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Acid ceramidase 1 expression correlates with a better prognosis in ER-positive breast cancer

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ABSTRACT

Objectives Ceramide and sphingosine mediate response to cancer therapy, inhibit cell growth and induce apoptosis *in vitro*. Only a few clinical data about the impact of ceramide and sphingosine *in vivo* are available. We investigated the relevance of ceramide- and sphingosine-generating enzymes in breast cancer (acid ceramidase 1 (ASAH1), ceramide synthases 4 (LASS4) and 6 (LASS6)) by means of gene expression analysis.

Methods We analyzed differences in ASAH1, LASS4 and LASS6 on mRNA level between breast cancer subgroups using microarray data from 1581 tumor samples.

Results High ASAH1, LASS4 and LASS6 expression correlates with pathohistological grading ($p < 0.001$) and estrogen receptor (ER) status ($p < 0.001$). High ASAH1 expression was associated with a larger tumor size >2 cm ($p = 0.003$), while high LASS6 expression was correlated with ErbB2 negativity ($p < 0.001$). In survival analysis, we detected a significant better prognosis of patients with higher ASAH1 expression ($p = 0.002$) in the ER-positive subgroup. In contrast, expression of LASS4 or LASS6 did not show any prognostic impact. In the multivariate analysis, only ASAH1 expression ($p = 0.002$), tumor size ($p < 0.0001$) and ErbB2 positivity ($p = 0.041$) remained significant.

Conclusion ASAH1 is an estrogen-dependent member of the sphingolipid metabolism, which might provide further prognostic information in ER-positive breast cancers.

INTRODUCTION

Sphingolipids are a family of membrane lipids with important structural roles in the lipid bilayer, but also act as effector molecules and second messengers[1,2]. Many sphingolipid-regulated functions have significant and specific links to various aspects of cancer initiation, progression and response to anticancer treatments. The main sphingolipids are ceramide, sphingosine and sphingosine 1 phosphate (S1P). Ceramide is the key hub in sphingolipid metabolism. It is intimately involved in the

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regulation of cancer cell growth, differentiation, senescence and apoptosis[3]. Many cytokines, anti-cancer drugs and other stress-causing agonists result in an increase of endogenous ceramide levels[1]. More recently, exogenous ceramide or ceramide analoga were shown to have therapeutic potential in various cancers *in vitro* (e.g. breast cancer[4], colon cancer[5] or head and neck squamous cell cancer[6]). Ceramide can be synthesized *de novo* by palmitoyl CoA and serine involving serine palmitoyl transferase (SPT) and (dihydro)-ceramide synthase (LASS1–6) and dihydroceramide desaturase (Figure 1) or via hydrolysis of sphingomyelin by sphingomyelinase[7]. Ceramide can be degraded by ceramidases (e.g. acid ceramidase, ASAH1) to form sphingosine. Sphingosine and its related sphingoid bases have roles in regulating the actin cytoskeleton, endocytosis, the cell cycle and apoptosis[8]. In contrast to ceramide and sphingosine, S1P is emerging as a key regulator of proliferation, inflammation, vasculogenesis and resistance to apoptotic cell death. It has been suggested that the dynamic equilibrium between the various sphingolipid metabolites (the so-called sphingolipid rheostat) and balanced regulation of opposing signaling pathways is an important factor that determines the fate of cells[9].

In previous investigations, we have observed a prognostic impact of sphingosine kinase 1 (Sphk1) but not of glucosylceramide synthase in breast cancer[10]. Since Sphk1 produces S1P, the counterplayer of ceramide and sphingosine in the rheostat, we investigated two ceramide metabolizing enzymes according to their prognostic impact on breast cancer. Both enzymes are highly expressed in ER-positive tumors. The first enzyme is the dihydroceramide synthase (LASS) which produces dihydroceramide by acylation of dihydrosphingosine. The second one is acid ceramidase. It metabolizes ceramide to sphingosine. So far, no clinical data about their clinical impact in breast cancer are available.

