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ORIGINAL PAPER

Predictive value of sphingosine kinase 1 expression in neoadjuvant treatment of breast cancer

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Abstract
Purpose Sphingolipids play important roles in apoptosis and cell proliferation. Sphingosine kinase 1 (SphK1) expression has a prognostic impact in primary breast cancer, but its predictive value is currently unknown.
Methods A total of 112 breast cancer specimens from a prospective neoadjuvant chemotherapy trial (GeparDuo) were studied. Using tissue microarrays of pre-treatment core cut biopsies, we determined the expression of SphK1 by immunohistochemistry. The upper quartile of the cohort according to an immune reactive score of SphK1 was used as cutoff for high expression.
Results We observed a larger number of samples with high SphK1 expression among ER-negative cancers (36.8 vs. 20.5 % among ER-positive cancers; Fisher test $p = 0.073$). Eighteen of the 112 patients demonstrated a pathological complete response. A significant predictive value for pathological complete response was observed for ER negativity ($p = 0.003$), young age ($p = 0.037$), and high tumor grade ($p = 0.049$). An increased pCR rate was observed in tumors with high SphK1 expression within the luminal subtype (26.7 vs. 5.8 %; Fisher test $p = 0.040$). No significant difference in survival was detected according to SphK1 expression.
Conclusions Our results suggest that SphK1 may be a predictive factor for pCR after neoadjuvant treatment in luminal type breast cancers and warrants further investigation.

Keywords Breast cancer · Neoadjuvant systemic therapy · Sphingolipids · Prediction of response

Introduction

In a recent study on gene pathways associated with prognosis and chemotherapy sensitivity, all nine statistically significant prognostic gene sets that were consistently identified for ER-negative cancer were involved in glyco- and sphingo-lipid metabolism (Iwamoto et al. 2011). Sphingolipids are crucial regulators of cell function and play important roles in the regulation of angiogenesis,

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apoptosis, cell proliferation, senescence, differentiation, migration, and inflammation. Simple bioactive sphingolipids include ceramide, sphingosine, and their phosphorylated forms sphingosine-1-phosphate (S1P) and ceramide-1-phosphate. The balance between the proapoptotic sphingosine and ceramide and the antiapoptotic sphingosine-1-phosphate is called "sphingolipid rheostat" (Spiegel 1999). More recent work has established a "sphingodynamic" model that reflects the complexity of the sphingolipid-mediated cellular processes and accounts for changes within a greater number of bioactive sphingolipids (Fyrst and Saba 2010). An imbalance in this equilibrium for what reason could lead to uncontrolled tumor progression or cell death (Hait et al. 2006). The two major players in the rheostat are ceramide and S1P. The latter is a bioactive mediator that plays an essential role in the regulation of cell motility and cell growth, chemoresistance as well as in the paracrine angiogenesis and lymphangiogenesis in vitro (Ponnusamy et al. 2010; Meacham et al. 2009). S1P is generated from sphingosine catalyzed by one of the two isoforms of sphingosine kinase 1 (SphK1). SphK1 is overexpressed in many tumors (Ogretmen and Hannun 2004; Furuya et al. 2011). In breast cancer cells, SphK1 has been implicated in the link between estrogen and growth factor signaling (Sukocheva et al. 2003, 2006, 2009a; Takabe et al. 2010). In a previous study, we were able to demonstrate a significant higher gene expression for SphK1 in ER-negative tumors as compared to ER-positive cancers. Moreover, we demonstrated that SphK1 expression is associated with poor prognosis in both the full cohort of 968 clinical breast cancer samples as well as in the subcohort of 750 ER-positive cases (Ruckhäberle et al. 2008). In addition, a prognostic role was also observed in independent studies applying immunohistochemical detection of SphK1 (Long et al. 2010; Watson et al. 2010; Ohotski et al. 2012, 2013).

Neoadjuvant systemic treatment represents a valuable therapeutic option for certain cases of breast cancer (e.g., large, inoperable triple-negative tumors with grading 3) (Kaufmann et al. 2012). Beyond achievement of breast conservative surgery, decrease in loco-regional therapy, an improvement in prognosis, an in vivo response monitoring, neoadjuvant systemic therapy provide the opportunity of identification of surrogate prognostic and predictive biomarkers (Makhoul and Kiwan 2011). So far only a few factors (tumor type, estrogen receptor status, Ki-67 levels, Her2 status) are available for prediction of response to chemotherapy.

