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CERKL is upregulated in cutaneous squamous cell carcinoma and maintains cellular sphingolipids and resistance to oxidative stress*

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Summary

Background Ceramide kinase-like protein (CERKL) was originally described in retinal tissue. CERKL has been shown to protect cells from oxidative stress, and mutations in CERKL underlie the inherited disease retinitis pigmentosa. CERKL expression maintains cellular sphingolipids via an unknown mechanism.

Objectives To determine whether CERKL is expressed in epidermis and cutaneous squamous cell carcinoma (cSCC) and whether CERKL expression affects cSCC sphingolipid metabolism and susceptibility to oxidative stress.

Methods CERKL expression was determined by RNA-Seq, quantitative polymerase chain reaction and immunohistochemistry. CERKL was knocked down in cSCC cells using small interfering RNA. Sphingolipid content was analysed by liquid chromatography–mass spectrometry. Oxidative stress was induced by treatment with H_2O_2, and apoptosis was measured using flow cytometry to determine annexin V binding.

Results CERKL mRNA and protein are highly expressed in actinic keratosis and cSCC in comparison with normal epidermis. CERKL is also expressed in metabolically active epithelial cells in normal hair bulbs and sebaceous glands. CERKL knockdown in cultured cSCC cells reduces cellular sphingolipid content and enhances susceptibility to oxidative stress.

Conclusions These findings suggest that CERKL may be important in cSCC progression and could lead to novel strategies for prevention and treatment of cSCC.

What is already known about this topic?

- Ceramide kinase-like protein (CERKL) is thought to protect cells from oxidative stress, and mutations in CERKL underlie the inherited disease retinitis pigmentosa.

What does this study add?

- We found that CERKL is upregulated in cutaneous squamous cell carcinoma (cSCC) compared with normal epidermis.
- CERKL protects cSCC cells from oxidative stress and maintains cellular sphingolipids.

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Conflicts of interest
The authors declare they have no conflicts of interest.

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What is the translational message?

- These findings demonstrate that CERKL may be important in cSCC progression by increasing the resistance of tumours to oxidative stress.
- Further investigation of CERKL could lead to novel strategies for prevention and treatment of cSCC.

Cancer is associated with oxidative stress and increased levels of reactive oxygen species (ROS).[1] While modestly elevated ROS promote cancer initiation by enhancing mutagenesis and activating cell proliferation and survival responses, excessive oxidative damage can also kill cancer cells and thus hinder cancer progression.[2] Therefore, cancer cells require protective mechanisms to limit or reduce ROS, especially during invasion and metastasis.[3] This is accomplished in part by modified cellular metabolism, including adaptations in energy substrates, metabolic intermediates and organelles (e.g. autophagy). Additional research is needed to determine how cancer cells control these pathways, which could provide new strategies for cancer treatment.

Ceramide kinase-like protein (CERKL) is an oxidative stress-associated protein, first described in retinal tissue. Loss-of-function mutations in CERKL underlie retinitis pigmentosa, an autosomal recessive disease with progressive vision loss due to photoreceptor apoptosis.[4] CERKL expression is normally restricted to retina, neural tissues, kidney, trachea, testis and lung.[5] CERKL expression has been reported in malignant cell lines,[5] but it has not previously been linked *in vivo* with cutaneous squamous cell carcinoma (cSCC) or other cancers. Although CERKL is structurally related to ceramide kinase enzymes, it appears to protect cells from oxidative stress-induced apoptosis via a mechanism unrelated to kinase activity.[6–8] cSCC growth and survival must proceed in the face of increased oxidative stress. We therefore examined whether CERKL might be upregulated in cSCC, as a mechanism for enhanced survival in the face of oxidative stress.

