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Increased ceramide synthase 2 and 6 mRNA levels in breast cancer tissues and correlation with sphingosine kinase expression

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ABSTRACT

Intervention in the ceramide metabolic pathway is emerging as a novel means to regulate cancer and to modify the activity of chemotherapeutic drugs. We now study mRNA expression levels of the six ceramide synthase (CerS) genes in breast cancer tissue. CerS2 and CerS6 mRNA was significantly elevated in breast cancer tissue compared to paired normal tissue, with approximately half of the individuals showing elevated CerS2 and CerS6 mRNA. A significant correlation was found between CerS2 and CerS6 expression, and between CerS4 and CerS2/CerS6 expression. Moreover, patients that expressed higher CerS2 or 4 mRNA levels tended to show no changes in sphingosine kinase 1 levels, and likewise patients that expressed no change in CerS2 or CerS4 mRNA levels tended to express higher levels of sphingosine kinase 1. Together these results suggest an important role for the CerS genes in breast cancer etiology or diagnosis.

Introduction

Over the past decade, significant interest has been shown in elucidating the roles of sphingolipids in cancer and in cancer therapy [1]. This is because levels of both the enzymes that regulate sphingolipid metabolism, as well as levels of sphingolipid metabolites, change significantly in various cancer tissues and in response to chemotherapeutic agents [2–5]. Of all the sphingolipids, ceramide has received particularly wide attention due to its role in regulating a number of cellular events, including apoptosis and differentiation [6]. Ceramide levels have been shown to change in a number of tumor tissues, such as head and neck squamous cell carcinoma (HNSCC) [7–9].

Although ceramide is often referred to as if it is one lipid, it actually exists as a number of different species which differ in both their sphingoid base composition and in the acyl chain length of the fatty acid that is attached to the sphingoid base [10]. Recently, a gene family encoding for six distinct Ceramide Synthase (CerS) enzymes has been discovered [11] (previously known as longevity assurance gene homologs, Lass genes), with each member of the CerS family synthesizing ceramide with a relatively restricted acyl chain composition. Thus, CerS1 and CerS5 synthesize C18- and C16-ceramides, respectively [12–14] (where C18 and C16 refer to the acyl chain length of the ceramide), CerS2 synthesizes very long acyl chain ceramides (C22–24-ceramide) [15], CerS3 synthesizes C26-ceramides [16] and CerS4 synthesizes C18–22-ceramides [13].

In the current study, we examine mRNA expression levels, by real time quantitative polymerase chain reaction (qPCR), of the six CerS genes, in breast cancer tissues and compare levels with paired normal tissue from the same patients. We describe some significant differences in the cancer tissues, which showed elevated CerS2 and 6 mRNA expression. Moreover, an interesting correlation was obtained between CerS expression and levels of sphingosine kinase (SK) 1; sphingosine kinase is the enzyme that synthesizes the important first and second messenger, sphingosine 1-phosphate (S1P), which is believed to act in an opposite manner to ceramide such that ceramide is a pro-apoptotic lipid whereas S1P is a pro-survival lipid [17]. We discuss the relevance of these findings to breast cancer etiology and diagnosis.

Material and methods

RNA samples. RNA samples from 20 invasive ductal carcinoma patients were obtained from the Cancer Tissue Bank Research Centre, University of Liverpool, England. Patient data is shown in Table 1. RNA quality was evaluated using an Agilent Bioanalyser instrument, and only RNA with an integrity level above 7, or with a good running profile, was used.

cDNA synthesis. cDNA was synthesized using 200 ng RNA and the Reverse-iT first strand synthesis kit (Thermo Scientific), using random hexamers, with 30 min incubation at 42 °C, followed by 30 min incubation at 47 °C and 2 min incubation at 95 °C.

Real time qPCR. Real time qPCR reactions were performed using a SYBR Green PCR Master Mix (Finnzyme) and a 7300 Sequence Detection System (Applied Biosystems). Relative CerS expression

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Table 1
Clinical data of patient samples used in this study.

