Influence of chemotherapeutic drug-related gene polymorphisms on toxicity and survival of early breast cancer patients receiving adjuvant chemotherapy

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Abstract

Background: We investigated whether GSTT1 (*null* allele), GSTM1 (*null* allele), GSTP1 (A313G), RFC1 (G80A), MTHFR (C677T), TS (2R/3R) polymorphisms were associated with toxicity and survival in patients with early breast cancer (EBC) treated with adjuvant chemotherapy (CT).

Methods: This prospective trial included patients with stage I–III BC subjected to CT with CMF or FEC regimens. PCR-RFLP was performed for MTHFR, RFC1 and GSTP1, while PCR for TS, GSTT1 and GSTM1 genes.

Results: Among the 244 patients consecutively enrolled, 48.7% were treated with FEC and 51.3% with CMF. Patients with TS2R/3R genotype showed less frequently severe neutropenia (G3/G4) than those with TS2R/2R and 3R/3R genotype (p = 0.038). Patients with MTHFRCCT genotype had a higher probability of developing severe neutropenia than those with MTHFR CC genotype (p = 0.043). Patients with RFC1GG or GSTT1-null genotype or their combination (GSTT1-null/RFC1GG) were significantly associated with a shorter disease free survival (DFS) (p = 0.009, p = 0.053, p = 0.003, respectively) and overall survival (OS) (p = 0.036, p = 0.015, p = 0.005, respectively). Multivariate analysis confirmed the association of RFC1GG genotype with a shorter DFS (p = 0.018) and of GSTT1-null genotype of a worse OS (p = 0.003), as well as for the combined genotypes GSTT1-null/RFC1GG, (DFS: p = 0.004 and OS: p = 0.003).

Conclusions: Our data suggest that TS2R/2R and 3R/3R or MTHFR CT genotypes have a potential role in identifying patients with greater risk of toxicity to CMF/FEC and that RFC1 GG and GSTT1-null genotypes alone or in combination could be important markers in predicting clinical outcome in EBC patients.

Keywords: Early breast cancer, Polymorphisms, Adjuvant chemotherapy, Toxicity, Prognosis

Background

Breast cancer (BC) currently accounts for 20% of all female cancers worldwide and is the most frequent malignancy occurring in women [1]. There is convincing evidence that adjuvant systemic chemotherapy (AC) increases survival of patients with BC [2]. AC imparted a statistically significant reduction in the risk of BC relapse and death at 5 years of follow-up (with a hazard reduction of approximately 25%), and combination chemotherapy was found to be significantly more effective than single-agent therapy [3]. Trials included more than 15 years of follow-up and led to the conclusion that AC conferred benefit to both premenopausal and postmenopausal patients and also to node-positive and node-negative patients [4]. In general, approximately one of every four recurrences and one of seven deaths is avoided annually by adjuvant chemotherapy [5].

Among the treatments used in this adjuvant setting, the combination of cyclophosphamide (CP), methotrexate (MTX) and 5-fluorouracil (5-FU) (CMF treatment)
or the combination of 5-FU, anthracycline-based chemotherapy (adriamycin or its analogue epirubicin) and CP (FAC/FEC treatment) are the most commonly used. Although the benefit of BC chemotherapy has been demonstrated, these drugs have shown the ability to induce DNA damage in eukaryotic cells [6, 7] and, consequently, chemotherapy treatment involves a risk of provoking DNA damage even in proliferative non-cancer cells [8] therefore leading to a marked toxicity state. Adverse events represent an important physical, psychological and financial burden for the patient and society since up to 15% of the patients receiving FEC will experience at least one serious adverse event [9, 10]. Besides toxicity, another major clinical problem encountered during adjuvant CMF or FEC treatments is BC recurrence of therapeutically resistant disease and thus affecting the long-term outcome of the patient. Significant variability in drug response may occur among cancer patients treated with the same medications [11].