In a recent study, it has been shown that gene expression data from enzymes involved in sphingolipid metabolism in combination with a mitotic gene module are predictive for response to neoadjuvant treatment of breast cancer with taxanes (Juul et al. 2010; Lee et al. 2012). In addition, SphK1 was shown to be a predictive marker for daunorubicin (Sobue et al. 2008), doxorubicin (Sarkar et al. 2005), and docetaxel (Sauer et al. 2009) sensitivity in cell lines. However, no clinical data on SphK1 as predictive marker in breast cancer exist. The aim of the present study was (1) the evaluation of the predictive value in a well-defined neoadjuvant group of patients from a clinical trial and (2) the validation of the prognostic role of SphK1 expression in this cohort.

Materials and methods

Study population and histopathological examination

All analyses were performed according to the "*REporting recommendations for tumor MARKer prognostic studies*" (REMARK) (McShane et al. 2005). A The CONSORT (Schulz et al. 2010) diagram in Fig. 1 presents the flow of patients through the study. The multicenter randomized prospective neoadjuvant phase III GeparDuo trial (NCT00793377) investigated 913 patients with operable breast cancer (T2-3, N0-2, M0) between June 1999 and September 2001 comparing doxorubicin 50 mg/m^2 plus docetaxel 75 mg/m^2 every 14 days for four cycles with filgrastim support (ddADOC, $n = 451$) or four cycles doxorubicin 60 mg/m^2 plus cyclophosphamide 600 mg/m^2 every 21 days followed by docetaxel 100 mg/m^2 every 21 days for four cycles (AC-DOC, $n = 453$) (von Minckwitz et al. 2005). The trial was conducted in compliance with the Helsinki Declaration. The protocol was reviewed and approved by all responsible ethics committees. Consent of patient, pathologist, and investigator to supply tumor material of biopsy and surgery for central pathologic evaluation and examination of predictive factors was available. All patients received tamoxifen simultaneously to all chemotherapy cycles, irrespective of HR status. The primary endpoint was the incidence of pCR in the breast and axillary nodes (absence of invasive and non-invasive (carcinoma in situ) tumor cells in the surgical specimen including lymph nodes). A statistical analysis using a pCR definition that also includes cases with residual in situ carcinoma yielded similar results (not shown). For 219 patients, tissue from the pre-surgical biopsy containing more than 30 % tumor tissue was available in our tissue bank. These samples were used to construct a tissue microarray. As the method of TMA construction from breast cancer core biopsies was a quite new development of the Charité pathology team at the time the GeparDuo TMA was constructed, dropout of tissue spots on the slides was relatively high, and for 112 of these samples, successful staining for SphK1 expression by immunohistochemistry was obtained. For clinicopathological characteristics of our

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study cohort see Table 1. Supplementary table S1 presents a comparison of the patient characteristics between the overall study population and the subgroup for which the biomarker assessment was available. Data according to clinical tumor stage (cT) and clinical lymph node state (cN), patient age, pCR, and outcome data were derived from the clinical study database. Core biopsies were re-evaluated according to tumor histology and grading (Bloom-Richardson modified by Elston and Ellis 1991) by two experienced pathologists. DFS data were available from 105 patients for a median follow-up time without event of 57.6 months.

Immunohistochemical staining

Immunohistochemical staining was performed using a polyclonal SphK1 antibody (Imgenex, IMG-72025, San Diego, CA, USA). Antigens were retrieved by microwaving sections in 10 mM citrate buffer (pH 6.0) for 20 min at 800 W. Blocking was performed using antibody dilution buffer (DCS-Diagnostics, Hamburg, Germany) at room temperature for 15 min. Subsequently, primary antibody was diluted 1:100 individually in this buffer. Sections were incubated with the antibody 1 h at room temperature. For negative controls, the primary antibody was replaced with PBS. For secondary antibody incubation and detection, the Dako REAL Detection System Alkaline Phosphatase/RED (Dako, Danmark) was used following the protocol of the supplier and sections were slightly counterstained with Mayer's hematoxylin. Stained slides were digitized by a slide scanner (Mirax Scan, Zeiss, Jena, Germany) and were subsequently evaluated by a senior pathologist who was blinded toward the patients' outcome (SDE) using a custom-made software for whole slide imaging (TMA Evaluator, VM Scope GmbH, Berlin, Germany). Semi-quantitative evaluation of the rate of stained tumor cells and the staining intensity was registered as followed: The percentage of positive cells was scored as 0 (0 %), 1 (<10 %), 2 (11–50 %), 3 (51–80 %), or 4 (>80 %), and the staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). Multiplication of both parameters resulted in the immunoreactivity score (IRS), which ranged from 0 to 12.