Patient number	Bloom Richardson stage	Tumor size (mm)	Number of lymph nodes	Lymph nodes plus vascular endothelium	ER[a] (%)	Age at surgery	Dead
1	II	30	3	1	75	42	No
2	II	20	n.a.[b]	n.a.	50	47	Yes
3	II	25	20	0	75	39	No
4	III	10	15	0	90	56	No
5	III	20	14	0	90	52	No
6	II	25	13	0	75	36	No
7	II	35	10	2	75	48	No
8	II	45	9	0	90	60	No
9	II	38	16	2	90	52	No
10	III	18	7	1	50	53	No
11	I	15	5	n.a.	n.a.	49	No
12	II	80	13	3	90	45	No
13	III	140	19	12	90	43	No
14	II	19	16	1	Negative	66	Yes
15	II	14	18	0	75	47	No
16	II	35	14	5	90	78	No
17	II	18	13	1	90	54	No
18	III	40	16	0	Negative	43	No
19	II	50	12	4	90	55	No
20	I	12	7	0	90	75	No

[a] ER, estrogen receptor.
[b] n.a., not available.

levels were determined by comparison to CerS levels in human embryonic kidney (HEK) cells. Quantitative analysis was performed in HEK cells using a standard curve generated using serial dilutions of expression plasmids for each CerS gene. To control for variability of RNA input, PCR reactions were normalized to the amount of TATA box binding protein (TBP) mRNA. Primers sequences are listed in Table 2. Thermal cycling conditions included initial denaturation at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 30 s at 56 °C and 30 s at 68 °C, and then a dissociation step. qPCR reactions were linear over 7 orders of magnitude.

Statistics. The significance of the relative expression of mRNA levels in cancer versus normal tissues was analyzed by the Wilcoxon test-paired sample. The significance of the correlations between mRNA levels of various genes was analyzed by the Pearson correlation.

Results

CerS expression in breast cancer tissue

Paired tissue samples were obtained from healthy and cancerous tissues from the same patients, and were chosen according to histological markers detected by microscopy (Table 1). Of the 20 RNA samples used, two were from patients in Bloom Richardson stage I, 13 were in stage II, and 5 were in stage III. The tumor size of nine of the patients was below or equal to 20 mm, whereas 11 patients had bigger tumors ranging from 25 to 140 mm. All of the patients were lymph node positive (ranging from 3 to 20 lymph nodes), and all except three were estrogen receptor – (ER) positive. The average age of the patients was 52 years-old.

Table 2
Primers used for qPCR.

Genes	Sequence
CerS1	F: 5′-CACTGCGCGCCTCTTTCG-3′ R: 5′-ATTGTGGTACCGGAAGGCG-3′
CerS2	F: 5′-GCTGGAGATTCACATTTTAC-3′ R: 5′-GAAGACGATGAAGATGTTGT-3′
CerS3	F: 5′-GCTATATGACTTATGGGAGG-3′ R: 5′-AAGATCATGAGCTGTAGGTT-3′
CerS4	F: 5′-GTTTCAACGAGTGGTTTTG-3′ R: 5′-TGAATCTCTCAAAGGCAAG-3′
CerS5	F: 5′-ATCTTCTTCGTGAGGCTG-3′ R: 5′-ATGTCCCAGAACCAAGGT-3′
CerS6	F: 5′-ATCAGGAGAAGCCAAGCACG-3′ R: 5′-AGTAGTGAAGGTCAGTTGTG-3′
SK1	F: 5′-CAGCTTCCTTGAACCATTA-3′ R: 5′-GCCGATACTTCTCACTCTCT-3′
SK2	F: 5′-CTTCAACCTCATCCAGACA-3′ R: 5′-ATATCCACATCTGACACGA-3′
TBP	F: 5′-GAGTTCTGGGATTGTACCG-3′ R: 5′-ACTGTTCTTCACTCTTGGCT-3′

Expression of two of the six CerS genes, namely CerS1 and CerS3, could not be detected by qPCR in either healthy or cancerous tissues from any of the patients. The most abundant CerS gene was CerS2 (Fig. 1A), which was expressed at levels of 68.6 ± 8.4 RNA molecules/ng of total RNA; previously, CerS2 was shown to be the most abundant CerS gene in a variety of mouse tissues, but breast tissue was not examined [15]. In the paired cancer tissues, CerS2 levels were significantly ($p < 0.05$) increased, and occurred at levels of 174.3 ± 11.5 RNA molecules/ng of total RNA (Fig. 1A).

