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GSTT1, *GSTM1*, and *GSTP1* polymorphisms and chemotherapy response in locally advanced breast cancer

A.L. Oliveira[1], F.F.O. Rodrigues[1], R.E. Santos[1], T. Aoki[1], M.N. Rocha[2], C.A. Longui[2] and M.B. Melo[3]

[1]Departamento de Ginecologia e Obstetrícia,
Irmandade da Santa Casa de Misericórdia de São Paulo,
Faculdade de Ciências Médicas, São Paulo, SP, Brasil
[2]Laboratório de Medicina Molecular, Departamento de Fisiologia,
Irmandade da Santa Casa de Misericódia de São Paulo,
Faculdade de Ciências Médicas, São Paulo, SP, Brasil
[3]Laboratório de Genética Molecular Humana,
Centro de Biologia Molecular e Engenharia Genética,
Universidade Estadual de Campinas, Campinas, SP, Brasil

Corresponding author: A.L. Oliveira
E-mail: drandrel@terra.com.br

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ABSTRACT. The glutathione S-transferase (GST) family consists of phase II detoxification enzymes that catalyze the conjugation of toxic substances, such as chemotherapeutic agents, to glutathione. We examined whether *GSTT1/GSTT1*"null", *GSTM1/GSTM1*"null" and *GSTP1*Ile105Ile/*GSTP1*Ile105Val polymorphisms are associated with different response rates to neoadjuvant chemotherapy in the treatment of stage II and III breast cancer. Forty Brazilian women with invasive ductal adenocarcinoma of the breast submitted to neoadjuvant chemotherapy, using 5-fluorouracil, epirubicin and cyclophosphamide, were genotyped for the *GSTT1*, *GSTM1* and *GSTP1* genes. Clinical

response was assessed by RECIST criteria. Comparisons were made for the three genes alone and in pairs, as polymorphic and as wild-type combinations and polymorphic/wild-type combinations. We analyzed all possible combinations and their response rate. Patients with the *GSTT1/GSTP1*105Ile combination were found to have a significantly better response than *GSTT1*"null"*/GSTP1*105Val (P = 0.0209) and *GSTT1/GSTM1* (P = 0.0376) combinations. Analysis of all possible combinations showed the *GSTM1*"null" polymorphic genotype to be present in four, and the wild-type *GSTP1*105Ile in six of the combinations associated with the largest number of responding patients. We found that patients with the *GSTT1/GSTP1*105Ile wild-type combination had a significantly higher response rate to chemotherapy than patients with the respective polymorphic *GSTT1*"null"*/GSTP1*105Val combination or patients with the wild-type *GSTT1/GSTM1*. The six gene combinations associated with the largest number of responding patients were found to contain the wild-type *GSTP1*105Ile and the polymorphic-type *GSTM1*"null". These specific combinations were virtually absent in the combinations with few responding patients.

Key words: Breast cancer; Polymorphism; Genetic; Drug therapy; Glutathione transferase

INTRODUCTION

Breast cancer is the most prevalent gynecological neoplasm with the highest incidence in women worldwide (Parkin et al., 2005). Late diagnosis of the disease and mechanisms of resistance to chemotherapeutic drugs are the greatest obstacles in treating breast cancer (Leonessa and Clarke, 2003). Different responses to similar chemotherapy schemes in breast cancer patients, having the same biologic characteristics and stage, suggest different mechanisms of resistance to this therapy, some of which are induced by genetic pathways (Hayes and Pulford, 1995; O'Brien and Tew, 1996; Pakunlu et al., 2003; Rodrigues et al., 2008). Molecular and biochemical aspects of the cellular resistance process have been described, where the genes of the glutathione S-transferase (GST) family have been shown to play an important role (Hayes and Pulford, 1995; O'Brien and Tew, 1996; Burg and Mulder, 2002; L'Ecuyer et al., 2004). These mechanisms of cellular resistance include metabolic detoxification by the GST family (Schisselbauer et al., 1990; Tew, 1994; Hayes and Pulford, 1995). The *GSTP1*, *GSTM1* and *GSTT1* genes, which belong to the GST family, encode the most important phase II detoxifying proteins involved in the conjugation of substrates that are toxic to cancer cells, including chemotherapeutic agents used in breast cancer treatment such as anthracyclines (Arrick and Nathan, 1984; Russo and Mitchell, 1985; Townsend and Cowan, 1989; Shea et al., 1990; Tew, 1994; Adler et al., 1999; Burg and Mulder, 2002; Townsend and Tew, 2003a,b; Daly, 2003; McIlwain et al., 2006).

