



GSTM1 polymorphism in Oncotype DX assay is a potential predictive factor for taxane-based neoadjuvant chemotherapy in estrogen receptor-positive Chinese breast cancer patients

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Background: Investigations of the 21-gene Oncotype DX assay in neoadjuvant settings for breast cancer patients are currently underdeveloped. It is still unclear whether any individual gene of the 16 cancer-related genes can be used to predict response to taxane-based neoadjuvant chemotherapy.

Methods: Here, we performed TaqMan RT-PCR reactions to profile the expression patterns of these genes in 66 Chinese ER-positive breast cancer patients' tumor tissues who underwent taxane-based neoadjuvant chemotherapies. Only GSTM1 has distinct non-expression/expression polymorphism, and we observed an almost 2.5-fold increment in the possibility of achieving pathological complete response (pCR) was defined as no histological evidence of residual invasive cancer both in the breast and in the axilla. in GSTM1 non-expression patients. We then analyzed the expression levels of 4 main GST isozymes (GSTA1, GSTT1, GSTP1 and GSTT1) and docetaxel cytotoxicity in various breast cancer cell lines (MCF7, MDA-MB-231, BT474, and SKBR3). Furthermore, we knocked down 4 GST isozymes with siRNA against each isoform in resistant MDA-MB-231. We then observed the changes of docetaxel sensitivity through WST-1 assay, in addition to apoptosis by Hoechst stain and TUNEL assay.

Results: Docetaxel IC₅₀ values ranging from 33.0 nM (MDA-MB-231) to 7.20 μM (resistant MDA-MB-231) revealed a close correlation with the expression levels of 4 main GST isozymes. Significantly, depletion of GSTM1 and GSTA1 but not GSTP1 nor GSTT1, suppressed cell viability by 174% and 155% respectively, and was able to induce obvious apoptosis in resistant MDA-MB-132.

Conclusions: Taken together, our results suggest that GSTM1 polymorphism could be useful in the prediction of taxane-based neoadjuvant chemotherapy in ER-positive Chinese breast cancer patients, and that GSTM1 and GSTA1 expression are linked to docetaxel resistance.

Keywords: Breast cancer; GSTM1 polymorphism; taxane; neoadjuvant chemotherapy; estrogen receptor-positive (ER-positive)

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Introduction

With the development and application of molecular biology, breast cancer can be classified into levels of malignant biological behavior via multiple gene expression detections into molecular subtypes. The 21-gene Oncotype Dx was reported as one of the multigene assays providing significant predictive power to breast cancer prognosis and grading the patients' benefit from chemotherapy. The recurrence score has been demonstrated to be effective and accurate in ER-positive patients in various clinical studies (1-3). For neoadjuvant settings, however, the prediction value of RS for response has not been adequately investigated, and it is still unclear whether any individual gene of the 16 cancer related genes out of the 21-gene panel can be used to predict response to taxane-based neoadjuvant chemotherapy.

GSTM1 in Oncotype DX assay is one of the members in the glutathione-S-transferase (GST) family that can catalyze the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds for Phase II detoxification, including antineoplastic agents (4-6). Human GSTs are categorized into 6 classes (α , μ , π , σ , ω , θ , and ζ) (4). However, the role of GSTs in the protection of cells against taxane remains unclear despite various studies (5-10).

This study aimed to investigate whether GSTM1 expression levels could act as a predictive marker for responses to taxane-based chemotherapy in breast cancer patients. First, we measured GSTM1 expression levels from tumor tissues and tested their potential links to chemotherapy responses within a cohort of Chinese breast cancer patients. Second, we set up cell models to further explore the possible molecular mechanisms to accredit such correlations biologically. By such a two-step designed study, the hypothesis was adequately tested and the results were solid to suggest the predictive role of GSTM1.

Methods

Patients and therapy response

The study retrospectively included 66 Chinese patients diagnosed with stage I to III invasive breast cancer, who were submitted to the taxane-based neoadjuvant chemotherapies at the Department of Breast Cancer in Guangdong Provincial People's Hospital, Guangzhou, China, between February 2012 and December 2014. 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; (III) estrogen- and/or progesterone-receptor-positive (ER⁺/PgR⁺); and (IV) previously stored fresh frozen tumor tissues before treatment. All patients underwent 4 cycles of taxane-based regimens at 21-day intervals before surgery. For HER2-positive patients, trastuzumab was incorporated concurrently with chemotherapy. The clinical response to chemotherapy was evaluated according to RECIST criteria. Pathological complete response (pCR) was defined as no histological evidence of residual invasive cancer both in the breast and in the axilla.

