Ceramide Kinase Promotes Tumor Cell Survival and Mammary Tumor Recurrence

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Abstract

Recurrent breast cancer is typically an incurable disease and, as such, is disproportionately responsible for deaths from this disease. Recurrent breast cancers arise from the pool of disseminated tumor cells (DTC) that survive adjuvant or neoadjuvant therapy, and patients with detectable DTCs following therapy are at substantially increased risk for recurrence. Consequently, the identification of pathways that contribute to the survival of breast cancer cells following therapy could aid in the development of more effective therapies that decrease the burden of residual disease and thereby reduce the risk of breast cancer recurrence. We now report that ceramide kinase (Cerk) is required for mammary tumor recurrence following HER2/neu pathway inhibition and is spontaneously upregulated during tumor recurrence in multiple genetically engineered mouse models for breast cancer. We find that Cerk is rapidly upregulated in tumor cells following HER2/neu downregulation or treatment with Adriamycin and that Cerk is required for tumor cell survival following HER2/neu downregulation. Consistent with our observations in mouse models, analysis of gene expression profiles from more than 2,200 patients revealed that elevated Cerk expression is associated with an increased risk of recurrence in women with breast cancer. In addition, although Cerk expression is associated with aggressive subtypes of breast cancer, including those that are estrogen receptor-negative, HER2+, basal-like, or high grade, its association with poor clinical outcome is independent of these clinicopathologic variables. Together, our findings identify a functional role for Cerk in breast cancer recurrence and suggest the clinical utility of agents targeted against this prosurvival pathway.

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Introduction

Breast cancer is the most common cancer and the leading cause of cancer-related mortality among women worldwide (1). In general, primary tumors can be effectively treated by the combination of surgery, radiotherapy, and adjuvant treatment with hormonal, chemotherapeutic, and/or targeted therapies, as reflected in 5-year survival rates for this disease that approach 90% (1). Despite this favorable short-term prognosis, however, many breast cancer survivors relapse with recurrent disease, sometimes up to 20 years after their initial diagnosis and treatment. Because recurrent breast cancers can be treated, but not cured, the reemergence of tumors following therapy is principally responsible for the morbidity and mortality associated with this disease.

Recurrent breast cancers arise from the pool of disseminated tumor cells (DTC), termed minimal residual disease, which survive therapy in their host, and DTCs can be identified in a substantial fraction of patients with breast cancer at the time of diagnosis, even those with early-stage disease (2). Nevertheless, despite the clinical importance of DTCs, the mechanisms that underlie their survival and recurrence following therapy are largely unknown.

To dissect the mechanisms contributing to breast cancer recurrence, we have used inducible transgenic mouse models of breast cancer that recapitulate critical features of disease progression as it occurs in patients. In this system, the doxycycline-dependent, mammary-specific expression of oncogenes relevant to human breast cancer, including HER2/neu, c-MYC, Wnt1, and Akt1, results in the formation of invasive mammary adenocarcinomas (3–7). Furthermore, in a manner analogous to the treatment of cancers with targeted therapies, such as trastuzumab, mammary tumors regress to a nonpalpable state following oncogene downregulation induced by doxycycline withdrawal. Similar to patients with breast cancer, however, mice bearing primary tumors that have spontaneously regressed often develop recurrent tumors following a variable period of dormancy. Moreover, because recurrent tumors typically arise in the absence of reexpression of the initiating oncogene, the survival and growth of recurrent tumor cells is ostensibly driven by the activation of alternate escape pathways, as is commonly observed in patients with cancer treated with targeted therapies. As such, genetically
engineered mouse models can provide a platform with which to investigate the cellular and molecular mechanisms that contribute to the survival and recurrence of residual tumor cells, thereby identifying essential pathways that may be amenable to therapeutic targeting.

