Prognostic value of using glucosylceramide synthase and cytochrome P450 family 1 subfamily A1 expression levels for patients with triple-negative breast cancer following neoadjuvant chemotherapy

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Abstract. Neoadjuvant chemotherapy (NACT) has been considered to be the preferred treatment option for early operable triple-negative breast cancer (TNBC). However, resistance to drugs remains to be the barrier to the efficacy of NACT. Glucosylceramide synthase (GCS) and cytochrome P450 family 1 subfamily A1 (CYP1A1) have been previously associated with drug resistance in breast cancer. The present study aimed to explore whether the expression levels of GCS and/or CYP1A1 are associated with the prognosis of TNBC after NACT. Immunohistochemistry was used to detect and measure GCS and CYP1A1 expression. Associations between GCS or CYP1A1 expression and the clinicopathological characteristics, pathological complete response (pCR), clinical complete response (cCR) and disease-free survival (DFS) were analyzed. GCS expression was found to be associated with tumor size (P=0.021) and TNM staging (P=0.042), whilst CYP1A1 expression was associated with lymph node metastasis (P=0.026) and TNM staging (P=0.034). The expression levels of GCS (P=0.024) and CYP1A1 (P=0.027) were upregulated after NACT. GCS and CYP1A1 expression were positively correlated (P=0.003; r=0.327). No difference was observed between the GCS^+ (P=0.188) or $CYP1A1^+$ group (P=0.073) and the GCS^- or $CYP1A1^-$ group in terms of pCR. However, compared with that in the $GCS^+CYP1A1^+$ group, the pCR was markedly increased in the $GCS^-CYP1A1^-$ group (P=0.031). The cCR was lower in the GCS^+ (P=0.021) and $CYP1A1^+$ groups (P=0.016) compared with in the GCS^- or $CYP1A1^-$ group. The DFS rate (57.9 vs. 65.4%; P=0.049) was lower in the $GCS^+CYP1A1^+$ group compared with that in the $GCS^-CYP1A1^-$ group. However, there was no statistical significance after P-value was adjusted for multiple comparisons using Bonferroni correction. In conclusion, co-expression of GCS and CYP1A1 was associated with pCR and DFS in TNBC, which may serve a role in the prediction of the prognosis of patients with TNBC following treatment with NACT.

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Abbreviations: GCS, glucosylceramide synthase; CYP1A1, cytochrome P450 family 1 subfamily A1; TNBC, triple-negative breast cancer; NACT, neoadjuvant chemotherapy; pCR, pathological complete response; cCR, clinical complete response; DFS, disease-free survival

Key words: glucosylceramide synthase, cytochrome P450 family 1 subfamily A1, neoadjuvant chemotherapy, triple-negative breast cancer

Introduction

Breast cancer is the second leading cause of cancer-associated mortality after lung cancer in women worldwide (1). One of the breast cancer subtypes, triple-negative breast cancer (TNBC), which is characterized by the lack of estrogen and progesterone receptors and HER-2 expression, accounts for 15-20% of all breast cancers (2,3). TNBC typically exhibits aggressive behaviors, including a high recurrence rate and early metastasis, resulting in poor prognoses (4,5). Neoadjuvant chemotherapy (NACT) can facilitate breast conservation, render inoperable tumors operable and provide important prognostic information based on the response to therapy (6,7). In addition, NACT is considered to be a treatment option for patients with early operable TNBC (6). The criterion for determining the response to NACT is the tumor pathological complete response (pCR), which is defined as the absence of residual cancer in the primary breast tumor and lymph nodes (8). Achievement of pCR following NACT is associated with good long-term outcomes (9-11). Although TNBC is initially sensitive to chemotherapy, it rapidly develops drug resistance such that only 10-40% of patients can achieve pCR (12,13). A number of clinical trials have previously attempted to apply novel agents, such as immunotherapy and anti-angiogenetic agents to increase the pCR but adverse side effects have limited their potential application in clinical

practice (14,15). Additionally, not all increases in pCR lead to an improvement of long-term outcomes (11,16). Therefore, development of novel specific biomarkers may help to identify patients who would benefit from NACT.

Glucosylceramide synthase (GCS) is a glycosyltransferase that transfers a glucose group from uridine diphospho-glucose to ceramide to produces glucosylceramide (17,18). GCS expression is upregulated in a number of multidrug resistance (MDR) cancer cell lines, including breast cancer (18,19), leukemia (20) and renal cell cancer (21) cell lines. Previous studies have reported that GCS was associated with MDR to anthracycline drugs in breast cancer, where GCS expression was upregulated in 30% of patients with TNBC (22,23). Although there is at present no standard regimen for NACT, anthracycline and taxanes are commonly used for NACT treatment (24).

