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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 *MTHFRCT* 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)

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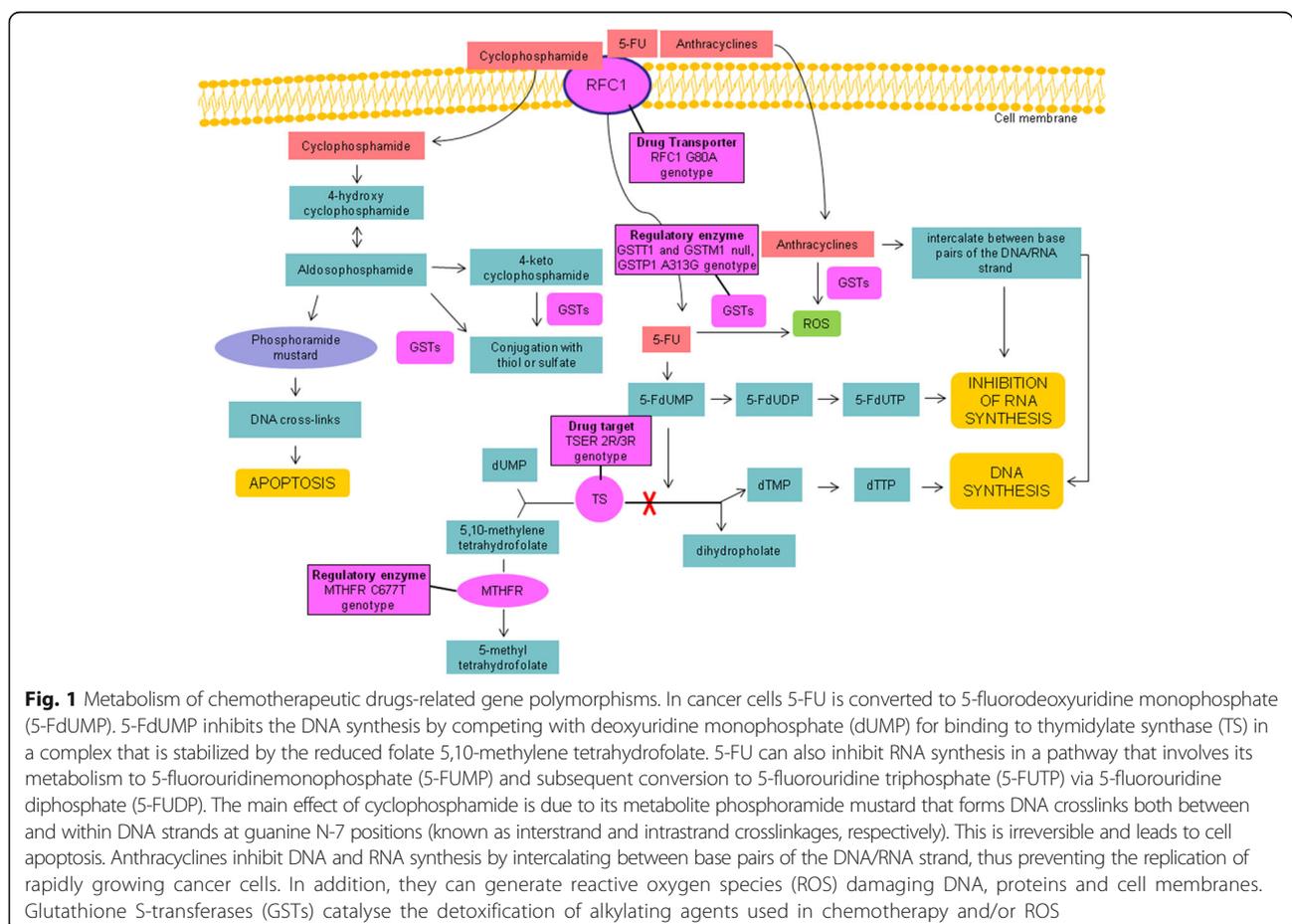
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or the combination of 5-FU, anthracycline-based chemotherapy (adriamycin or its analogue epirubicin) and CP (FAC/FEC treatment) are the most commonly used. Although the benefit of BC chemotherapy has been demonstrated, these drugs have shown the ability 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



metabolization 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 \geq 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 μ L 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

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 $\geq 1\%$)	34 (13.9)
Positive HER2 ^a (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)