Of the different classes of genes found in the GST family, the *GSTT1* form located on chromosome 22 is the most studied in the *GSTT* class, while the *GSTM1* form located on chromosome 1 has been the most researched in the *GSTM* class. Both classes have polymorphic null forms (*GSTT1*"null" and *GSTM1*"null"), which do not have their two alleles and are therefore unable to encode the detoxifying enzymes (Cho et al., 2001; Townsend and Tew, 2003a,b; McIlwain et al., 2006). The *GSTP* class gene located on chromosome 11 has a wild-type form known as *GSTP1*A* (Ile105Ile/Ala113Ala) and two polymorphic forms known as *GSTP1*B* (Ile105Val/Ala113Ala) and *GSTP1*C* (Ile105Val/Ala113Val), which, akin to the "null" forms, do not have their two alleles and therefore are unable to encode the detoxifying enzymes. The *GSTM1*"null" and *GSTP1**B forms are also unable to inhibit their respective apoptosis pathways (ASK1 and JNK1) (Adler et al., 1999; Townsend and Tew, 2003a; Dang et al., 2005).

A number of different studies have yielded variable, inconsistent results regarding the relationship between the presence of polymorphic forms of GSTs and chemotherapeutic response (Riddick et al., 2005). Consequently, the aim of this study was to evaluate the clinical response to chemotherapy in patients with stage II and III invasive ductal adenocarcinoma of the breast in the presence of the *GSTT1/GSTT1*"null", *GSTM1/GSTM1*"null" and *GSTP1*Ile105Ile*/GSTP1*Ile105Val polymorphisms.

MATERIAL AND METHODS

Patients and samples

The study included 40 Brazilian patients diagnosed with stage II or III invasive ductal adenocarcinoma of the breast, who were consecutively referred to the chemotherapy outpatient unit of the Department of Obstetrics and Gynecology at Santa Casa de São Paulo Hospital, between February 2004 and December 2006. Inclusion criteria were as follows: i) a single, unilateral tumor without clinical or radiological signs of metastasis; ii) patient age between 35 and 70 years, and iii) absence of cardiomyopathy. Table 1 lists the patients and tumor characteristics.

The greater diameter of clinical margins of the patients' tumor was measured with pachymeter. It was tattooed before the first session of chemotherapy. Thirty days after the third session (just before surgery) we remeasured the major diameter using the same pachymeter and compared it to the tattooed initial margins. The clinical response to chemotherapy was evaluated according to RECIST criteria, adopted since 2002 for assessing chemotherapeutic response to solid tumor treatment. Individuals showing at least 30% tumor reduction are deemed responders and those showing less than 30% tumor reduction are considered to be non-responders (Therasse et al., 2000).

Chemotherapy treatment consisted of three neoadjuvant administrations at 21-day intervals, following a 5-fluorouracil, epirubicin and cyclophosphamide scheme (FEC therapy).

Multiplex polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism

The genotypes of the *GSTT1* and *GSTM1* genes were determined by co-amplification

Table 1. Demographic, clinical, pathologic, and genetic characteristics of the 40 patients enrolled in this study.