Among a number of factors which can induce drug resistance, one proposed mechanism is the activation or inactivation of drug-metabolizing enzymes. The cytochrome P450 (CYP) family is a multigene family of enzymes that has been implicated in the metabolism of a diverse range of drugs (25). In particular, CYP family 1 subfamily A1 (CYP1A1) belongs to the CYP family, which is an enzyme that is involved in the bioactivation of endogenous and environmentally reactive compounds, such as dimethylbenz(a)anthracene and heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. In addition, CYP1A1 can also bind to DNA to mediate carcinogenesis (26). CYP1A1 colocalizes with P-glycoprotein and contributes to tumor cell chemoresistance by enhancing the metabolism of numerous drugs (27-30).

Therefore, the present study explored the association between GCS and CYP1A1 expression in TNBC, by analyzing their association with clinicopathological parameters, pCR and disease-free survival (DFS) following NACT. In addition, the present study also focused on the possibility that GCS is associated with responses to NACT and that in can be used to predict prognosis following anthracycline- or taxanes-based NACT regimen in TNBC.

Materials and methods

Patients. In total, 80 female patients with a median age of 56 years (range, 24-72 years), who met the following inclusion criteria between January 1, 2012 and February 31, 2014 at Yuhuangding Hospital Affiliated to Qingdao University (Yantai, China) were eligible for the present study: i) Age ≥18 years, core needle biopsy diagnosis of invasive breast cancer and immunohistochemistry-confirmed estrogen receptor (ER) expression to be <1% positive, progesterone receptor (PR) expression to be <1% positive and HER-2 score 0 or 1-2+, if a Her-2 score of 2+ was found, fluorescence *in situ* hybridization was used to further test for Her-2 negativity as previously described (31); ii) patients underwent ≥2 cycles of NACT and were demonstrated to have operable breast cancer (stage IIA-IIIB); iii) all tumors of the patients were deemed by at least a CT scan as having one measurable lesion; iv) Eastern Cooperative Oncology Group score of 0-2 (32); v) normal routine blood tests reporting hemoglobin levels of ≥100 g/l, leukocyte count ≥$4x10^9$/l, neutrophil count ≥$1.5x10^9$/l, thrombocyte count ≥$100x10^9$/l and liver and kidney functions within ≤1.5X of the normal range; vi) no previous therapy, including chemotherapy, radiotherapy, endocrine therapy, immunotherapy or surgery, for breast cancer; and vii) life expectancy >6 months.

The exclusion criteria were as follows: i) Patients with active concomitant malignancy; ii) patients with active infection and serious concomitant diseases, including heart failure, severe diabetes, liver failure, severe peripheral neuropathy or severe drug allergy; and iii) pregnant or lactating. The characteristics of all patients are presented in Table I.

Written informed consent was obtained from all individual participants included in the present study before treatment. The present study was conducted in accordance with the Declaration of Helsinki. The present study was approved by the Institutional Review Board, Medical Ethics Committee of Yantai Yuhuangding Hospital (Yantai, China).

NACT treatment. NACT (33-36) was administered every 21 days. The present study used an AT regimen (doxorubicin 50 mg/m^2 + docetaxel 75 mg/m^2 or paclitaxel 175 mg/m^2) in the majority of the cases. An AC regimen (doxorubicin 60 mg/m^2 + cyclophosphamide 600 mg/m^2), TC regimen (docetaxel 75 mg/m^2 + cyclophosphamide 600 mg/m^2) or AC-Follow T regimen (four cycles of doxorubicin 60 mg/m^2 + cyclophosphamide 600 mg/m^2, followed by four cycles of docetaxel 75 mg/m^2 or paclitaxel 175 mg/m^2) was used in the remaining cases. Most of patients received 6 cycles of chemotherapy.

Response to NACT assessment. Tumor staging was performed according to the Eighth Edition of the guidelines of the American Joint Committee on Cancer (37). The primary objective was to evaluate the pCR rate, which is defined as no histological evidence of residual invasive tumor cells in the breast and axillary lymph nodes (ypT0/TisypN0) (38). Residual tumors were defined as non-pCR and were classified using a pathological TNM system (36). The secondary objective was to evaluate the clinical response rate and DFS. Clinical tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors version 1.1 (39): i) Complete response (cCR) was defined as the disappearance of all tumor foci after chemotherapy; ii) partial response was defined as ≥30% decline in the maximum tumor diameters; iii) progressive disease was defined as ≥20% increase in the cumulative measurement of all tumor diameters from the baseline; and iv) stable disease was confirmed when complete response, partial response or progressive disease was not noted. DFS was defined as the time from the date of surgery to the first observation of tumor recurrence (metastatic recurrence and/or local relapse) or death. Patients who remained alive without recurrence and/or metastasis were administratively censored at the last follow-up date. All patients who received chemotherapy (>one cycle of each regimen) were evaluated. All patients were followed up for a median period of 68.8 months (range, 33.0-84.0 months) after surgery.

Immunohistochemistry. Tumor specimens were obtained from patients included in this study before NACT and after surgery. All specimens were fixed with 10% formalin for 6-24 h at room temperature, paraffin embedded and cut into 4-μm sections. The slides were allowed to dry overnight at

Table I. Clinicopathological characteristics of patients with triple-negative breast cancer in the present study.

