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# Prognostic relevance of glucosylceramide synthase (GCS) expression in breast cancer

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**Abstract**

*Purpose*   Multidrug resistance (MDR) has been linked to sphingolipid metabolism and preclinical data ascribe glucosylceramide synthase (GCS) a major role for MDR especially in breast cancer cells but no profound data are available on the expression of this potential therapeutic target in clinical breast cancer specimens.

*Methods*   We analyzed microarray data of GCS expression in a large cohort of 1,681 breast tumors.

*Results*   Expression of GCS was associated with a positive estrogen receptor (ER) status, lower histological grading, low Ki67 levels and ErbB2 negativity ($P < 0.001$ for all). In univariate analysis there was a benefit for disease free survival for patients with tumors displaying low levels of GCS expression but this significance was lost in multivariate Cox regression.

*Conclusions*   Our results suggest ER positive tumors may be the most promising candidates for a potential therapeutic application of GCS inhibitors.

**Keywords**   Multidrug resistance · Breast cancer · Ceramide · Sphingolipids · Microarray

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## Introduction

Breast cancer is the most frequent cancer entity worldwide. Beside surgical methods chemotherapy and endocrine therapy are fundamentals of breast cancer treatment. In particular response to chemotherapy in advanced disease is limited by the multidrug resistance (MDR) phenomenon. Several mechanisms have been suggested to be responsible for this resistance with increased expression of drug resistance genes like MDR 1 and bcl 2 to be the most important (Makin and Dive 2001; Shabbits et al. 2001). Knowledge about chemotherapy induced drug resistance is increasing. Recent research focused on the role of glucosylceramide in MDR (Lucci et al. 1998; Gouaze-Andersson and Cabot 2006; Liu et al. 2008). Glucosylceramide belongs to the sphingolipids, a family of membrane lipids that play key roles in apoptosis, senescence, proliferation, MDR, and neo angiogenesis (Ogretmen and Hannun 2004; Hannun and Obeid 2008, see Fig. 1). Accumulation of glucosylceramide is a characteristic finding in multi drug resistant ovarian, breast, colon, and epitheloid cells in vitro (Lucci et al. 1998; Kok et al. 2000; Lavie et al. 1996; Nicholson et al. 1999). Glucosylceramide synthase (GCS) transfers a glucose residue from UDP-glucose to ceramide to synthesize glucosylceramide (Liu et al. 2008; Jeckel et al. 1992). In several in vitro studies (Liu et al. 2008; Ogretmen and Hannun 2004; Reynolds et al. 2004; Gouaze et al. 2004; Liu et al. 1999a, b) glycosylation of ceramide was combined with resistance to drugs like adriamycin and paclitaxel. Transfection with GCS genes conferred resistance to daunorubicin, doxorubicin and TNF-α to breast cancer cells (Liu et al. 1999a, b; Ogretmen et al. 2001). Inhibition of GCS resensitized tumor cells to chemotherapy (Radin et al. 1993; Norris-Cervetto et al. 2004; Liu et al. 2004). In addition it has been proposed that drugs that reverse drug
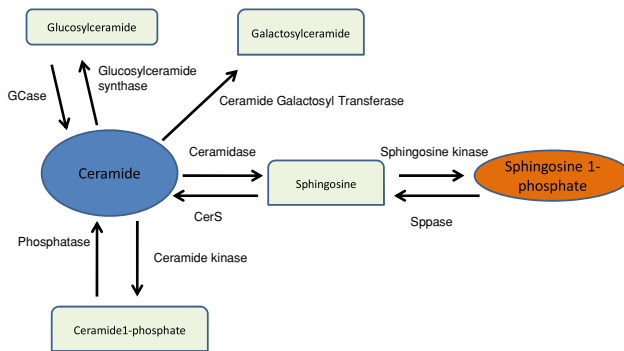
**Fig. 1** Glucosylceramide synthase and sphingolipid metabolism

resistance like verapamil, tamoxifen, and cyclosporin A might act via inhibition of ceramide glycosylation (Lavie et al. 1997).

Only very little data is available about the expression of GCS in clinical breast cancer samples and it is not known, whether differences exist between levels of GCS expression in clinical subgroups of breast cancer, for example, according to the estrogen receptor (ER) or ErbB2 status. Since we could previously demonstrate a prognostic impact of sphingolipid metabolism in breast cancer (Ruckhäberle et al. 2007) we set out to analyze possible prognostic effects of GCS. Here we present results of microarray analysis of 1,681 breast cancer samples according to GCS expression.