In addition to the increased levels of CerS2 in cancer tissues, mRNA levels of CerS4 and CerS6 were also increased, but only CerS6 reached statistical significance upon analyses of the average mRNA levels of all the samples (CerS4, 30.5 ± 1.8 molecules RNA/ng total RNA in healthy patients versus 51.4 ± 2.0 molecules RNA/ng total RNA in cancer tissues; CerS6, 28.4 ± 2.8 in healthy patients versus 76.1 ± 5.9 molecules RNA/ng total RNA in cancer tissues, $p < 0.02$) (Fig. 1A). Levels of CerS5 mRNA were much lower than the other three CerS, and no significant changes in CerS5 expression were detected (Fig. 1A, inset).

We next compared levels of CerS2, 4, 5 and 6 in the paired tissue samples taken from individual patients. Eleven of the 20 patients expressed more CerS2 in the cancer tissues compared to normal tissues, 10 of the 20 expressed more CerS4 (with 3 of the 20 expressing less CerS4), and 14 expressed more CerS6 (Fig. 1B). To determine if individual patients express increased levels of more than one CerS gene, correlation analyses of levels of the relative expression of CerS in individual patients was performed. A highly significant linear correlation was detected between relative expression levels of CerS2 and CerS6 (with a correlation coefficient of 0.71, and $p < 0.0005$) (Fig. 2A); thus, patients with higher CerS2 expression levels show a statistically significant preponderance towards higher CerS6 levels. A correlation was also obtained between CerS4 and CerS2 (with a corre-

