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PRECLINICAL STUDY

Microarray analysis of altered sphingolipid metabolism reveals prognostic significance of sphingosine kinase 1 in breast cancer

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Abstract Beside their structural role for the cell membrane the family of sphingolipids act as effector molecules in signal transduction with links to various aspects of cancer initiation, progression and treatment response. The "sphingolipid rheostat" balances between apoptosis inducing ceramid and growth promoting sphingosine-1-phosphate. We analyzed gene expression of 43 proteins from this pathway in different subtypes of breast cancer using microarray data of 1,269 tumor samples (test set $n = 171$; validation sets $n = 1098$) and observed significant differences for several genes. Sphingosine kinase 1 (SPHK1), ceramide galactosyltransferase (UGT8), and Ganglioside GD3-Synthase (ST8SIA1) displayed higher expression among ER negative tumors. In contrast, glucosylceramidsynthase (GCS), dihydroceramidsynthases (LASS4, LASS 6) and acid ceramidase (ASAH1) were higher expressed in ER positive samples. Survival analysis revealed a worse outcome of patients with high SPHK1 expression. To avoid a confounding effect of the ER status we also restricted the analysis to 750 patients with ER positive tumors. Again a worse outcome was observed for tumors displaying high SPHK1 expression. While $75.8 \pm 1.9\%$ of the patients with tumors low in SPHK1 expression were free of metastasis at 5 years, this was the case for only $64.9 \pm 3.6\%$ of patients with tumors displaying high SPHK1 expression ($P = 0.008$). Immunohistochemistry identified the carcinoma cells as the major source of SPHK1 expression in the tumor. The correlation of SPHK1 with a poor prognosis as well as its high expression among ER negative tumors are in line with the antiapoptotic and proliferative properties of its product sphingosine-1-phosphate. Targeting of the sphingolipid rheostat may thus open new treatment options.

Keywords Breast cancer · Ceramide · Sphingolipid metabolism · SPHK1

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Introduction

Breast cancer is the most frequent cancer of women worldwide and the knowledge of the complexity of this disease is steadily increasing. A topic that has gained interest quite recently in breast cancer research is the role of the sphingolipid metabolism in this disease [1]. The sphingolipids are a family of membrane lipids with important structural roles in the regulation of the fluidity and subdomain structure of the lipid bilayer [2]. Molecular studies of sphingolipid metabolism and function during the past two decades however revealed that the sphingolipids ceramide, ceramide-1-phosphate, glucosylceramide, lactosylceramide, galactosylceramide, sphingosine, sphingosylphosphocholine, psychosine and sphingosine-1-phosphate (S1P) also act as effector molecules and not only as inert precursors and products of sphingolipid metabolism (see Ref. 3). Many sphingolipid-regulated functions have significant and specific links to various aspects of cancer initiation, progression and response to anticancer

treatments. Ceramide in particular is intimately involved in the regulation of cancer-cell growth, differentiation, senescence and apoptosis. Many cytokines, anticancer drugs and other stress-causing agonists result in increases in endogenous ceramide levels through de novo synthesis and/or the hydrolysis of sphingomyelin. Reciprocally, decreased levels of endogenous ceramide caused by increased expression of glucosylceramide synthase (GCS), which clears ceramide levels by incorporating it into glucosylceramide, might be involved in the development of a multidrug resistance (MDR) phenotype in many cancer cells [3–5]. In contrast to the actions of ceramide, S1P is emerging as a key regulator of proliferation, inflammation, vasculogenesis and resistance to apoptotic cell death. S1P was shown to stimulate invasiveness of human glioblastoma cells and to promote estrogen-dependent tumorigenesis of MCF 7 human breast cancer cells [6, 7]. Small-molecule mimetics of ceramide as well as enzyme inhibitors of ceramide clearance or S1P generation might represent novel targets for anticancer therapeutics [8–10].

Our understanding of sphingolipid function has been hindered by the great complexities of the networks of sphingolipid metabolism and their compartmentalization, coupled with inherent experimental difficulties in studying lipid metabolism and function (see Fig. 1). The analysis of these networks can be addressed at different levels. Firstly, at the level of the respective enzymes controlling the sphingolipid metabolism or secondly at the level of the metabolites themselves. The “enzyme level” can be analyzed by methods directed either towards gene or protein expression where genomic and proteomic approaches would allow a global view on the network. The “metabolite level” can be addressed by chromatographic and spectroscopic methods which allow detection of the lipids themselves.

Aim of our study presented here was to investigate sphingolipid metabolism in breast cancer on the level of gene expression of the respective enzymes. Since the estrogen receptor (ER) status is a major determinant of breast cancer subtypes with important clinical implications we first examined the influence of the ER status on expression of several enzymes from the sphingolipid metabolism network using the microarray technology. Secondary objective was a possible impact of gene expression of these enzymes on the prognosis of the patients.