In recent years, sphingolipids have emerged as important regulators of tumor cell survival as well as a number of other pathophysiologic processes relevant to cancer, including proliferation, migration, and inflammatory signaling. Ceramide is a central molecule in sphingolipid signaling and its accumulation has been demonstrated to play an important role in mediating apoptosis in tumor cells in response to chemotherapy, radiotherapy and, more recently, targeted therapy (8–18).

Ceramide can mediate apoptosis in cancer cells through both the extrinsic and intrinsic pathways with structural and signaling roles. Ceramide-enriched membrane platforms in the plasma membrane facilitate the clustering of death receptors and increase formation of death-inducing signaling complexes, resulting in apoptotic signaling (19, 20). Ceramide can also induce mitochondrial outer membrane permeabilization—a hallmark of the intrinsic pathway of apoptotic signaling—by forming ceramide channels (21, 22). Many signaling pathways downstream of ceramide, involving BAX, p38 MAPK, AKT, PP2A, GSK3β, can also enhance apoptotic signaling (reviewed in ref. 23). Conversely, decreased ceramide levels—either through decreased production or accelerated metabolism—have been described as a mechanism of resistance to cancer therapies (16, 24). To this end, a number of ceramide mimetics and inhibitors of enzymes that metabolize ceramide are currently under development as antineoplastic agents (23, 25, 26).

In light of the importance of ceramide in apoptosis induced by antineoplastic therapies, ceramide metabolites, such as glucosylceramide, sphingo-1-phosphate (SIP), and ceramide-1-phosphate (C1P), have been the focus of increasing attention (26, 27). In addition to decreasing levels of proapoptotic ceramide by virtue of their generation, ceramide metabolites exert antiapoptotic functions of their own (24).

Glucosylceramide is synthesized by glucosylceramide synthase (GCS), which catalyzes the transfer of a glucose molecule to ceramide, the first step in glycolipid biosynthesis. GCS has been shown to impart resistance to apoptosis to cancer cell lines in the context of cancer therapy in vitro (reviewed in ref. 28). However, it has not been shown to be explicitly involved in tumorigenesis or tumor progression in vivo.

SIP is generated by the cleavage of ceramide into sphingosine by ceramidase followed by phosphorylation by sphingosine kinase (SphK). The lysosomal ceramidase, acid ceramidase (AC), is overexpressed in many cancers including prostate and head and neck cancers, and has been implicated in resistance to apoptosis induced by treatment with chemotherapeutic agents and C6-ceramide in vitro (29). However, its role in breast cancer remains unclear, as high AC expression has been shown to correlate with better recurrence-free survival in patients with estrogen receptor (ER)-positive breast cancer (30). SIP and SphK have been implicated in many cellular processes, including cell growth, proliferation, survival, and migration. The functions of SIP, AC, and SphK in cancer, including in tumorigenesis and resistance to therapy, have been extensively characterized and have led to the development of a number of inhibitors of that pathway (reviewed in refs. 29, 31, 32). These findings suggest the potential utility of targeting ceramide metabolism in cancer therapy.

C1P and its synthetic enzyme ceramide kinase (Cerk) have been implicated in a number of protumorigenic cellular processes, including inflammation, proliferation, and cell survival. Several studies have shown that Cerk is cytoprotective by virtue of its generation of C1P, which signals through the PI3K–Akt pathway (33, 34). C1P has also been reported to activate cPLA2, recruiting it to Golgi and plasma membranes, resulting in the cleavage of arachidonic acid that is then converted into a variety of prostaglandins. Prostaglandins have, in turn, been associated with many inflammatory processes as well as regulation of cell growth (35) and cancer (reviewed in ref. 36). High CERK expression has been reported to be associated with poor recurrence-free survival in women with ER-negative breast cancer (37). Consistent with this, CERK expression has been reported to be associated with aggressive subtypes of breast cancer, including ER-negative status, HER2-positive status, and high tumor grade (37).