Germline genetic variation in drug metabolizing enzymes and transporters is thought to contribute to the observed inter-individual variation in treatment toxicity and/or efficacy [12]. Recently, pharmacogenomic studies have elucidated the inherited nature of these differences in drug disposition and effects, thereby providing a stronger scientific basis for optimizing drug therapy according to each patient’s genetic constitution. Candidate genes are thymidylate synthase (TS), 5,10-methylenetetrahydrofolate reductase (MTHFR), the reducer folate carrier (RFC1) and glutathione-S-transferases (GSTs), involved in CMF or FEC adjuvant chemotherapies transport and/or metabolism, or being targets of such drugs, as it is shown in Fig. 1. TS is an enzyme implicated in the conversion of deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (dTMP), which is essential in DNA synthesis. The human TS gene (hTS) is polymorphic with either double (2R) or triple (3R) tandem repeats of a 28 base-pair sequence downstream of the cap site in the 5’ terminal regulatory region [13]. In vitro studies, the activity of a reporter gene linked to the 5’ terminal fragment of the hTS gene with triple (3R) tandem repeats was 2.6 times higher than that with double (2R) tandem repeats [14]. Thus, this polymorphic region TS 2R/3R appears to be functional and may modulate TS gene expression. MTHFR is an enzyme responsible for the metabolism of chemotherapeutic drugs-related gene polymorphisms. In cancer cells 5-FU is converted to 5-fluorodeoxyuridine monophosphate (5-FdUMP). 5-FdUMP inhibits the DNA synthesis by competing with deoxyuridine monophosphate (dUMP) for binding to thymidylate synthase (TS) in a complex that is stabilized by the reduced folate 5,10-methylene tetrahydrofolate. 5-FU can also inhibit RNA synthesis in a pathway that involves its metabolism to 5-fluorouridine monophosphate (5-FUMP) and subsequent conversion to 5-fluorouridine triphosphate (5-FUTP) via 5-fluorouridine diphosphate (5-FUDP). The main effect of cyclophosphamide is due to its metabolite phosphoramide mustard that forms DNA crosslinks both between and within DNA strands at guanine N-7 positions (known as interstrand and intrastrand crosslinkages, respectively). This is irreversible and leads to cell apoptosis. Anthracyclines inhibit DNA and RNA synthesis by intercalating between base pairs of the DNA/RNA strand, thus preventing the replication of rapidly growing cancer cells. In addition, they can generate reactive oxygen species (ROS) damaging DNA, proteins and cell membranes. Glutathione S-transferases (GSTs) catalyse the detoxification of alkylating agents used in chemotherapy and/or ROS.
metabolism of vitamin B9 (folate), which is required for DNA synthesis. A known 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 [15]. RFC1 is a major MTX transporter whose impaired function has been recognized as a frequent mechanism of antifolate resistance [16]. Different gene alterations affecting RFC1 transport properties were found in cell lines selected for antifolate resistance [17]. A polymorphism G > A at position 80 in exon 2 of RFC1 gene which replaces His by Arg at position 27 of the RFC1 protein was identified. A recent study implied an effect of G > A80 in combination with C > T677 in MTHFR on plasma folate levels and homocysteine pools [18]. It is known that the mechanism of cytotoxicity with chemotherapy is through the generation of reactive oxygen species (ROS) and their by-products. The reactive molecules responsible for cytotoxicity of these therapies are subject to enzymatic removal, and variability of cells in sensitivity to therapy could depend, at least in part, on the availability and activity of specific metabolizing enzymes. GSTs enzymes are an important cellular defence system that protects cells from chemical injury by catalyzing conjugation of reactive electrophilic molecules with glutathione (GSH). GSTs catalyze the detoxification of some alkylating agents used in chemotherapy and detoxification of products of reactive oxidation [19]. GSTs M1 and T1 have been shown to have activity toward lipid hydroperoxides [20], and individuals lacking each of these enzymes (null allele) may have reduced removal of secondary organic oxidation products produced by cancer therapy and thus may have better prognoses. The pi-class human GST (GSTP1) besides playing a role in protection from oxidative damage was shown to catalyze GSH conjugation of reactive cyclophosphamide metabolites in vitro assays [21]. The present study aimed at investigating the association between TS 2R/3R, MTHFR C677T, RFC1 G80A and GSTT1 null, GSTM1 null or GSTP1 A313G polymorphisms with toxicity, disease free survival (DFS) and overall survival (OS) in Caucasian patients with early BC treated with CMF or FEC regimens.

Methods
Study population
This prospective study was conducted in patients with a histological diagnosis of stage I-III BC treated with conservative surgery or mastectomy, and subjected to adjuvant chemotherapy with CMF or FEC regimens. Tumor staging followed the TNM-AJCC classification [22] and the pTNM was obtained after classical pathological examination. Patients with metastatic disease and with other previous tumors were excluded from this study. Recorded clinical and pathological features for each patient included: age, menopausal status, histology, grade, stage, estrogen receptors (ER) and progesterone receptor (PgR) status, Ki67, p53, HER2 and medical adjuvant therapy. ER, PgR, Ki67, p53 and HER2 status were assessed at the time of surgery on formalin-fixed paraffin-embedded tissue blocks of the primary tumor in the Pathology Department of the University of Perugia. We used the following cut-off for considering Ki 67 positive >14%, [23] p53 positive ≥ 1%, Her2 positive IHC 3+ or IHC 2+ and FISH amplified. Written informed consent was obtained by all patients and the study was reviewed and approved by the institution’s Ethics Committee in accordance with the principles established in the Helsinki declaration.

Chemotherapy regimen
Treatment combined regimen was as follows: CMF (cyclophosphamide 600 mg/m², MTX 40 mg/m² and 5-fluorouracil 600 mg/m²) administered on day 1 and 8 each 4 weeks, for 6 cycles; FEC (5-fluorouracil 600 mg/m², 4-epirubicin 90 mg/m² and cyclophosphamide 600 mg/m²) administered on day 1, every 21 days, for 6 cycles. Physical examination and a full blood counts were performed after each chemotherapy cycle. Hepatic and renal function tests were assessed at baseline and repeated before each cycle of treatment. All patients who had received at least one course of chemotherapy were evaluated for toxicity. Toxicity was scored every 3 weeks according to the Common Toxicity Criteria of the National Cancer Institute (NCI-CTC, version 2.0) [24].