## Materials and methods

A database of 1,681 Affymetrix microarray experiments from primary breast cancer patients was created. 220 samples from our own institutions were included (datasets Frankfurt and Hamburg) which have been described previously (Rody et al. 2007; Ruckhäberle et al. 2007; Rody et al. 2006; ASCO, Ahr et al. 2002) as well as 1,461 samples from nine different publicly available datasets (Table 1): Uppsala (Miller et al. 2005), Stockholm (Pawitan et al. 2005), Rotterdam (Wang et al. 2005; Minn et al. 2007), Oxford-Untreated (Sotiriou et al. 2006), Oxford-Tamoxifen and London (Loi et al. 2007), NewYork (Minn et al. 2005), Villejuif (Desmedt et al. 2007), and expO (http://www.intgen.org). For comparability only data from Affymetrix HG-U133A microarrays were used. The clinical characteristics of the patients in the different datasets are given in Table 1. For 1,363 of the 1,681 patients follow up information was available (no follow up data has been reported for dataset expO). The median follow-up time was 76 months. 1,200 of the 1,681 samples (71.4%) were ER positive. Treatment information could be obtained for 878 ER positive and 262 ER negative patients. Since methods of Affymetrix microarray normali-

zation can have significant effects on the levels for individual probe sets, several uniform normalization methods (Li and wong 2001; Irizarry et al. 2003) of CEL file data has been developed to allow the analysis of sets of multiple arrays. However, important discrepancies between different datasets depend on the dynamics of the measurements originating from different hybridization efficiencies and even uniform normalization methods are incapable in compensating those experimental differences. In addition, no CEL files are available for some studies (e.g., the Rotterdam dataset). Therefore, we used a conservative strategy for dataset stratification which relies on a ranking of samples in each cohort. Each dataset of microarrays was normalized separately using the originally proposed method in the respective study (see Table 1). Log transformed expression values were median centered over each array. For genes the normalization, ranking of expression values and median splits were done separately in each dataset.

### Assessment of ER, ErbB2, proliferative status, and GCS expression of the samples

Since standard pathology for ER and ErbB2 was not available for all samples and to allow comparison of different datasets, receptor status was determined based on Affymetrix expression data as previously described (Foekens et al. 2006; Gong et al. 2007; Bonnefoi et al. 2007; Alexe et al. 2007). ER status was based on Affymetrix ProbeSet 205225_at, the ErbB2 status on ProbeSet 216836_s_at. A specificity of 86.1% and a sensitivity of 92.2% was observed when the chip based ER status was compared to immunohistochemical obtained ER status (available for 1,333 samples), while the specificity and sensitivity of chip based ErbB2 status was 98.6 and 45.8%, respectively, compared to 3+ staining in immunohistochemistry with HER2 antibody (data available for 206 samples). As a surrogate marker for cellular proliferation we used the expression of the proliferation marker Ki67 (ProbeSets 212020-212023_s_at). Appropriate cut off values that distinguish between high and low proliferative activity in a clinically relevant manner using Ki67 immunohistochemistry in breast cancer have not been universally established (de Azambuja et al. 2007). Thus, a conservative median split according to Ki67 gene expression was applied which corresponds to a percentage of MIB-1 positive cells of 16–17% (Spyratos et al. 2002). To allow comparison of GCS expression between different datasets we used a median split of each dataset according to GCS (UGCG) using the ProbeSet 204881_s_at from the Affymetrix HG-U133A array. Samples were characterized as high or low expressing based on a median split of the cohorts according to this ProbeSet.