Immunohistochemical determination of ER, PgR, and Her2 was performed as described previously (Darb-Esfahani et al. 2009).

Intrinsic breast cancer subtypes were determined according to clinicopathologic criteria recently recommended by the St. Gallen panelists (Goldhirsch et al. 2011). Because information on Ki-67 was not available, we used grade to capture cell proliferation. The following definitions were used (von Minckwitz et al. 2012): *Luminal A-like tumors:* ER positive and/or PgR positive, Her2 negative, grade 1 or 2. *Luminal B-like tumors* included all tumors of the following two groups: (1) luminal B-like/Her2-negative tumors: ER positive and/or PgR positive, Her2 negative, grade 3; and (2) luminal B-like/Her2-positive tumors: ER positive and/or PgR positive, Her2 positive, all grades. *Her2-positive (non-luminal)-like tumors:* ER negative and PgR negative, Her2 positive, all grades. *Triple-negative (TN) tumors:* ER negative, PgR negative, Her2 negative, all grades.

Statistical evaluation

All statistical analyses were performed following a pre-defined statistical analysis plan (SAP) using the upper quartile of immunoreactivity scores (IRS) of the cohort as cutoff value for SphK1 positivity. This cutoff has been pre-defined based on previous observations (Ruckhäberle et al. 2008). The distribution of SphK1 in the subgroups defined by the standard baseline parameters was assessed using cross-tabulation and two-sided exact test of Fisher. The impact of SphK1 and standard baseline parameters on pCR

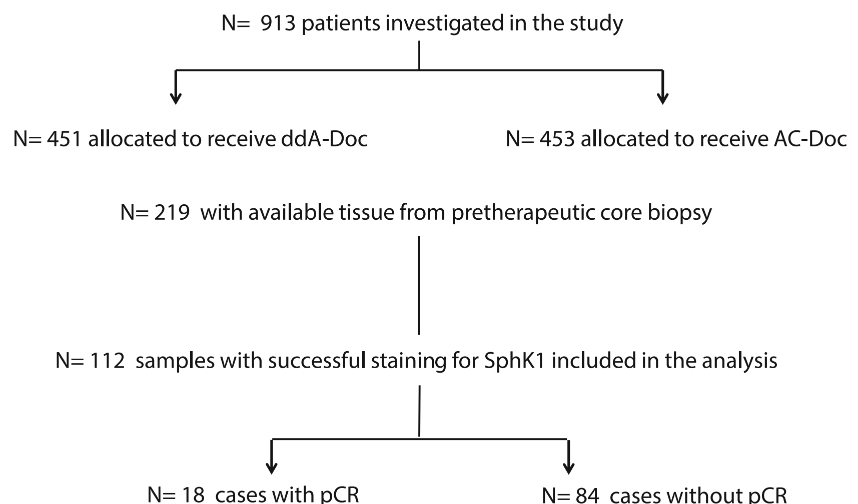

Fig. 1 Consort diagram of the flow of patients and samples through the study

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Table 1 Clinical parameters of 112 patients with tumors displaying low or high SphK1 expression