We defined “severe toxicity” as hematological or gastrointestinal toxicity of grade 3–4.

Genotyping analysis
Genomic DNA was extracted from 200 μL of whole blood using the Qiamp blood kit (Qiagen, Milan, Italy) according to the manufacturer’s instructions. Polymorphisms were characterized using the PCR-RFLP for genotyping analyses of MTHFR, RFC1 and GSTP1, while PCR was used for TS polymorphism determination. Multiplex PCR was used to simultaneously amplify GSTT1 and GSTM1, with albumin as a control gene. All primers used in this study were designed by using Primer express 2.0 software (Applied Biosystems, Italy). The primer sequences, restriction enzymes and PCR conditions used in the study are shown in Additional file 1: Table S1.

Statistical analysis
Allele and genotype frequencies for each polymorphism were calculated and tested as to whether they were
distributed according to the Hardy-Weinberg equilibrium. A chi-square test for deviation from Hardy-Weinberg equilibrium was used to estimate differences in allele frequencies. The association of each polymorphism and clinical-pathological features of the patients was assessed by means of a chi-square test. A univariate logistic regression model was used to assess the effect of the same variables, included as dummy variables on incidence of toxicity (0–1-2 grade vs. 3–4), expressing results as odds ratios (OR) and relative 95% confidence intervals (95% CIs). Disease free survival (DFS) was defined as the time from the treatment start up to the date of first progression or death from any cause, whichever came first. Patients who had not died or had disease progression at the date of analysis were censored at the last available information on status. Overall survival (OS) was defined as the time from the treatment start to the date of death from any cause. Time-to-event data were described by the Kaplan-Meier curves. Cox proportional hazards models were used for univariate and multivariate analyses to estimate and test clinical-pathological features and polymorphisms for their associations with DFS and OS. Variables statistically significant at univariate analysis (at a level of \( p < 0.10 \)) were included in the multivariate models. Results were expressed as hazard ratio (HRs) and their 95% CIs. Due to the explorative nature of the study, no adjustment of the significance level to make allowance for multiple tests has been made. Statistical significance was set at \( p < 0.05 \). All statistical analyses were carried out using SAS version 9.2 (SAS Institute, Cary, NC).

Results

Patient characteristics

From June 2000 to September 2005 a total of 244 consecutive Caucasian patients with conservative surgery or mastectomy for primary BC, referred to the Breast Unit Surgical Department of the University of Perugia, Italy, were recruited. Histological diagnosis was confirmed by a pathologist at the Institute of Pathology, University of Perugia. The main clinical-pathological characteristics of the patients are summarized in Table 1.

Frequencies and associations among the polymorphisms and clinical-pathological features

The associations between genetic polymorphisms and the patient clinical-pathological features are reported in Additional file 2: Table S2.

The frequencies of genotypes GSTT1-null e GSTM1-null were 20.5% and 54.1%, respectively and GSTM1-null allele was significantly higher in stage I than the GSTM1-present allele (\( p = 0.042 \)). The frequencies of the genotypes GSTP1 AA, AG, and GG were 59.4%, 39.3%, and 1.2%, respectively. GSTP1 AA genotype was significantly higher in stage III, in positive lymph nodes and in negative p53, than the GSTP1 AG or GG genotype (\( p = 0.006, p = 0.027 \) and \( p = 0.033 \), respectively). For MTHFR the frequencies of CC, CT, and TT were 27.5%, 47.5%, and 25.0%, respectively and the MTHFR CT or TT genotypes were significantly higher in stage III or in positive lymph nodes than the MTHFR CC genotype (\( p = 0.025 \) and \( p = 0.011 \), respectively). For RFCI polymorphism, the frequencies of GG, GA, and AA were 30.3%, 46.3%, and 23.4%, respectively. The frequencies of TS tandem repeat genotype distribution were 32.8% in 3R3R, 35.2% in 3R2R, and 32.0% in 2R2R. There was no statistically significant association among genotype distributions and tumor size, grading, ER, PgR, Ki67 and HER2 status. The genotype