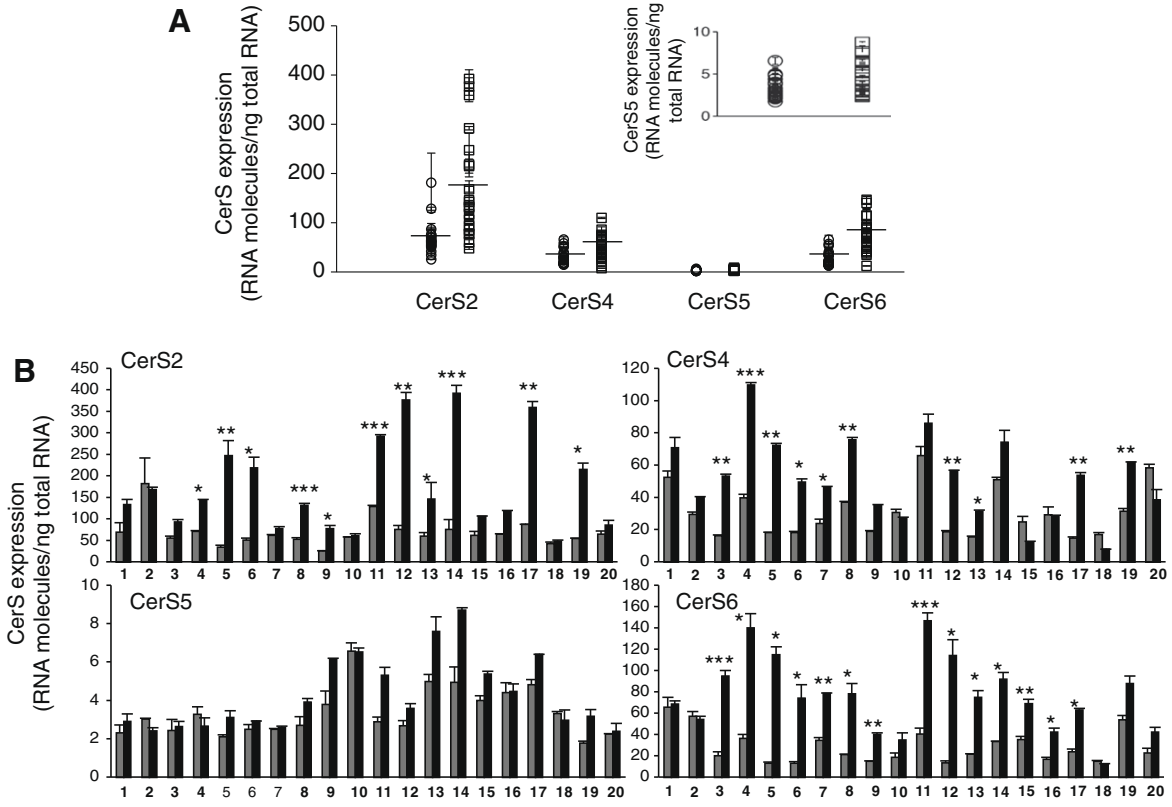

Fig. 1. Levels of CerS mRNA expression in breast cancer tissues. (A) Equal amounts of total RNA from 20 healthy (circles) and breast cancer (squares) tissues were used to determine expression levels of CerS2 ($p < 0.05$), CerS4, CerS5 and CerS6 ($p < 0.02$) by qPCR. Mean values for each CerS are indicated by horizontal lines. The inset shows levels of CerS5 gene expression. (B) Data are shown for each individual patient. Healthy tissue, *grey* columns cancer tissue, *black* columns. Patient numbers correspond to those given in Table 1. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

lation coefficient of 0.65, and $p < 0.002$) (Fig. 2B) and CerS6 (with a correlation coefficient of 0.74, and $p < 0.0002$) (Fig. 2C). No other statistically significant correlations were found between the four CerS genes expressed in breast tissue.

SK expression in breast cancer tissue

To determine whether there is a relationship between CerS and SK expression, qPCR for SK1 and SK2 was performed on the 20 tissue samples. SK1 mRNA was elevated in 7 of the 20 samples, and increased by an average of 2.3-fold, whereas SK2 mRNA was also elevated in 7 of the 20 samples but to a smaller extent (1.8-fold) (Fig. 3). There was no correlation between relative expression levels of SK1 and SK2 in paired tissue samples from the same patients.

Interestingly, there appears to be a correlation between relative expression levels of SK1 and CerS2 and CerS4 (Fig. 4). Thus, 6 patients show higher SK1 levels and no or small changes in CerS2 levels, whereas 6 other patients show no change of SK1 and higher CerS2 levels; 7 of the patients showed no major changes in either CerS2 or SK1. Similarly, 6 patients showed higher SK1 levels and no or little change in CerS4 levels, whereas 5 patients showed higher CerS4 and no change in SK1 levels, with 8 patients showing no major change in either SK1 or CerS4. One outlying patient showed high levels of both CerS2 and SK1, and of CerS4 and SK1. The relatively small number of samples precludes rigorous statistical analysis of these correlations, but the tendency towards a reverse correlation between CerS2/CerS4 and SK1 mRNA levels in paired breast cancer tissues is worthy of further study.

Discussion

The main finding of the current study is that CerS mRNA expression is altered in human breast cancer tissue compared to paired normal tissue from the same patients. Thus, breast cancer joins the growing list of cancers in which the sphingolipid/ceramide pathway is altered.

While this study was in preparation, a study was published showing changes in ceramide levels, measured by liquid chromatography tandem mass spectrometry (LC–MS/MS), in 43 malignant breast tumors, 21 benign breast tumors and 12 normal breast tissue samples [18]. Ceramide levels were elevated in the cancer tissues, with significant levels of elevation of C16-, C24:1- and C24-ceramides. Furthermore, changes in mRNA levels of CerS genes in patient samples were observed, and correlations were analyzed between CerS gene expression and changes in ceramide levels in the same patient samples. However, there was no obvious correlation between levels of elevation of specific CerS genes and elevation of the ceramide species made by the particular CerS. Thus, elevation of CerS2 expression correlated with levels of C16-ceramide, although CerS2 does not synthesize C16-ceramide [15], and levels of CerS6 expression correlated with C18-ceramide, although CerS6 does not make C18-ceramide [19]. Interestingly, the changes reported by Schiffmann et al. [18] in ceramide acyl chain length correlates very closely with the qPCR data obtained in our current study, which shows elevation in CerS6, which synthesizes C16-ceramide, and elevation in CerS2, which synthesizes C24:1- and C24-ceramides.