Patient gene profiling in by qRT-PCR

Tumor tissues in 66 enrolled Chinese ER-positive breast cancer patients were subjected to TaqMan RT-PCR reactions, performed exactly as described previously for the assay for the 21-gene panel (3,11). Expression levels were normalized relative to the set of 5 reference genes. All reactions were repeated 3 times. The 16 cancer-related genes were Ki67, STK15, survivin, cyclin B1, MYBL2, GRB7, HER2, ER, PgR, BCL2, SCUBE2, MMP11, CTSL2, GSTM1, CD68, and BAG1, and the 5 reference genes were ACTB, GAPDH, PLPO, GUS, and TFRC.

Statistics analysis

The analysis consisted in comparing clinical response with isolated genes. Data were input into the Statistical Program for the Social Sciences (SPSS, version 13.0, SPSS Inc., Chicago, IL, USA) and analyzed for statistical significance using the univariate/multivariate logistic regression analysis as well as the chi-square test. Overall significance of each gene expression was determined by calculating the odds ratios (OR). The odds ratio was expressed with a corresponding 95% confidence interval (CI). $P < 0.05$ was considered to be statistically significant.

Cell culture and experimental reagents

Human breast cancer cell lines MDA-MB-231, docetaxel (TXT)-resistant SKBR3, TXT-resistant BT474, TXT-resistant MCF7, and TXT-resistant MDA-MB-231 were obtained from Dr. Wang HH. All of these cell lines were cultured according to the ATCC instructions. siRNA and LipofectamineTM 2000 were purchased from Thermo Scientific Dharmacon[®]. SYBR Green Real-time PCR Master Mix was purchased from Toyobo (Osaka, Japan).

RevertAid™ First Strand cDNA Synthesis kit was obtained from MBI (Fermentas, Hanover, MD, USA). Docetaxel (Aventis Pharmaceuticals, Bridgewater, NJ, USA) was stored at a concentration of 10 mg/mL (12.6 mM) in 13% w/w ethanol at 4 °C and diluted in medium before use. Cell Counting Kit-8 was obtained from Roche. TUNEL assay was performed by use of In Situ Cell Death Detection Kit (Fluorescein, Roche, Switzerland). DAPI (4,6-diamidino-2-phenylindole) was purchased from Beyotime (Shanghai, China). Hoechst 33342 was purchased from Sigma-Aldrich China (Shanghai, China).

Cell fractionation and Western blot analysis

The expression of GST isozymes in 5 breast cancer cells was investigated as described previously (12). Cytosolic proteins (20 µg) were separated by 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membrane. The membranes were blocked overnight followed by incubation with the mouse monoclonal primary antibodies anti-GSTA1, M1, P1, or T1 1:500 (Sigma), and mouse monoclonal primary antibody anti-β-actin 1:2,000 (Sigma). Then, membranes were incubated with anti-mouse peroxidase-conjugated secondary antibodies 1:5,000 for GSTs and β-actin. Specific proteins were visualized using X-ray films after incubation with enhanced chemiluminescence reagents (Amersham Biosciences, Corston, UK).

Real-time RT-PCR

Extraction of total cellular RNA was carried out using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and expression was analyzed by RT-PCR using standard procedures as described previously (13). The primer sequences for β-actin, GSTA1, GSTP1, GSTM1, GSTT1, which were purchased from Santa Cruz, are available on request. PCR products were run on agarose gels supplemented with ethidium bromide and visualized by ultraviolet illumination. Band intensities were quantified by densitometry analysis using Bio1D software (Vilber Lourmat, Marne La Vallée, France).