Name	Race	IM	FM	CS	APM	CR	*GSTT1*	*GSTM1*	*GSTP1*
ACFS	W	7	5	IIB	6	Nr	Wild	Wild	Ile105Val
AMJP	W	5	3	IIA	0.5	Pr	Wild	"Null"	Ile105Val
ALO	W	4.5	2	IIA	1.5	Pr	Wild	Wild	Ile105Ile
BAC	W	8	7	IIIA	3	Nr	Wild	"Null"	Ile105Val
CRN	B	5	3	IIIA	4	Pr	Wild	Wild	Ile105Ile
DAA	W	6	2	IIB	5	Pr	"Null"	Wild	Ile105Ile
DFM	B	7	5.5	IIIA	8	Nr	Wild	"Null"	Ile105Ile
EL	W	4	2	IIB	3	Pr	"Null"	Wild	Ile105Val
ERCM	B	5	3.5	IIB	5.5	Pr	Wild	"Null"	Ile105Ile
EMFS	W	4.5	2	IIB	2	Pr	Wild	Wild	Ile105Val
ESN	B	6	2	IIIA	3.5	Pr	"Null"	Wild	Ile105Ile
FGG	W	4.5	3.5	IIB	5	Nr	"Null"	Wild	Ile105Val
GMS	W	4	4	IIB	3	Nr	"Null"	Wild	Ile105Ile
GMS	B	5.5	2	IIIA	4	Pr	Wild	Wild	Ile105Ile
IMF	B	9	4.5	IIIA	6	Pr	Wild	Wild	Ile105Val
IAS	B	8	6	IIB	9	Nr	"Null"	"Null"	Ile105Ile
JSS	B	4	4	IIB	5	Nr	Wild	"Null"	Ile105Val
JP	W	4	3.5	IIA	2.2	Nr	Wild	Wild	Ile105Ile
LSB	W	8	6	IIB	6	Nr	"Null"	"Null"	Ile105Ile
MAEN	W	10	8	IIIA	4	Nr	Wild	Wild	Ile105Val
MCRA	W	5	2.5	IIB	2	Pr	"Null"	Wild	Ile105Ile
MAA	W	8	7	IIB	4.5	Nr	"Null"	"Null"	Ile105Ile
MFG	W	4	3	IIA	2	Nr	Wild	Wild	Ile105Val
MGS	B	3	1.5	IIB	2	Pr	Wild	Wild	Ile105Val
NMP	W	10	4.5	IIB	2	Pr	"Null"	Wild	Ile105Val
NOSS	W	8	2	IIIA	4	Pr	"Null"	Wild	Ile105Val
OFS	B	3.5	2	IIB	0.8	Pr	Wild	Wild	Ile105Val
RR	W	4	3	IIA	0	Nr	Wild	Wild	Ile105Val
SM	B	2.5	0	IIB	0	Cr	"Null"	Wild	Ile105Ile
TR	W	4	2.5	IIB	3	Pr	"Null"	Wild	Ile105Val
ZLJ	W	5.5	3	IIIA	0	Pr	Wild	Wild	Ile105Ile
MPA	B	5	6	IIB	3.5	Nr	"Null"	"Null"	Ile105Val
MNA	W	4	0	IIB	0	Cr	"Null"	"Null"	Ile105Ile
VOM	W	6	0	IIB	0	Cr	"Null"	Wild	Ile105Ile
MJSA	W	5	3	IIB	2.5	Pr	Wild	"Null"	Ile105Val
FPS	W	6	7	IIIA	4.5	Nr	Wild	Wild	Ile105Ile
CMS	W	2.5	1.5	IIA	2	Pr	Wild	"Null"	Ile105Val
EBSP	B	8	6	IIIA	2	Nr	"Null"	"Null"	Ile105Val
DFS	B	15	8.5	IIIA	7.5	Pr	"Null"	"Null"	Ile105Val
NCC	W	4	2	IIIA	0.5	Pr	Wild	Wild	Ile105Val
Mean	Na	5.77	3.37	Na	3.43	Na			
Median	Na	5	3	Na	3	Na			
SD	Na	2.45	1.86	Na	2.22	Na			

Nr = no response; Cr = complete response; Pr = partial response; Na = not applicable. Correlation of 40 patients submitted to neoadjuvant chemotherapy by race (W = Write; B = Black), initial clinical measurement of tumor (IM) and final clinical measurement of tumor (FM) in centimeters, clinical state (CS), anatomo-pathologic measurement (APM), clinical response (CR), and alleles identified.