^aIHC 3+ or IHC 2+ and FISH amplified
 ER estrogen receptor; PgR, progesterone receptor
 CMF cyclophosphamide, methotrexate, 5-fluorouracil
 FEC 5-fluorouracil, epirubicin, cyclophosphamide

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 the *RFC1* 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

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 regimens 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: 0.06–0.93, $p = 0.038$). 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-GA/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

Table 2 Association among gene polymorphisms and risk of severe toxicity (grade 3–4 vs. 0–1–2)

Genotype	HEMATOLOGIC TOXICITY				NON-HEMATOLOGIC TOXICITY				p							
	LEUCOPENIA		NEUTROPENIA		STOMATITIS		HEPATIC									
	0–1–2	3–4	0–1–2	3–4	0–1–2	3–4	0–1–2	3–4								
GSTT1																
null	47	3	1 (reference)	0.155	45	5	1 (reference)	0.349	49	1	1 (reference)	0.822	49	1	1 (reference)	0.335
Present	190	4	0.33 (0.07–1.52)		182	12	0.59 (0.30–1.77)		191	3	0.77 (0.08–7.56)		193	1	0.25 (0.02–4.13)	
GSTM1																
null	129	3	1 (reference)	0.548	122	10	1 (reference)		131	1	a		132	0	a	
Present	108	4	1.59 (0.35–7.27)		105	7	0.81 (0.30–2.21)	0.686	109	3			110	2		
GSTP1																
AA	141	4	1 (reference)	0.832	134	11	1 (reference)	0.150	142	3	1 (reference)	0.569	144	1	a	
AG	91	3	1.18 (0.26–5.39)		89	5	0.70 (0.23–2.07)		93	1	0.52 (0.05–5.04)		93	1		
GG	3	0			2	1			3	0			3	0		
RCF1																
GG	71	3	1 (reference)	0.598	69	5	1 (reference)	0.759	73	1	1 (reference)	0.824	74	0	a	
GA	110	3	0.64 (0.13–3.29)		104	9	1.19 (0.38–3.72)		111	2	1.32 (0.12–14.8)		113	0		
AA	56	1	0.42 (0.04–4.17)	0.461	54	3	0.77 (0.18–3.35)	0.724	56	1	1.30 (0.08–21.3)	0.852	55	2		
AA vs. GA + GG	0.54 (0.06–4.57)			0.571	0.69 (0.19–2.48)			0.566	1.10 (0.11–10.74)			0.938				
MTHFR																
CC	64	1	1 (reference)		66	1	1 (reference)		67	0	a		67	0	a	
CT	113	3	0.57 (0.11–2.89)	0.494	103	13	8.32 (1.06–65.2)	0.043	114	2			115	1		
TT	60	1	0.36 (0.04–3.51)	0.376	58	3	3.41 (0.35–33.7)	0.294	59	2			60	1		
TT vs. CT + CC	0.49 (0.06–4.17)			0.515	0.62 (0.17–2.25)			0.472	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 (0.08–2.47)	0.352	68	10	0.25 (0.06–0.93)	0.038	76	2	0.45 (0.04–5.03)	0.514	78	0		
3R/3R	79	1	0.23 (0.03–2.14)	0.199	76	4	0.36 (0.11–1.19)	0.095	79	1	0.48 (0.04–5.42)	0.553	78	2		
3/3R vs. 2/3R + 2/2R	0.33 (0.04–2.82)			0.313	0.61 (0.19–1.94)			0.403	0.68 (0.07–6.64)			0.740				

OR Odds Ratio, CI Confidence Intervals

^aDue to the low number of events it was not always possible to perform the comparison test

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

Variable	Univariate analysis - DFS				Univariate analysis - OS			
	HR	95% CI		<i>p</i>	HR	95% CI		<i>p</i>
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 – 2R/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*								
Group 1	1 (reference)				1 (reference)			
Group 2	4.20	1.52	11.56	0.006	4.54	1.09	18.92	0.038
Group 3	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