Knockdown of GSTA1, GSTP1, GSM1, and GSTT1 with siRNA oligos

GSTA1si, GSTP1si, GSTM1si, and GSTT1si stock solutions (20 µM) were diluted with 1× siRNA buffer

to form 5 µM solutions. Lipofectamine RNAiMAX transfection reagent was mixed with 5 µM siGST incubated for 20 min. The final concentration of siRNA was 50 nM. The cells were incubated with siRNA for 24 and 48 h for subsequent experiments.

Measurement of docetaxel sensitivity

Cells (1×10^4) were seeded into a flat bottomed 96-well plate and incubated with various concentrations of docetaxel for 24 h at 37 °C. Cell viability was determined by the -(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl) (2H)tetrazolium monosodium salt (WST) assay using Cell Counting Kit-8 according to the manufacturer's instructions, and the percentage of cell death was calculated as described previously (14). To measure docetaxel sensitivity after GST isozyme knockdown, docetaxel was added to the culture medium at 24 h post-transfection with siRNA treatment.

Hoechst staining

Following treatment with 100 µM docetaxel for 24 h, the MDA-MB-231 cells were harvested by centrifugation at 800 rpm for 5 min, washed with PBS, and fixed with methanol acetic acid for 10 min. Fixed cells were washed with PBS followed by staining with 200 µM Hoechst 3325 (1 mg/mL) at room temperature in the dark for 5 min. Hoechst 33258 stained cells were examined and immediately photographed with a fluorescence microscope with an excitation wavelength of 330–380 nm.

TUNEL assay

TUNEL assay was performed as previously described (15). Briefly, following treatment with 100 µM docetaxel for 24 h, the MDA-MB-231 cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed 3 times with PBS, and then stained with TUNEL following manufacturer's instructions or with DAPI (0.5 µg/mL) for 10 min at room temperature. Images of the cells were then acquired using fluorescence microscope.

Results

GSTM1 mRNA expression correlates with taxane-neoadjuvant therapy response

The 66 patients, with an average age 51.7 years, were ER

Table 1 Distribution of HER2 and GSTM1 genotypes in pathological complete response (pCR) and non-pCR patients who received taxane-based neoadjuvant chemotherapy

Genotype	pCR (n=25)	Non-pCR (n=41)	Odds ratio (95% CI)	P
GSTM1			0.349 (0.121–1.009)	0.048
Expression	13	31		
No-expression	12	10		
HER2			3.945 (1.362–11.431)	0.01
Positive	14	10		
Negative	11	31		

positive by IHC, and 36.4% (n=24) were HER2 positive. Twenty-five patients achieved pCR, 14 of whom were HER2-positive patients, and 11 of whom were HER2-negative patients (58.3% vs. 26.2%, chi-square value =6.706, odds ratio =3.945, 95% CI: 1.362–11.431, P=0.01). Analysis of the results of patients is summarized in *Table 1*. It was noted that only GSTM1 has distinct non-expression/expression polymorphism, with 33.3% (n=22) non-expression vs. 66.7% expression; this polymorphism does not correlate to any other assayed genes in this study. Univariate analysis showed that the expression level of HER2 and GSTM1 non-expression (rather than expression level) correlate to pCR at the significance level 0.05 (GSTM1: odds ratio 0.349 and P=0.048; HER2: odds ratio 3.945 and P=0.010; GRB7: odds ratio 1.0 and P=0.043; cyclin B: odds ratio 1.0 and P=0.024). We further included HER2 and GSTM1 in multivariate logistic regression analysis. However, none of these 2 genes showed statistical significance for pCR.

Differential expression of GST isoenzymes

The expression of GSTM1, GSTA1, GSTP1, and GSTT1 varied across the 5 breast cancer cell lines (*Figure 1A*). Our results showed TXT-resistant MDA-MB-231 expresses all the 4 GST isozymes at higher levels compared to other lines. TXT-resistant MCF7 cells express GSTM1 at lower levels; GSTT1 levels are barely detectable whereas GSTA1 and GSTP1 were not detected. SKBR3 and BT474, the HER2-positive cells, express GSTM1 at lower levels compared to TXT-resistant MDA-MB-231 and also express GSTT1. Interestingly, our results showed a significant increase in GSTM1, GSTP1, GSTA1, and GSTT1 expression, measured at mRNA levels and protein levels (*Figure 1B,C*), in TXT-resistant MDA-MB-231 cells

compared to non-resistant MDA-MB-231 cells.