Materials and methods

Patient cohort

Tissue samples of 171 invasive breast cancer cases were obtained with IRB approval and informed consent from consecutive patients undergoing surgical resection between December 1996 and July 2003 at the Department of Gynecology and Obstetrics at the J. W. Goethe-University in Frankfurt. All tissue samples were stored in liquid nitrogen. Samples were characterized according to standard

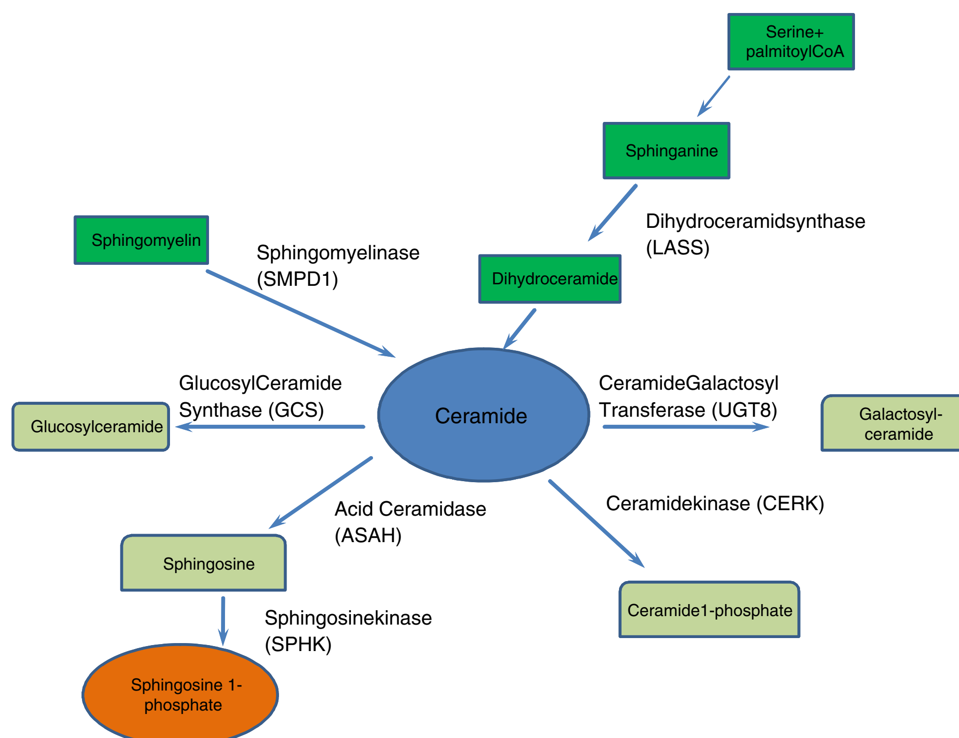

Fig. 1 Pathways of sphingolipid metabolism. Substrates and intermediates of the metabolism of ceramide and sphingosine-1-phosphate are schematically shown as boxes and the respective enzyme names are given beside the arrows

Table 1 Clinical charateristics of the patients of the primary dataset

Clinical variable		Number of patients ($n = 171$)
Age	≤50	68
	>50	103
Tumor size	≤2 cm	91
	>2 cm	80
Lymph node status	Negative	97
	Positive	71
	Unknown	3
Histology	Ductal	116
	Lobular	42
	Mixed	8
	Other	5
Tumor Grade	G1	13
	G2	97
	G3	61
ER status	Positive	129
	Negative	42

pathology including the estrogen receptor status by ligand binding assays or immunohistochemistry (IHC). Clinical characteristics of the patients are given in Table 1.

Microarray data analysis

Isolation of RNA and expression profiling using Affymetrix Human Genome U133A microarrays was performed as described elsewhere [11]. Briefly, hybridization intensity data were automatically acquired and processed by Affymetrix Microarray Suite 5.0 software. The expression level of each gene was determined by calculating the average of differences in intensity (perfect match-mismatch) between its probe pairs. Scans were rejected if the scaling factor exceeded two or "chip surface scan" revealed scratches, specks or gradients affecting overall data quality (Refiner, GeneData AG, Basel, Switzerland). Additional Affymetrix gene expression raw data of 1,098 breast cancers from five additional breast cancer studies [12–16] were downloaded from the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/) and used for validation. Table 2 gives details of all six datasets used. ER status of these validation sets was determined using Affymetrix ProbeSet 205225_at. To allow comparison of different datasets, a cutoff of 1,000 was applied to this ProbeSet [17, 18]. Tumors with expression values ≥1,000 were classified as ER^{+}, those below 1,000 as ER^{-}. This cutoff which had been specified per protocol prior to the analyses had a concordance of 94.5% with the results of IHC, performed on 164 of the "Frankfurt" samples.

Immunohistochemistry

Polyclonal SPHK1 antibody was purchased from Imgenex (IMG-72025, San Diego, CA). Paraffin sections (2 μm) were mounted on Superfrost Plus slides, dewaxed in xylene and rehydrated through graduated ethanol to water. 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, antibodies were diluted 1:100 individually in this buffer. Sections were incubated with antibodies 1 h at room temperature. For negative controls, the primary antibodies were replaced with PBS. For secondary antibody incubations 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.