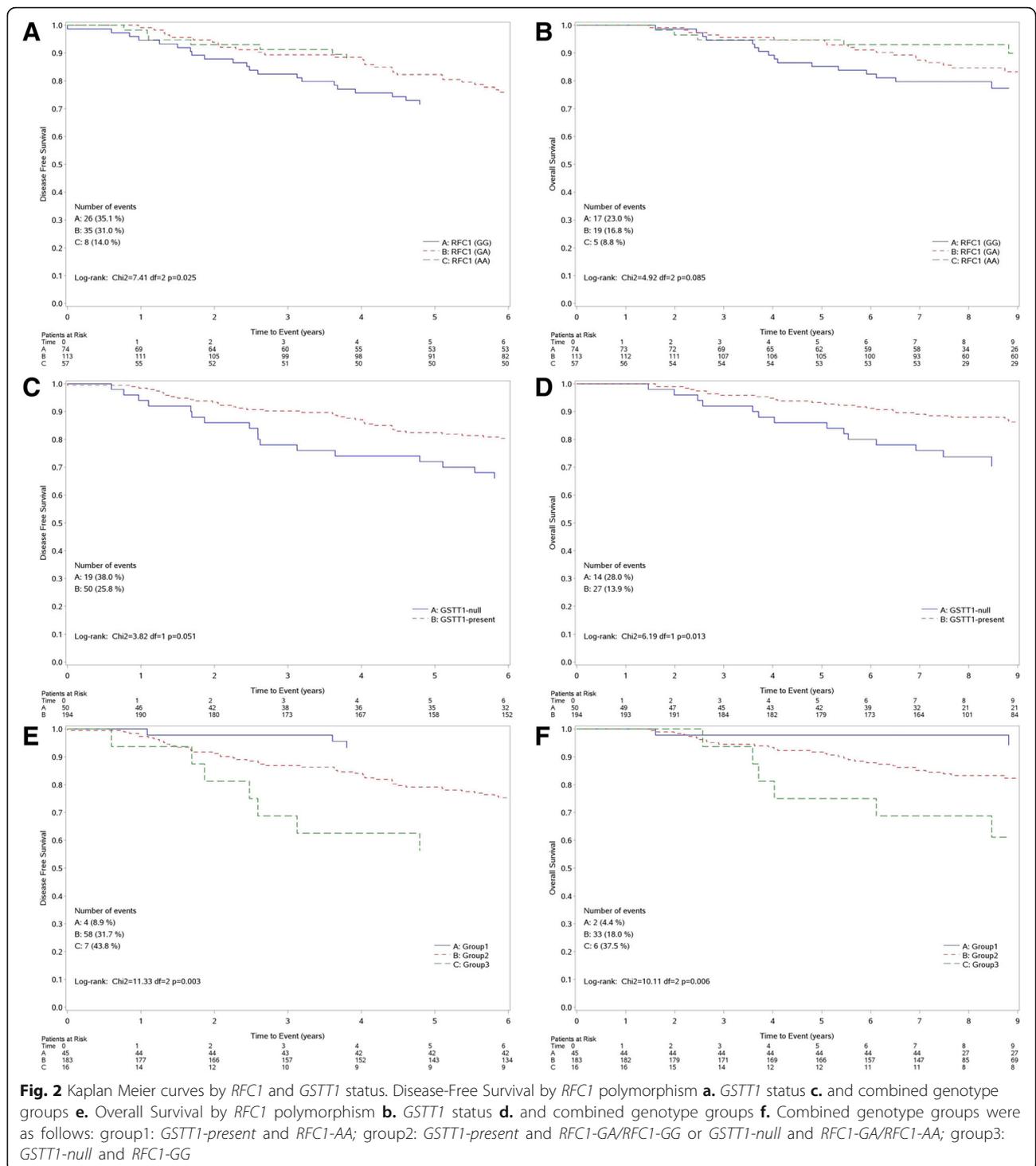
*group1: *GSTT1*-present and *RFC1*-AA

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

group3: *GSTT1*-null and *RFC1*-GG

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. There are a few studies on the role of GSTs isoenzymes on mortality in BC survivors drawn from community practice. The majority of these studies



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 *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 *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

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

Variable	Multivariate analysis* - DFS				Multivariate analysis*– OS			
	HR	95% CI		<i>p</i>	HR	95% CI		<i>p</i>
GSTT1 (nullvs. 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		1 (reference)				1 (reference)		
Group 2	3.93	1.42	10.86	0.008	3.87	0.92	16.20	0.064
Group 3	6.35	1.82	22.17	0.004	11.53	2.26	58.71	0.003

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

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 gene-dosage 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 Drozdik 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].

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 v.2.0. (DOC 55 kb)

Additional file 4: Table S4. Association among gene polymorphisms and risk of toxicity of any grade (grade1–2–3–4 vs 0). (DOC 83 kb)

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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, GN, 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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