**Table 1** Clinical characteristics of breast cancer patients from Affymetrix microarray datasets used in this study

| Dataset | Data source | Array | Norm. method | No. of samples | % of samples | | | | | System treatment | Median follow up months | No. of relapses | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Age ≤50 | Tumor size ≤2 cm | LNN | ER pos. | G3 | | | | |
| Frankfurt | This study | U133A | MAS5 | 120 | 54 | 50 | 57 | 66 | 47 | Chemotherapy | 39 | 29 | Rody et al. (2007), Ruckhäberle et al. (2007) |
| Hamburg | This study | U133A | MAS5 | 100 | 46 | 24 | 59 | 65 | 59 | Chemotherapy | 57 | 31 | Rody et al. (2006) |
| Rotterdam | GSE2034, GSE5327 | U133A | MAS5 | 344 | NA | NA | NA | 61 | NA | 286 untreated, 58 NA | 86 | 118 | Wang et al. (2005), Minn et al. (2007) |
| Uppsala | GSE3494 | U133A | MAS5 | 251 | 22 | 51 | 65 | 80 | 22 | Yes/no | 118 | 91 | Miller et al. (2005) |
| Stockholm | GSE1456 | U133A | MAS5 | 159 | NA | Na | NA | 82 | 42 | Yes/no | 85 | 40 | Pawitan et al. (2005) |
| Oxford-Untreated | GSE2990 | U133A | RMA | 61 | 44 | 64 | 100 | 69 | 41 | Untreated | 121 | 29 | Sotiriou et al. (2006) |
| Oxford-Tamoxifen | GSE6532 | U133A | RMA | 109 | 14 | 34 | 64 | 95 | 19 | Endocrine | 61 | 30 | Loi et al. (2007) |
| London | GSE6532 | U133+ | RMA | 87 | 6 | 35 | 33 | 98 | 23 | Endocrine | 137 | 28 | Loi et al. (2007) |
| New York | GSE2603 | U133A | MAS5 | 99 | 37 | 9 | 34 | 58 | NA | NA | 65 | 27 | Minn et al. (2005) |
| Villejuif | GSE7390 | U133A | RMA | 50 | 80 | 26 | 100 | 72 | 38 | Untreated | 108 | 22 | Desmedt et al. (2007) |
| expO | GSE2109 | U133A | MAS5 | 301 | 31 | 32 | 47 | 65 | 49 | NA | NA | NA | http://www.intgen.org |
| Total | | | | 1,681 | 33 | 38 | 69 | 71 | 37 | | 76 | 445 | |

## Statistical analysis

All reported *P* values are two sided and *P* values of less than 0.05 were considered to indicate a significant result. Subjects with missing values were excluded from the analyses. Chi-square test was used for categorical parameters. Survival intervals were measured from the time of surgery to the time of death from disease or of the first clinical or radiographic evidence of disease recurrence. Data for women in whom the envisaged end point was not reached were censored as of the last follow-up date or at 120 months. We constructed Kaplan-Meier curves and used the log rank test to determine the univariate significance of the variables. A Cox proportional-hazards regression model was used to examine simultaneously the effects of multiple covariates on survival. The effect of each variable was assessed with the use of the Wald test and described by the hazard ratio, with a 95% confidence interval. The model included age, tumor size, lymph node status, ER, ErbB2, Ki67 as well as GCS expression. All analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL).

## Results

A database of Affymetrix microarray hybridizations from 1,681 primary breast cancer samples was established which was derived from eleven microarray datasets as given in Table 1. All experiments enclosed in this database were performed using the Affymetrix HG-U133A microarray. The GCS mRNA is represented by two different ProbeSets on this array. Consistency of normalized expression values obtained from these two ProbeSets was verified as shown in Fig. 2. Subsequently, all samples were stratified according to GCS expression based on a median split in each dataset. The clinical parameters of tumors with high and low expression of GCS, respectively, are presented in Table 2. There were no significant differences according to age and lymph node status of the patients when comparing the two groups with high and low GCS expression. In contrast, the analysis revealed a higher GCS expression in smaller tumors (*P* < 0.001 for tumors smaller than 2 cm vs. larger tumors). Tumors with lobular histology were preferentially found in the group with low GCS expression (72%) but the overall difference in histological subtypes between tumors with low and high GCS expression was not statistically significant. Interestingly, high expression of GCS was associated with a lower histological grade (*P* < 0.001) as well as low Ki67 expression (*P* < 0.001). On the other hand, low GCS expression was clearly associated with a negative ER status with 79.4% of ER the negative tumors stratified to the group with low GCS expression (*P* < 0.001). Breast cancer samples with ErbB2 overexpression were associated
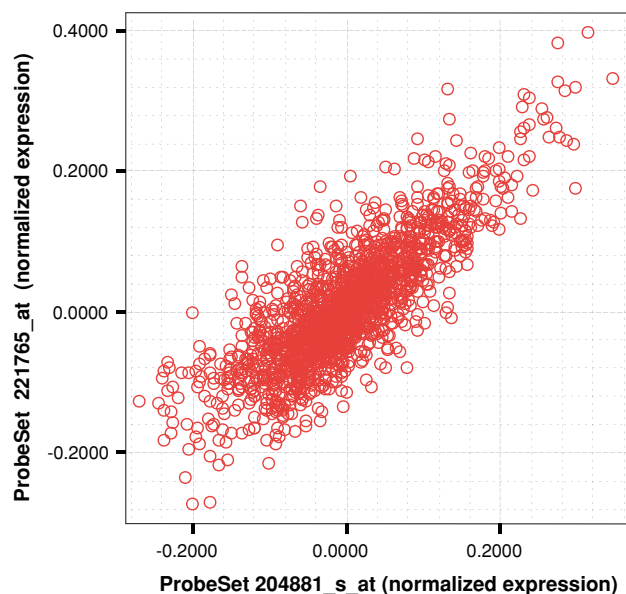