	SphK1 expression						p value
	Total ($n = 112$)		Low ($n = 83$)		High ($n = 29$)		
Age							
≤50	49	43.8 %	33	39.8 %	16	55.2 %	
>50	63	56.3 %	50	60.2 %	13	44.8 %	0.193
Clinical tumor size							
≤2 cm	8	7.1 %	4	4.8 %	4	13.8 %	
>2 cm	104	92.9 %	79	95.2 %	25	86.2 %	0.201
Clinical lymph node status							
LNN	80	71.4 %	60	72.3 %	20	69.0 %	
N1	32	28.6 %	23	27.7 %	9	31.0 %	0.812
ER status							
Positive	73	65.8 %	58	70.7 %	15	51.7 %	
Negative	38	34.2 %	24	29.3 %	14	48.3 %	0.073
Her 2 neu status							
Negative	81	81.0 %	62	84.9 %	19	70.4 %	
Positive	19	19.0 %	11	15.1 %	8	29.6 %	0.149
Histological grading							
G1–2	73	65.2 %	54	65.1 %	19	65.5 %	
G3	39	34.8 %	29	34.9 %	10	34.5 %	1.000
Chemotherapy type							
ADoc	62	55.4 %	42	50.6 %	20	69.0 %	
AC-Doc	50	44.6 %	41	49.4 %	9	31.0 %	0.128
Molecular subtype							
Her2-positive-like (ER−/PR−/Her2+)	9	9.9 %	4	5.8 %	5	22.7 %	
Triple-negative (ER−/PR−/Her2−)	15	16.5 %	13	18.8 %	2	9.1 %	
Luminal A-like (ER +/PR +/Her2−)	47	51.6 %	36	52.2 %	11	50.0 %	
Luminal B-like (ER +/G3 or ER +/Her2+)	20	22.0 %	16	23.2 %	4	18.2 %	0.112

was analyzed with the univariate and bivariate binary logistic regressions. The impact of SphK1 and of the standard baseline parameters on disease-free and overall survival was assessed using the Kaplan–Meier product-limit method with logrank test and Cox proportional hazards model (univariate and bivariate). All statistical tests are two-sided. The significance level was taken as $p \leq 0.05$. All p values are to be considered exploratory and are reported as is, without adjustment for the multiple testing. The analysis was performed with the software package SPSS v14.0 (Chicago, IL, USA).

Results

Within the GEPARDUO study, 913 patients were enrolled; 451 women were randomized to receive ddADOC, and 453 were randomly assigned to AC-DOC. As part of the translational research program, 219 pre-therapeutic core cut specimens were processed to tissue microarrays and investigated by immunohistochemistry for SphK1 expression. SphK1 was expressed in the cytoplasm of tumor cells and, if present, revealed a diffuse staining pattern in most cases. ER, PgR, and Her2 expression had been determined as described previously (Darb-Esfahani et al. 2009). Successful staining for SphK1 was available for 112 of the samples. Table 1 presents the clinical parameters of the samples subdivided according to the expression of SphK1. We detected a larger proportion of samples with high SphK1 expression among ER-negative cancers (36.8 vs. 20.5 %; $p = 0.073$). No significant correlations between patient's age, tumor size, grading, lymph node status, chemotherapy regimen nor Her2 status, and expression of SphK 1 were observed.

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Eighteen out of the 112 patients (16.1 %) achieved a pathologic complete remission. In univariate logistic regression, negative hormone receptor status (OR = 5.15, 95 % CI 1.75–15.7, $p = 0.003$), age under 40 years (OR = 3.42, 95 % CI 1.08, 10.8, $p = 0.037$), and tumor grade 3 (OR = 2.86, 95 % CI 1.01–8.13, $p = 0.049$) were significant predictors for a pCR (Table 2). No significant correlation with pCR rate was detected for Her2 status, tumor size, nodal status, and the treatment regimen (Table 2). Tumors with high SphK1 expression showed only a small trend toward a higher pCR rate (24.1 vs. 13.3 %; OR = 2.08, 95 % CI 0.72–6.02; $p = 0.175$, Table 2). We also evaluated the correlation of pCR and SphK1 expression separately in the different breast cancer subtypes. As shown in Table 3, we detected no difference in pCR rates when ER-negative tumors were analyzed. In contrast, within the subgroup of ER-positive ("luminal") breast cancers, the pCR rate among the tumors with strong SphK1 expression was significantly higher than for those with low SphK1 expression (26.7 vs. 5.8 %; $p = 0.040$; Table 3). When we further stratified tumors into molecular subtypes still a trend was observed within the luminal A-like subgroup ($p = 0.076$), but no significance was detected for luminal B-like cancers ($p = 0.37$). However, the percentages of pCR rates for high versus low SphK1 were still similar in both subgroups (27.3 vs. 5.6 % and 25.0 vs. 6.3 %, in luminal A-like and luminal B-like groups, respectively; Table 3). Thus, the lack of significance may be attributed to the small samples size of the luminal B-like subgroup ($n = 20$).