Clinical characteristics	Number (%)
Age, years	
<35	6 (7.50)
35-60	46 (57.50)
>60	28 (35.00)
Grade	
I	9 (11.25)
II	45 (56.25)
III	26 (32.50)
Node	
0	25 (31.25)
0-3	19 (23.75)
4	36 (45.00)
Tumor size	
T1-2	44 (55.00)
T3-T4	36 (45.00)
Ki67	
<14%	16 (20.00)
≥14%	64 (80.00)
TNM stage	
IIA-B	29 (36.25)
IIIA-B	51 (63.75)

room temperature. To deparaffinize the sections, they were placed in two containers of xylene at room temperature for 5 min each. To start rehydration, the sections were placed in three containers of 100% ethanol, 95% ethanol and 85% ethanol at room temperature for 5 min each. Antigen retrieval was performed in a microwave oven at 100˚C for 15 min in 10 mM citrate buffer (pH 6.0). For all samples, endogenous peroxidase activity was blocked for 10 min using a 3% H_2O_2-methanol solution at room temperature. The slides were blocked with 10% normal goat serum (Dako; Agilent Technologies, Inc.) at room temperature for 10 min and incubated with appropriately diluted primary antibodies overnight at 4˚C. The antibodies against ER (ready-to use, no dilution; cat. no. 790-4325), PR (ready-to use, no dilution; cat. no. 790-4296), HER-2 (ready-to use, no dilution; cat. no. 790-4493) and Ki67 (ready-to use, no dilution; cat. no. 790-4286) were purchased from Roche Diagnostics. CYP1A1 antibody was purchased from LifeSpan BioSciences, Inc. (dilution, 1:50; cat. no. LS-C99804). GCS antibody was purchased from BIOSS (dilution, 1:300; cat. no. bs-0701R). Subsequently, the slides were probed with a horseradish peroxidase-labeled polymer conjugated to an appropriate secondary antibody (DAB + substrate chromogen system; cat. no. GK600505; Dako; Agilent Technologies, Inc.) for 30 min. The slides were incubated DAB+ (DAB + substrate chromogen system cat. no. GK600505; Dako; Agilent Technologies, Inc.) at 25˚C for 5-15 min until there were yellow to brown granules visible when viewed under a light microscope. The stained sections were then observed under a light microscope (magnification, x400; Nikon eclipse 80i; Nikon Corporation).

A dual semi-quantitative scale combining staining intensity and percentage of positive cells was used to evaluate GCS and CYP1A1 protein staining. Each index counted 10 high power microscopic fields, containing at least 1,000 tumor cells. The staining intensity of the cell plasma was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). The percentage of positive cells was scored as follows: i) 0, no staining or staining in <5% of tumor cells; ii) 1, staining in 5-25% of cells; iii) 2, staining in 26-50% of cells; iv) 3, staining in 51-75% of cells; and v) 4, staining in >75% of cells. The staining intensity and the percentage were then multiplied to obtain a final score (range, 0-12). For GCS and CYP1A1 expression, cytoplasmic staining was considered positive with a score >4, or negative with an immunohistochemical score ≤4 (22,23,40).

Transfection with GCS plasmid. The breast cancer cell lines MDA-MB-453 (ATCC HTB-131; https://www.atcc.org/products/all/HTB-131.aspx) and MDA-MB-231 (ATCC HTB-26; https://www.atcc.org/products/all/HTB-26.aspx) were obtained from the ATCC. The full-length GCS vector pcDNA3.1-GCS was synthesized and purified by Shanghai GenePharma Co., Ltd. Prior to transfection, cells were cultured in PRMI-1640 with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and seeded into six-well plates at the density of $1x10^6$ cells per well and incubated at 37˚C in an atmosphere with 5% CO_2 for 12 h. For each well, 10 μl (2 mg/ml) Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and 5 μl (1 mg/ml) vector were diluted into 250 μl RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) culture medium without serum. After incubation for 10 min at room temperature, the diluted vector and Lipofectamine were mixed together and incubated for 20 min at 25˚C. The mixture was then added to the cells. The medium was replaced with 1 ml complete RPMI-1640 culture medium 6 h later, so that the final concentration of the plasmid was 5 μg/ml. As negative control, 10 μl Lipofectamine and 5 μl (1 mg/ml) pcDNA3.1 were also transfected into the two cell lines. Forty-eighrt hours after the transfection, the subsequent experiments were performed.