MATERIAL AND METHODS

Microarray data

We established a database containing 1581 Affymetrix microarray experiments from primary breast cancer patients; 120 of the included samples came from our own institution (dataset Frankfurt) and have been described previously[11,12]). In addition, we included 1461 samples from nine different, publicly available datasets (Table 1): Uppsala[13], Stockholm[14], Rotterdam[15,16], Oxford-Untreated[17], Oxford-Tamoxifen and London[18], NewYork[19], Villejuif[20], and ExpO[21]. For comparability, only data from Affymetrix HG-U133A microarrays were used. Table 1 presents the clinical characteristics of the patients from the different datasets. Follow-up information was available for 1263 of the 1581 patients (no follow-up data have been reported for dataset ExpO), with a median follow-up time of 79 months; 1135 of the 1581 samples (71.9%) were ER-positive. Since methods of Affymetrix microarray normalization can have significant effects on the levels for individual probe sets, several uniform

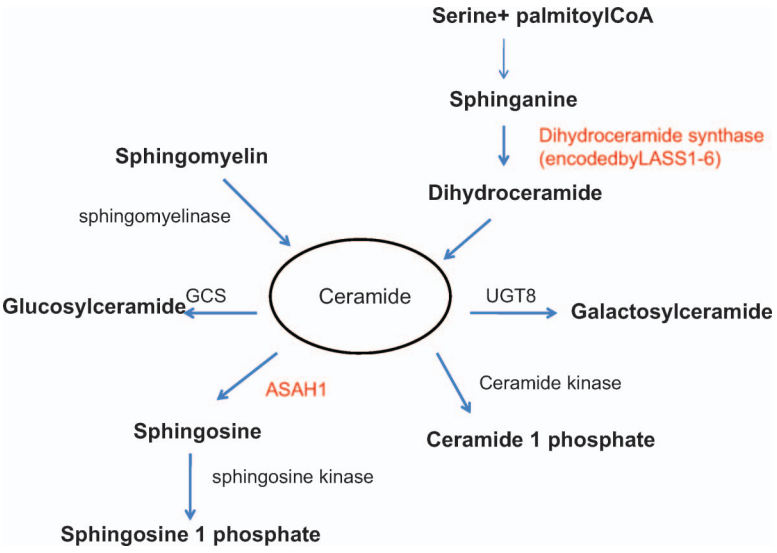

Figure 1 Main metabolic pathways in sphingolipid metabolism

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

					% of samples								
Dataset	*Data source*	*Array*	*Norm. method*	*Number of samples*	*Age ≤50*	*Tumor size ≤2 cm*	*LNN*	*ER-positive*	*G3*	*System of treatment*	*Median follow-up (months)*	*Number of relapses*	*Reference*
Frankfurt	this study	U133A	MAS5	120	54	50	57	66	47	chemotherapy	39	29	10, 11
Rotterdam	GSE2034, GSE5327	U133A	MAS5	344	n.a.	n.a.	n.a.	61	n.a.	286 untreated, 58 n.a.	86	118	15, 16
Uppsala	GSE3494	U133A	MAS5	251	22	51	65	80	22	yes/no	118	91	13
Stockholm	GSE1456	U133A	MAS5	159	n.a.	n.a.	n.a.	82	42	yes/no	85	40	14
Oxford-Untreated	GSE2990	U133A	RMA	61	44	64	100	69	41	untreated	121	29	17
Oxford-Tamoxifen	GSE6532	U133A	RMA	109	14	34	64	95	19	endocrine	61	30	18
London	GSE6532	U133+	RMA	87	6	35	33	98	23	endocrine	137	28	18
New York	GSE2603	U133A	MAS5	99	37	9	34	58	n.a.	n.a.	65	27	19
Villejuif	GSE7390	U133A	RMA	50	80	26	100	72	38	untreated	108	22	20
ExpO	GSE2109	U133A	MAS5	301	31	32	47	65	49	n.a.	n.a.	n.a.	http://www.intgen.org
Total				1581	31	39	69	72	35		79	414	

LNN, lymph node-negative; ER, estrogen receptor; n.a., not available; Norm. method, normalization method

normalization methods[22,23] of CEL file data have 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 of compensating for 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. 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 and subsequent stratification relied on a ranking of samples in each cohort.