Table 2 Clinical characteristics of breast cancer patients from Affymetrix microarray datasets used in this study

Variable	Dataset					
	Frankfurt	Uppsala	Stockholm	Oxford	Rotterdam	expO
Data source	This study	Validation GSE3494	Validation GSE1456	Validation GSE2990	Validation GSE2034	Validation GSE2109
Number of samples	171	251	159	101	286	301
Age ≤50 (%)	39.8%	21.9%	n.a.	32.7%	49% premenop.	31.1%
Tumor size ≤ 2 cm (%)	53.2%	51.0%	n.a.	58.4%	51.0%	32.3%
Lymph node positive	41.5%	34.7%	n.a.	15.2%	0%	53.4%
ER positive	75.4%	80.9%	79.2 %	84.2%	72.4%	67.4%
Reference	(This study)	Miller et al. [13]	Pawitan et al. [14]	Sotiriou et al. [15]	Wang et al. [12]	http://www.intgen.org/

Statistical analysis

P values of less than 0.05 were considered to indicate a significant result and all reported P values are two-sided. The Mann–Whitney U-Test was used to determine significant differences in expression values between two sample groups. Chi-square test was used to test for associations between expression of markers and standard clinical and molecular parameters. Patients with missing values for clinical variables were excluded from the analyses. Survival intervals were measured from the time of surgery to the time of death from disease or the first evidence of disease recurrence. Data for women in whom the clinical end point was not reached were censored as of the last follow-up date. The method of Kaplan and Meier was used to estimate survival rates and the Log Rank test for comparison of survival curves. All analyses were performed using SPSS 14 (SPSS Inc., Chicago, IL).

Results

The mRNA expression of 43 genes involved in sphinogolipid metabolism (Table 3) was analyzed using microarray data of 171 invasive breast cancer samples (dataset "Frankfurt"). Tumor samples were stratified in two groups based on their estrogen receptor status and these groups were analyzed for differences in expression. We observed significant differences in the expression of several genes. As shown in Fig. 2, sphingosine kinase 1 (SPHK1, $P < 0.001$), ceramide galactosyltransferase (UGT8, $P < 0.001$), Ganglioside GD3-Synthase (ST8SIA1, $P < 0.001$) and the Bcl2 interacting protein BNIP3 ($P < 0.005$) displayed higher expression among ER negative tumors. In contrast, the analysis of glucosylceramidsynthase (GCS), dihydroceramidsynthases (LASS4, LASS 6), and acid ceramidase (ASAH1) revealed higher expression of those enzymes in ER positive samples ($P < 0.001$ for all).

To validate these results in independent cohorts we obtained microarray data of five independent published datasets representing a total of 1,098 additional samples. Figure 3 presents the data of the four markers which were found to be higher expressed among ER negative tumors in the primary dataset (SPHK1, UGT8, ST8SIA1, BNIP3). While the differences of SPHK1 and UGT8 expression were significant among all five datasets, significant differences of ST8SIA1 expression were obtained for four of the five cohorts using the Mann–Whitney test. In contrast, for BNIP3 a significant difference was detected only in two of the five validation cohorts (Fig. 3). Figure 4 displays the higher expression of GCS, LASS4, LASS6 and AHSA1 in ER positive tumors among the different datasets. As given in the figure highly significant differences were obtained in all comparisons.

Because of the complexity of the metabolic network the effects of most of the observed expression differences on the sphingolipid metabolism are difficult to estimate (see Fig. 1). Relatively straightforward would be the differences for SPHK1 expression, since this enzyme directly effects the "sphingolipid rheostat" which is suggested to balance between apoptosis inducing ceramide and proliferation stimulating S1P. In accordance, higher levels of SPHK1 were found in ER negative tumors, which are known for their higher proliferative activity. Thus we further concentrated on the analysis of the expression of this enzyme. To define a cutoff for SPHK1 microarray data we compared the numerical expression values of all 171 samples with the ranking of these values in the scatter plot presented in Fig. 5. A clear rise was seen among those samples with highest expression. The cutoff adapted from this graph (dotted blue line in Fig. 5) discriminates 30 samples (17.5%) with high and 141 samples (82.5%) with low SPHK1 expression, respectively. Table 4 presents the clinical parameters of the patients with tumors stratified by expression of SPHK1 according to this cutoff. There was no significant difference of patients age, tumor size, and lymph node status between the two groups. In contrast a significant correlation of high SPHK1 expression with ER negativity and higher histological grading was found ($P < 0.001$ for both). Moreover, a lobular histological subtype was correlated with low SPHK1 expression ($P = 0.035$). To analyze the prognostic significance of SPHK1 we performed a Kaplan–Meier analysis of the disease free survival of the patients stratified according to the expression of SPHK1 which is given in Fig. 6. Patients with high SPHK1 expression tended to have a worse outcome, but this difference was not yet significant in the sample cohort of 171 patients ($P = 0.25$). Combining the different datasets given in Table 2 would result in 1,269 patients. However, follow up data are available only for five of the six datasets encompassing 968 total patients. To allow comparison we applied a simple stratification of each dataset according to the proportion of 17.5% samples with highest SPHK1 expression, which was adopted from the primary dataset above. The characteristics of the 968 patients with available follow up data expression are given in Table 5 stratified according to this cutoff for SPHK1. Again, significant correlations of both ER status and histological grading with SPHK1 expression were detected in the combined datasets ($P < 0.001$ for both) while tumor size and lymph node status displayed no significant difference. In addition, a higher percentage of younger patients was seen in the group with high SPHK1 expression ($P = 0.023$). Figure 7 gives the Kaplan–Meier analysis of