RNA extraction and quantitative PCR (qPCR). Total RNA from the cell lines was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. Reverse transcription was performed using a Toyobo First Strand cDNA Synthesis kit (cat. no. FSQ-201; Toyobo Life Science). A total of 1 μl RNA was added into 10 μl reaction solutions according to the manufacturer's protocol and the reaction conditions were as follows: 37˚C for 15 min and 95˚C for 5 min. qPCR was performed using a SYBR® Green Real-Time PCR Master Mix (Toyobo Life Science). The primers for GCS were forward, 5'-CCTTTCCTCTCCCCACCTTCC TCT-3' and reverse, 5'-GGTTTCAGAAGAGAGACACCT GGG-3' (41). The primers for CYP1A1 were forward, 5'-CTC AGCTCAGTACCTCAGCCAC-3' and reverse, 5'-CCCCAT ACTGCTGGCTCATC-3'. The primers for the β-actin were forward, 5'-ACCCCCACTGAAAAAGATGA-3' and reverse, 5'-ATCTTCAAACCTCCATGATG-3', which was used as an internal control. The final volume was 25 μl and an iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories,

Table II. Association between GCS or CYP1A1 expression and clinical features.

Clinical characteristics	GCS^+, n (%)	GCS^-, n (%)	P-value	$CYP1A1^+$, n (%)	$CYP1A1^-$, n (%)	P-value
Age, years						
<35	4 (5.00)	2 (2.50)	0.137	3 (3.75)	3 (3.75)	0.284
>35	21 (26.25)	53 (66.25)		16 (20.00)	58 (72.50)	
Grade						
I	2 (2.50)	7 (8.75)	0.811	3 (3.75)	6 (7.50)	0.763
II-III	23 (28.75)	48 (60.00)		16 (20.00)	55 (68.75)	
Node						
N0	5 (6.25)	20 (25.00)	0.143	2 (2.50)	23 (28.75)	0.026
N1-N3	20 (25.00)	35 (43.75)		17 (21.25)	38 (47.50)	
Tumor size						
T1-T2	9 (11.25)	35 (43.75)	0.021	7 (8.75)	37 (46.25)	0.068
T3-T4	16 (20.00)	20 (25.00)		12 (15.00)	24 (30.00)	
Ki67						
<14%	7 (8.75)	9 (11.25)	0.228	6 (7.50)	10 (12.50)	0.264
≥14%	18 (22.50)	46 (57.50)		13 (16.25)	51 (63.75)	
TNM stage						
IIA-IIB	5 (6.25)	24 (30.00)	0.042	3 (3.75)	26 (32.50)	0.034
IIIA-IIIB	20 (25.00)	31 (38.75)		16 (20.00)	35 (43.75)	
NACT						
Before	25 (31.25)	55 (68.75)	0.024	19 (23.75)	61 (76.25)	0.027
After	39 (48.75)	41 (51.25)		32 (40.00)	48 (60.00)	
pCR						
Yes	9 (11.25)	15 (18.75)	0.188	6 (7.50)	18 (22.50)	0.073
No	30 (37.50)	26 (32.50)		26 (32.50)	30 (37.50)	
cCR						
Yes	11 (13.75)	22 (27.50)	0.021	8 (10.00)	25 (31.25)	0.016
No	28 (35.00)	19 (23.75)		24 (30.00)	23 (28.75)	

GCS, glucosylceramide synthase; CYP1A1, cytochrome P450 family 1 subfamily A1; NACT, neoadjuvant chemotherapy; pCR, pathological complete response; cCR, clinical complete response.

Inc.) was used for qPCR. The thermocycling conditions for the qPCR reaction were as follows: Initial denaturation for 5 min at 94˚C; followed by 35 cycles of denaturation for 30 sec at 94˚C, primer annealing for 30 sec at 60˚C and polymerization for 30 sec at 72˚C; and a final extension for 10 min at 72˚C. The relative mRNA expressions were calculated using the $2^{-\Delta\Delta}$Cq method (42).

Statistical analysis. Data were analyzed using the SPSS software (version 18.0; SPSS, Inc.). χ^2 test was used in the table presented in Tables I and II. In Table III, P1 represented a comparison between the $GCS^+CYP1A1^-$ and $GCS^+CYP1A1^+$ group; P2 represented a comparison between the $GCS^-CYP1A1^+$ and $GCS^+CYP1A1^+$ group; and P3 represented a comparison between the $GCS^-CYP1A1^-$ and $GCS^+CYP1A1^+$ group. Spearman's rank correlation coefficient was used to analyze the correlation between the immunohistochemical scores of GCS and CYP1A1. DFS curves were generated according to the Kaplan-Meier method and the survival between groups was compared using log-rank test. P-values were adjusted for multiple comparisons using the Bonferroni correction. For the multiple comparisons of DFS rate, P<0.017 (0.05/3) was defined as statistically significant. For other tests, P<0.05 was considered to indicate a statistically significant difference. All P-values were the results of two-sided tests.