using multiplex PCR, with the β-globin gene as an internal control, as described by Wilson et al. (2000). The primers used were 5'- CTT CCT TAC TGG TCC TCA CAT CTC -3' (sense) and 5'- TCA CCG GAT CAT GGC CAG CA -3' (anti-sense) for the *GSTT1* gene, resulting in a 480-bp fragment; 5'- GAA CTC CCT GAA AAG CTA AAG C -3' (sense) and 5'- CTT GGG CTC AAA TAT ACG GTG G -3' (anti-sense) for the *GSTM1* gene, resulting in a 215-bp fragment, and 5'- GAA GAG CCA AGG ACA GGT AC -3' (sense) and 5'- CAA CTT CAT CCA CGT TCA CC -3' (anti-sense) for the β-globin gene, resulting in a 268-bp fragment. The *GSTT1*"null" and *GSTM1*"null" variant forms were defined by the absence of the 480- and 215-bp fragments, respectively.

GSTP1 gene-related products were obtained by PCR-restriction fragment length polymorphism, with 5'- ACC CCA GGG CTC TAT GGG AA -3' (sense) and 5'- TGA GGG CAC AAG AAG CCC CT -3' (anti-sense) primer, which generates a 176-bp product. The amplified product was then submitted to digestion with the *Alw*26I enzyme (New England Biolabs, Ipswich, MA, USA), yielding 91- and 86-bp fragments for the *GSTP1*105Val homozygous genotypes, 176-, 91- and 86-bp fragments for the *GSTP1*Ile105Val heterozygous genotypes, and a 176-bp fragment for the wild-type *GSTP1*105Ile (Wilson et al., 2000).

Statistical analysis

The first part of the analysis consisted in comparing clinical response with any isolated gene (wild-type and polymorphic) and all possible combinations of genes in pairs (wild-type and/or polymorphic). The hypothesis test was used, where the null hypothesis was when there is no association between clinical response (RECIST) and the isolated or combined gene, i.e., these variables are independent. Cramer correlations and a non-parametric test of the chi-square test were used for categorical variables. The maximum value of this test is 1 and the minimum is 0. When the variables are independent, the value approaches 0. The data were input to the Statistical Program for the Social Sciences (SPSS, version 13.0, SPSS Inc., Chicago, IL, USA).

In the second part of the analysis, the test of equal proportions was used to analyze the variable clinical response (RECIST) between all possible combinations of genes in pairs (wild-type and/or polymorphic). We used the Microsoft Excel® software. $P < 0.05$ was considered to be statistically significant for both tests.

RESULTS

Among the 40 patients studied, 24 (60%) were considered to be responders and 16 (40%) non-responders according to RECIST criteria. Twenty-two patients (55%) showed the *GSTT1* genotype, 18 (45%) the *GSTT1*"null" genotype; 26 (65%) the *GSTM1* genotype, 14 (35%) the *GSTM1*"null" genotype, 18 (45%) the *GSTP1* genotype, and 22 (55%) the *GSTP1*Ile105Val genotype.

Comparison of RECIST data for the genotype (individual genes) revealed no statistically significant difference in response between patients harboring either the polymorphic or the wild-type genotype (Table 2).

Table 2. Frequencies of the wild-type and polymorphic-type *GSTT1*, *GSTM1*, and *GSTP1* genes according to the RECIST classification.

RECIST	*GSTT1*		P	*GSTM1*		P	*GSTP1*		P
	"Null" (%)	Wild-type (%)		"Null" (%)	Wild-type (%)		Ile105Val (%)	Ile105Ile (%)	
R	9 (22.5)	15 (37.5)	0.622172	11 (27.5)	13 (32.5)	0.068842	11 (27.5)	13 (32.5)	0.143526
NR	9 (22.5)	7 (17.5)		3 (7.5)	13 (32.5)		11 (27.5)	5 (12.5)	
Total	18	22		14	26		22	18	

R = responders; NR = non-responders.