Docetaxel sensitivity in human breast cancer cell lines with differential expression of GST isozymes

We next tested if variation in sensitivity to docetaxel of human breast cancer cells could be correlated with expression of specific GST isoenzymes utilizing CCK8 assay for the analysis of cytotoxicity (*Figure 2*). Our results revealed a wide range of sensitivity among the 4 TXT-resistant cell lines for docetaxel IC50 values. Interestingly, taxane-resistant MDA-MB-231, with the highest expression of 4 GST isozymes, showed the highest IC50 for docetaxel (~7.3 μ M). Resistant MCF7, which expresses relatively lower levels of GSTM1, was more sensitive to docetaxel (IC50: ~3.8 μ M). Cell lines showing relatively more resistance to docetaxel (SKBR3 and BT474) were associated with higher GSTM1 levels and GSTT1 than the most sensitive cell lines (MCF-7). Moreover, normal MDA-MB-231 (IC50: ~33.0 nM) cells express significantly lower levels GST isozymes GSTM1, GSTP1, GSTA1, and GSTT1 (*Figure 1B,C*) compared to docetaxel resistant MDA-MB-231. Taken together, the breast cancer tumor cells expressing higher levels of GST isoenzyme(s) were more resistant to docetaxel, suggesting the levels of the GST isoenzymes correlate with sensitivity to docetaxel.

Depletion of GST isozymes in taxane-resistant MDA-MB-231 cells

Since our results showed taxane-resistant MDA-MB-231 cells express all 4 GST isozymes, we utilized these cells to study the individual contribution of each isozyme to docetaxel resistance. We used 2 different siRNA oligos targeting each of the 4 GST isozymes in order to achieve specific knockdown of their mRNA. By analyzing both protein lysates with enzyme specific antibodies in western blotting and mRNA message quantified by RT-PCR, we found a significant depletion of 4 isozymes 24 h post siRNA treatment (*Figure 3*). For docetaxel resistance assays, siRNA oligos that achieved maximum knockdown (si-GSTA1 02, si-GSTP1 02, si-GSTM1 01, and si-GSTT1 02) of each isozyme in resistant-MDA-MB-231 were selected.

GSTM1 and GSTA1 depletion increased docetaxel sensitivity of taxane-resistant MDA-MB-231 cells

Taxane-resistant MDA-MB-231 were most resistant to

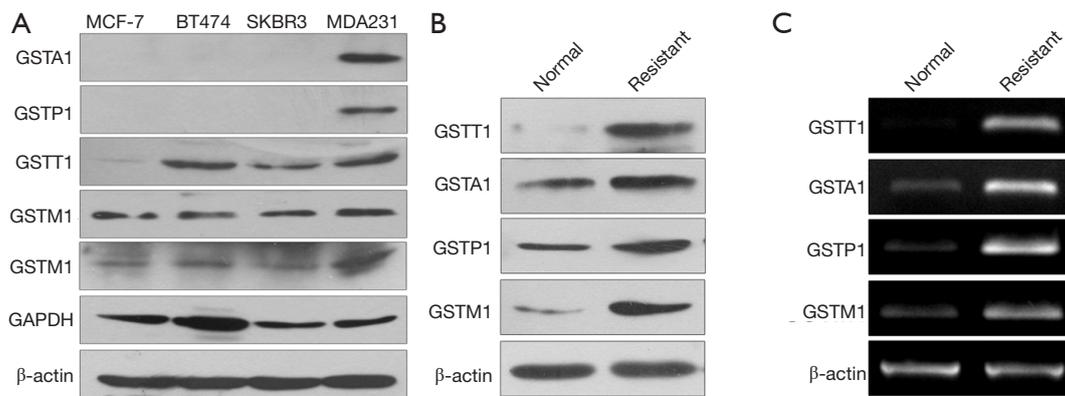


Figure 1 Expression level of GSTA1, GSTM1, GSTP1, and GSTT1 proteins and mRNAs in 5 breast cancer cell lines. (A) Western blot analysis of GSTA1, GSTM1, GSTP1, and GSTT1 expression in docetaxel (TXT)-resistant MCF-7, TXT-resistant SKBR3, TXT-resistant BT-474, and TXT-resistant MDA-MB-231; (B) Western blot analysis of GSTA1, GSTM1, GSTP1, and GSTT1 expression in normal MDA-MB-231 and TXT-resistant MDA-MB-231; (C) RT-PCR analysis of GSTA1, GSTM1, GSTP1, and GSTT1 expression in normal MDA-MB-231 and TXT-resistant MDA-MB-231. Results are from 3 representative, independent experiments.