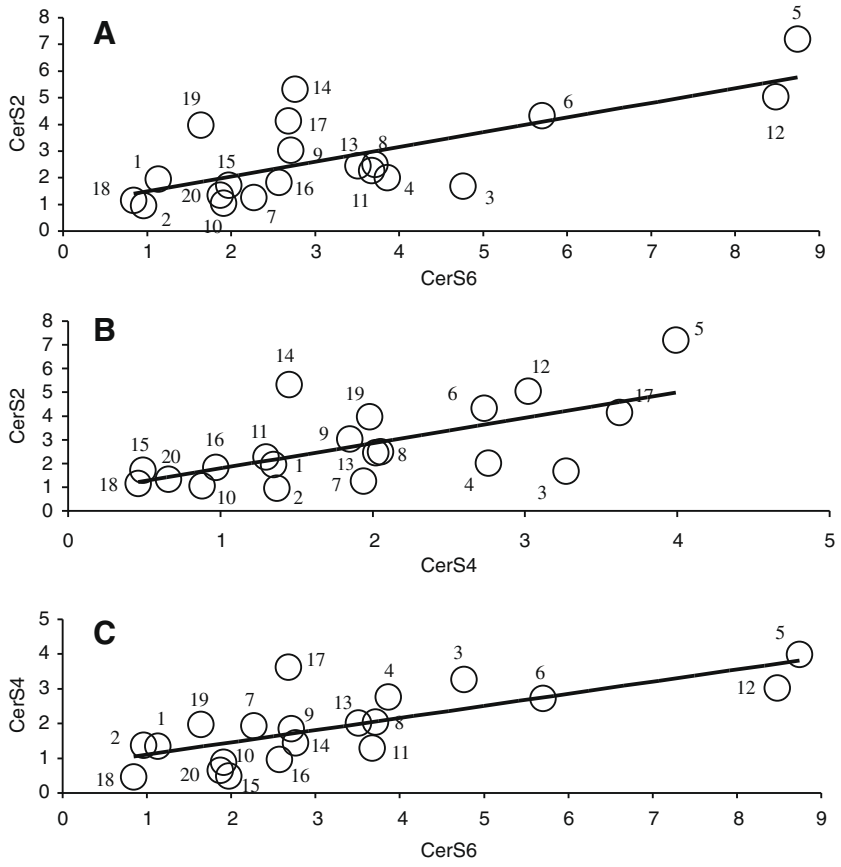

Fig. 2. Correlation between expression levels of different CerS. Linear regression analysis was performed to compare the relative expression in individual patients of (A) CerS2 and CerS6 (correlation coefficients (r) of 0.71; $p < 0.0005$), of (B) CerS2 and CerS4 ($r = 0.65$, and $p < 0.002$), and of (C) CerS4 and CerS6 ($r = 0.74$, and $p < 0.0002$); the numbers correspond to patient numbers as given in Table 1.

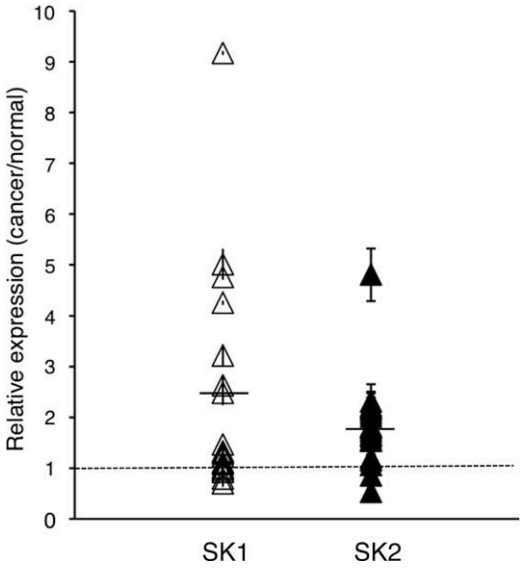

Fig. 3. SK mRNA relative expression in breast cancer tissues. Levels of SK1 and SK2 in breast cancer tissues. Means are indicated by horizontal lines. Changes in the mean values were not statistically significant.