Comparison of data on the wild-type combinations against the respective polymorphic-type combinations showed that eight patients (100%) with the *GSTT1*/*GSTP1*105Ile combination responded to treatment as did four patients (50%) with the *GSTT1/GSTP1*105Ile"null" combination, according to RECIST criteria ($P = 0.0209$) (Table 3). When wild-type geno-

type combinations were compared with each other, and polymorphic genotype combinations were also compared with each other, according to the "responder" RECIST variable, nine patients (60%) who had the *GSTT1*/*GSTM1* combination and eight patients (100%) who had the *GSTT1*/*GSTP1*105Ile combination were found to be responders (P = 0.0376) (Table 4). Finally, two-by-two analysis of all possible combinations of genes according to the number of responders, showed the polymorphic-type *GSTM1*"null" to be present in four (66.6%), and the wild-type *GSTP1*105Ile in three (50%) of the six combinations associated with the largest number of responders. In addition, the polymorphic-type *GSTM1*"null" was not present in any of the six combinations in patients with poor response to chemotherapy, while the wild-type *GSTP1*105Ile was only present in one of these six combinations (17%) (Table 5).

Table 3. Correlations between the numbers of responding patients according to the RECIST classification as to polymorphic- and their respective wild-type combinations of the *GSTT1*, *GSTM1*, and *GSTP1* genes when combined in pairs.

Group 1	AN	R	Group 2	AN	R	P
GSTT1"null"/*GSTM1*"null"	7	5	*GSTT1/GSTM1*	15	9	0.6037
GSTT1"null"/*GSTP1*Ile105Val	8	4	*GSTT1/GSTP1*Ile105Ile	8	8	0.0209
GSTM1"null"/*GSTP1*Ile105Val	8	7	*GSTM1/GSTP1*Ile105Ile	12	9	0.4936

AN = absolute number of cases; R = number of responders.

Table 4. Correlations between all possible polymorphic type combinations and between all possible wild-type combinations according to the number of responding patients based on the RECIST classification.

Group 1	N	R (%)	Group 2	N	R (%)	P
GSTT1"null"/*GSTM1*"null"	7	5 (71.4)	*GSTT1*"null"/*GSTP1*Ile105Val	8	4 (50.0)	0.3960
GSTT1/GSTM1	15	9 (60.0)	*GSTT1/GSTP1*Ile105Ile	8	8 (100.0)	0.0376
GSTT1"null"/*GSTP1*Ile105Val	8	4 (50.0)	*GSTM1*"null"/*GSTP1*Ile105Val	8	7 (87.5)	0.1052
*GSTT1/GSTP1*Ile105Ile	8	8 (100.0)	*GSTM1/GSTP1*Ile105Ile	12	9 (75.0)	0.1260
GSTT1"null"/*GSTM1*"null"	7	5 (71.4)	*GSTM1*"null"/*GSTP1*Ile105Val	8	7 (87.5)	0.4412
GSTT1/GSTM1	15	9 (60.0)	*GSTM1/GSTP1*Ile105Ile	12	9 (75.0)	0.4122

N = number of cases; R = number of responders.

Table 5. Percentages of responders according to combinations of genes in pairs in decreasing order of frequency.

Gene combination	N	R	% of responders
*GSTT1/GSTP1*Ile105Ile	8	8	100
GSTM1"null"/*GSTP1*Ile105Val	8	7	87.5
GSTT1/GSTM1"null"	7	6	85.7
*GSTM1/GSTP1*Ile105Ile	12	9	75
GSTT1"null"/*GSTM1*"null"	7	5	71.4
GSTM1"null"/*GSTP1*Ile105Ile	6	4	66.7
GSTT1/GSTM1	15	9	60
*GSTT1/GSTP1*Ile105Val	14	7	50
GSTT1"null"/*GSTP1*Ile105Val	8	4	50
GSTT1"null"/*GSTP1*Ile105ILe	10	5	50
GSTM1/GSTT1"null"	11	4	36.4
*GSTM1/GSTP1*Ile105Val	14	4	28.6

N = absolute number of patients observed for that combination; R = number of responders for that combination.