Materials and methods

Tissue acquisition and cell culture

Skin tumours and adjacent normal skin were obtained from surgery during cSCC excisions. The cSCC cell line SCC12F2 was cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, 2% glutamine and 1% penicillin–streptomycin. To induce oxidative stress, SCC12F2 cells were seeded into six-well culture plates at a density of 25 000 cells per cm^2, then treated on day 4 with 250 mmol L^{-1} H_2O_2 for 18 h. Cells were then rinsed with phosphate-buffered saline and collected by dissociation with trypsin–ethylenediaminetetraacetic acid. Apoptosis was quantified as annexin V staining by flow cytometry,[9] using the Annexin Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Flow cytometry on control and experimental cells was done using the FACSCanto instrument (BD Biosciences, San Jose, CA, USA).

Gene expression analysis

RNA expression levels in patient-matched primary human SCC, actinic keratosis and healthy skin were derived from a previously published dataset using RNA-Seq.[10] Relative mRNA expression was assessed by quantitative reverse-transcriptase polymerase chain reaction using SensiMix SYBR PCR Master Mix (Bioline, Taunton, MA, USA) as described previously.[11] Total RNA was extracted from cells in Buffer RLT with 1% β-mercaptoethanol, and isolated using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA), followed by preparation of cDNA using the SensiFAST cDNA synthesis kit (Bioline). The primer sets used are listed in Table 1.

The thermal cycling conditions were 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s, on an ABI Prism 7900 (Applied Biosystems, Foster City, CA, USA). mRNA expression was normalized to levels of *GAPDH*, used as a control. Values shown represent the mean and SD of three independent assays.

After harvest, cells were lysed using the QIAshredder kit and purified following the protocols of the RNeasy kit by Qiagen. The amount of RNA produced was then quantified using a nanodrop. Quantitative polymerase chain reaction was run with the cDNA using the primers listed in Table 2.

Immunohistochemistry

Skin sections of 8 μm were obtained from paraffin-embedded tissue, and immunostained with an antibody to CERKL (ab198918, rabbit; Abcam, Cambridge, UK) at a dilution of 1 : 100 or 1 : 200, with a secondary antibody of biotinylated goat antirabbit IgG (Vector BA-1000; Vector Laboratories Inc., Burlingame, CA, USA). Samples without the secondary antibody and normal interfollicular skin were used as controls.

Table 1 Primer sets for gene expression analysis

CERKL forward	5′-CAGGGATCTCCCAAATCTGA-3′
CERKL reverse	5′-AGCCTCTAGGTGCCACTGAA-3′
GAPDH forward	5′-GGAGTCAACGGATTTGGTCGTA-3′
GAPDH reverse	5′-GCAACAATATCCACTTTACCAGAGTTAA-3′

Table 2 Primers for quantitative polymerase chain reaction

Human *GAPDH* forward	GAAGGTGAAGGTCGGAGTC
Human *GADPH* reverse	GAAGATGGTGATGGGATTTC
Human *CERKL* forward	CAGGGATCTCCCAAATCTGA
Human *CERKL* reverse	AGCCTCTAGGTGCCACTGAA

Sections were counterstained with haematoxylin and eosin. Samples were photographed on a Leica DM4000B microscope (Leica, Wetzlar, Germany).

Gene expression knockdown using small interfering RNA

On day 2 of culture, SCC12F2 cells were transfected using Lipofectamine RNAiMAX with 20 nmol L^{-1} anti-CERKL or scrambled small interfering (si)RNA, obtained as silencer predesigned siRNA from Ambion (Thermo Fisher). The sequences for the *CERKL* siRNA were sense: 5′-GCAUCAGAGGUCCAUAUUAtt-3′; and antisense: 5′-UAAUAUGGACCUCUGAUGCaa-3′. Control experiments demonstrated approximately 40% *CERKL* knockdown in cSCC expression after siRNA treatment, and did not show an increase in apoptosis after *CERKL* siRNA treatment alone.