Since standard pathology for ER and ErbB2 (the ErbB2 protein is named for its similarity to ErbB, avian erythroblastosis oncogene B) was not available for all samples and to allow comparison of different datasets, the receptor status of all samples was determined based on Affymetrix expression data. This has been applied in several studies, resulting in good concordance with biochemical/IHC methods[24–27]. Optimal cut-off values for ER and ErbB2 were derived using a method that we have described previously[28] by fitting two normal distributions to the observed expression data. ER status was based on Affymetrix ProbeSet 205225_at, the ErbB2 status on ProbeSet 216836_s_at. A remarkable high specificity of 86.1% and sensitivity of 92.2% were observed when the chip-based ER status was compared to the immunohistochemically/biochemically obtained ER status (available for 1233 samples). The specificity and sensitivity of chip-based ErbB2 status were 98.6% and 45.8%, respectively, compared to 3+ staining in immunohistochemistry with HER2 antibody (data available for 206 samples). To allow stratification based on the expression of ASAH1 (ProbeSets 210980_s_at and 213702_x_at), LASS4 (ProbeSet 218922_s_at), and LASS6 (ProbeSets 212442_ s_at and 212446_s_at) in the different datasets, we used a median or quartile split among each dataset. Samples were characterized as high- or low-expressing based on a split of the cohorts (see Results).

Immunohistochemistry

We used immunohistochemistry as a proof that microarray measurements of the analyzed markers originate from tumor cells in the sample. 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 mmol/l 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 for 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. The monoclonal LASS6 antibody was obtained from Abnova (H00253787 M01). The ASAH1 antibody was purchased from Acris Antibodies GmbH (H00000427-M01). A LASS4 antibody is currently not commercially available. Ten randomly chosen tumor samples from the Frankfurt microarray cohort were stained by LASS6 and ASAH1, respectively. Representative immunohistochemistry results are given in Figure 2.

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. The χ^2 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 endpoint 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. Cox regression analyses were applied to determine the univariate and multivariate significance of different parameters on the prognosis of patients. A Cox proportional-hazards regression model was used to examine simultaneously the effects of multiple covariates on disease-free 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, grading, ER and ErbB2 status expression. All analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Gene expression in ER-positive and -negative breast cancer subgroups

Initially, the mRNA expression of three genes that synthesize or metabolize ceramide (Figure 1) was analyzed in our microarray database of 1581 invasive breast cancer samples. Because of the predictive and prognostic value of the ER status, tumor samples were stratified into groups based on their estrogen receptor status and these groups were analyzed for differences in expression. The analysis of the dihydroceramide synthases LASS4 and LASS6, as well as the acid ceramidase (ASAH1) revealed higher expression of all three enzymes in ER-positive samples (Mann–Whitney U test, $p < 0.001$ for all). To verify that the tumors cells are the source of the expression of the analyzed genes, we performed immunohistochemistry using a monoclonal antibody against LASS6 and ASAH1 on samples from the Frankfurt cohort. As shown in Figure 2, specific staining of LASS6 (Figure 2a) and ASAH1 (Figure 2b) protein was observed only in the cancer cells of ER-positive breast tumors.

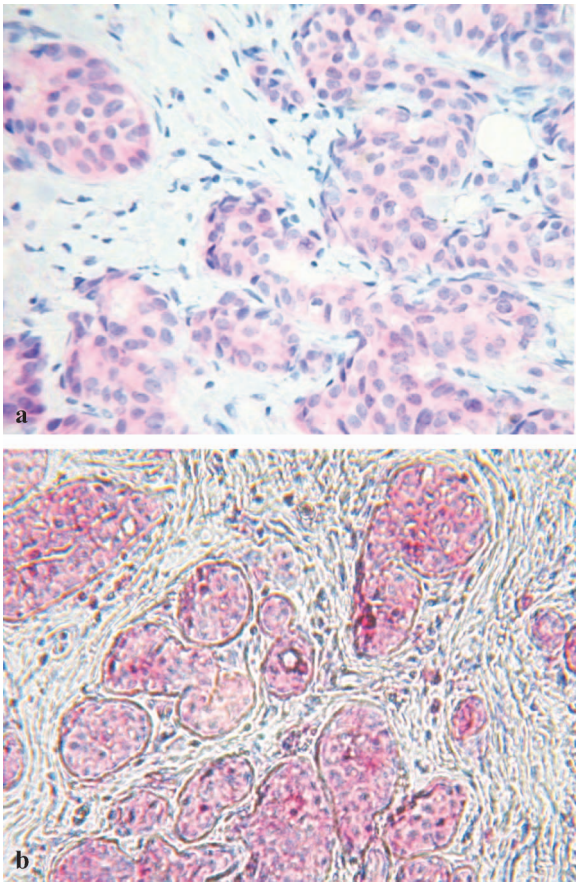

Figure 2 Immunohistochemical analysis of LASS6 and ASAH1 expression. Shown are representative stainings (red) of (a) LASS6 and (b) ASAH1, respectively, in breast cancer tissue (both grade 2 tumors). Magnification: 25 × and 10 ×; counterstain: Mayer's hematoxylin (blue)