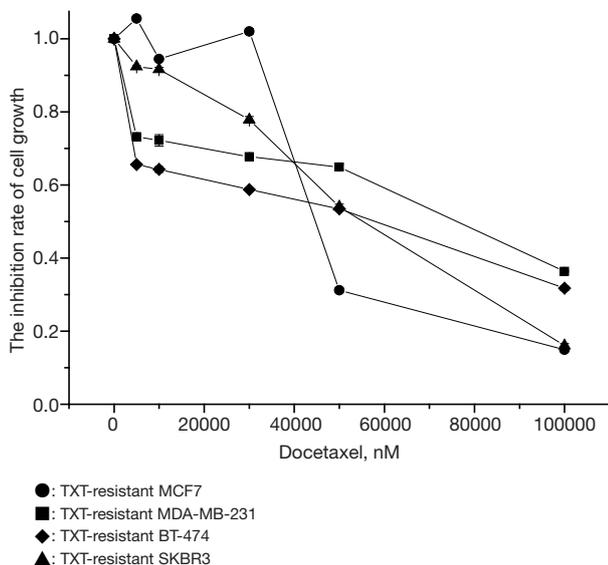


Figure 2 Docetaxel sensitivity of 4 breast cancer cell lines. Cells plated in 96-well plates (1×10^4 cells/well/100 μ L of medium) were treated with various concentrations of docetaxel (0–100 μ M) for 24 h. Cell death was determined by the WST assay as described under “Experimental Procedures”. Data are expressed as the mean S.D. of triplicate samples. Similar results were obtained in 3 independent experiments. Sensitivity of MDA-MB-231, docetaxel (TXT)-resistant MDA-MB-231, TXT-resistant MCF7, TXT-resistant BT474, and TXT-resistant SKBR3 cells to docetaxel.

docetaxel which, interestingly expressed higher levels of all 4 GST isozymes compared with normal MDA-MB-231 cells (Figure 3). In order to identify the role of each isozyme in docetaxel resistance, we analyzed the sensitivity to docetaxel using WST assay over a period of 24 h (Figure 4) after knocking down each isozyme (Figure 3) in MDA-MB-231 cells. Our results showed that the depletion of GSTA1 (oligo: si-GSTA1 02) enhanced the docetaxel sensitivity by 138% in resistant MDA-MB-231 cells compared with scrambled siRNA-treated or cells with no siRNA treatment (i.e., decrease in IC₅₀ value by 0.5-fold, from $\sim 7.3 \mu$ M to $\sim 4.7 \mu$ M). GSTM1 knockdown (oligo: si-GSTM1 02) resulted in a docetaxel IC₅₀ of $\sim 4.2 \mu$ M compared to a control cell IC₅₀ of $\sim 2 \mu$ M. In notable contrast, GSTP1 and GSTT1 depletion had no effect on docetaxel sensitivity compared to controls (IC₅₀ $\sim 0.9 \mu$ M for both). Collectively, the results demonstrate that GSTA1 and GSTM1 levels, but not GSTP1 or GSTT1 levels, are inversely correlated with docetaxel-induced cell cytotoxicity.

Evaluation of apoptosis by Hoechst staining in GSTA1 and GSTM1-depleted resistant-MDA-MB-231 cells

Apoptosis is one of the principal mechanisms of docetaxel induced cytotoxicity. We evaluated the apoptotic morphology in GSTA1- and GSTM1-depleted resistant-MDA-MB-231 cells after treatment with docetaxel