Sphingolipid analysis by liquid chromatography–tandem mass spectrometry

To assess cellular sphingolipid levels, cells were harvested 2 days after siRNA treatment using a cell scraper and lysed in RIPA buffer. This was followed by extraction of sphingolipids, as reported previously.[12] Extracted lipids, dried using a vacuum system (Vision, Seoul, Korea), were redissolved in methanol and analysed by liquid chromatography–electrospray ionization–tandem mass spectrometry (API 3200 QTRAP; AB Sciex, Concord, ON, Canada) in selective ion monitoring mode. Ceramide tandem mass spectrometry transitions (m/z) were 510→264 for C14-ceramide, 538→264 for C16-ceramide, 552→264 for C17-ceramide, 566→264 for C18-ceramide, 594→264 for C20-ceramide, 648→264 for C24:1-ceramide and 650→264 for C24-ceramide. The sphingoid bases' tandem mass spectrometry transitions (m/z) were 286→238 for C17 sphingosine as an internal standard, 300→252 for C18 sphingosine and 302→60 for C18 sphinganine. Data were acquired using Analyst 1·4·2 software (Applied Biosystems).

Statistical analysis

Data were analysed using unpaired, two-tailed Student's *t*-tests.

Data availability statement

The RNA sequencing data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus with SuperSeries accession code GSE84194. Additional data from this study are available upon request from the corresponding author (T.M.M.).

Results

To investigate whether CERKL is expressed in cSCC, we first quantified *CERKL* gene expression in patient-matched normal skin, precancerous actinic dysplasia (actinic keratosis) and cSCC. *CERKL* expression was significantly greater in actinic keratosis and cSCC than in patient-matched normal skin (Figure 1). Immunohistochemistry confirmed CERKL protein expression in cSCC and demonstrated that CERKL was highly expressed in cSCC near areas of necrosis (Figure 2a). CERKL was also expressed in actinic keratoses (Figure 2b) and in metabolically active sites such as normal hair bulbs (Figure 2c) and sebaceous glands (Figure 2d), but CERKL expression was not seen in normal interfollicular epidermis adjacent to cSCC (Figure 2e). Consistently with previous reports,[8] CERKL was localized to the cytoplasm of cells rather than the nucleus or plasma membrane.

In order to investigate functional roles for CERKL in cSCC, we treated the SCC cell line SCC12F2 with anti-CERKL siRNA, which reduced CERKL expression by around 40% without any increase in apoptosis or loss of cell viability (data not shown). Similarly to previous reports in mouse retina,[8] we observed that *CERKL* gene silencing reduced SCC12F2 sphingolipid content, as determined by liquid chromatography–mass spectrometry (Figure 3). All analysed sphingolipids were significantly reduced by anti-CERKL siRNA compared with control scrambled siRNA, but reductions were greatest for dihydroceramide, ceramide 1-phosphate and sphingoid bases (including sphingosine, sphinganine, sphingosine 1-phosphate and dihydrosphingosine 1-phosphate), while ceramide and sphingomyelin were only modestly reduced. Sphingolipid reductions were not specific to N-acyl chain length, as all species within each class were reduced similarly (Figures S1–S5; see Supporting Information).

We then tested whether CERKL is required for the resistance of cSCC to oxidative stress. When CERKL expression was reduced by siRNA, SCC12F2 cells were more susceptible to H_2O_2-induced apoptosis (Figure 4; and Figure S6). These findings suggest that CERKL contributes to maintenance of cSCC sphingolipids and resistance to oxidative stress.

Discussion

This report is the first to show that CERKL is expressed in cSCC and protects cultured cSCC cells from cell death induced by oxidative stress. Our results are consistent with those reported in retinas of fish, mice and humans, as well as diverse cultured cell lines,[6,7,13] suggesting that CERKL can protect against oxidative stress in a variety of contexts. CERKL upregulation in cSCC might be an adaption to the increased oxidative stress in tumours and could promote cancer progression. As CERKL protein is expressed in both cSCC tumours and precancerous actinic keratoses, but not in normal