Clinical-pathological characteristics and prognostic value of ASAH1

There are several ProbeSets for ASAH1 on the Affymetrix U133A Chip. Analysis of their correlation demonstrated highest consistency ($R = 0.86$) between ProbeSets 210980_s_at and 213702_x_at (Figure 3). ProbeSets 210980_s_at was used for further analyses. Since ASAH1 is highly expressed in ER-positive tumors and those cancers are known to have a better prognosis than ER-negative cancers, it is important to analyze a possible prognostic value of ASAH1 separately in the subgroups of ER-positive and -negative samples to avoid confounding effects. When we perfomed a simple median split of the sample cohorts according to ASAH1 expression, we did not observe a significant prognostic value of ASAH1 in the ER-positive or in the ER-negative subgroup of samples. To analyze tumors with highest ASAH1 expression and to allow comparability between the different datasets, we next used a split of expression values using quartiles. Those tumors with expression values in the lowest quartile were classified as ASAH1-negative, all

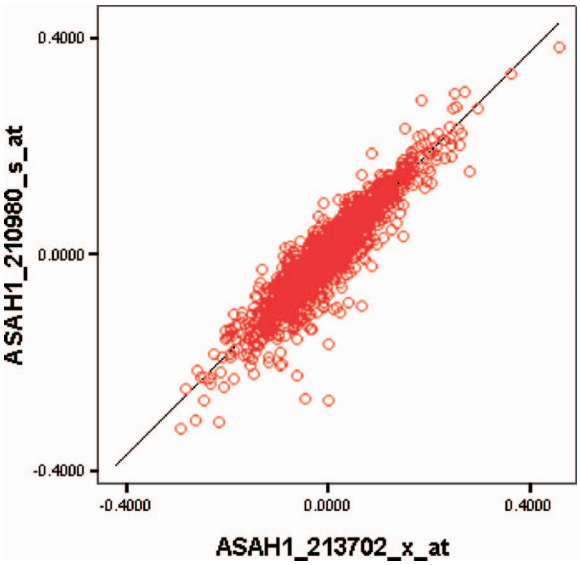

Figure 3 Analysis of the correlation between the two ASAH1 ProbeSets 210980_s_at and 213702_x_at; consistency was high with $R = 0.86$

Table 2 Clinical characteristics of patients ($n = 1581$) in relation to expression of AHSA1, LASS4, and LASS6. Data are given as *n* (%)

		ASAH1			*LASS4*			*LASS6*		
Parameter	*n*	*Low*	*High*	*p Value*	*Low*	*High*	*p Value*	*Low*	*High*	*p Value*
Age†† (years)										
≤50	328	88 (34.0)	240 (30.5)	0.32	176 (33.5)	152 (29.2)	0.14	176 (33.7)	152 (29.0)	0.11
>50	719	171 (66.0)	548 (69.5)		350 (66.5)	369 (70.8)		346 (66.3)	373 (71.0)	
*Lymph node status**										
LNN	899	236 (71.1)	663 (68.7)	0.45	454 (69.5)	445 (69.1)	0.9	455 (70.9)	444 (67.8)	0.23
N1	398	96 (28.9)	302 (31.1)		199 (30.5)	199 (30.9)		187 (29.1)	211 (32.2)	
*Tumor size*** (cm)										
≤2	377	76 (31)	301 (41.7)	0.003	194 (39.5)	183 (38.5)	0.79	189 (39.2)	188 (38.8)	0.95
>2	589	169 (69)	420 (58.3)		297 (60.5)	292 (61.5)		293 (60.8)	296 (61.2)	
Tumor grade†										
Low grade (G1 + G2)	624	107 (44)	517 (72.5)	<0.001	282 (58.5)	342 (72.2)	0.001	285 (59.4)	339 (71.2)	<0.001
High grade (G3)	332	136 (56)	169 (27.5)		200 (58.5)	132 (27.8)		195 (40.6)	137 (28.8)	
ER status										
Positive	1135	190 (48.3)	945 (79.5)	<0.001	460 (58.3)	675 (85.2)	<0.001	449 (56.9)	686 (86.6)	<0.001
Negative	446	203 (51.7)	243 (20.5)		329 (41.7)	117 (14.8)		340 (43.1)	106 (13.4)	
ErbB2 status										
Negative	1358	335 (85.2)	1023 (86.1)	0.68	675 (85.6)	683 (86.2)	0.72	701 (88.8)	657 (83.0)	0.001
Positive	223	58 (14.8)	165 (13.9)		114 (14.4)	109 (13.8)		88 (11.2)	135 (17.0)	