Table 3 List of the analyzed genes involved in sphingolipid metabolism

Gene symbol	Affymetrix ProbeSet IDs	Protein
A4GALT	219488_at	GB3 Synthase
ASAH1	213902_at, 213702_x_at, 210979_at, 210980_s_at	Acid ceramidase 1
ASAHL	213702_x_at, 215178_x_at	Acid ceramidase-like gene
B4GALT6	206233_at, 206232_s_at, 216286_at	Beta-1 4-galactosyltransferase
BECN1	208945_s_at, 208946_s_at	Beclin 1
BNIP3	201848_s_at, 201849_at	BCL2/adenovirus E1B 19 kDa interacting protein 3
CERK	218421_at	Ceramide kinase
DEGS1	209250_at, 207431_s_at	Dihydroceramide desaturase
EDG1	204642_at	Sphingolipid G-protein-coupled receptor 1
EDG2	204037_at, 204036_at, 204038_s_at	Sphingolipid G-protein-coupled receptor 2
EDG4	206723_s_at, 206722_s_at	Sphingolipid G-protein-coupled receptor 4
EDG5	208537_at	Sphingolipid G-protein-coupled receptor 5
EDG6	206437_at	Sphingolipid G-protein-coupled receptor 6
FVT1	202419_at, 222359_x_at	3-ketodihydrosphingosine reductase
GAL3ST1	205670_at	Cerebroside sulfotransferase
GALC	211810_s_at, 204417_at	Galactocerebrosidase/Galactosylceramidase
GALGT	206435_at	Beta-1 4 N-acetylgalactosaminyltransferase
GBA	216400_at, 210589_s_at, 209093_s_at	Beta-Glucosidase
HPGD	211549_s_at, 203913_s_at, 203914_x_at, 211548_s_at	Hydroxyprostaglandin dehydrogenase 15
LASS2	222212_s_at	Dihydroceramidsynthase LASS2
LASS4	218922_s_at	Dihydroceramidsynthase LASS4
LASS6	212442_s_at, 212446_s_at	Dihydroceramidsynthase LASS6
LCT	206945_at	Lactase glycosylceramidase
LOC51190	221405_at	Neutral sphingomyelinase
NSMAF	203269_at	Neutral sphingomyelinase activation associated factor FAN
SFTPB	214354_x_at, 213936_x_at, 37004_at, 209810_at	N-acylsphingosine amidohydrolase (acid ceramidase)-like
SLC26A10	214951_at	N-acetylgalactosaminyltransferase
SMPD1	209420_s_at, 217171_at, 216230_x_at, 216571_at	Neutral sphingomyelinase
SMPD2	205622_at, 214206_at	Neutral sphingomyelinase
SMPDL3A	213624_at	Acid sphingomyelinase-like phosphodiesterase
SMPDL3B	205309_at	Acid sphingomyelinase-like phosphodiesterase
SPHK1	219257_s_at	Sphingosinkinase 1
SPHK2	40273_at, 209857_s_at	Sphingosinkinase 2
SPTLC1	202277_at, 202278_s_at	Serin Palmitoyltransferase 1
SPTLC2	216202_s_at, 216203_at, 203127_s_at, 203128_at	Serin Palmitoyltransferase 2
ST3GAL5	203217_s_at	Sialyl-Transferase I
ST6GALNAC5	220979_s_at	Sialyl-Transferase V
ST8SIA1	210073_at	GD3 synthase
ST8SIA3	208064_s_at, 208065_at	GT3 synthase (Sialyl-Transferase III)
UGCG	204881_s_at, 221765_at	Glukosylceramidsynthase (GCS)
UGCGL1	218257_s_at	UDP-glucose ceramide glucosyltransferase
UGCGL2	218801_at	UDP-glucose ceramide glucosyltransferase
UGT8	208358_s_at	UDP-galactose ceramide galactosyltransferase

the disease free survival of these 968 patients. The 5 year disease free survival of patients with high SPHK1 was 65.7 ± 3.7% while those of the patients with lower SPHK1 expression was 72.0 ± 1.6% ($P = 0.05$). Since SPHK1 is clearly correlated with ER negativity the different proportions of ER negative tumors in the groups with high and low SPHK1 expression could confound the clinical results obtained so far. We thus were interested whether