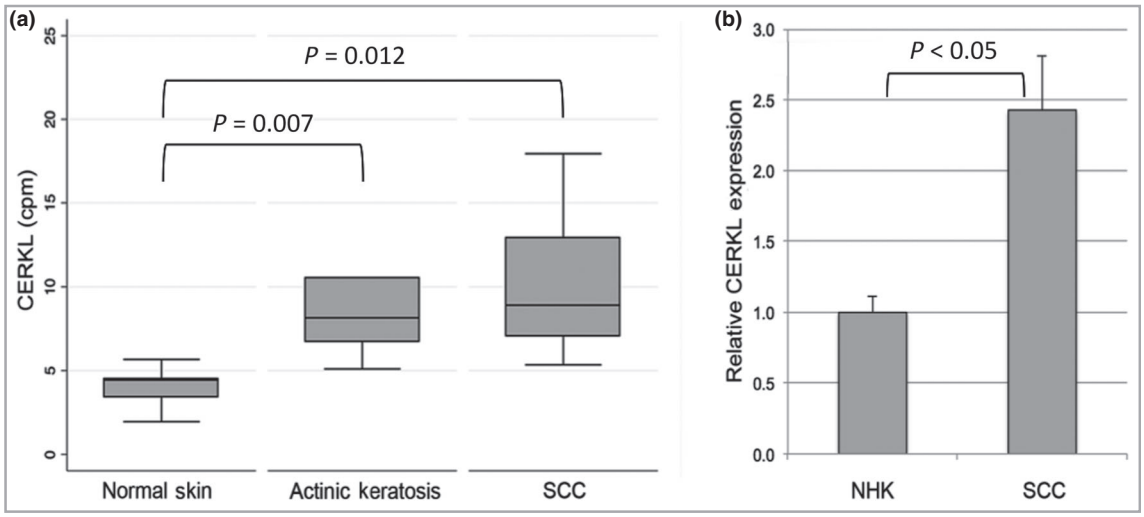

Figure 1 *CERKL* gene expression is upregulated in actinic keratosis (AK) and cutaneous squamous cell carcinoma (cSCC), compared with patient-matched normal skin. (a) RNA-seq expression data are shown for *CERKL* from nine sets of patient-matched normal skin (n = 7), AK (n = 10) and cSCC (n = 9). Mean counts per million (cpm) mapped reads are 4·1 for normal skin, 10·0 for AK and 11·2 for cSCC. There was no difference in expression between AK and SCC (P = 0·6). (b) *CERKL* expression was upregulated in cSCC compared with normal human keratinocytes (NHKs).