Although ceramide and its metabolites have been demonstrated to contribute to cell death following therapy in vitro, whether this pathway plays a functional role in tumor progression in vivo has not been addressed. Moreover, despite being cytoprotective in tumor cell lines in vitro and predicting poor recurrence-free survival in patients with ER-negative breast cancer, functional roles for Cerk and C1P have not been established in tumorigenesis or tumor recurrence.

In this study, we identify a functional role for Cerk in breast cancer recurrence. We demonstrate that Cerk is spontaneously upregulated during tumor recurrence in mice and is rapidly upregulated in response to HER2/neu pathway inhibition or treatment with Adriamycin. We further establish that Cerk is required for tumor cell survival following HER2/neu down-regulation and is sufficient to promote mammary tumor recurrence. Consistent with observations in mice and with an analogous role for this enzyme in human breast cancer, we find that elevated CERK expression in primary tumors is strongly associated with an increased risk of relapse in patients with breast cancer. Taken together, these observations provide the first functional evidence for a role for Cerk in cancer and identify a potential target for the treatment and prevention of breast cancer recurrence.

Materials and Methods

Animals and orthotopic recurrence assays

All animal experiments were performed in accordance with guidelines of the Institutional Animal Care and Use Committee at the University of Pennsylvania (Philadelphia, PA). MTB/TAN, MTB/TOM, MTB/TWNTp53+/−, and MTB/TAKT mice were bred and tumors were generated as previously described (3–6). Orthotopic recurrence assays and competition assays were performed as described previously (7, 38). Athymic (nu/nu) mice were obtained from Taconic.
Tissue culture and reagents

Primary MTB/TAV tumor cells were cultured as described in the presence of doxycycline (7, 38). Primary tumor cells were transduced with retrovirally packaged mCerk cDNA or shRNAs targeting mCerk.

BT474, SKBR3, and BT20 cells were purchased from the ATCC. All cells were cultured at 37°C with 5% CO2. BT474 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine. SKBR3 cells were cultured in McCoy’s 5A Modified Medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine. BT20 cells were cultured in EMEM medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine.

Plasmids and RNAi

A cDNA for mCerk (MM1013-202798251; Open Biosystems) was subcloned into the pk1 vector. shRNAs targeting mCerk were designed using RNAi Central (http://katahdin.cshl.org/siRNA) and purchased from Open Biosystems. The following sequences were used: shCerk1 TGGTGTTGAGGTGATGACATGTGTTACCTGTGTATATTACATCAAAAGGCAAGCAGATGATGTATTCACTACCGCAAGTGCCTTGTGGACTCCTGCGA; shCerk2 TGCTGTGACAGTGAGCGCGCACTTGCTGGTATTCATCAATGAGCGCTGTCGCAACATGTGATTGAGTGGTGTGTCTTCACTACTAGTGAAAGGCACATGACGTATGATGTATTCACTACCGCAAGTGCCTTGTGGACTCCTGCGA. Oligonucleotides were cloned into the LMP vector (Open Biosystems) as described previously (39). cDNA and shRNA plasmids were virally packaged using Plat-E cells (40), which were transfected with retroviral constructs using Lipofectamine 2000 (Life Technologies). Supernatant-containing viral particles were collected 48 hours posttransfection, filtered, and used to infect primary tumor cell lines. Transduction was performed in the presence of 4 μg/mL polybrene (Millipore).

Antibodies

The following antibodies were used as indicated throughout the text: cleaved caspase-3 (Cell Signaling Technology), cleaved PARP (Cell Signaling Technology), Ki67 (Dako), and β-tubulin (Biogenex).

Immunoblotting

Protein lysates were generated by homogenizing cells in RIPA buffer (50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 1% NP40, 0.5% NaDOC, and 0.1% SDS) supplemented with the HALT Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Secondary antibodies conjugated to IRDye 800CW (LI-COR Biosciences) were used. Odyssey V3.0 system (LI-COR Biosciences) was used to visualize and quantify proteins of interest. Quantification was performed using the ImageStudio software (LI-COR Biosciences).