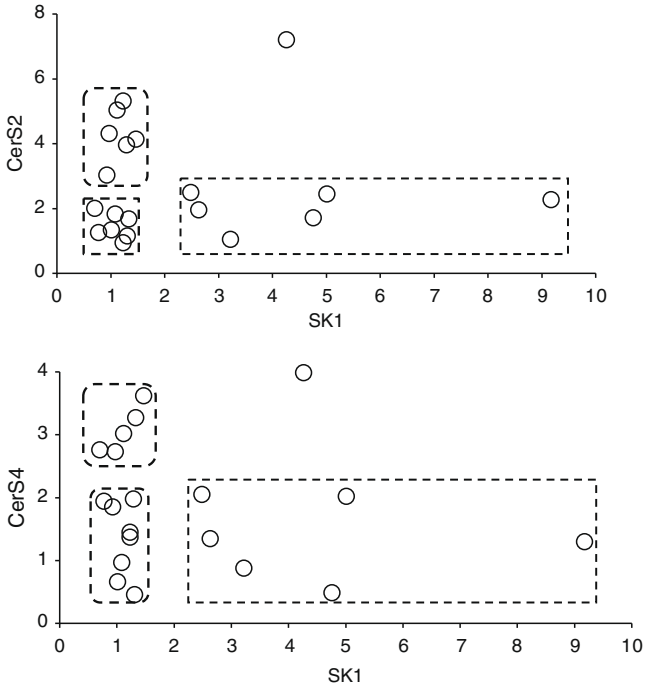

Fig. 4. Correlation between CerS2/CerS4 and SK1 expression. The relative expression of CerS2 (upper panel) and CerS4 (lower panel) compared to SK1 in individual patients is shown.

Thus, the results in the current study extend and complement the data obtained by Schiffmann et al. [18], but there are also some important differences between the studies. First, our data was obtained using paired samples from the same patient; paired analyses were not performed by Schifmmann et al. Secondly, we analyzed SK expression, in addition to CerS expression. Although only small changes were observed in SK levels per se, a correlation

was obtained between SK1 and CerS2 and CerS4 relative expression. Although these changes were not statistically significant, perhaps due to the small number of samples available to us in our study, this correlation might be of some consequence due to the great interest shown in sphingosine 1-phosphate, and in SK, as therapeutic targets in a number of diseases, including cancer [20–22]. Thirdly, we did not detect any correlation between CerS mRNA levels and any of the clinical parameters, unlike Schifmmann et al. who did show some clinical correlations; this may be due to the larger sample size available to Schifmmann et al.

It should be stressed that levels of ceramides can be regulated by multiple pathways, including de novo synthesis via the CerS [11], but also by hydrolysis of sphingomyelin via sphingomyelinases [23]. Moreover, levels of CerS mRNA may not directly reflect levels of CerS enzyme activity as there are likely to be multiple modes of regulation of CerS activity, including mRNA half-life and stability, as well as post-translational modifications of the CerS proteins [11]. Whether it is more useful to measure ceramide levels [18], or to measure CerS mRNA levels (this study) as a possible diagnostic tool for breast cancer, is open to debate, but analyses of ceramide levels requires a dedicated facility for LC–MS/MS analyses, whereas qPCR is a relatively simple technique available in many laboratories, including those that are not specialized for sphingolipid biochemistry.

A number of other studies have recently been performed examining possible roles of ceramide, and enzymes that metabolize ceramide, in breast cancer. For instance, a study showed higher expression among ER negative tumors of SK1 and ceramide galactosyl transferase, with somewhat higher levels of CerS4 and 6 in ER positive samples [24], and high expression levels of ceramide kinase (CERK) were shown to correlate with ErbB2 status [25]. In another study, deoxycholate was shown to promote the survival of murine mammacarcinoma 4T1 cells by reducing ceramide levels [26]. Finally, breast cancer tissues are being targeted by nanoliposomal ceramide as a means to regulate tumor growth [27,28]. Together with the current study, and with that of Schifmmann et al., a role for ceramide in regulating breast cancer growth, etiology and diagnosis is becoming apparent. Future studies will determine whether breast cancer tissue is refractive to intervention in the ceramide metabolism pathway, or whether the kind of analyses performed in the current study, measuring CerS mRNA levels, can be used as a prognostic or diagnostic tool.

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