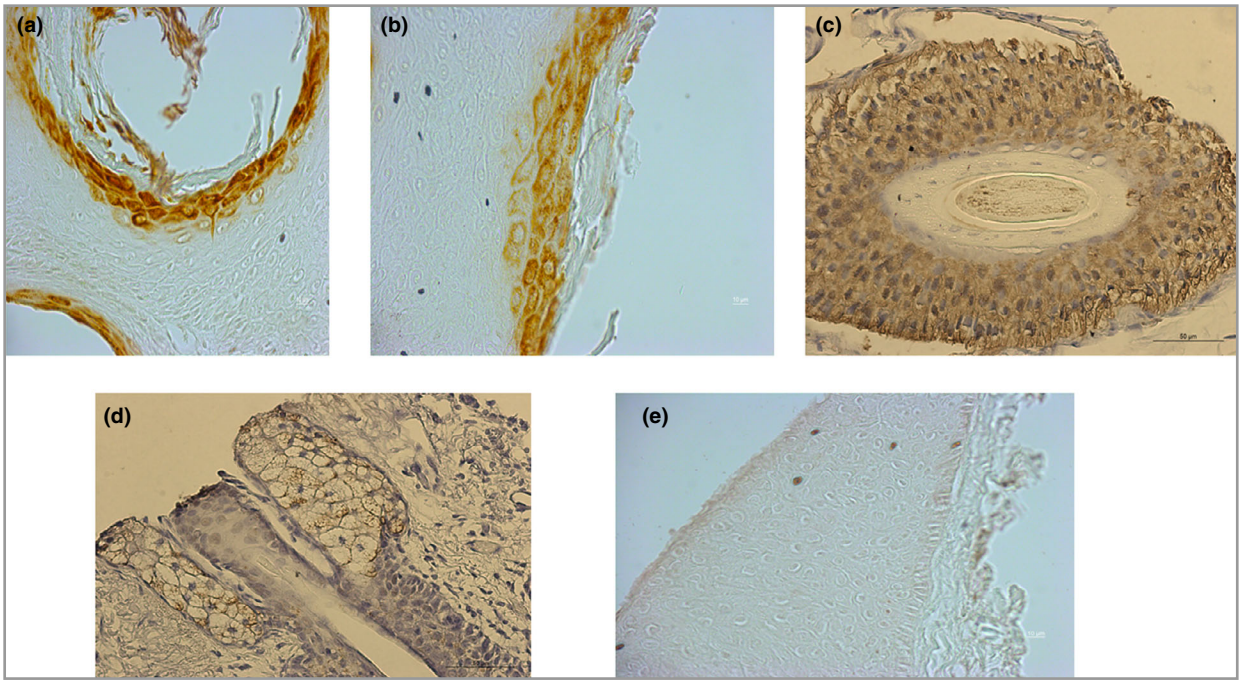

Figure 2 CERKL protein is expressed in cutaneous squamous cell carcinoma (cSCC), actinic keratosis (AK) and appendages but not normal epidermis. CERKL expression was assessed by immunostaining of (a) cSCC, (b) AK, (c) hair bulb, (d) sebaceous gland and (e) normal interfollicular epidermis. Original magnification ×400 (a–d) and ×200 (e).

interfollicular epidermis, CERKL inhibition could be an effective approach for prevention or treatment of cSCC.

Several mechanisms have been proposed for CERKL-mediated protection from oxidative stress, including its role as a reducing agent for the mitochondrial antioxidant TRX2,[14] and by promoting autophagy.[15] Autophagy protects against cell death by converting damaged organelles into additional energy.[16] CERKL has recently been shown to control autophagy by direct molecular interaction with the autophagy regulator sirtuin 1.[15] As sphingosine 1-phosphate and ceramide are also essential regulators of autophagy,[17] CERKL could also modulate autophagy indirectly via changes in sphingolipid