*, Information on lymph node status was available for 1297 patients; **, information on tumor size was available for 966 patients; †, information on tumor grade was available for 956 patients; ††, information on age was available for 1047 patients
ASAH1, acid ceramidase 1; LASS4, ceramide synthase 4; LASS6, ceramide synthase 6

other tumors as positive. As shown in Table 2, ASAH1 expression was associated with ER-positivity ($p < 0.001$), larger tumor size >2 cm ($p = 0.003$) and well-differentiated tumors or those with intermediate or moderate differentiation ($p < 0.001$; Table 2). The survival analysis revealed a significant better prognosis ($p = 0.002$) with a significant longer 5-year disease-free survival (77.1 ± 1.6% vs. 64.5 ± 4.0%) and 10-year disease-free survival (67.0 ± 1.9% vs. 54 ± 4.6%) for patients with higher ASAH1 expression in the ER-positive group (Figure 4a). In the ER-negative tumor subpopulation (Figure 4b), no significant difference was observed.

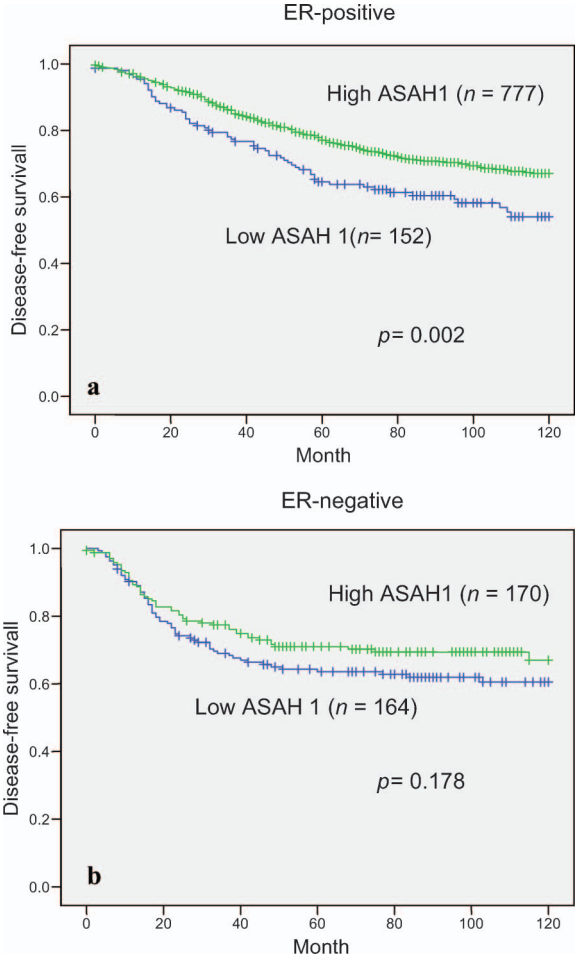

Figure 4 Kaplan–Meier analysis of the disease-free survival in the breast cancer subgroups according to their expression of ASAH1. (a) Significantly ($p = 0.002$) better prognosis with higher ASAH1 expression in estrogen receptor (ER)-positive breast cancer samples; (b) ER-negative samples without significant differences ($p = 0.178$)