**Fig. 2** Validation of consistency of GCS expression data from the Affymetrix microarray. A scatter plot of the normalized expression value from two different ProbeSets for glucosylceramide synthase (GCS) present on the Affymetrix HG U133A microarray (ProbeSet 221765_at and 204881_s_at) is shown for all *n* = 1,681 samples

with lower GCS levels (66.7 vs. 33.3%, *P* < 0.001). The strong correlation of ER status with higher GCS expression is demonstrated in Fig. 3 (*P* < 0.001 for the combined data in Fig. 3a as well as for all individual datasets in Fig. 3b; Mann Whitney test).

The method of Kaplan-Meier was used to analyze the prognostic value of high GCS expression. For 1,363 of the 1,681 patients follow up data were available. As shown in Fig. 4 Kaplan-Meier estimates of disease free survival (DFS) revealed a significant better prognosis for breast cancer patients with high GCS expression (75.4 ± 1.7% vs. 70.0 ± 1.8%, *P* = 0.005 for 5 year DFS; and 65.9 ± 2.1% vs. 63.1 ± 2.1%, *P* = 0.047 for 10 year DFS). Next we analyzed the simultaneous influence of GCS expression and standard parameters on the prognosis of the patients in a multivariate Cox regression model using *n* = 699 patients for which all parameters were available. As presented in Table 3, however, only tumor size and ErbB2 status remained significant factors for disease recurrence in this multivariate analysis but the contribution of GCS expression was no more significant. Since GCS expression of breast cancers displayed a strong correlation with the ER status of the tumor it could be hypothesized that the prognostic value of GCS expression observed in the univariate analysis above (see Fig. 4) and the loss of this effect in the multivariate analysis might result from a confounding effect of the ER status between the two groups. Thus we additionally analyzed the univariate prognostic value of GCS by Kaplan-Meier analysis separately for ER positive and ER

**Table 2** Clinical characteristics of patient with high and low GCS expression

| Parameter | | $n = 1{,}681$ | Low GCS ($n = 840$) | High GCS ($n = 841$) | $P$ value |
|---|---|---|---|---|---|
| Age[++] | ≤50 year | 374 | 179 (47.9%) | 195 (52.1%) | 0.45 |
| | >50 year | 773 | 389 (50.3%) | 384 (49.7%) | |
| Lymph node status** | LNN | 957 | 469 (49.0%) | 488 (51.0%) | 0.4 |
| | N1 | 439 | 219 (49.9%) | 220 (50.1%) | |
| Tumor size* | ≤2 cm | 401 | 180 (44.9%) | 221 (55.1%) | 0.032 |
| | <2 cm | 665 | 344 (51.7%) | 321 (48.3%) | |
| Tumor grade[+] | Low grade (G1 and G2) | 663 | 286 (43.1%) | 377 (56.9%) | <0.001 |
| | High grade (G3) | 387 | 239 (61.8%) | 148 (38.2%) | |
| Ki67 expression | Below median | 839 | 351 (41.8%) | 488 (58.2%) | <0.001 |
| | ≥ median | 842 | 489 (58.1%) | 353 (41.9%) | |
| ER status | Positive | 1,200 | 458 (38.2%) | 742 (61.8%) | <0.001 |
| | Negative | 481 | 382 (79.4%) | 99 (20.6%) | |
| ErbB2 status | Negative | 1,432 | 674 (47.1%) | 758 (52.9%) | <0.001 |
| | Positive | 249 | 166 (66.7%) | 83 (33.3%) | |
| Histological subtype | Ductal | 357 | 185 (51.8%) | 172 (48.2%) | 0.34 |
| | Lobular | 25 | 7 (28.0%) | 18 (72.0%) | |
| | Mixed | 3 | 0 (0%) | 3 (100%) | |
| | Others | 61 | 28 (45.9%) | 33 (54.1%) | |