| Table 1 Baseline characteristics of patients |
|-------------------------------|-----------------|------------------|
| Characteristics              | No. of patients (%) |
| All patients                  | 244 (100)        |
| Median age, years (min-max)   | 51.3 (26.6–75.6) |
| Stage                         |                  |
| I                             | 111 (45.5)       |
| II                            | 93 (38.1)        |
| III                           | 40 (16.4)        |
| Tumor size, ≤2 cm             | 49 (34.0)        |
| Positive lymph nodes status   | 107 (43.9)       |
| Tumor grade                   |                  |
| G1                             | 18 (7.4)         |
| G2                             | 143 (58.6)       |
| G3                             | 59 (24.2)        |
| Unknown                       | 24 (9.8)         |
| Histology                     |                  |
| Ductal infiltrating carcinoma | 212 (86.9)       |
| Other histology               | 32 (14.1)        |
| Positive ER status (cut-off > 10%) | 154 (63.1) |
| Positive PgR status (cut-off > 10%) | 137 (56.1) |
| Ki67 positive status (cut-off > 14%) | 112 (45.9) |
| Positive p53 status (cut-off ≥ 1%) | 34 (13.9) |
| Positive HER2 (IHC/FISH)      | 26 (10.7)        |
| Surgery                       |                  |
| Conservative                  | 201 (82.4)       |
| Mastectomy                    | 43 (17.6)        |
| Adjuvant chemotherapy         |                  |
| CMF                            | 124 (50.8)       |
| FEC                            | 120 (49.2)       |
| Endocrine therapy             | 148 (60.6)       |
| Radiotherapy                  | 205 (84.0)       |

*HC 3 + or IHC 2+ and FISH amplified

ER estrogen receptor; PgR, progesterone receptor

CMF cyclophosphamide, methotrexate, 5-fluorouracil

FEC 5-fluorouracil, epirubicin, cyclophosphamide
distribution observed was similar to that expected under Hardy-Weinberg equilibrium.

**Toxicity and effect of polymorphisms in whole BC group**

All 244 patients were evaluable for toxicity. Hematological and non-hematological toxicities to CMF/FEC regimen were evaluated and are summarized in Additional file 3: Table S3. Among patients with BC who developed toxicity the prevalence of hematologic and non-hematologic toxicities of any grade was as follows: 63 neutropenia (25.8%), 58 leucopenia (23.7%), 13 anemia (5.2%), 46 mucositis (18.8%) and 35 hepatic toxicity (14.3%). Among BC patients treated with CMF (n = 124) the prevalence of hematologic and non-hematologic toxicities of any grade was as follows: 28 neutropenia (22.5%), 27 leucopenia (21.7%), 6 anemia (4.8%), 27 mucositis (21.7%) and 18 hepatic (14.5%) toxicity. Among BC patients treated with FEC (n = 120) the prevalence of hematologic and non-hematologic toxicities of any grade was as follows: 24 neutropenia (20.0%), 20 leucopenia (16.6%), 8 anemia (6.6%), 18 mucositis (15.0%) and 15 hepatic (12.5%) toxicity. There were no statistically significant differences between Table S4:CMF and FEC regimens in terms of toxicity (Additional file 3: Table S3). Grade 3/4 toxicity was observed overall in 14.3% (35/244) of patients: 10% (24/244) for hematological toxicity, 4.5% (11/244) for non-hematological toxicity (alopecia not included). A few patients experienced cycle delay (n.5 patients) or dose reduction (n.8 patients). No toxic deaths were observed in this study. Associations between genotypes and toxicities are reported in Table 2. A significant association was detected between the number of 28-bp tandem repeats in the 5′-untranslated region of the TS gene and the severity of toxicity. The patients with 2R/3R TS genotype showed less frequently severe (G3/G4) neutropenia than patients with 2R/2R TS genotype (OR = 0.25, 95% CI: 0.06–0.93p = 0.038). The patients with CT MTHFR genotype had a higher probability of developing severe neutropenia than patients with CC MTHFR genotype (OR = 8.32 95% CI: 1.06–65.2, p = 0.043). When considering toxicity of any grade (G1–4), patients with 2R/3R TS genotype had a lower probability of developing oral mucositis (OR = 0.36 95% CI: 0.16–0.82, p = 0.015, Additional file 4: Table S4). No other statistically significant differences in toxicity were found with respect to the other polymorphisms.

**Survival analysis**

At a median follow-up of 9.2 years (interquartile range: 8.2–10.6), we observed 38 (15.6%) disease recurrences, 16 (6.6%) second tumors and 41 (16.8%) deaths. Overall the patients with recurrence and/or second tumor and/or deaths were 85 (34.8%). Loco-regional recurrence was observed in 13 patients (34.2%) and metastatic disease in 25 patients (65.8%): dominant site was visceral in 28 of 38 patients (76.7%). Results of univariate analysis for DFS and OS are reported in Table 3. Both patients with genotype RFC1 GG and genotype RFC1 GA had a shorter DFS in comparison to those with genotype AA (HR = 2.89, 95% CI: 1.31–6.38, p = 0.009; HR = 2.35, 95% CI: 1.09–5.07, p = 0.029 for GG and GA, respectively (Fig. 2a- DFS curves for RFC1). Patients with genotype RFC1 GG had a shorter OS in comparison to those with genotype AA (HR = 2.90, 95% CI: 1.07–7.88, p = 0.036) while patients with genotype RFC1 GA did not show a different survival when compared with genotype AA (HR = 1.95, 95% CI: 0.79–5.22, p = 0.184) (Fig. 2b- OS curves for RFC1). DFS was also shorter in patients with genotype GSTT1-null when compared to patients with genotype GSTT1-present (HR = 1.68, 95% CI: 0.99–2.86, p = 0.05) (Fig. 2c- DFS curves for GSTT1). OS was also shorter in patients with genotype GSTT1-null when compared to patients with genotype GSTT1-present (HR = 2.22, 95% CI: 1.17–4.24, p = 0.015). (Fig. 2d- OS curves for GSTT1). The multivariate model (including age, ER/PgR positive, stage, the genotypes GSTT1 and RFC1) for DFS and OS showed that the genotype RFC1 GG confirmed a shorter DFS when compared to RFC1 AA genotype (HR = 2.64, 95% CI: 1.18–5.90, p = 0.018), while genotype GSTT1-null was confirmed as an independent prognostic factor for a worse OS (HR = 2.82, 95% CI: 1.41–5.64, p = 0.003) (Table 4).