Immunofluorescence and microscopy

For immunofluorescence studies of tumor tissue from the fluorescent cell competition assay, tumors were snap-frozen in optimal cutting temperature (OCT; TissueTek) and cut into 8-μm frozen sections. Sections were fixed in 4% paraformaldehyde (PFA) for 10 minutes then stained with Hoechst 333258 (Sigma). For cleaved caspase-3 staining, tumors were fixed in 4% PFA in PBS overnight and then embedded in paraffin. Sections were prepared using a standard xylene-based de-waxing procedure, then subjected to antigen retrieval, and stained with appropriate primary and secondary antibodies.

For cell culture experiments, cells were seeded on four-chamber slides, fixed in 4% PFA for 10 minutes, and then permeabilized with 0.5% Triton in PBS for 20 minutes. Slides were then stained with corresponding primary and secondary antibodies. Fluorescence and immunofluorescence microscopy were performed on a DM 5000B Automated Upright Microscope (Leica). Images were captured with a DFC350 FX monochrome digital camera (Leica).

RNA isolation and quantitative RT-PCR

RNA was isolated from tumors using TRIzol (Invitrogen) followed by the RNasy RNA Mini Kit (Qiagen). RNA was isolated from cells using the RNasy RNA Mini Kit (Qiagen). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions. qPCR was performed on the Viia 7 Real-Time PCR System (Life Technologies) using 6-carboxyfluorescein–labeled TaqMan probes for mCerk, hCERK, mTPB, and hTPB (Applied Biosystems). Relative expression levels were calculated using the comparative Ct method.

Statistical analyses

Unpaired Student t tests were used to analyze normally distributed data. Mann–Whitney U tests were used when data were not normally distributed. Two-way ANOVAs were used to compare paired data. Log-rank tests were used to analyze survival curves. P values < 0.05 were considered statistically significant. Hazard ratios (HR) with 95% confidence intervals (CI) were calculated for all survival curves.

Human breast cancer microarray data analysis

We obtained publicly available microarray data and the corresponding clinical annotations from 14 datasets encompassing 2,224 patients with breast cancer (41–54). Normalized data were downloaded from NCBI Gene Expression Omnibus or original authors’ websites. Microarray data were converted to log2 scale where necessary. Affymetrix microarray data were renormalized using Robust Multiarray Average when .CEL files were available.

Association between CERK mRNA expression and 5-year relapse-free survival was estimated using meta-analysis of univariate Cox proportional hazards regression as previously described (38). Meta-analyses were performed in subsets of patients stratified by ER status, PR status, HER2 status, molecular subtype, and tumor grade as described previously (38). Influence analyses were performed on all significant findings derived by meta-analysis to determine whether the significant result was independent of any single dataset.

The association between CERK mRNA expression and known prognostic factors of human breast cancer, such as ER status, PR status, HER2 status, lymph node status, tumor
size, tumor grade, and molecular subtype, was assessed by ANOVA in pooled microarray datasets as previously described (38).

For each prognostic factor significantly associated with CERK mRNA expression, we re-assessed the association between CERK mRNA expression and relapse-free survival after adjusting for the prognostic factor in a multivariate Cox proportional hazards model. The adjusted HRs and CIs for CERK mRNA expression were aggregated across multiple datasets and assigned an overall significance using meta-analysis as described above.

Results

Cerk is spontaneously upregulated in recurrent mammary tumors in mice

As an initial approach to investigating the potential role of sphingolipid metabolites in tumor progression, we evaluated the expression of enzymes involved in sphingolipid metabolism in primary and recurrent mammary tumors generated by the inducible expression—and subsequent downregulation—of HER2/neu, c-MYC, Wnt1, and Akt1 in MMTV-rTA;TetO-HER2/neu, MMTV-rTA;TetO-HER2/neu, MMTV-rTA;TetO-MYC, MMTV-rTA;TetO-Wnt1; p53<sup>−/−</sup>, and MMTV-rTA;TetO-Akt1 transgenic mice (3–7). Expression of SphK1, which generates S1P from sphingosine, and GCS, which generates glucosylceramide from ceramide, was not consistently altered in recurrent tumors compared with primary tumors (data not shown). In contrast, expression of Cerk, which generates C1P from ceramide, was markedly upregulated in recurrent tumors compared with primary tumors generated by induction of each of the four oncogenes tested (Fig. 1A).