Table 2 Univariate logistic regression of pCR according to SphK1 and standard baseline parameters

Parameter	*p* value	OR with 95 % CI
SphK1 high versus low	0.175	2.08 (0.72–6.02)
Hormone receptor negative versus positive	**0.003**	5.15 (1.75–15.2)
Age ≤40 years versus >40 years	**0.037**	3.42 (1.08–10.8)
Tumor grade 3 versus 1–2	**0.049**	2.86 (1.01–8.13)
Treatment arm ddADoc versus AC-Doc	0.13	0.45 (0.16–1.27)
Nodal negative versus nodal positive	0.29	0.57 (0.20–1.63)
cT1 versus cT2–3	0.48	1.83 (0.34–9.9)
Her2 negative versus positive	0.98	1.02 (0.26–4.0)

Significant *p*-values are given in bold

We correlated the available follow-up information of the patients with either high or low SphK1 expression. However, we did detect a significant difference neither in the disease-free survival ($p = 0.65$, Fig. 2a) nor in the overall survival ($p = 0.43$, Fig. 2b) for these patients. Similarly, additional separate analyses of those tumors without a pathological complete remission (Fig. 3a) and those with a pCR (Fig. 3b) also revealed no significant prognostic differences between tumors with high or low SphK1 expression (Fig. 3).

Discussion

Recent studies have reported a predictive value of enzymes from sphingolipid metabolism for response to neoadjuvant chemotherapy in breast cancer (Juul et al. 2010; Lee et al. 2012) and SphK1 as a marker for sensitivity to several chemotherapeutic agents in cell lines (Sobue et al. 2008; Sarkar et al. 2005; Sauer et al. 2009). Since neoadjuvant treatment allows directly testing response prediction (Kaufmann and Pusztai 2011), we have analyzed the predictive

Table 3 Correlation of pCR and SphK1 expression among different intrinsic subtypes of breast cancer

Subgroup	pCR	Total ($n = 91$)	% pCR	SphK1 expression				*p* value
				Low ($n = 69$)	% pCR	High ($n = 22$)	% pCR	
ER positive	No	60		49		11		
	Yes	8	10.4 %	3	5.8 %	4	26.7 %	**0.040**
ER negative	No	16		11		5		
	Yes	8	33.3 %	6	35.3 %	2	28.6 %	1.0
Her2-positive-like	No	7		4		3		
(ER−/PR−/Her2+)	Yes	2	22.2 %	0	0 %	2	40.0 %	0.44
Triple-negative	No	9		7		2		
(ER−/PR−/Her2−)	Yes	6	40.0 %	6	46.2 %	0	0 %	0.49
Luminal A-like	No	42		34		8		
(ER+/PR+/Her2−)	Yes	5	10.6 %	2	5.6 %	3	27.3 %	0.076
Luminal B-like	No	18		15		3		
(ER+/G3 or ER+/Her2+)	Yes	2	10.0 %	1	6.3 %	1	25.0 %	0.37

Significant *p*-values are given in bold

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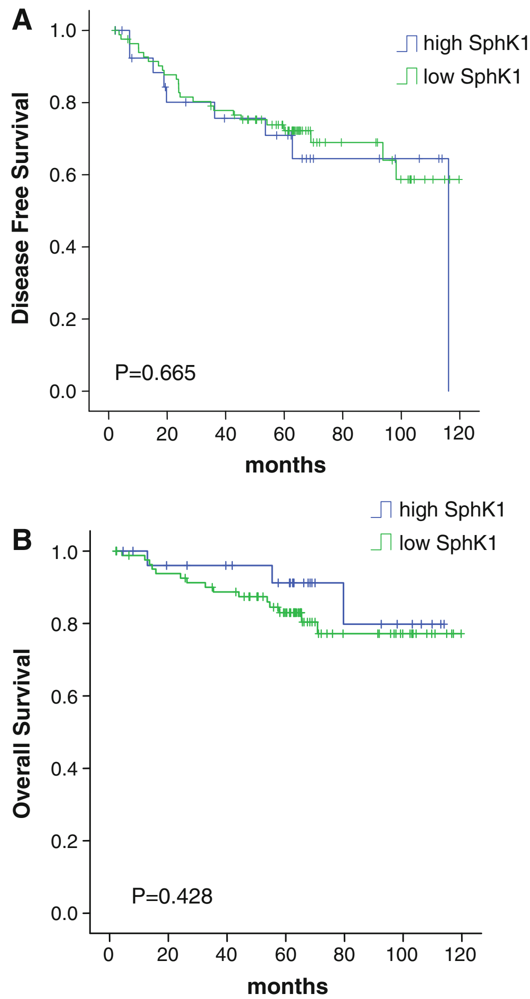