According to genotypes of GSTT1 and RFC1 genes we classified patients in three groups: the first with GSTT1-present and RFC1-AA (group1), the second with GSTT1-present and RFC1-GA/RFC1-GG or GSTT1-null and RFC1-AA (group2), and the third with GSTT1-null and RFC1-GG (group3).

Kaplan-Meier curves for DFS and OS are reported in Fig. 2e and f, respectively. At univariate analysis, confirmed at multivariate analysis (Table 4) both for DFS and OS, group2 showed a worse prognosis compared with group1 (HR = 4.20, 95% CI 1.52–11.56, p = 0.006; HR = 4.54, 95% CI 1.09–18.92, p = 0.038 for DFS and OS respectively). A greater difference was detected when compared group3 with group1 (HR = 6.61, 95% CI 1.93– 22.59, P = 0.003; HR = 10.12, 95% CI 2.04–50.19, P = 0.005 for DFS and OS respectively).

**Discussion**

In the present study, we demonstrated that among BC patients who received CMF or FEC, those possessing the TS 2R/3R variant showed a significantly lower risk of severe toxicity (grade 3–4) for neutropenia and, when considering toxicity of any grade (G1–4), the same variant conferred a lower probability of developing oral mucositis. Our data are in agreement with previously published
| Genotype | HEMATOLOGIC TOXICITY | NON-HEMATOLOGIC TOXICITY |
|----------|----------------------|--------------------------|
|          | LEUCOPENIA | NEUTROPENIA | STOMATITIS | HEPATIC |
| GSTT1    |            |            |            |         |
| null     | 47 3 1 (reference) | 190 4 0.33 (0.007–1.52) | 49 1 1 (reference) | 49 1 1 (reference) |
| Present  | 129 3 1 (reference) | 108 4 1.59 (0.35–7.27) | 131 1 a | 144 1 a |
| GSTM1    |            |            |            |         |
| null     | 141 4 1 (reference) | 91 3 1.18 (0.26–5.39) | 142 3 1 (reference) | 144 1 a |
| Present  | 71 3 1 (reference) | 56 1 0.42 (0.004–4.17) | 73 1 1 (reference) | 74 0 a |
| RCIF1    |            |            |            |         |
| null     | 64 1 1 (reference) | 113 3 0.57 (0.11–2.89) | 67 0 a | 67 0 a |
| Present  | 110 3 0.64 (0.13–3.29) | 56 1 0.36 (0.004–3.51) | 114 2 | 115 1 |
| MTHFR    |            |            |            |         |
| CC       | 64 1 1 (reference) | 103 13 8.32 (1.06–65.2) | 67 0 a | 67 0 a |
| CT       | 113 3 0.57 (0.11–2.89) | 376 58 3 3.41 (0.35–33.7) | 114 2 | 115 1 |
| TT       | 56 1 0.36 (0.004–3.51) | 54 3 0.77 (0.18–33.5) | 56 1 1.30 (0.08–21.3) | 55 2 |
| TT vs. CT + CC | 0.49 (0.006–4.17) | 0.515 0.62 (0.17–2.25) | 3.07 (0.42–22.3) | 0.268 |
| TS-TR    |            |            |            |         |
| 2R/2R    | 84 2 1 (reference) | 83 3 1 (reference) | 85 1 1 (reference) | 86 0 a |
| 2R/3R    | 74 4 0.44 (0.008–2.47) | 76 10 0.25 (0.006–0.93) | 76 2 0.45 (0.04–5.03) | 78 0 |
| 3R/3R    | 79 1 0.23 (0.003–2.14) | 76 4 0.36 (0.11–1.19) | 79 1 0.48 (0.04–5.42) | 78 2 |
| 3R/3R vs. 2R/3R + 2R/2R | 0.33 (0.004–2.82) | 0.313 0.61 (0.19–1.94) | 0.403 0.68 (0.07–6.64) | 0.740 |