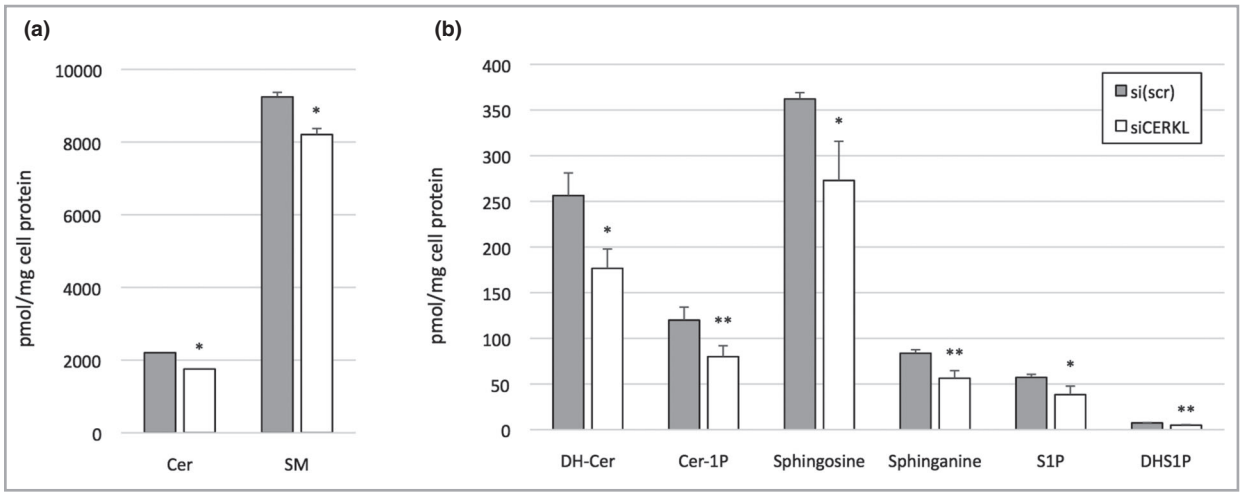

Figure 3 CERKL knockdown reduces the cell sphingolipid content of cutaneous squamous cell carcinoma. SCC12F2 cells were treated with anti-CERKL small interfering (si)RNA for 48 h, then collected and analysed for content of protein (bicinchoninic acid method) and sphingolipid (by liquid chromatography–tandem mass spectrometry). Cer, ceramide; Cer-1P, ceramide 1-phosphate; DH-Cer, dihydroceramide; DHS1P, dihydrosphingosine 1-phosphate; S1P, sphingosine 1-phosphate; si(scr), scrambled control siRNA; siCERKL, anti-CERKL siRNA; SM, sphingomyelin. Data are presented as the mean and SEM; n = 3 for each group. *P < 0·05 vs. si(scr), **P < 0·01 vs. si(scr).

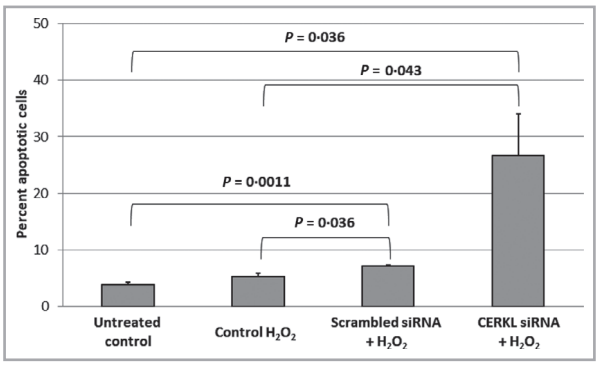

Figure 4 CERKL enhances squamous cell carcinoma survival in response to oxidative stress. SCC12F2 cells were treated with scrambled control or anti-CERKL small interfering (si)RNA for 48 h, then treated with or without 250 μmolL^{-1} H_2O_2 for an additional 18 h. SCC12F2 cells not treated with siRNA were also treated with H_2O_2 in parallel for comparison. Cells were then collected, and apoptosis was quantified as annexin V staining by flow cytometry.[9] Data are presented as the mean and SEM (n = 3 per group).

metabolism. Indeed, we found that CERKL knockdown broadly reduced the concentration of sphingolipid in cSCC, similarly to that reported in CERKL knockdown in retina.[18]

Unlike ceramide kinases, CERKL is not required for ceramide phosphorylation during ceramide 1-phosphate and sphingosine 1-phosphate synthesis,[19] and how CERKL affects sphingolipid metabolism is unclear. In addition to regulating autophagy and cell death, sphingolipid metabolites have been shown to control keratinocyte cell-to-cell adhesion via endoplasmic reticulum Ca^{2+} stores,[20] and thus abnormal CERKL function may lead to acantholysis, a common pathological finding in SCC. Further studies are needed to determine how CERKL carries out its function in cSCC and other tissues.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1 Dihydroceramide quantification in scrambled vs. anti-CERKL small interfering RNA-treated SCC12F2 cells.

Figure S2 Ceramide quantification in scrambled vs. anti-CERKL small interfering RNA-treated SCC12F2 cells.

Figure S3 Sphingomyelin quantification in scrambled vs. anti-CERKL small interfering RNA-treated SCC12F2 cells.

Figure S4 Ceramide 1-phosphate quantification in scrambled vs. anti-CERKL small interfering RNA-treated SCC12F2 cells.

Figure S5 Sphingoid base quantification in scrambled vs. anti-CERKL small interfering RNA-treated SCC12F2 cells.

Figure S6 Representative scatter plots showing the distribution of fluorescein isothiocyanate A (annexin V staining indicating apoptosis) vs. PerCP-Cy5 5-A (cell death).

Powerpoint S1 Journal Club Slide Set.