Fig. 2 **a** Disease-free survival of neoadjuvant treated patients according to SphK1 expression. Kaplan–Meier curves of the disease-free survival in the complete cohort of 112 patients treated with neoadjuvant chemotherapy. Samples are stratified according to high or low SphK1 expression. **b** Overall survival of neoadjuvant treated patients according to SphK1 expression. Kaplan–Meier curves of the overall survival in the complete cohort of 112 patients treated with neoadjuvant chemotherapy. Samples are stratified according to high or low SphK1 expression

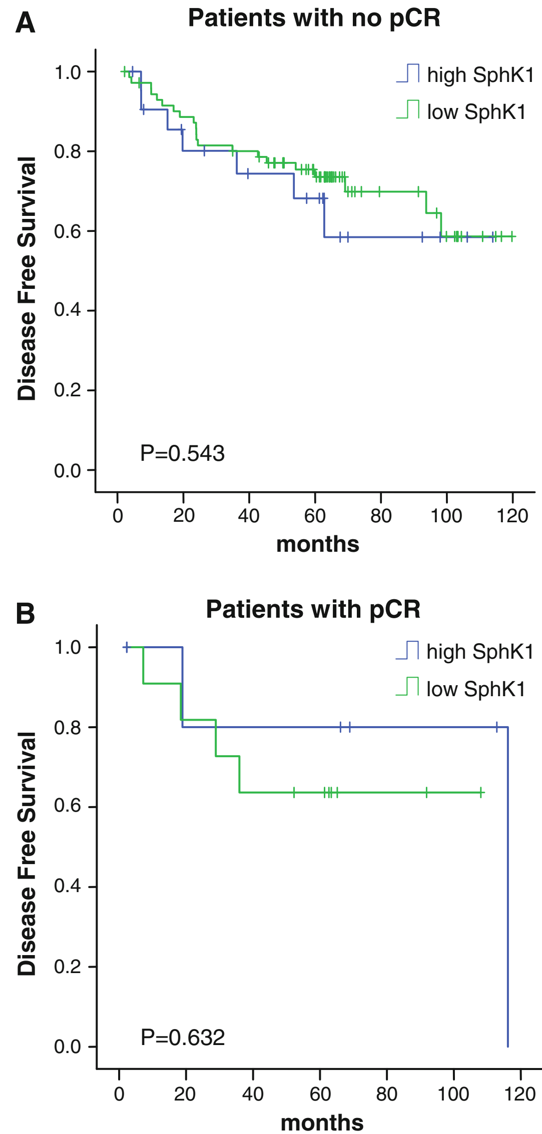

Fig. 3 **a** Disease-free survival of patients without pCR according to SphK1 expression. Kaplan–Meier curves of the disease-free survival of 94 patients which did not accomplish a pathological complete response after neoadjuvant chemotherapy. Samples are stratified according to high or low SphK1 expression. **b** Disease-free survival of patients with pCR according to SphK1 expression. Kaplan–Meier curves of the disease-free survival of 18 patients which accomplished a pathological complete response after neoadjuvant chemotherapy. Samples are stratified according to high or low SphK1 expression

value of SphK1 expression in samples from the neoadjuvant GeparDuo trial.

Our study failed to establish a significant predictive value of SphK1 expression for pCR among the total analyzed cohort of 112 patients (OR 2.08; 95 % CI 0.72–6.02, $p = 0.175$; Table 2). However, in a subgroup analysis, we detected a significant higher pCR rate for tumors with high SphK1 expression within the ER-positive luminal subtype of tumors (26.7 vs. 5.8 %; $p = 0.040$; Table 3). The respective pCR rates were similar for both luminal A-like (27.3 vs. 5.6 %, $p = 0.076$; Table 3) and luminal B-like (25.0 vs. 6.3 %, $p = 0.37$; Table 3) types of tumors even when the results of these small subgroups were not significant which may be attributed to reduced samples sizes ($n = 11$ vs. 36 and $n = 4$ vs. 16, respectively; Table 3).