OR Odds Ratio, CI Confidence Intervals

*Due to the low number of events it was not always possible to perform the comparison test.
studies [25–27] confirming a significant inverse association of \( TS \ 2R/3R \) polymorphism and severity toxicity. However, whereas in the study by Lecomte et al. patients with the \( 2R/2R \) genotype were 20 times more likely to have severe toxicity compared with \( 3R/3R \) carriers, this effect was much less pronounced in our study and more similar to the results of Schwab’s study [28]. However, the role of other 5-FU catabolism-involved polymorphisms, such as dihydropyrimidine dehydrogenase (DPYD), should be explored to improve prediction of 5-FU toxicity [29]. At present, the real predictive value of \( MTHFR \) \( C677T \) polymorphism on MTX and 5-FU toxicity is not completely established. In our study, we found that the patients with \( MTHFR \) \( CT \) genotype had a higher probability of developing severe neutropenia than patients with \( MTHFR \) \( CC \) genotype. Some recent studies have shown increased toxicity in \( 677 \ T \)–carriers treated with methotrexate [30–32], although other studies did not confirm such an association [33, 34]. Different methotrexate doses and schemes as well as diverse nutritional/folate status might account, at least in part, for these discrepant results. Probably, the heterozygous effects of \( MTHFR \) \( CT \) and \( TS \ 2R/3R \) genotypes as compared to each homozygous effect might be justified by considering that exogen factors, environmental conditions, dietary habits and lifestyle might play an important role [25–27, 35, 36]. No other significant differences in toxicity were found with respect to the other polymorphisms.

### Table 3: Cox models for DFS and OS (univariate analysis)

| Variable                | Univariate analysis - DFS | Univariate analysis - OS |
|-------------------------|---------------------------|--------------------------|
|                         | HR | 95% CI | \( p \)   | HR | 95% CI | \( p \)   |
| Age (per years)         | 1.01 | 0.99 | 1.04 | 0.270 | 1.05 | 1.01 | 1.08 | 0.005 |
| ER- PgR-                | 1 (reference) |          |          | 1 (reference) |          |          |
| ER+ PgR/- ER- PgR+      | 0.72 | 0.40 | 1.30 | 0.273 | 0.64 | 0.30 | 1.40 | 0.269 |
| ER+ PgR+                | 0.51 | 0.29 | 0.89 | 0.018 | 0.51 | 0.25 | 1.04 | 0.066 |
| Stage I                 | 1 (reference) |          |          | 1 (reference) |          |          |
| Stage II                | 2.01 | 1.13 | 3.56 | 0.018 | 3.73 | 1.48 | 9.41 | 0.005 |
| Stage III               | 3.77 | 2.01 | 7.08 | <0.001 | 9.77 | 3.85 | 24.82 | <0.001 |
| LN (pos vs. neg)        | 1.79 | 1.11 | 2.88 | 0.016 | 2.61 | 1.37 | 4.98 | 0.004 |
| HER2 (pos vs. neg)      | 1.51 | 0.75 | 3.04 | 0.251 | 1.67 | 0.70 | 3.97 | 0.248 |
| GSTT1 (null vs. present)| 1.68 | 0.99 | 2.86 | 0.053 | 2.22 | 1.17 | 4.24 | 0.015 |
| GSTM1 (present vs. null)| 1.23 | 0.77 | 1.98 | 0.383 | 1.68 | 0.90 | 3.12 | 0.103 |
| RFC1 – AA               | 1 (reference) |          |          | 1 (reference) |          |          |
| RFC1 – GA               | 2.35 | 1.09 | 5.07 | 0.029 | 1.95 | 0.73 | 5.22 | 0.184 |
| RFC1 – GG               | 2.89 | 1.31 | 6.38 | 0.009 | 2.90 | 1.07 | 7.88 | 0.036 |
| GSTP1 – AA              | 1 (reference) |          |          | 1 (reference) |          |          |
| GSTP1 – AG              | 0.77 | 0.46 | 1.26 | 0.297 | -    | -    | -    | 0.989 |
| GSTP1 – GG              | -    | -    | -    | 0.985 | 0.80 | 0.42 | 1.53 | 0.500 |
| MTHFR – CC              | 1 (reference) |          |          | 1 (reference) |          |          |
| MTHFR – CT              | 1.28 | 0.72 | 2.27 | 0.394 | 1.02 | 0.49 | 2.13 | 0.957 |
| MTHFR – TT              | 0.85 | 0.42 | 1.71 | 0.642 | 0.96 | 0.41 | 2.25 | 0.920 |
| TS-TR – 2R/2R           | 1 (reference) |          |          | 1 (reference) |          |          |
| TS-TR – 3R/3R           | 0.62 | 0.35 | 1.11 | 0.105 | 0.67 | 0.31 | 1.48 | 0.327 |
| TS-TR – 3R/3R           | 0.80 | 0.46 | 1.41 | 0.439 | 1.11 | 0.54 | 2.28 | 0.767 |
| Combined genotype groups* | 1 (reference) |          |          | 1 (reference) |          |          |
| Group 1                 | 4.20 | 1.52 | 11.56 | 0.006 | 4.54 | 1.09 | 18.92 | 0.038 |
| Group 2                 | 6.61 | 1.93 | 22.59 | 0.003 | 10.12 | 2.04 | 50.19 | 0.005 |