* information on tumor size was not available for $n = 615$ patients

** information on lymph node status was not available for $n = 285$ patients

[+] information on tumor grade was not available for $n = 631$ patients

[++] information on age was not available for $n = 534$

negative subgroups of cancers. As presented in Fig. 5 tumors with high GCS expression seem to have a better survival in both subgroups but this benefit is no more significant.

Recent in vitro data suggested a possible co activation of the genes for GCS and MDR1 in breast cancer cells (Gouaze-Andersson et al. 2007). Thus we analyzed if a correlation of these two parameters might also be apparent in our clinical sample collective. However, as demonstrated in Fig. 6 there was no clear relationship of MDR1 and GCS expression, suggesting that in clinical samples such co activation might only be observable after pretreatment with chemotherapeutic agents and not in our treatment naïve cohort.

## Discussion

Anticancer chemotherapy is mainly limited by the MDR phenomenon. Multidrug resistance can be caused by several mechanisms like an increase in cellular gluthathione S-transferase (Morrow and Cowan 1990), a decrease in topoisomerase II alpha activity (Deffie et al. 1989), increased expression of proteins of the bcl 2 family (Reed 1995) as well as loss of the tumor suppressor protein p53 (Mueller and Eppenberger 1996). Of particular importance is the transporter P-glycoprotein (P-gp, the product of the MDR1 gene) which reduces the intracellular concentration of antitumor agents as well as certain glycosylated sphingolipids.

Several recent reports have demonstrated an association between glycosylated sphingolipids and MDR for various types of cancer (Liu et al. 1999b; Gouaze-Andersson and Cabot 2006). Accumulation of glucosylceramide and increased activity of GCS is a characteristic finding in multi drug resistant ovarian, breast, colon, and epitheloid cells in vitro (Nicholson et al. 1999; Kok et al. 2000; Lavie et al. 1996; Morjani et al. 2001). An elevated GCS activity might prevent the accumulation of ceramide, which is thought to precede, and trigger apoptosis in response to at least some cytotoxic drugs (Simstein et al. 2003; Gomez-Munoz et al. 2006). Liu and colleagues could demonstrate, that doxorubicin enhances GCS gene expression (Liu et al. 2008) mediated by ceramide as second messenger. Furthermore, MDR reversing drugs like tamoxifen, verapamil, and cyclosporin A were shown to act by inhibition of ceramide glucosylation (Lucci et al. 1998; Lavie et al. 1997). Overexpression of GCS in MCF 7 cells conferred MDR to adriamycin (Liu et al. 1999b). Several authors have demonstrated that transfection of antisense GCS DNA depresses GCS expression, reduces drug resistance in breast cancer cells (Liu et al. 2000), inhibits neuroepithelioma cell growth (Di Sano et al. 2002), and inhibits melanoma growth in mice (Deng et al.