DISCUSSION

Since the first evidence that glutathione S-transferases are involved in response to chemotherapy (Schisselbauer et al., 1990; Tew, 1994; Hayes and Pulford, 1995), the results of various subsequent studies have shown the inconsistent nature of this relationship (Riddick et al., 2005). In the present study, we investigated the possible association between polymorphisms in the *GSTT1*, *GSTM1* and *GSTP1* genes and response to chemotherapy in Brazilian women with stage II and III breast cancer submitted to neoadjuvant FEC therapy. When analyzed individually, none of the genes showed a statistically significant relationship with response to chemotherapy according to the Cramer test. Similar results were found by other authors (Moscow et al., 1989; Leyland-Jones et al., 1991; Alpert et al., 1997; Morrow et al., 1998; Allan et al., 2001; Yang et al., 2005). Lizard-Nacol et al., in 1999, studied 92 patients with breast cancer treated with neoadjuvant chemotherapy and did not find any association of clinical reduction of tumor size among patients with the wild-type or polymorphic form of *GSTM1* genes. Yang et al., in 2005, studied 1602 women with breast cancer submitted to adjuvant chemotherapy and also did not observe a statistical difference in response between wild-type and polymorphic forms of the *GSTM1* and *GSTT1* genes. Allan et al., in 2001, studied the presence of acute myeloid leukemia in patients submitted to chemotherapy and did not observe a greater incidence of that disease when compared to patients with wild-type or polymorphic forms of the *GSTT1* and *GSTM1* genes.

However, when we combined the genes in pairs, the variant-type *GSTM1*"null" was present in four and the wild-type *GSTP1*105Ile in six of the combinations with the largest percentage of responders. Furthermore, in the six combinations with the lowest percentage of responders, the polymorphic-type *GSTM1*"null" was not present, while the wild-type *GSTP1*105Ile was present in only one combination. These results were also found by other authors when the *GSTM1* gene was analyzed individually (Hamada et al., 1994; Morrow et al., 1998; O'Brien et al., 2000; Allan et al., 2001; Naoe et al., 2002; Huang et al., 2003; Yang et al., 2005), but were not replicated in the case of the *GSTP1* gene. When the "responder" variable in the RECIST classification was used in comparing wild-type combinations with their respective variant polymorphic combinations, eight patients (100%) with the *GSTT1/GSTP1*105Ile combination and four patients (50%) with the *GSTT1*"null"/*GSTP1*105Val combination proved to be responders according to an analysis based on the test of equal proportions ($P = 0.0209$). When the wild-type combinations were compared with each other, and the variant-type combinations were also compared with each other, nine patients (60%) with the *GSTT1/GSTM1* combination and eight patients (100%) with the *GSTT1/GSTP1*105Ile combination proved to be responders based on the test of equal proportions ($P = 0.0376$). These results suggest that the wild-type combination *GSTT1/GSTP1*105Ile was more sensitive to the chemotherapy used in this study. Our results are thus in contrast with those of other authors (Dirven, 1994; Tew, 1994; Howells et al., 2001; Paumi et al., 2001; Naoe et al., 2002; Khedhaier et al., 2003; Townsend and Tew, 2003a,b) who either found that the wild-types were more resistant than polymorphic forms or found no significant differences. Ambrosone et al., in 2001, conducted a retrospective study of breast cancer patients submitted to chemotherapy and noted greater recurrence-free survival among women who showed the polymorphic form of the *GSTT1* and *GSTM1* genes when compared to that with wild-type forms, thus in contrast with findings of other authors. Allan et al., in 2001, noted that acute myeloid leukemia was significantly more severe among patients who had the polymorphic

form of the *GSTP1* gene than patients with the wild-type form of the gene. Khedhaier et al., in 2003, studied 309 breast cancer patients submitted to neoadjuvant chemotherapy and noted greater recurrence-free survival among patients who had the polymorphic form of the *GSTT1* and *GSTM1* genes, than patients with the wild form of genes. Yang et al., in 2005, studied 1602 breast cancer patients submitted to adjuvant chemotherapy and also noted greater recurrence-free survival among women with the polymorphic form of the *GSTP1* gene.

As the wild-type genes are able to produce detoxifying enzymes that act in the metabolism of chemotherapeutic drugs and do take part in the inhibition of the JNK1 apoptosis pathway (Adler et al., 1999; Dang et al., 2005; McIlwain et al., 2006), our results were unexpected. Maybe a highly mixed racial origin represents a unique response to chemotherapy by Brazilian women, or other genetic factors related or not to the metabolism of drugs are involved. When interpreting these results, it should be borne in mind that this was a prospective study, and a larger sample is needed to guarantee statistical validity. Our results are drawn from an initial study, and further studies are warranted for confirmation.

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