HR = Hazard Ratio, CI = Confidence Interval, DFS = Disease free Survival, OS = Overall Survival, LN = lymph nodes

*group1: GSTT1-present and RFC1-AA

*group2: GSTT1-present and RFC1-GA/RFC1-GG or GSTT1-null and RFC1-GA/RFC1-AA

*group3: GSTT1-null and RFC1-GG
have small sample sizes, are based on participants diagnosed prior to 1999 and on women undergoing chemotherapy and/or radiotherapy. In addition, most of them examined only one GST gene (usually GSTP1). In our study, we showed that genotype \textit{GSTT1-null} was associated with worse DFS and OS in EBC patients. This association was maintained in the multivariate model only for OS independently of age and other traditional predictors of prognosis. Our results are based on the assumption that the individuals with \textit{GSTT1-null} genotype, that is associated with an absence of enzyme activity, are considered to be at increased risk for malignancies due to reduced efficiency in protection against environmental carcinogens [37, 38]. Conversely, Ambrosone et al. [39], showed that

**Fig. 2** Kaplan Meier curves by \textit{RFC1} and \textit{GSTT1} status. Disease-Free Survival by \textit{RFC1} polymorphism \textbf{a}. \textit{GSTT1} status \textbf{c}. and combined genotype groups \textbf{e}. Overall Survival by \textit{RFC1} polymorphism \textbf{b}. \textit{GSTT1} status \textbf{d}. and combined genotype groups \textbf{f}. Combined genotype groups were as follows: group1: GSTT1-present and RFC1-AA; group2: GSTT1-present and RFC1-GA/RFC1-GG or GSTT1-null and RFC1-GA/RFC1-AA; group3: GSTT1-null and RFC1-GG
**GSTM1-null** and **GSTT1-null** genotypes predicted significantly better DFS and OS, both individually or in combination. Our results on **GSTM1** genotype are in agreement with those of Lizard-Nacol et al. [40] who, showed no effect of **GSTM1-null** genotype on DFS or OS among 92 women with advanced BC who had received cyclophosphamide, doxorubicin, and 5-FU. Whereas, Kristensen et al. [41] found that patients with **GSTM1-null** allele had a significantly shorter OS. Moreover, Yu Ke-Da et al. [42] showed a more complicated role for **GSTM1** that should be considered in breast cancer risk prediction. The results of this study indicated a U-shaped association of **GSTM1** with breast cancer, which challenges the linear genodose effect of **GSTM1** that was previously proposed. This effect was due to a new SNP, rs412543 (−498C > G) located in the promoter region that decreased gene transcription by 30–40% via reducing the DNA-binding affinity of AP-2. In contrast to these previous studies, our study is the only one to examine adjuvant therapy in a population of patients with a relatively uniform recurrence risk, with a longer follow-up (9.2 years), providing a homogeneous patient population in which to study treatment related genotypes and outcomes. Genetic background differences among races account for differences in the frequencies of allelic variants so that the association of polymorphic variants with a disease risk can significantly vary among populations. As far as we know, scanty information is available on the association of chemotherapeutic drug-related gene polymorphisms on toxicity and survival of breast cancer patients in non Caucasian populations. The results of Yang et al. showed no association between any of the **GSTM1** or **GSTT1** genotypes in patients with breast carcinoma who were treated with chemotherapy [43].

**RFC1** genotypes, as predictors of BC treatment efficacy, have not been previously reported. Recent evidence suggests that **G80A** polymorphism in **RFC1** is associated with altered folate/antifolate levels and may influence the efficacy of therapy with MTX [39]. Data suggest that subjects carrying the homozygous mutant **AA** genotype tend to have higher plasma folate and MTX levels and higher erythrocyte polyglutamate levels compared with those with the wild type or heterozygous genotype. In our study, for the first time to our knowledge, we showed that patients with **RFC1 GG** genotype had a shorter DFS and OS than carriers of the **AA** genotype. These observations are in keeping with previous studies on rheumatoid arthritis (RA). The work of Drozdzik et al. [44] showed that patients with **RFC1 AA** genotype responded to the therapy more effectively than carriers of **AG** and **GG** genotypes. The remission of RA symptoms was significantly higher (3.32-fold) in **AA** carriers in comparison to **GG** individuals. In contrast to RA patients, the study on acute lymphoblastic leukemia of Laverdiere et al. [45] showed children with **AA** genotype had worse prognoses than patients with **GG** genotype, and **AA** genotype was associated with higher plasma levels of MTX than other genotypes. Moreover, we showed, in an explorative analysis, that the combined genotypes (**GSTT1-null/ RFC1-GG**) had a negative prognostic effect on DFS and OS. This subgroup of tumors could have a more aggressive clinical course and the availability of a non-invasive, repeatable and reproducible technique to detect polymorphisms in the blood appears to be a useful tool for identifying high-risk BC patients. Therefore, further large sample size and well designed studies are greatly needed to confirm these preliminary results. Limitations of our study include relatively small sample size and low number of events, thus we were not able to evaluate the association with outcome by subgroups, such as menopausal status. Nevertheless, the association between GST polymorphisms and BC survival, showed by our results seems to be in agreement with those of the literature [39, 40].