**A**



**B**
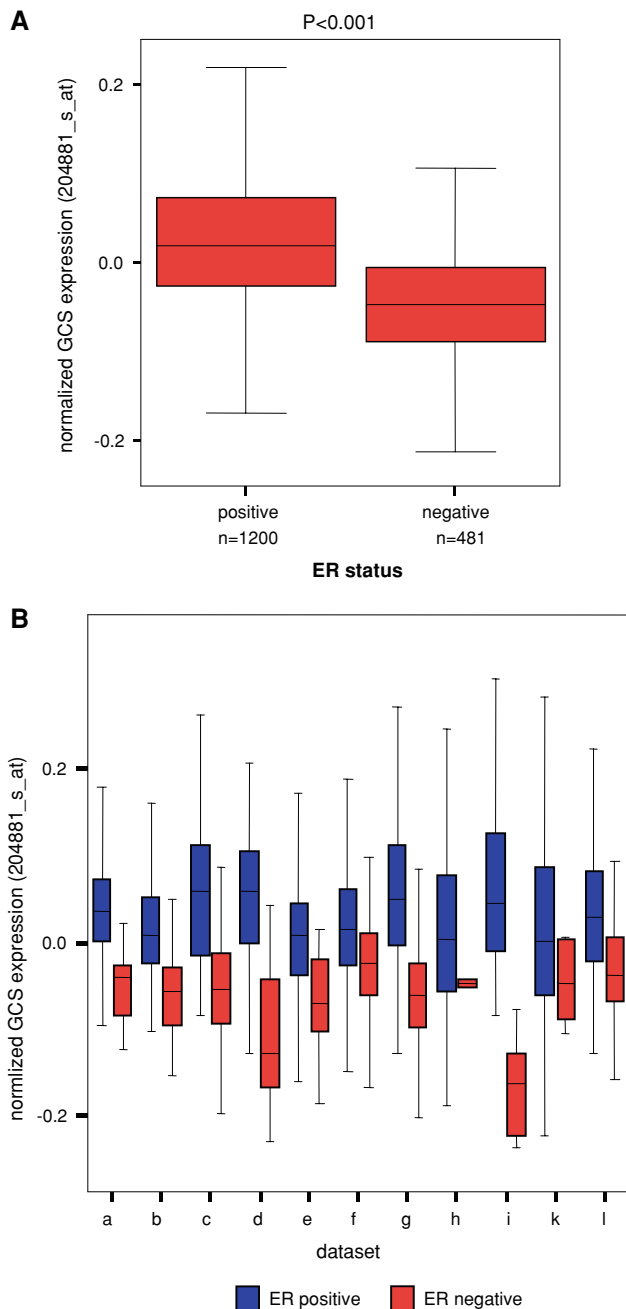


**Fig. 3** Correlation of normalized GCS expression with the ER status of the tumor. **a** Box plots of normalized GCS expression values of breast cancers stratified by their ER status from a combined analysis of all $n = 1,681$ samples. **b** Results from individual datasets (*a* Frankfurt, *b* Uppsala, *c* Hamburg, *d* Oxford-Untreated, *e* Stockholm, *f* expO, *g* New York, *h* London, *i* Villejuif, *k* Oxford-Tam, *l* Rotterdam)

2002; Weiss et al. 2003). In adriamycin resistant MCF 7 cells selective antisense oligodeoxyribonucleotides (ODNs) to GCS were able to substantially reverse the MDR phenotype (Liu et al. 2000). GCS antisense substantially restored cellular sensitivity to many anticancer drugs, including anthracyclines, taxanes, vinca alkaloids, and actinomycin D. All of these agents are substrates for the transporter
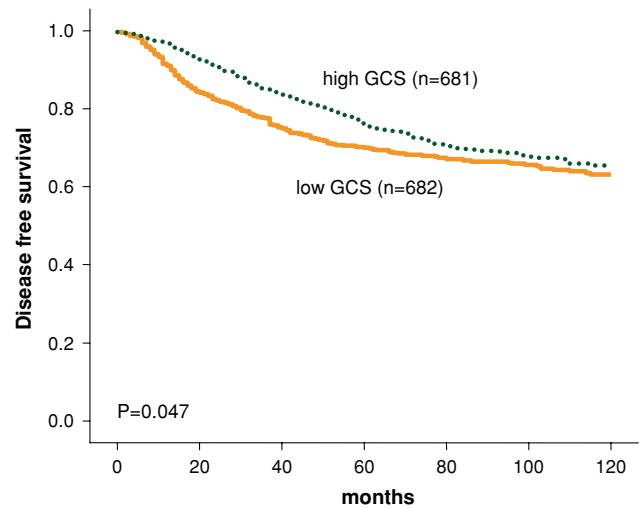