Cerk is upregulated following HER2/neu pathway inhibition

To identify the stage of tumor progression at which Cerk upregulation occurs, tumors were harvested from doxycycline-induced MMTV-rTA;TetO-HER2/neu (MTB/TAN) mice bearing primary tumors, MTB/TAN mice bearing primary tumors in which the HER2/neu transgene had been de-induced for 48 or 96 hours, or MTB/TAN mice bearing primary tumors that had fully regressed and then spontaneously recurred following doxycycline withdrawal and HER2/neu downregulation.

Quantitative RT-PCR analysis demonstrated Cerk upregulation in mammary tumors within 48 hours following doxycycline withdrawal, which reached statistical significance by 96 hours and remained upregulated 4.1-fold in recurrent tumors (Fig. 1B). To determine whether alterations in Cerk expression occur in tumor cells subject to HER2/neu downregulation, we next evaluated Cerk mRNA levels following oncogene downregulation in vitro in doxycycline-dependent primary tumor cells derived from a tumor-bearing MTB/TAN mouse. Doxycycline withdrawal resulted in Cerk upregulation within 24 hours that became more pronounced over time (Fig. 1C). This finding suggests that Cerk may be acutely upregulated in primary tumor cells in response to HER2/neu pathway downregulation.

To determine whether Cerk upregulation is an evolutionarily conserved response to HER2/neu pathway inhibition in breast cancer cells, we treated two HER2/neu–amplified human breast cancer cell lines, BT474 and SKBR3, with the dual inhibitor of HER2/neu and EGFR, lapatinib. Treatment with lapatinib resulted in upregulation of Cerk in each of the HER2/neu–amplified cell lines relative to controls within 24 hours (Fig. 1D and E), demonstrating that Cerk upregulation is conserved in human and mouse breast cancer cells following HER2/neu pathway inhibition.

To determine whether Cerk upregulation is also seen in response to other types of antineoplastic therapy, we treated BT20 human breast cancer cells with the chemotherapeutic agent Adriamycin for increasing periods of time. Adriamycin treatment resulted in marked upregulation of Cerk within 8 hours, peaking at 6.5-fold at 24 hours relative to vehicle-treated controls (Fig. 1F). Consistent with this, Cerk was upregulated within 4 hours in primary MTB/TAN mouse tumors treated with Adriamycin, with levels peaking at 4.5-fold at 16 hours (Fig. 1G). Together, these data indicate that Cerk is rapidly upregulated in mouse and human mammary tumor cells following HER2/neu pathway inhibition or treatment with Adriamycin.

Cerk promotes tumor cell survival in vitro following HER2/neu downregulation

Having observed acute Cerk upregulation following HER2/neu pathway inhibition in vivo and in vitro, we hypothesized that Cerk upregulation might contribute to the survival of tumor cells following HER2/neu pathway inhibition. Doxycycline was removed from the culture media of primary MTB/TAN tumor cell lines transduced with expression constructs for wild-type Cerk or the kinase-dead–mutant G198D-Cerk, which are expressed at comparable levels (data not shown), or a control vector. Cells were then stained for cleaved caspase-3 and Ki67.