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**Table 4** Cox models for DFS and OS (multivariate analysis)

| Variable | Multivariate analysis* - DFS | | Multivariate analysis* - OS | |
|----------|-------------------------------|-------------------|-------------------|-------------------|
|          | HR 95% CI p                    |                   | HR 95% CI p       |                   |
| GSTT1 (null vs. Present) | 1.67 0.96 2.91 0.071 | | 2.82 1.41 5.64 0.003 | |
| RFC1 – AA | 1 (reference) | | 1 (reference) | |
| RFC1 – GA | 2.15 1.00 4.65 0.051 | | 1.53 0.57 4.14 0.402 | |
| RFC1 – GG | 2.64 1.18 5.90 0.018 | | 2.62 0.94 7.31 0.066 | |

Combined genotype groups**

Group 1

| Variable | HR 95% CI p | |
|----------|-------------|-------------|
| 1 (reference) | | |

Group 2

| Variable | HR 95% CI p | |
|----------|-------------|-------------|
| 3.93 1.42 10.86 0.008 | | |

Group 3

| Variable | HR 95% CI p | |
|----------|-------------|-------------|
| 6.35 1.82 22.17 0.004 | | |

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**HR** Hazard Ratio, **CI** Confidence Intervals, **DFS** Disease free Survival, **OS** Overall Survival, **LN** lymph nodes

*multivariate model includes the combination of GSTT1 and RFC1 genes adjusted for age, ER/PGR, stage

**group1: GSTT1-present and RFC1-AA; group2: GSTT1-present and RFC1-GA/RFC1-GG or GSTT1-null and RFC1-GA/RFC1-AA

group3: GSTT1-null and RFC1-GG
The cohort was established before some current treatments, such as aromatase inhibitors, and Her2/neu targeted therapies were available. Therefore, we cannot estimate what associations GST isoenzymes might have with survival in women using these treatments. However, our study has a larger sample size than most prior studies examining the association between GST polymorphisms and survival and it is the first study to evaluate RFC1 genotypes as predictors of BC treatment efficacy.

Conclusions
In conclusion, our study provides important novel information about the potential role of drug-transporter enzyme polymorphisms in the outcome after adjuvant therapy for EBC. Confirmation of these findings in a large sample size and well designed studies and supportive mechanistic data will ultimately allow the potential for drug-transporter genotyping to be realized in the clinic to individualize and optimize EBC therapy.

Additional files

- **Additional file 1**: Table S1. Characteristics of the studied polymorphisms. (DOC 46 kb)
- **Additional file 2**: Table S2. Association among gene polymorphisms and clinical-pathological features. (DOC 99 kb)
- **Additional file 3**: Table S3. CMF/FEC treatment-related toxicity graded according to the NCI-CTC v2.0. (DOC 55 kb)
- **Additional file 4**: Table S4. Association among gene polymorphisms and risk of toxicity of any grade (grade 1–2–3–4 vs 0). (DOC 83 kb)

Acknowledgements
The authors would like to remember Irene Floriani for her technical support and to dedicate this work to her, who deceased. She was head of the Clinical Research Laboratory of Mario Negri institute in Milan, Italy. They also want to remember her commitment, dedication and professionalism as well as the human talent that she had and which led us to reallocate many oncological research projects. Her loss is tremendous to our Society and especially to our hearts. The authors also express their gratitude to the patients who participated in this study.

Funding
This work was supported in part (reagents for gene polymorphism analysis) by Consiglio Nazionale delle Ricerche (CNR), by the Umbria Association Against Cancer (AUCC) and by “Conoscere per Vincere” charities.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
Conception and design: VL, SG; MT; Manuscript writing: VL; Statistical analysis: ER, IF; Patient management/enrolment: AR, JF, EL, SG, LC; genotyping analysis: LP, GM, FRT, SP; Histological diagnosis and biomolecular characterization: AS; Review of the manuscript: VL, CA, VNT. All authors approved the final version of this article.

Ethics approval and consent to participate
The study is in compliance with the Helsinki declaration. Ethical approval has been granted by the Institutional Review Board of the Comitato Etico Aziende Sanitarie (CEAS) Umbria (reference-number: 9440). Upon inclusion, a written informed consent is obtained from all participants.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 18 December 2015 Accepted: 12 July 2017
Published online: 26 July 2017

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