Clinical-pathological characteristics and prognostic value of LASS4 and LASS6

We obtained similar expression profiles among the breast cancer samples for LASS4 and LASS6, for which ProbeSets were available on the Affymetrix U133A microarray. For correlation with clinical parameters, a median split in LASS4 and LASS6 expression was performed. There was no significant difference according to patient's age, tumor size, histological subtype and lymph node status between the two groups (Table 2). In contrast, we observed a significantly higher expression of LASS6 in ER-positive tumors ($p < 0.0001$) and in well-differentiated tumors ($p < 0.001$). Moreover, ErbB2-positive samples have shown higher expression of LASS6 ($p = 0.046$). The investigation of the prognosis of the patients according to expression of LASS4 and LASS6 revealed no significant differences between the two groups. The 5-year disease-free survival was comparable for both enzymes irrespective of the expression level of LASS4 (73.7 ± 1.9% vs. 76.9 ± 2.3%; Figure 5a) and for LASS6 (73.1 ± 1.9% vs. 77.9 ± 2.2%; Figure 6a) in the ER-positive subgroup. In the ER-negative subgroup, a tendency was observed towards a better 5-year disease-free survival in those tumors with high expression of LASS4 (75 ± 5.1% vs. 65.2 ± 3.0%; Figure 5b). Since probe sets for LASS1, 3 and 5 are not available on Affymetrix U133A but only on U133B, we were only able to look for association of those three markers with ER in one single dataset (Stockholm). However, analysis of these other three members of the LASS family in this dataset revealed no significant correlation with the estrogen receptor status in contrast to LASS4 and 6.

Univariate and multivariate Cox regression analysis

Cox regression analysis was used to assess the univariate and multivariate significance of standard parameters and expression of ASAH1, LASS4, and LASS6 in relation to disease-free survival. The multivariate model included only a subset of 605 patients for whom data on all standard parameters (tumor size, lymph node status, grading, age, ER status and ErbB2) were available. The results of these analyses are presented in Table 3. In the univariate analyses, significant values were obtained for ER ($p = 0.05$) and lymph node status ($p = 0.033$), histological grading ($p < 0.001$), tumor size ($p < 0.001$) and

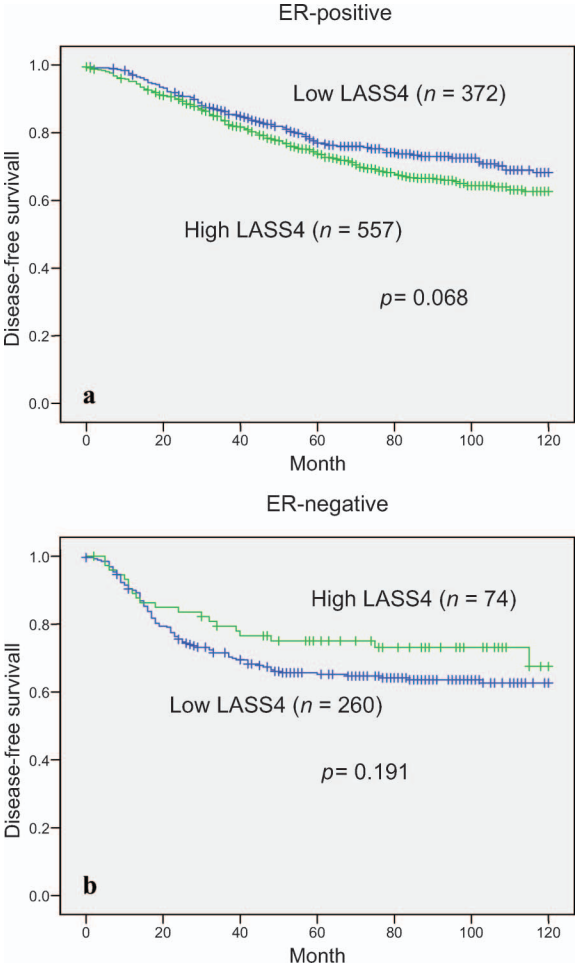

Figure 5 Kaplan–Meier analysis of the disease-free survival in dependence of the expression of LASS4 in (a) estrogen receptor (ER)-positive and (b) ER-negative samples