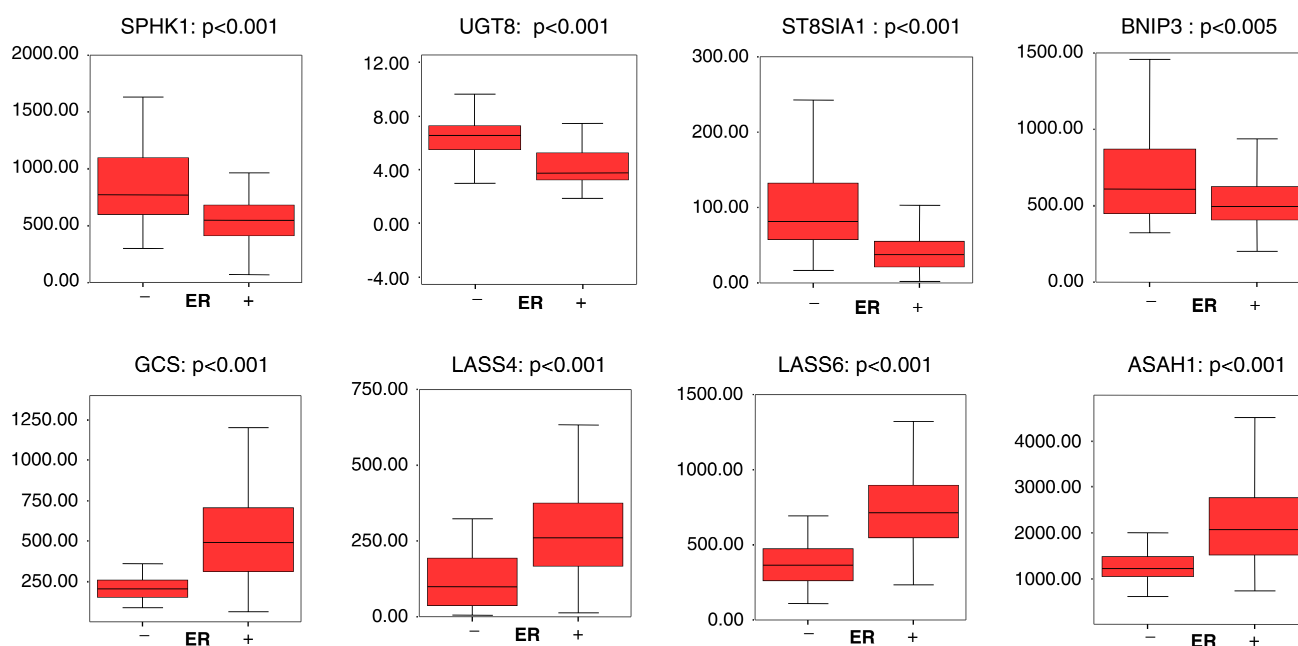

Fig. 2 ER status and expression of genes from the sphingolipid metabolism in the primary dataset. Box plots of eight genes with significant differences in expression between ER positive and negative tumors in the primary datasets

Dataset	SPHK1	P-Value	UGT8	P-Value	ST8SIA1	P-Value	BNIP3	P-Value
Uppsala		0.002		0.018		<0.001		0.068
Stockholm		0.012		<0.001		0.004		0.052
Oxford		<0.001		0.011		0.480		0.622
Rotterdam		<0.001		<0.001		<0.001		<0.001
expO		<0.001		<0.001		<0.001		<0.001

Fig. 3 Validation of genes associated with ER negative tumors in independent datasets. Genes higher expressed among ER negative tumors in Fig. 2 were analyzed in five published microarray datasets

Dataset	GCS	P-Value	LASS4	P-Value	LASS6	P-Value	AHSA1	P-Value
Uppsala		<0.001		<0.001		<0.001		<0.001
Stockholm		<0.001		<0.001		<0.001		<0.001
Oxford		<0.001		<0.001		<0.001		<0.001
Rotterdam		<0.001		<0.001		<0.001		<0.001
expO		<0.001		0.002		<0.001		<0.001

Fig. 4 Validation of genes associated with ER positive tumors in independent datasets. Genes higher expressed among ER positive tumors in Fig. 2 were analyzed in five published microarray datasets

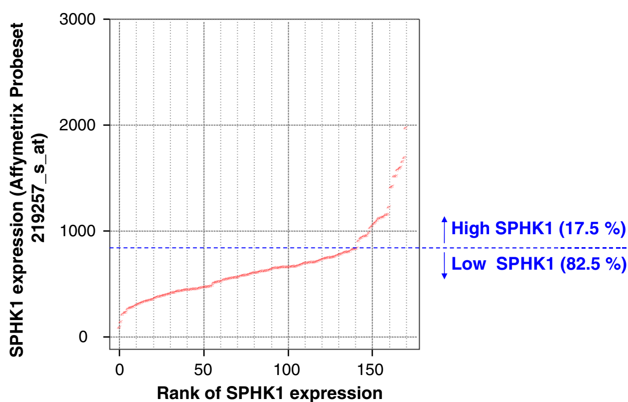

Fig. 5 Determination of a cutoff for SPHK1 expression on microarray. Scatter plot of the expression values of SPHK1 from microarrays of the primary dataset compared with their ranks. The adapted cutoff is represented by the dotted line

differences in SPHK1 expression would have also an impact on outcome of a cohort of patients with ER positive tumors only. Seven hundred and fifty of the patients with follow up had ER positive tumors. We stratified these tumors in two groups by delineating the upper quartile (25%) in each dataset displaying highest SPHK1 expression. As shown in Table 6, no significant correlation with age, tumor size, lymph node status or histological grading was found. In contrast, as presented in Fig. 8 those ER positive patients with tumors displaying high SPHK1 expression were characterized by a worse prognosis. While $75.8 \pm 1.9\%$ of the 562 patients with tumors with low SPHK1 expression were free of metastasis at 5 years follow up, this was the case for only $64.9 \pm 3.6\%$ of those 188 patients with tumors displaying high SPHK1 expression ($P = 0.008$; log rank test).