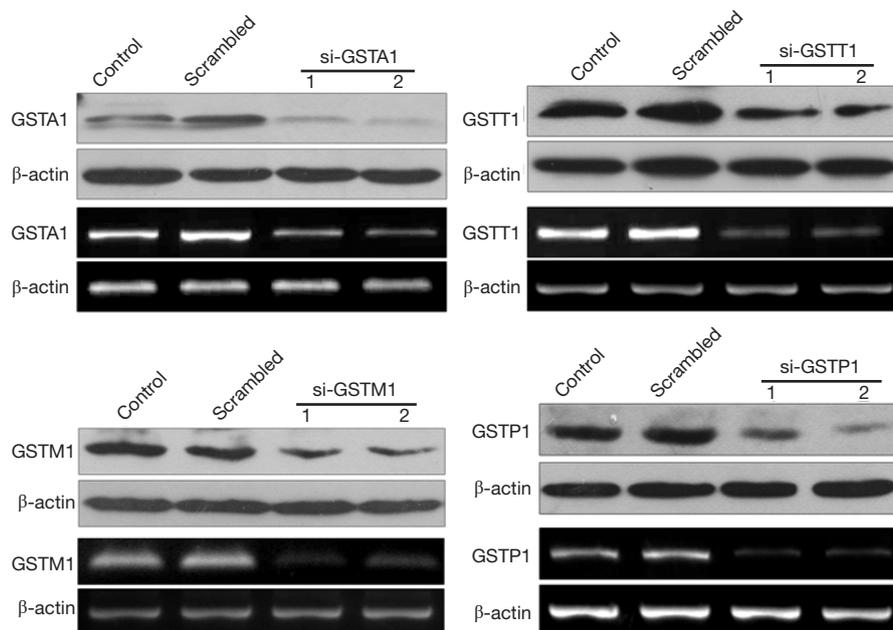


Figure 3 Sequence-specific knockdown of glutathione-S-transferase (GST) isozymes on docetaxel (TXT)-resistant MDA-MB-231 cells. Specific inhibition of each GST isozymes protein by 2 different siRNAs (1,2). Fifty nM GST siRNA, or 10 µg/mL of agent alone (control, Ctrl), or 50 nM scrambled siRNA (negative control, NC) was transfected into TXT-resistant MDA-MB-231 cells, at 48 h after transfection, total protein was extracted, and GST isozymes and β-actin protein levels were quantified by western blot analysis (upper part of each panel). At 24 h after transfection, GST isozymes and β-actin mRNA levels were analyzed by RT-PCR (lower part of each panel).

(100 µM) for 24 h using membrane-permeable blue Hoechst 33258 (Figure 5). Our results showed nuclei of docetaxel-treated GSTA1- and GSTM1-depleted cells appeared hypercondensed (brightly stained). The number of apoptotic nuclei containing nuclear fragmentation and condensed chromatin induced by docetaxel increased significantly as the result of GSTA1 and GSTM1 knockdown. Similar treatment in control cultures showed nuclei with regular contours that were round and large in size. Cells with smaller nuclei and condensed chromatin were rarely seen. Scrambled siRNA treatment did not change the nuclear morphology of docetaxel-treated cells.

Evaluation of apoptosis by TUNEL staining in GSTA1- and GSTM1-depleted resistant-MDA-MB-231 cells

We next used TUNEL assay for quantification of the docetaxel-induced apoptosis in GSTM1 and GSTA1 knock-down cells. Consistent with Hoechst staining results, TUNEL analysis of GSTM1 and GSTA1 knock-down cells after treatment with docetaxel (100 µM) for 24 h resulted in significant TUNEL-positive nuclei (green spots) resulting

from staining of nicked DNA by FL-dUTP (Figure 6). DAPI staining of GSTM1 and GSTA1 knock-down cells showed fragmented nuclei and enhanced DAPI fluorescence by densely stained nuclear granular bodies (apoptotic bodies). Control cells (blank and scramble siRNA) displayed a homogenous morphology with nuclei lightly and evenly stained by DAPI (Figure 6).

