptor status	
ER-negative	33 (35.5%)
ER-positive	52 (55.9%)
Unknown	8 (8.6%)
Nodal involvement	
Negative	47 (50.5%)
1–3	33 (35.5%)
4–9	7 (7.5%)
>10	6 (6.5%)
Tumour size	
T1	36 (38.7%)
T2	45 (48.4%)
T3	6 (6.5%)
T4	6 (6.5%)
Histology	
C. ductal infiltrative	78 (83.9%)
C. lobulillar infiltrative	9 (9.7%)
C. intraductal	1 (1.1%)
Others	5 (5.4%)
Histological grade	
I	9 (9.7%)
II	24 (25.8%)
III	35 (37.6%)
Unknown	25 (26.9%)
Chemotherapy regimen	
CMF	50 (53.8%)
FEC	43 (46.2%)
Radiotherapy	
Yes	67 (72%)
No	26 (28%)
Endocrine therapy	
Yes	45 (48.4%)
No	48 (51.6%)

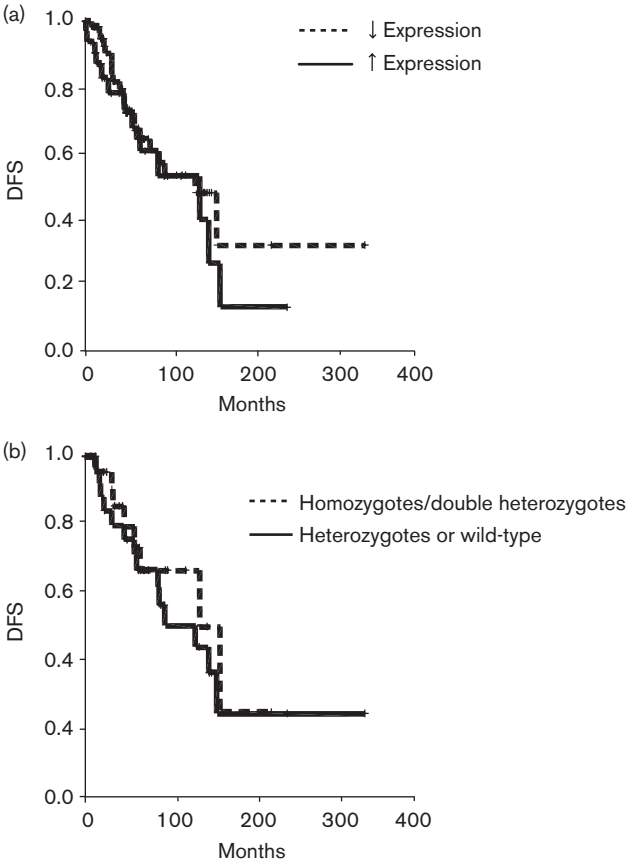
CMF, cyclophosphamide, methotrexate and 5-fluorouracil; ER, estrogen receptor.

Table 2 TS (a) and MTHFR (b) genotypes

	N	Median DFS (months)	*P*
(a)			
TS genotype			0.24
2R/2R	25	134	
2R/3R	42	87	
3R/3R	26	149	
TS genotype			0.42
Low expression	60	134	
High expression	33	139	
(b)			
MTHFR C677T			0.1
Homozygous C/C	21	97	
Heterozygous C/T	21	163	
Homozygous T/T	8	66	
MTHFR A1298C			0.6
Homozygous A/A	22	134	
Heterozygous A/C	24	150	
Homozygous C/C	4	139	
MTHFR genotype			0.5
Low expression	23	138	
High expression	27	134	

Distribution of patients and univariate disease-free survival (DFS) analysis.

Fig. 1



Disease-free survival (DFS) according to the TS (a) and MTHFR (b) genotypes.

A Cox regression analysis was performed to study the influence of genetic determinants in DFS adjusted for the relevant clinical variables (age, histological grade, tumour size, lymph node involvement, hormone receptor status, radiotherapy treatment and endocrine therapy). No differences were found ($P = 0.9$ for TS; $P = 0.9$ for C677T and $P = 0.9$ for A1298C).