The relationship between the p53 key regulator of apoptosis and the sphingolipid metabolism is not yet clear. However, the worse prognosis of patients with high SPHK1 expression might be linked to a mutated p53 tumor suppressor. Miller et al. [13] have determined the p53 mutation status of each tumor in their sample cohort. Thus the data from this cohort allow the analysis of gene expression in relation to the p53 status of the tumor. However, we found no difference in SPHK1 expression between p53 mutated and wildtype samples (data not shown).

Table 4 Clinical parameters of patients with tumors displaying high SPHK1 expression in the primary dataset ($n = 171$)

		Total ($n = 171$)	SPHK1 expression		*P*-value
			Low ($n = 141$)	High ($n = 30$)	
Age	≤50	68	53	15	n.s.
	>50	103	88	15	
Tumor size	≤2 cm	91	76	15	n.s.
	>2 cm	80	65	15	
Lymph node status	LNN	97	79	18	n.s.
	N1	71	16	11	
ER status	Positive	129	119	10	<0.001
	Negative	42	22	20	
Histological grading	G1	13	12	1	<0.001
	G2	97	88	9	
	G3	61	41	20	
Histological subtype	Ductal	116	89	27	0.035
	Lobular	42	40	2	
	Mixed	8	7	1	
	Other	5	5	0	

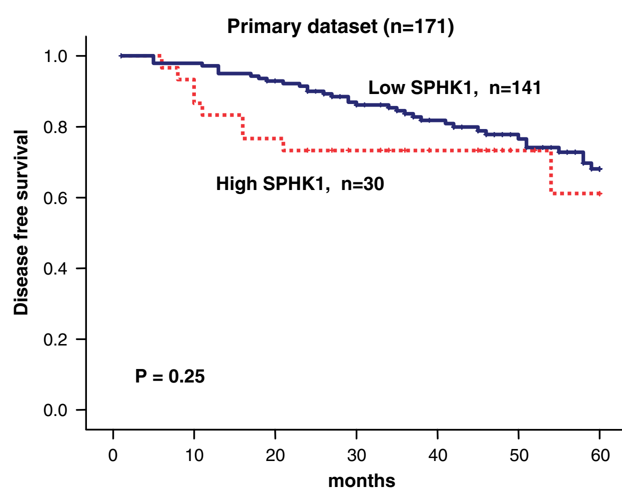

Fig. 6 Prognostic significance of SPHK1 expression in the primary dataset. Kaplan-Meier analysis of disease free survival in the primary dataset using the cutoff derived from Fig. 5

A variety of different cell types like monocytes [19] and mast cells [20] has been reported as a source of SPHK1 expression. Thus an important point is the cellular origin of SPHK1 expression in those tumors positive for high SPHK1 expression as detected by microarray analysis. To identify the cells expressing SPHK1 we performed immunohistochemical analysis of SPHK1 expression of samples previously profiled on Affymetrix microarrays. Figure 9a presents an example of a tumor displaying high expression of SPHK1 while a sample with low SPHK1 expression is shown in Fig. 9b. The immunohistochemical analyses clearly identified the carcinoma cells as the major source of SPHK1 expression in the tumor tissue.

Discussion

There is evidence that sphingolipids play not only roles in the structure and regulation of the cellular membranes, but also have key roles in human cancers as effectors and inducers of apoptosis, senescence, cell growth, drug resistance and vasculogenesis [2]. Major representatives of those sphingolipids are ceramide, sphingosine, sphingosine-1-phosphate and glucosylceramide. Figure 1 shows the pathways of sphingolipid metabolism with metabolites and corresponding enzymes. Ceramide and sphingosine 1 phosphate represent the main players in this pathway. The effects of ceramide are pleiotropic, but for the most part growth inhibiting. Sphingosin 1 phosphate, the opponent of ceramide has antiapoptotic effects and influences neovascularisation as well as multi drug resistance [2]. Ceramide can be formed de novo from serine and palmitoyl CoA as well as from hydrolysis of sphingomyelin or cerebrosides (glucosylceramide, galactosylceramide). Ceramide is produced in response to stress stimuli including chemotherapeutic drug treatment, factor withdrawal, or irradiation. The mechanisms by which ceramides exert their biological actions include modulation of diverse signal transduction pathways and key regulatory enzyme activities such as specific serine/ threonine kinases like protein kinase C, stress-activated protein kinases (SAPKs) also known as Jun nuclear kinases (JNKs), the mitogen activated protein kinases p42/44, and protein kinase B (PKB), as well as protein phosphatases , phospholipase A2, and phospholipase D (PLD) [1, 2, 21].