**Fig. 4** Prognostic value of GCS expression among breast cancer. Kaplan-Meier analysis of disease free survival of all $n = 1,363$ samples with follow up information stratified according to GCS expression

protein P-gp, whereas 5-flurouracil and cisplatin, for which toxicities were not substantially modified by GCS antisense, are not classified as pump efflux drugs.
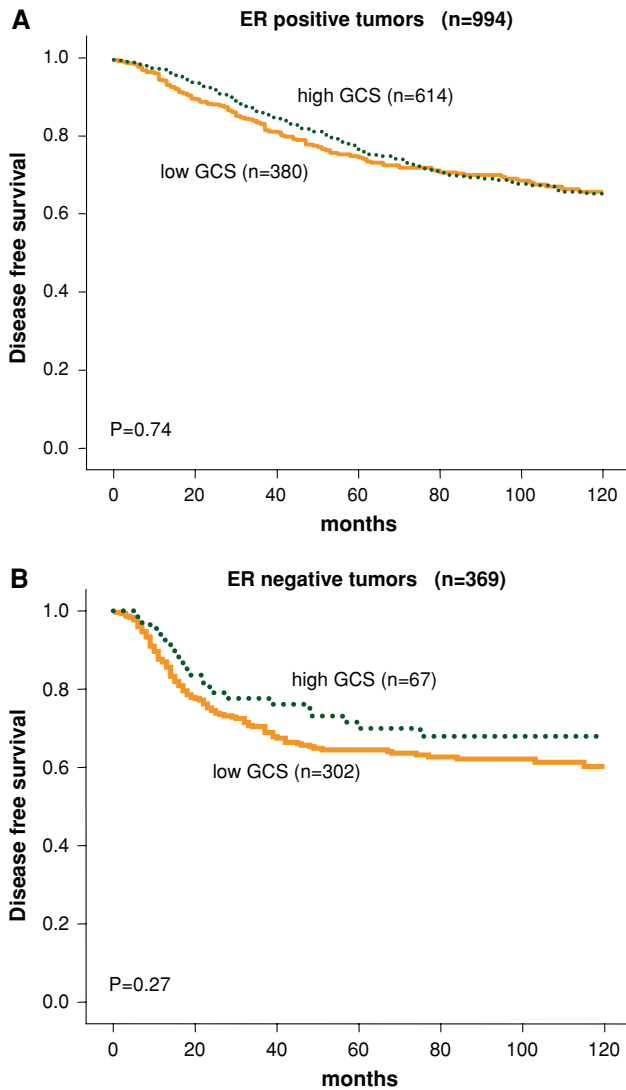
However, all of these data on GCS and chemotherapy resistance were so far obtained either in vitro using cultured cells. There are no firm data on GCS in clinical specimens especially for breast cancer. Therefore, we analyzed microarray data of a cohort of 1,681 clinical breast cancer samples and investigated GCS expression in subtypes of breast cancer and its prognostic relevance for disease recurrence. To our knowledge, this is the first report of clinical data from a large cohort on the expression of GCS in breast cancer and its impact on the course of disease. Expression of GCS was higher in the ER positive subgroup while ErbB2 positive tumors displayed lower GCS expression. In addition significant higher expression was revealed in smaller tumors ($P < 0.001$), breast cancers with low grading ($P < 0.001$) as well as low Ki67 levels ($P < 0.001$). The DFS of patients with tumors displaying low levels of GCS expression was significantly higher in univariate analysis. In multivariate Cox regression analysis, however, this benefit for survival was no more significant. This is in line with separate analysis of the univariate prognostic values of GCS in ER positive and ER negative subgroups, which were also not significant.

There seems to be a link between glycosphingolipids and multi drug resistance caused by P-gp. It is well known that doxorubicin, vinblastine, etoposide, cytarabine, methotrexate, and paclitaxel can induce MDR through direct activation of the MDR1 gene, which codes for the P-gp protein (Chaudhary and Roninson 1993; Kohno et al. 1989). More recently Anderson-Gouaze et al. could demonstrate that elevated levels of ceramide and GCS enhance expression of

**Table 3** Multivariate Cox regression analysis of standard parameters and GCS expression in relation to disease free survival among breast cancers patients

| Parameter | | $n$ | $P$ value | Hazard ratio | (95% CI) |
|---|---|---|---|---|---|
| GCS expression | Low vs. high | 354 vs. 345 | 0. 90 | 0.98 | (0.73–1.32) |
| Age | >50 vs. ≤50 | 456 vs. 243 | 0. 58 | 0.92 | (0.69–1.23) |
| Lymph node status | LNN vs. N1 | 447 vs. 252 | 0.17 | 0.82 | (0.63–1.09) |
| Tumor size | ≤2 cm vs. >2 cm | 296 vs. 403 | **<0.001** | 0.50 | (0.36–0.68) |
| Histological grading | Poor vs. well/intermediate | 230 vs. 469 | 0.43 | 1.14 | (0.83f–1.58) |
| ER status | Positive vs. negative | 541 vs. 158 | 0.62 | 0.91 | (0.62–1.33) |
| ErbB2 status | High vs. low | 104 vs. 595 | **0.044** | 1.44 | (1.01–2.05) |
| Ki67 expression | Low vs. high | 346 vs. 353 | **0.001** | 0.59 | (0.43–0.80) |