Results

Association between GCS and CYP1A1 and the clinicopathologic parameters in TNBC. Cell experiments were performed to assess the potential association between GCS and CYP1A1 (Fig. S1). A total of 80 patients with TNBC who had undertaken NACT were enrolled into the present study. Clinical and pathological TNM classifications of patients were evaluated according to the Eighth Edition American Joint Committee on Cancer Staging Criteria (37). Immunohistochemical staining was performed to detect the expression levels of GCS and CYP1A1. GCS and CYP1A1 staining were mainly observed in the cytoplasm of cancer cells (Fig. 1). The association

Table III. Association between GCS and CYP1A1 co-expression and pCR.

Outcome	$GCS^+CYP1A1^+$, n (%)	$GCS^+CYP1A1^-$, n (%)	P1-value	$GCS^-CYP1A1^+$, n (%)	P2-value	$GCS^-CYP1A1^-$, n (%)	P3-value
pCR	2 (2.50)	7 (8.75)	0.127	3 (3.75)	0.374	11 (13.75)	0.031
Non-pCR	17 (21.25)	13 (16.25)		10 (12.50)		17 (21.25)	

GCS, glucosylceramide synthase; CYP1A1, cytochrome P450 family 1 subfamily A1; pCR, pathological complete response.

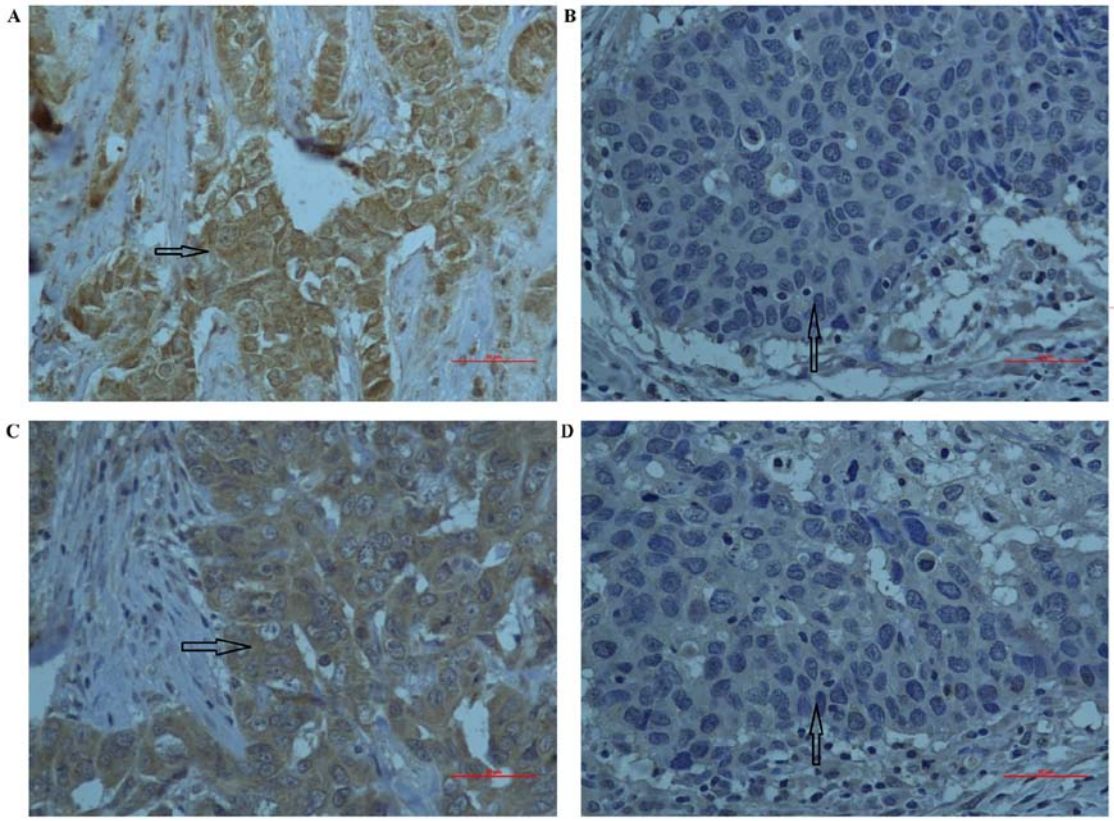

Figure 1. Expression levels of GCS and CYP1A1 in patients with triple-negative breast cancer. Representative immunohistochemistry images of (A) GCS^+, (B) GCS^-, (C) $CYP1A1^+$ and (D) $CYP1A1^-$ tissue sections. Scale bars, 50 μm. CYP1A1, cytochrome P450 family 1 subfamily A1; GCS, glucosylceramide synthase.

between GCS or CYP1A1 expression and each of the clinicopathological parameters were subsequently analyzed. The expression levels of GCS were found to be associated with tumor size (P=0.021) and TNM stage (P=0.042). In addition, the expression levels of CYP1A1 were associated with lymph node metastasis (P=0.026) and TNM stage (P=0.034). No other clinicopathologic parameters were associated with GCS or CYP1A1 expression (Table II).