In the concept of a "sphingolipid rheostat" ceramide represent the substrate in the synthesis of sphingomyelin

Table 5 Clinical parameters of patients with tumors displaying high SPHK1 expression in the combined datasets (patients with follow up data only; $n = 968$)

		Total ($n = 968$)	SPHK1 expression		P-value
			Low ($n = 799$)	High ($n = 169$)	
Age	≤50	156	119 (27.6%)	37 (40.2%)	0.023
	>50	367	312 (72.4%)	55 (59.8%)	
	Data missing	445			
Tumor size	≤2 cm	278	228 (52.9%)	50 (54.3%)	n.s.
	>2 cm	245	203 (47.1%)	42 (45.7%)	
	Data missing	445			
Lymph node status	LNN	625	515 (78.5%)	110 (79.1%)	n.s.
	N1	170	141 (21.5%)	29 (20.9%)	
	Data missing	173			
ER status	Positive	750	669 (83.7%)	81 (47.9%)	<0.001
	Negative	218	130 (60.3%)	88 (52.1%)	
Histological Grading	G1	135	123 (22.9%)	12 (10.6%)	<0.001
	G2		255 (46.6%)	48 (42.5%)	
	G3		158 (29.5%)	53 (46.9%)	
	Data missing	319			

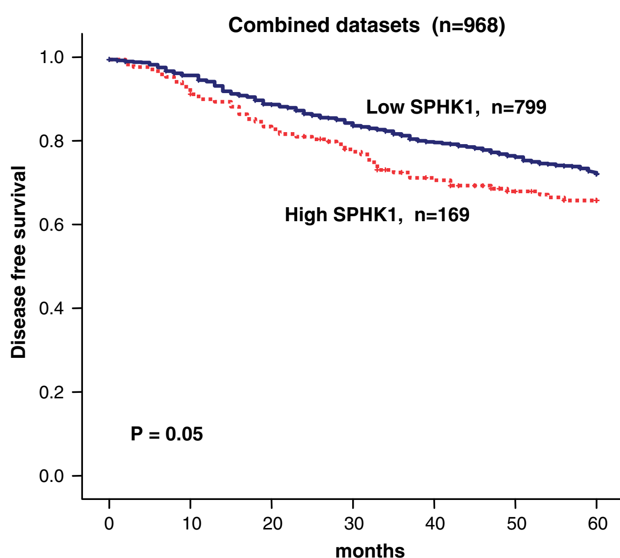

Fig. 7 Prognostic significance of SPHK1 expression in the combined datasets. Kaplan-Meier analysis of the disease free survival in the combined cohorts

and glucolipids or can be metabolized to ceramid-1 phosphate as well as to sphingosine which is further phosphorylated to shingosin-1-phosphate by sphingosine kinase [21].

Aim of our work was to investigate a possible correlation between these elements of sphingolipid metabolism and the estrogen receptor status of breast cancers. We observed significant differences between estrogen receptor positive and negative tumors and the expression of several proteins involved in these pathways. Sphingosine kinase 1, ceramide galactosyltransferase (UGT8), ganglioside GD3 Synthase, and BNIP 3 displayed higher expression in receptor negative breast cancers while the dihydroceramidsynthases (LASS 4+6), ASAH1 and GCS were higher expressed among estrogen receptor positive tumors. The alteration of these three latter enzymes could have several implications. Dihydroceramide synthases acylate sphinganine to form dihydroceramide but can also acylate sphingosine to ceramide. The hypothesis that higher levels of dihydroceramide synthase would directly or indirectly lead to higher levels of ceramides in ER positive tumor cells could be one explanation for the better prognosis of patients with ER positive tumors. The second enzyme glucosylceramide synthase is responsible for the conversion of ceramide to glucosylceramide. It was demonstrated to be a branch point enzyme in the formation of cerebrosides and gangliosides [1]. Several authors pointed out the role of GCS in multi drug resistance against chemotherapeutic agents. Thus it could be speculated that higher expression of GCS in estrogen receptor positive tumor cells might be one explanation for the worse response to chemotherapy of these tumors [22, 23].

Regarding those genes with higher expression among ER negative cancers there is yet little information about the impact of GD3 synthase, BNIP3 and UGT8 on cancer development. Omran and colleques described an involvement of GD3 in apoptosis in U-1242 glioma cells [24]. Zeng et al. [25] found evidence for an association between GD3 Ganglioside and tumor progression and even development of metastatic potential. There might be a role of BNIP3 in cell death but is not yet clearly understood. Loss

Table 6 Clinical parameters of patients with follow up data and ER positive tumors displaying high SPHK1 expression