Discussion

In 66 ER-positive Chinese breast cancer patients, GSTM1 had a distinct loss or variation in expression, which was significantly associated with increased response rate to taxane-based neoadjuvant chemotherapy. Our findings are consistent with a retrospective study by Ambrosone *et al.* that showed greater recurrence-free survival among patients with GSTM1-null genotypes than patients with wild GSTM1 (16). Similarly, the observations of Khedhaier *et al.* in 309 breast cancer patients are consistent with our results (17). However, studies by Lizard-Nacol *et al.* and Yang *et al.* showed no association of treatment response among patients with the wild type or polymorphic GSTM1 in women treated with

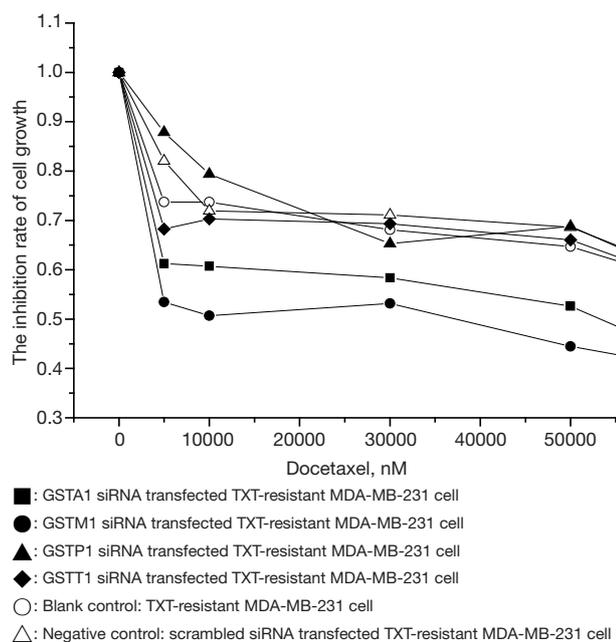


Figure 4 Docetaxel sensitivity of GST-siRNA-transfected-resistant MDA-MB-231 cells. Cells plated in 96-well plates (1×10^4 cells/well/100 μ L of medium) were treated with various concentrations of docetaxel (0–100 μ M) for 24 h. Cell death was determined by the WST assay as described under “Experimental Procedures”. Data are expressed as the mean S.D. of triplicate samples. Similar results were obtained in 3 independent experiments. Sensitivity of the transfectants, blank-control- and negative-control-resistant MDA-MB-231 cells (scrambled siRNA transfected cells) to docetaxel.

neoadjuvant or adjuvant chemotherapy (18,19). Although the observed association between GSTM1 polymorphisms and neoadjuvant outcomes in our study did reach significance, a multivariate logistic regression analysis of HER2 and GSTM1 for pCR did not show statistical significance, most probably due to the small number of patients enrolled in this study to guarantee statistical validity.

Our results from 5 breast cancer cell lines showed an inverse correlation with GST isoenzymes expression level and sensitivity to docetaxel. This observation is in agreement with recent findings by Armstrong *et al.* for 6 human breast cancer cell lines (20). We then used TXT-resistant MDA-MB-231 cells that expressed all 4 GST isozymes to study the functional role of each isozyme in docetaxel resistance by siRNA. Our data showed a 1.5-fold increase in docetaxel sensitivity upon GSTA1 and GSTM1 knockdown in comparison to controls, and observed significantly enhanced docetaxel-induced apoptosis, suggesting the role of GSTM1 and GSTA1 as the resistance factors responsible for intrinsic resistance to docetaxel. In contrast, GSTP1 and GSTT1 knockdown resulted in no significant change to docetaxel sensitivity.

In summary, our findings strongly supported the current understanding of GSTM1 involvement in docetaxel detoxification and for the first time demonstrated that inhibition of GSTM1 and GSTA1 contributes to overcoming docetaxel resistance in breast cancer lines. GSTM1 expression was identified as a predictive marker