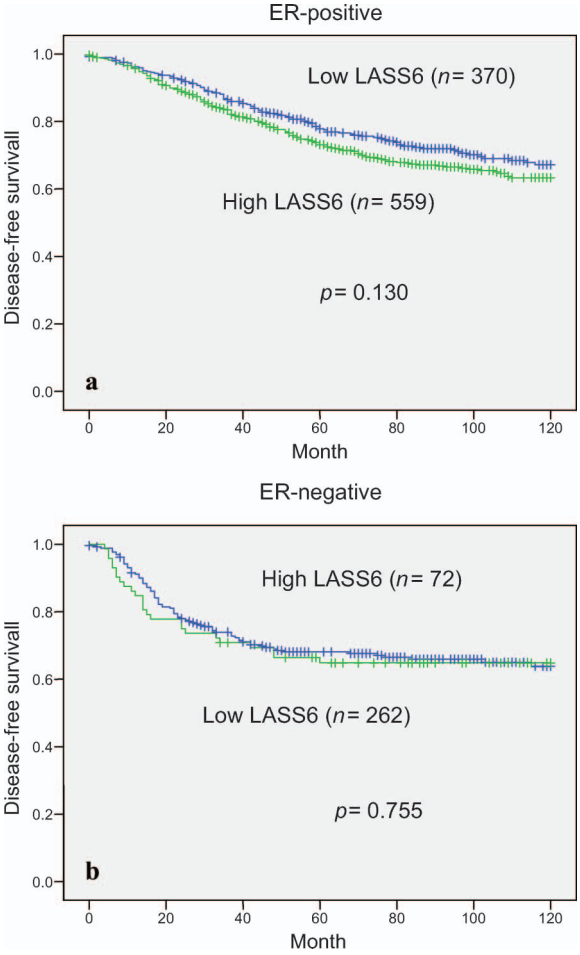

Figure 6 Kaplan–Meier analysis of disease-free survival in dependence of the expression of LASS6 in (a) estrogen receptor (ER)-positive and (b) ER-negative samples

ErbB2 ($p < 0.018$) as well as expression of ASAH1 ($p < 0.001$). In the multivariate model including all parameters in parallel, the significance of ER status and grading was lost and lymph node status only revealed a trend to significance ($p = 0.09$). Only ASAH1 (hazard ratio (HR) 1.66; 95% confidence interval (CI) 1.20–2.30, $p = 0.002$) remained a significant prognostic marker in addition to tumor size (HR 0.49; 95% CI 0.35–0.68, $p < 0.001$) and ErbB2 status (HR 1.53; 95% CI 1.02–1.53; $p = 0.041$).

DISCUSSION

There is recent evidence that sphingolipids are not only required for the subdomain structure and regulation of cellular membranes, but also have key functions in human cancers as effectors and inducers of apoptosis, senescence, cell growth, distant recurrence, drug resistance and vasculogenesis[1,7]. In the normal cell, there exists a balance between proapoptotic and anti-apoptotic sphingolipids, called the sphingolipid rheostat[29]. The two key players are ceramide and S1P.

We investigated ceramide metabolizing enzymes on the level of gene expression. Our results suggest that the gene expression of ASAH1, LASS4 and LASS6 strongly correlates with ER status. Furthermore, our investigations revealed ASAH1 as a strong prognostic marker which remains significant in multivariate analysis. These data confirm earlier results that sphingolipids play an important role in the clinical behavior of breast cancer cells[2,9,10]. The better prognosis of samples with higher ASAH1 expression could be explained by higher levels of the proapoptotic sphingosine in the ER-positive subgroup. Sphingosine has functional roles in regulating the actin cytoskeleton,

endocytosis, cell cycle and apoptosis[30]. Additionally, Ahn and co-workers observed a chemotherapeutic and chemopreventive effect of sphingosine and sphinganine in human breast epithelial cells[31]. These observations might provide a protective effect of normal breast cells against uncontrolled proliferation. Furthermore, ceramide and sphingosine have been shown to inhibit EGF receptor kinase in epidermoid carcinoma cells A431[32,33]. Riboni and co-workers[34] found an inverse correlation between ceramide levels and malignancy in human astrocytomas. Similarly, the total content of ceramide was decreased in ovarian tumors compared to normal ovarian tissues[35]. The hypothesis that higher levels of dihydroceramide synthase would directly or indirectly lead to higher levels of ceramides in ER-positive tumor cells may explain the better prognosis seen in patients with ER-positive tumors.