		Total (n = 750)	SPHK1 expression		*P*-value
			Low (n = 562)	High (n = 188)	
Age	≤50	118	82 (26.2%)	36 (34.6%)	0.104
	>50	299	231 (73.8%)	68 (64.4%)	
	Data missing	333			
Tumor size	≤2 cm	232	169 (54.0%)	63 (60.6%)	0.26
	>2 cm	185	144 (46.0%)	41 (39.4%)	
	Data missing	333			
Lymph node status	LNN	475	351 (76.6%)	124 (79.5%)	0.51
	N1	139	107 (23.4%)	32 (20.5%)	
	Data missing	136			
Histological Grading	G1	128	102 (26.3%)	26 (20.5%)	0.41
	G2	273	201 (51.8%)	72 (56.7%)	
	G3	114	85 (21.9%)	29 (22.8%)	
	Data missing	235			

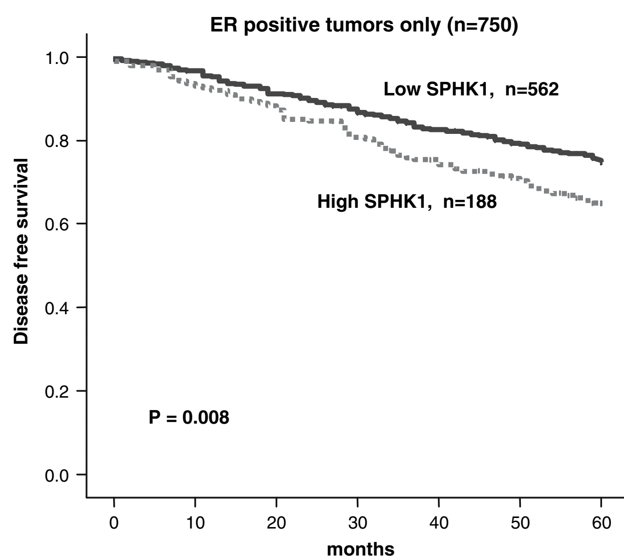

Fig. 8 Prognostic significance of SPHK1 expression among a cohort of ER positive tumors only. Kaplan-Meier analysis of the disease free survival in all ER positive tumors from the combined cohorts

or knockout of BNIP3 expression seem to enable metastatic growth [26]. UGT8 has already been described by Yang et al. [27] as one of the genes displaying highest correlation with an ER negative tumor type.

The most easily interpretable difference seems to be the higher expression of sphingosine kinase 1 (SPHK1) in ER negative tumors. Sphingosine kinase is a crucial regulator of the ceramide/S1P balance and exists in two isoenzymes (SPHK1 and SPHK2) [28, 29]. SPHK1 modulates the ceramide and S1P balance by producing the proliferative, antiapoptotic S1P and decreasing the intracellular levels of ceramide [10]. The content of SPHK1 mRNA in tumor tissues (breast, brain, colon, lung) is higher than it is in healthy reference tissues [30]. Additionally, there was a correlation between expression of SPHK1 and tumor stage in colon cancer [31]. In our data we saw correlations between higher SPHK1 expression and worse prognosis both for the full cohort as wells for estrogen receptor positive breast cancers only. These results agree with the report of Van Brooklyn et al. [32] who observed a

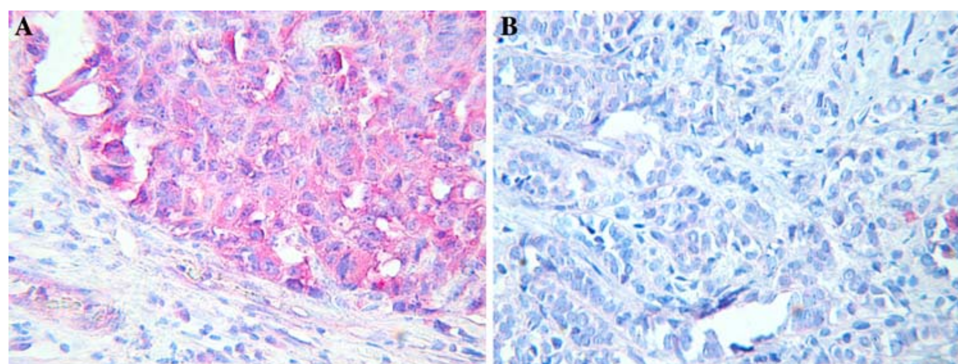

Fig. 9 Immunohistochemical detection of SPHK1 protein expression. Representative examples of a tumor displaying high expression of SPHK1 (**A**) as well as a sample with low SPHK1 expression (**B**) on microarray are shown. Red indicates staining with SPHK1 antibody, blue counterstain

correlation between high SPHK1 expression and poor survival in glioblastoma cell lines. Possible explanations for the worse prognosis of tumors overexpressing SPHK1 could be higher levels of S1P leading to drug resistance to chemotherapy and/or increased cell migration and invasion, phenomena that were shown to be correlated to overexpression of SPHK1 and higher S1P levels [33–36].

In conclusion, certain sphingolipids seem to affect the onset, dissemination and formation of metastases in different types of cancer. We were able to demonstrate that several enzymes from the ceramide/S1P rheostat are differentially expressed in different subtypes of breast cancer and seem to have a prognostic impact for the course of disease.

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