Data were available from $n = 699$ patients, significant $P$ values are given in bold



**A** ER positive tumors (n=994)

high GCS (n=614)

low GCS (n=380)

P=0.74

**B** ER negative tumors (n=369)

high GCS (n=67)

low GCS (n=302)

P=0.27

**Fig. 5** Prognostic value of GCS expression among ER positive and negative breast cancers. Kaplan-Meier analysis of disease free survival of breast cancers stratified according to GCS expression in the subgroups of **a** ER positive and **b** ER negative tumors
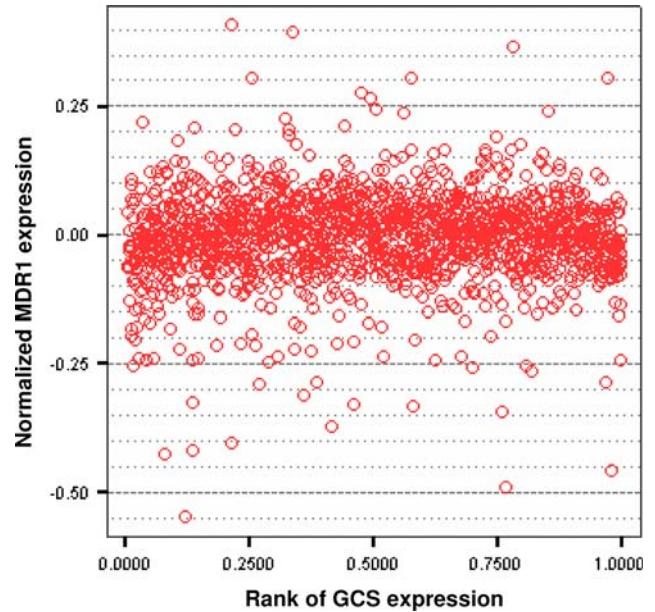


**Fig. 6** Relationship of MDR1 and GCS gene expression among breast cancers. Scatter plot of MDR1 and GCS gene expression values from Affymetrix microarrays

the MDR1 gene (Gouaze-Andersson et al. 2007). On the other hand P-gp was shown to modulate ceramide mediated sensitivity to paclitaxel and vincristin (Shabbits and Mayer 2002). Even if those data were obtained in vitro a correlation of GCS and MDR1 expression might also be observable in clinical samples. In our treatment naïve collective we found no evidence for such correlation, but pretreatment with chemotherapy might well induce a co activation of both genes in patients. Thus further analysis of tumors from neoadjuvant treated patients could give important insights in this regard.

Inhibition of GCS is possible by specific inhibitors like PDMP and Miglustat or selective antisense oligideoxyribonucleotides (Weiss et al. 2003). Inhibition of GCS activity

is evaluated as a possible medical treatment for several lipid-storage diseases and proven in vitro for some types of cancer (Butters 2007; Tifft and Proia 2000; Lachmann 2003; Jeyakumar et al. 2002). Glucosyl ceramide synthase inhibitors have already been approved for treatment of type I Gaucher disease and used in trials for Fabry disease, the GM2 gangliosidoses and Niemann-Pick disease (Patterson et al. 2007). They have been proven in principle in Tay-Sachs and Sandhoff mice models (Jeyakumar et al. 2002) and investigated in a clinical setting recently (Bembi et al. 2006). These clinical as well as the in vitro data might also propose a possible application of these drugs in breast cancer. In this context it will be crucial to define those subgroups of patients which might benefit from such treatment. According to our data, patients with ER positive and Her-2-neu negative tumors would be the most adequate starting point for trials of GCS inhibiting drugs since those tumors display high GCS expression.

In conclusion our results suggest that there seems to be no prognostic value of GCS expression in a treatment naïve cohort but ER positive tumors may be the most promising candidates for a potential therapeutic application of GCS inhibitors.

**Conflicts of interest statement**   There are no conflicts of interest to declare.

**Ethical approval**   Ethical approval was not required.

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