Association between GCS and CYP1A1 expression and NACT in TNBC. The present study also measured the expression levels of GCS and CYP1A1 before NACT and after surgery using immunohistochemistry. The positive expression of GCS was increased from 31.25 to 48.75% after NACT. The positive expression of CYP1A1 was also increased from 23.75 to 40.0% after NACT. Upregulation of both GCS (P=0.024) and CYP1A1 (P=0.027) expression were found to be associated with NACT (Table II). Furthermore, Spearman's rank correlation analysis revealed that there was a significant but weak correlation between GCS and CYP1A1 expression in the TNBC tissues (P=0.003; r=0.327; Fig. 2).

Association of GCS and CYP1A1 expression with the pathological response to NACT in TNBC. pCR was defined as no histological evidence of residual invasive tumor cells in the breast and axillary lymph nodes (ypT0/TisypN0). Due to the upregulation of GCS and CYP1A1 expression, the present study next analyzed the possible association between the expression levels of GCS or CYP1A1 and pCR. There was no difference in pCR in the GCS^+ (P=0.188) or $CYP1A1^+$ group (P=0.073) compared with that in the GCS^- or $CYP1A1^-$ group (Table II).

cCR was defined as the disappearance of all tumor foci after chemotherapy. cCR rate in the GCS^+ group was 28.20% (11/39), which was lower compared with 53.7% (22/41) in the

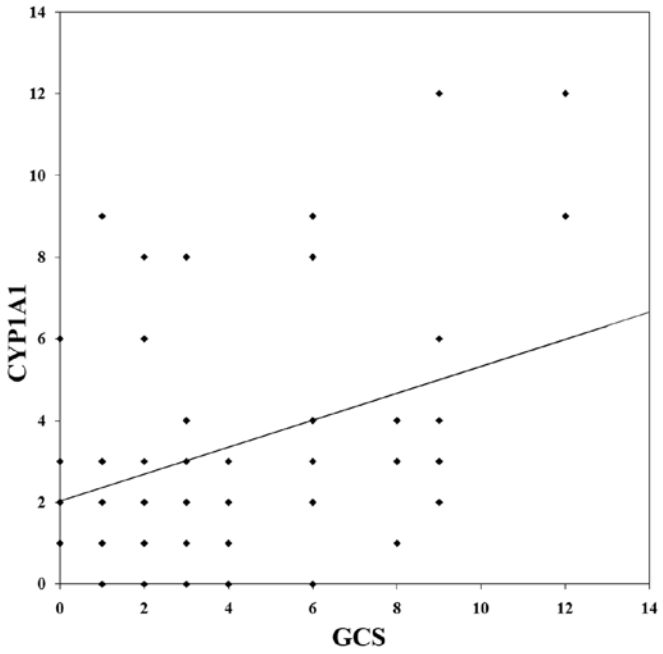

Figure 2. Correlation analysis between GCS and CYP1A1 expression in triple-negative breast cancer. CYP1A1, cytochrome P450 family 1 subfamily A1; GCS, glucosylceramide synthase.

GCS$^-$ group (P=0.021). The cCR rate in the CYP1A1$^+$ group was 25.0% (8/32), which was lower compared with 52.1% (25/48) in the CYP1A1$^-$ group (P=0.016; Table II).

Among the 80 cases of TNBC, 19 cases were GCS$^+$CYP1A1$^+$, 20 cases were GCS$^+$CYP1A1$^-$, 13 cases were GCS$^-$CYP1A1$^+$ and 28 cases were GCS$^-$CYP1A1$^-$. Compared with that in the GCS$^+$CYP1A1$^+$ group, incidences of pCR was increased in the GCS$^-$CYP1A1$^-$ group (P=0.031). However, no significant association was observed between the incidences of pCR between the GCS$^+$CYP1A1$^+$ and the GCS$^+$CYP1A1$^-$ or GCS$^-$CYP1A1$^+$ groups (Table III).

Association of GCS and CYP1A1 expression with the prognosis following NACT in TNBC. The association between GCS or CYP1A1 and DFS was subsequently analyzed by Kaplan-Meier survival analysis. There was no difference between the DFS of patients in the GCS$^+$ (P=0.301; Fig. 3A) or CYP1A1$^+$ (P=0.099; Fig. 3B) groups and their corresponding negative groups. DFS of patients in the GCS$^-$CYP1A1$^-$ group was compared with the other three groups. No statistically significant difference was observed between the DFS of GCS$^-$CYP1A1$^+$ and GCS$^-$CYP1A1$^-$ group (61.5 vs. 65.4%; P=0.497). Similar result was observed between the DFS of GCS$^+$CYP1A1$^-$ and GCS$^-$CYP1A1$^-$ group (65.0 vs. 65.4%; P=0.734). The DFS of patients in the GCS$^+$CYP1A1$^+$ group exhibited markedly worse DFS rate compared with that in the GCS$^-$CYP1A1$^-$ group (57.9 vs. 65.4%; P=0.049). However, after the significance threshold was corrected using Bonferroni correction, there was no statistical significance between the two groups (Fig. 3C).