Dihydroceramide synthases are encoded by the LASS genes. Six mammalian homologues of LASS proteins exist[36]. Each LASS exerts specificity for the metabolization of distinct endogenous ceramides with high fatty-acid chain length[7]. For instance, LASS1 has been shown to specifically generate C18 ceramide[37], whereas LASS5 and 6 mainly generate C16-ceramide[38,39]. Moreover, expression of the different LASS genes and isoforms varies in different tissues[40–43]. LASS1 plays an important role in the pathogenesis of head and neck squamous cell cancer (HNSCC)[44]. Increased LASS1 expression was seen *in vivo* in gemcitabine/doxorubicin-treated HNSCC cells[45]. Furthermore, significantly lower C18 ceramide levels compared to normal tissue in HNSCC and an association between C18 levels, lymphovascular invasion and nodal disease were observed[46]. We found higher expression of LASS4 and 6 on mRNA level in ER-positive specimens and could verify LASS6 protein expression in tumor cells by means of immunohistochemistry. However, we failed to detect a prognostic value of LASS4 or LASS6 expression. Further investigation is needed to explore whether other LASS genes have a prognostic or predictive impact.

A potential weakness of our study might be the applied pooling of different datasets. Pooling and meta-analyses are common in cancer research[47,48] since sample sizes of thousands of patients are often needed to analyze treatment effects or the prognosis of specific subgroups. Still, these methods can have severe disadvantages caused by the inhomogeneity of the data, patient groups and treatment modalities. Since microarray datasets generally contain only tens or hundreds of patients

Table 3 Univariate and multivariate Cox regression analysis of standard parameters and ASAH1, LASS4 and LASS6 expression in relation to disease-free survival

		Univariate analysis					*Multivariate analysis*				
Parameter		*n1*	*n2*	*p Value*	*HR*	*95% CI*	*n1*	*n2*	*p Value*	*HR*	*95% CI*
ASAH1 expression	low vs. high	316	947	<0.001	1.49	1.20–1.85	151	454	0.002	1.66	1.20–2.30
LASS4 expression	low vs. high	632	631	0.78	0.97	0.80–1.19	299	306	0.178	1.24	0.91–1.70
LASS6 expression	low vs. high	632	631	0.50	0.93	0.77–1.14	302	303	0.289	0.84	0.62–1.15
ER status	pos. vs. neg.	334	929	0.05	0.80	0.65–1.00	481	124	0.933	0.98	0.64–1.50
Lymph node status	LNN vs. N1	803	284	0.033	0.78	0.62–0.98	393	212	0.091	0.77	0.57–1.04
Age	>50 vs. ≤50	521	239	0.74	0.96	0.73–1.26	406	199	0.561	0.91	0.67–1.24
Grading	G3 vs. G1 + 2	241	528	<0.001	1.77	1.36–2.31	175	430	0.204	1.25	0.88–1.78
Tumor size	≤2 cm vs. >2 cm	314	446	<0.001	0.45	0.33–0.59	274	331	<0.001	0.49	0.35–0.68
ErbB2 status	pos. vs. neg.	179	1084	0.018	1.37	1.06–1.79	82	523	0.041	1.53	1.02–2.29

ASAH1, acid ceramidase 1; LASS4, ceramide synthase 4; LASS6, ceramide synthase 6; HR, hazard ratio; 95% CI, 95% confidence interval; ER, estrogen receptor; LNN, lymph node-negative; pos., positive; neg., negative

because of the expenditure and complexity of this method, data-pooling has been increasingly applied[17,18,49–52]. Moreover, recent studies[53,54] suggest that the pooling microarray datasets generates more accurate results and advocate the analysis of new data within the context of a compendium, rather than analysis in isolation. To reduce the drawbacks of pooling and potential biases, we first adapted the different datasets by normalizing all individual arrays. Moreover, we used a conservative strategy by stratifying each individual cohort. Still, it should be noted that the significance of some well-known prognostic markers like grading and lymph node status was lost when we used a multivariate regression model including all parameters (Table 3). This might be attributed to the smaller number of cases for which all parameters were available and has been observed in other studies as well, but yet underscores the need for validation of the results in larger, prospective studies.

In conclusion, our results clearly demonstrate a relationship between ER status and expression of the main enzymes of the sphingolipid metabolism. Moreover, ASAH1 expression has a potential as a prognostic marker in breast cancer. Further validation of these preliminary results from prospective studies and with longer follow-up data is needed. In addition, it still remains unclear whether specific members of the sphingolipid rheostat are closely associated with treatment response in terms of endocrine or cytotoxic therapy. Furthermore, therapeutic targeting of sphingolipid metabolism may be an interesting approach in specific breast cancer subtypes. In this context, more detailed analysis of the predictive value of sphingolipids and their metabolizing enzymes for neoadjuvant breast cancer treatment is warranted.

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Conflict of interest Nil.

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