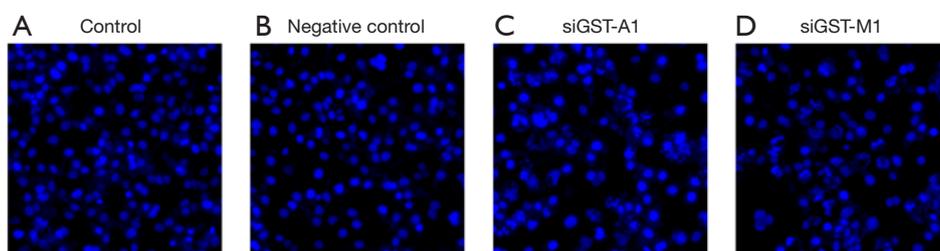


Figure 5 Fluorescence photomicrograph of GSTA1 and GSTM1 knockdown docetaxel (TXT)-resistant MDA-MB-231 cells, blank control cells, and scrambled siRNA transfected cells after docetaxel treatment stained with Hoechst 33258. Cells were exposed to 100 μ M docetaxel for 24 h, stained with Hoechst 33258 as described (Methods), and examined under fluorescence microscope. Selected fields illustrating occurrence of apoptosis are shown. (A,B) Blank control cells and negative control cells (scrambled siRNA transfected cells): the nuclei were stained homogeneously and less brightly, and had regular contours which were large in size. Cells with condensed chromatin were rarely seen. (C,D) GSTA1 and GSTM1 knockdown TXT-resistant MDA-MB-231 cells: the nuclei were bright, and hypercondensed nuclear fragmentation and condensed chromatin formed. Photograph was a representative experiment repeated 3 times (original magnification $\times 200$).

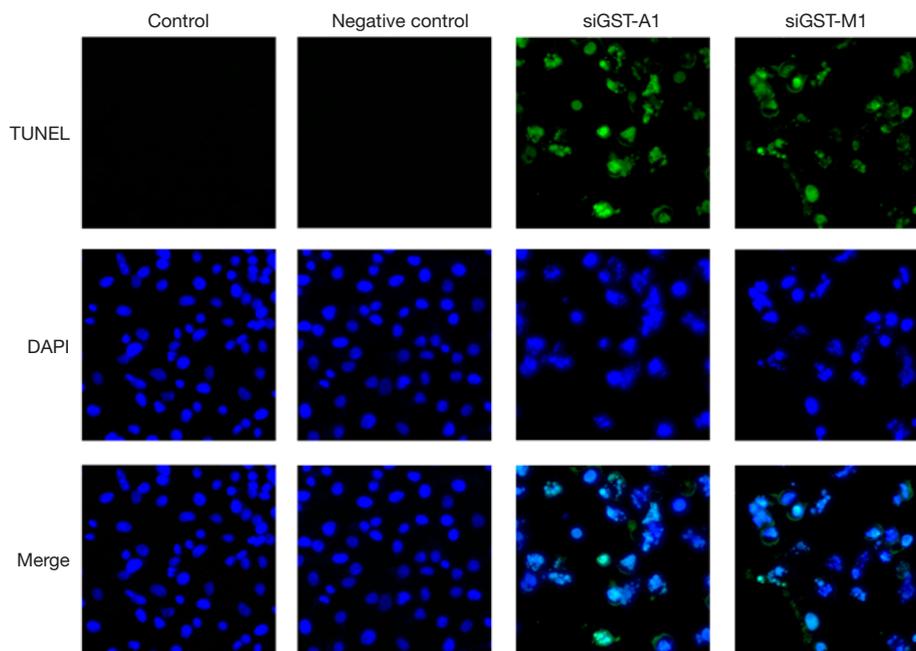


Figure 6 Representative fluorescence microscopic images for DNA fragmentation in GSTA1 and GSTM1 knockdown docetaxel (TXT)-resistant MDA-MB-231 cells, blank control cells, and scrambled siRNA transfected cells after docetaxel treatment. Cells were exposed to 100 μ M docetaxel for 24 h then stained with TUNEL or 4,6-diamidino-2-phenylindole (DAPI) according to the protocols described in the materials and methods. Images were analyzed using a fluorescence microscope. Green color represents positive TUNEL staining, which means existence of DNA fragmentation, and blue color is from DAPI, which stains only the nuclei. Significant population in siGSTM1 and siGSTA1 cells were TUNEL-positive whereas no TUNEL-positive signal was observed in blank control cells and negative control cells (scrambled siRNA-transfected cells). Enhanced DAPI fluorescence by condensed or fragmented nuclei was observed in siGSTM1 and siGSTA1 cells as compared to the blank control and negative control cells which show round and homogeneous nuclei. Photograph was a representative experiment repeated 3 times (original magnification $\times 200$).

in patients with breast cancer that underwent taxane-based neoadjuvant chemotherapy.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.04.03>).

The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was waived due to the retrospective nature of the study. Institutional Review Board approval was waived.

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