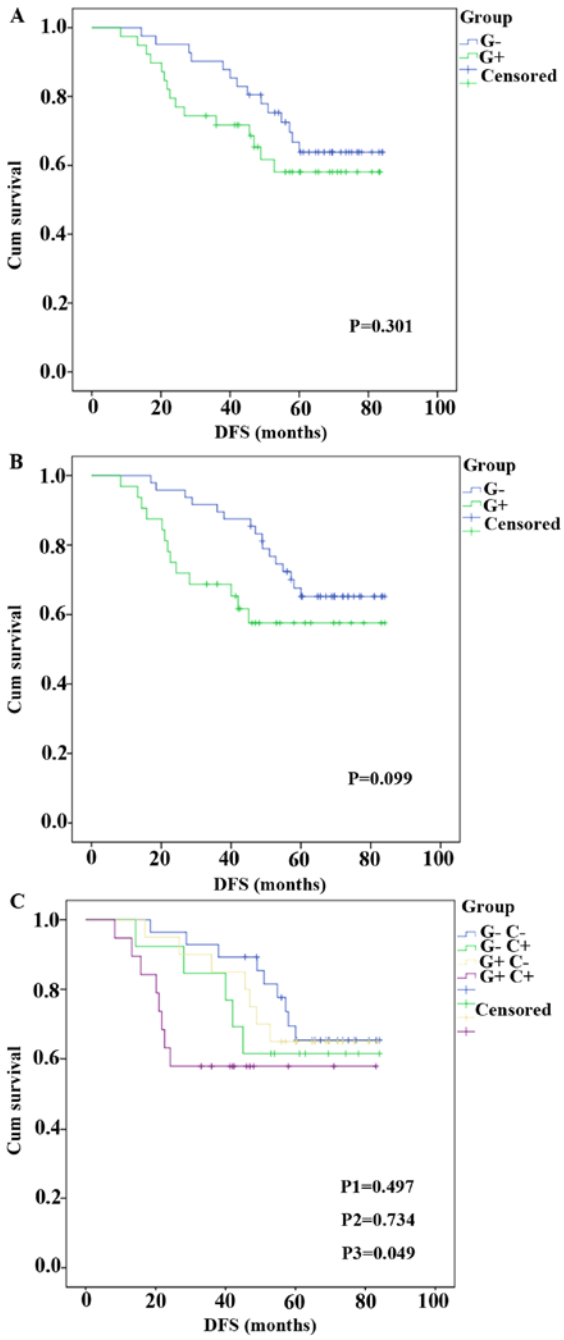

Figure 3. Association between GCS or CYP1A1 expression and DFS. DFS of patients in the (A) GCS$^+$ and GCS$^-$ groups, (B) CYP1A1$^+$ and CYP1A1$^-$ groups, and (C) the GCS$^-$CYP1A1$^-$, GCS$^+$CYP1A1$^+$, GCS$^-$CYP1A1$^+$ and GCS$^+$CYP1A1$^-$ groups. After adjustment using Bonferroni's correction, P<0.017 (0.05/3) was defined as statistically significant. P1=0.497, GCS$^-$CYP1A1$^+$ vs. GCS$^-$CYP1A1$^-$. P2=0.734, GCS$^+$CYP1A1$^-$ vs. GCS$^-$CYP1A1$^-$. P3=0.049, GCS$^+$CYP1A1$^+$ vs. GCS-CYP1A1$^-$. CYP1A1, cytochrome P450 family 1 subfamily A1; DFS, disease-free survival; GCS, glucosylceramide synthase.

Discussion

TNBC represents a heterogeneous group of tumors based on gene expression profiling (43). Results from the Capecitabine for Residual Cancer as Adjuvant Therapy (44) and KATERINE (45) clinical trials demonstrated that NACT has become the preferred treatment strategy for patients with TNBC and HER-2-positive breast cancer in clinical practice. Numerous studies have previously highlighted the prognostic significance of pathological complete response (pCR) (9-11). A number of drugs, including as poly(ADP-ribose) polymerase inhibitors (46), vascular endothelial growth factor

inhibitors (47) and immune checkpoint inhibitors (48,49), have been applied in clinical trials to improve the pCR rate in breast cancer. Compared with that in other breast cancer subtypes, TNBC has a relatively high possibility of achieving pCR. However, this advantage could not be clearly translated into improved DFS or overall survival (OS) due to poor outcomes in the non-pCR groups (50,51). Therefore, early identification of sensitive responders could provide definitive value for decision making with regards to the type of therapy for patients with TNBC. There is no universally approved marker for the prediction of the response to NACT (52). Therefore, the introduction of novel biomarkers could expand the repertoire of currently available clinical options and to accurately predict the response to NACT for patients with TNBC.