In the present study, we failed to confirm the previously reported prognostic impact of SphK1 expression (Ruckhäberle et al. 2008). Nonetheless, we could validate the higher SphK1 expression in ER-negative tumors (Ruckhäberle et al. 2008). One reason for the failure to validate the prognostic value in the present study could also be

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related to differences in sample size. In our previous study, a total cohort of 968 samples with follow-up were analyzed as compared to only 112 samples in the present study. Importantly, in that previous study, the prognostic value of SphK1 did equally not reach significance in a smaller cohort of 171 patients. Second, the analytical methods for SphK1 detection differ between the two studies (immunohistochemistry vs. microarray gene expression profiling) limiting direct comparability. Nevertheless, independent reports which have also applied immunohistochemical detection of SphK1 were able to verify its prognostic value (Long et al. 2010; Watson et al. 2010; Ohotski et al. 2012, 2013). However, differences between subtypes were observed in these studies. SphK1 expression was associated with shorter disease-free survival in 304 ER-positive patients treated with tamoxifen, but an inverse effect was detected within the subgroup of Her2-positive ER-positive samples (Long et al. 2010; Watson et al. 2010). In an analysis of 140 ER-negative patients, SphK1 was also associated with poor prognosis within 39 samples of the ER-negative Her2-positive subtype (Ohotski et al. 2012). However, in the 101 samples of the ER-negative Her2-negative group, the prognostic value of SphK1 alone did not reach significance, but was only associated with shorter disease-free survival if tumors also contained low levels of the S1P receptor $S1P_4$ (Ohotski et al. 2012). When stratifying patients in the present study according to breast cancer subtypes, the sample groups are very small with only 9, 15, and 47 patients in the Her2-positive-like, triple-negative, and luminal A-like groups, respectively (see Table 3). ER-positive Her2-positive patients in our classification are included in the luminal B-like subtype encompassing 20 samples in total (see Table 3; "Materials and methods" section). Thus, the differences to the above-mentioned studies which were also based on immunohistochemical detection may well originate from these reduced sample sizes. In addition, the patients in the larger referred study (Long et al. 2010) received solely tamoxifen as adjuvant treatment. Thus, the effect of chemotherapy treatment might also interfere with the prognostic value of SphK1, even if we did not detect differences when we separately analyzed patients which accomplished a pathological complete remission and those who have not (Fig. 3).

The recent study provoking sphingolipid metabolism as a predictor for response to neoadjuvant chemotherapy (Juul et al. 2010) did not particularly analyze SphK1 but rather different genes from the sphingolipid metabolism (UGCG and COL4A3BP). Moreover, a predictive value for response to taxane treatment was specifically found for triple-negative breast cancer in that study. In our cohort, 15 samples belonged to this subtype, but we did not detect a predictive value of SphK1 expression in this subgroup with no pCR among the two samples with high SphK1 expression (Table 3). In contrast, a predictive value of SphK1 was only observed for luminal ER-positive tumors in our study. Luminal tumors, especially of the luminal A subtype of breast cancer, have been reported to be less sensitive to chemotherapy than other molecular subtypes (Darb-Esfahani et al. 2009; Rouzier et al. 2005; Rody et al. 2007; Kaufmann et al. 2012). Thus, SphK1 expression may help to identify those patients who might still respond to chemotherapy within this subtype. However, it should be noted that all patients in our study received tamoxifen simultaneously to all chemotherapy cycles, irrespective of ER status. Thus, since the only association between SphK1 and pCR was observed in ER-positive tumors, a potential contribution on this association from tamoxifen cannot be ruled out. Interestingly in this respect, many recent results from basic research highlight the involvement of SphK1 in estrogen-induced signaling in breast cancer cells (Sukocheva et al. 2009b; Takabe et al. 2010; Antoon et al. 2011, 2012).

In conclusion, our study has main limitations in its relatively small sample size and thus the observed results are preliminary and only hypothesis generating. Nevertheless, an evaluation of the predictive potential of SphK1 in a larger number of luminal type tumors might be worth to be verified in additional studies.

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Conflict of interest The authors declare that they have no competing interests.

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