Discussion

Breast cancer mortality is decreasing in many countries despite a rising incidence. This decrease coincides with the widespread use of adjuvant combination chemotherapy and with increasing evidence of its benefit for survival. Adjuvant systemic therapy is, thus, considered an integral component of the management of the vast majority of women with primary breast cancer [17].

Clinical trials of adjuvant treatments that include chemotherapy have shown that (1) the benefits of the treatment are greatest in young women, (2) its use in older patients remains controversial mainly because tamoxifen is effective for postmenopausal women and (3) the absolute benefit is enhanced with increasing adverse prognostic factors in such a way that these clinical factors determine whether or not chemotherapy should be used [18,19].

A meta-analysis of worldwide adjuvant clinical trials by the Early Breast Cancer Trialists' Collaborative Group demonstrated statistically significant reductions in recurrence and death over a 10-year period resulting from adjuvant chemotherapy [20]. In a recent work, Bonadonna *et al.* [21] reported the results of their long-term analyses of the trials they started three decades ago. These authors showed that the significant advantage in both the relapse-free and the overall survival persisted over the years, and that adjuvant chemotherapy can suppress micrometastases to a moderate but worthwhile extent.

Earlier studies have confirmed the usefulness of the TS and MTHFR genotypes to predict to some extent the clinical outcome of patients with advanced neoplastic diseases treated with 5-FU and/or MTX [22,23]. Little doubt exists that polychemotherapy plays a more important role in the treatment of advanced disease than in adjuvant therapy. This suggests that the influence of the genotype of the patient would be more marked in the treatment of advanced disease than in adjuvant therapy. Nevertheless, the promising pharmacogenetic results obtained in the treatment of advanced colorectal cancer [24,25] prompted us to study the role of these genetic factors in the outcome of patients with breast cancer treated with adjuvant chemotherapy.

Few studies exist that have analysed the pharmacogenetic aspects of adjuvant chemotherapy. As regards colorectal cancer, only Tsuji *et al.* [26] have studied tumour tissues from 135 patients with stage I–III colorectal cancer who had undergone curative resection and tegafur-based adjuvant chemotherapy. They found that 5-year DFS rates did not vary according to the TS genotype. In breast cancer patients, a recent study [27] of the MTHFR genotype includes a large number of women that received adjuvant chemotherapy. The overall 5-year survival did not differ significantly across all genotypes, but the interpretation of these results is not easy owing to the fact that specific treatment data were unknown.

The loss of heterozygosity at the TS locus [28,29] as well as the TS copy number [30] in the tumour samples of patients with advanced colorectal cancer could influence the TS expression. In the pharmacogenetic studies of these cases both conditions must be taken into account. In the present study, which includes only patients under adjuvant chemotherapy in which there is no overt disease, the aforementioned factors (loss of heterozygosity and copy number) were not analysed owing to the lack of available tumour samples.

Our results (regardless of the performance of univariate or multivariate studies) show that the polymorphisms in the TS promoter enhancer region and in the MTHFR genetic variants play no role in the prediction of the DFS of patients undergoing adjuvant 5-FU/MTX-based chemotherapy, at least during the follow-up of these patients. The absence of statistically significant differences in DFS between genotypes can be attributed to the lack of statistical power of the study. Nevertheless, the resemblance in the DFS values between the different groups with any trend to significance allow us to assume that the inclusions of a large number of patients would not change the results.

The use of MTX in 50 of our patients could account in part for our negative results given that MTX has been described to decrease the ability of 5-FU to inhibit TS and that it helps to move 5-FU toxicity to a TS-independent pathway when given together (CMF regimen) [31].

The advantage reported in relapse-free survival owing to adjuvant therapy [21] is of the order of 15% at 10 years of follow-up and of the order of 8% at 30 years. These differences, albeit significant, are insufficient to enable us to appreciate the involvement of the genotype in the DFS should this exist. At any rate, this study shows that genotyping the TS or the MTHFR gene is of little value in the individual assessment of the use of adjuvant therapy in breast cancer patients.

Acknowledgement

This work was partially supported by FIS (05/1218).

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