Anthracycline-taxanes are commonly used in clinical practice due to the lack of a standard treatment regimen for NACT (24). However, development of drug resistance to the available treatments is the primary barrier to TNBC treatment with NACT (53). Both GCS and CYP1A1 expression levels are associated with P-glycoprotein expression and upregulate multidrug resistance protein 1 expression during the regulation of breast cancer drug resistance via β-catenin signaling (40,54-56). In a previous study, the expression levels of GCS were found to be associated with ER-positive (P=0.017) and HER-2-negative (P=0.007) invasive breast cancer (23). GCS is more highly expressed in younger patients (<35 years) (23). However, the present study did not identify an association between age and GCS upregulation in patients with TNBC, although GCS upregulation was associated with tumor size and TNM staging. These differences may be due to the heterogeneity of TNBC. Zhang *et al* (22) previously reported that GCS expression was upregulated after NACT in ER-positive invasive breast cancer. The present study also detected a change in GCS expression in TNBC after NACT, the upregulation of which was associated with NACT in TNBC. CYP encodes enzymes involved in the metabolism of pharmacological agents (57). By activating or inactivating carcinogens and anticancer drugs, CYP serves an important role for the study of cancer and cancer treatments (58). Due to the overlapping substrate specificity between CYP and P-glycoprotein, numerous drug interactions can involve both P-glycoprotein and CYP (59). CYP1A1 is one of the most important isoforms responsible for the metabolic activation of pre-carcinogens (60). In the present study, the expression levels of CYP1A1 were associated with lymph node metastasis and TNM staging. This was consistent with the results of Wang and Wang (61). Another report previously revealed that the expression levels of CYP1A1 are associated with age or tumor grade in breast cancer (62). The present study did not identify a relationship between CYP1A1 and these two parameters, but it did reveal that the upregulation of CYP1A1 were associated with NACT. This suggested that the upregulation of GCS and CYP1A1 may be the underlying reason for NACT resistance in patients with TNBC. The mechanisms of GCS- and CYP1A1-induced chemoresistance to NACT in TNBC need to be confirmed by further *in vitro* experiments.

It is of interest to explore whether these two biomarkers are associated with each other and can provide useful prognosis information for patients with TNBC after undergoing NACT. Therefore, the association between GCS and CYP1A1 expression was next detected. The results revealed that they were positively correlated. pCR in the primary tumor following NACT is a strong predictor of freedom from recurrence and long-term survival (38). cCR is the disappearance of all lesions with nodes measuring <10 mm and normal tumor markers. Measuring residual disease after neoadjuvant chemotherapy could improve the prognostic information that can be obtained from evaluating the pathologic complete response (pCR) (63). A number of studies have previously reported factors that affect the prognosis of patients with TNBC following NACT, including C-X-C motif chemokine ligand 8-C-X-C motif chemokine receptor 1/2 (64), pre-treatment neutrophil-lymphocyte ratio (65) and tumor-infiltrating lymphocytes (66). However, it remains to be difficult to introduce a biomarker into daily clinical use due to the lack of consistent evidence of clinical significance and operational barriers to clinical implementation (62,64,65). In the present study, both GCS and CYP1A1 were associated with cCR. However, neither GCS nor CYP1A1 upregulation was associated with pCR or DFS. When these two biomarkers were analyzed together, the results revealed that combined GCS and CYP1A1 upregulation was associated with pCR (P=0.031). The results also revealed a trend that patients in the $GCS^{+}CYP1A1^{+}$ group had a worse DFS rate, even if there was no statistically significance. The combination of these two biomarkers could also predict the prognosis of patients with TNBC undergoing NACT. If successful, it may differentiate patients who are at high risk of recurrence and need further therapy from patients with low risk of recurrence, avoiding the unnecessary long-term toxicity of chemotherapy. This will be of importance in the clinical setting in the future.

The present study has several limitations. The sample size was small and the follow-up time was relatively short, such that there was no statistical significance between the DFS rate of the $GCS^{+}CYP1A1^{+}$ group and the $GCS^{-}CYP1A1^{-}$ group. The present study was a retrospective single-center study. In addition, different NACT regimens, which may influence the pCR and DFS (67), were not evaluated. The follow-up time should be longer and the OS requires further analysis. Whether the β-catenin signaling pathway induced GCS and CYP1A1 expression after NACT needs to be explored in further *in vitro* experiments. Overall, future prospective studies with a large sample size and sufficient follow-up times are required to verify the results found in the present study.

In conclusion, the present study provided evidence that both GCS and CYP1A1 expression are important in patients with TNBC treated with NACT. Furthermore, they may help in classifying TNBC into subtypes with different responses to chemotherapy. Increased GCS and CYP1A1 expression after NACT could indicate a poor prognosis in patients with TNBC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JL and SW designed the study and analyzed the data and drafted the manuscript. CW performed the IHC. XK collected the clinical data. PS conceived and designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all individual participants included in the present study before treatment. The present study was conducted in accordance with the Declaration of Helsinki. The present study was approved by the Institutional Review Board, Medical Ethics Committee of Yantai Yuhuangding Hospital (Yantai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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