Cerk-overexpressing MTB/TAN cells exhibited markedly reduced staining for cleaved caspase-3 compared with control cells following HER2/neu downregulation induced by doxycycline withdrawal (Fig. 2A and B). In contrast, MTB/TAN cells transduced with kinase-dead G198D-Cerk exhibited an increase in cleaved caspase-3 staining following HER2/neu downregulation that was comparable with control cells (Fig. 2A and B). As anticipated, proliferation rates as measured by Ki67 staining fell dramatically upon HER2/neu downregulation, but did not differ between Cerk, G198D-Cerk, and empty vector controls (Supplementary Fig. S1A). These findings suggest that Cerk overexpression is sufficient to suppress apoptosis induced by HER2/neu pathway inhibition and that it does so in a kinase-dependent manner.

To determine whether loss of function of Cerk would sensitize cells to apoptosis following HER2/neu downregulation, we performed an analogous set of experiments in primary MTB/TAN tumor cells expressing either of two Cerk shRNAs, a scrambled control shRNA or an empty vector control. Cerk knockdown cells exhibited markedly increased staining for cleaved caspase-3 compared with control cells following doxycycline withdrawal (Fig. 2C and D). Proliferation rates fell dramatically upon HER2/neu downregulation, but were not significantly different between control and Cerk knockdown
cells (Supplementary Fig. S1B). These findings suggest that Cerk is required for the suppression of apoptosis following HER2/neu downregulation.

To confirm the observed increase in apoptosis, we performed Western blot analyses for cleaved caspase-3 and cleaved PARP under the same culture conditions. Consistent with immunofluorescence results, Cerk overexpression reduced levels of cleaved PARP and cleaved caspase-3 following doxycycline withdrawal (Fig. 2E–G). Kinase-dead G198D-Cerk exhibited comparable levels of cleaved PARP and cleaved caspase-3 as controls, indicating that kinase activity is required for Cerk to protect cells from apoptosis. Similarly, Cerk knockdown cell lines exhibited significantly higher levels of cleaved PARP and cleaved caspase-3 than controls following doxycycline withdrawal (Fig. 2H–J).

Cerk is required for tumor cell survival following HER2/neu downregulation

To expand our in vitro findings that Cerk protects tumor cells from apoptosis following HER2/neu pathway inhibition,
Figure 2. Cerk protects tumor cells from apoptosis upon HER2/neu downregulation in vitro. A and B, representative fluorescence images (A) and quantification (B) of MTB/TAN primary tumor cell lines expressing empty vector, Cerk, or G198D-Cerk withdrawn from doxycycline (Dox) for 72 hours and then stained for cleaved caspase-3 (red; Hoechst, blue). C and D, representative fluorescence images (C) and quantification (D) of MTB/TAN primary tumor cell lines expressing empty vector, a control scrambled shRNA, shCerk1 or shCerk2 withdrawn from doxycycline for 72 hours and then stained for cleaved caspase-3. E–G, Western blot analysis of MTB/TAN primary tumor cell lines expressing empty vector, Cerk, or G198D-Cerk withdrawn from doxycycline for 48 hours, blotted for cleaved PARP, quantified in F, and cleaved caspase-3, quantified in G. H–J, Western blot analysis of MTB/TAN primary tumor cell lines expressing empty vector, a control scrambled shRNA, or shCerk2 withdrawn from doxycycline for 48 hours, blotted for cleaved PARP, quantified in I, and cleaved caspase-3, quantified in J. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
we sought to test this in vivo. To determine whether Cerk is required for the survival of tumor cells following acute HER2/neu downregulation, we performed an orthotopic fluorescent cell competition assay using an isogenic pair of doxycycline-dependent MTB/TAN tumor cell lines that differed only in Cerk expression. MTB/TAN primary tumor cells expressing one of two shRNAs targeting Cerk were labeled with an H2B-eGFP reporter, whereas MTB/TAN cells transduced with a control vector were labeled with an H2B-mCherry reporter. These two fluorescent populations of cells were injected into the mammary glands of nu/nu mice at a 1:1 ratio and allowed to form orthotopic primary tumors in the presence of doxycycline and HER2/neu expression. Doxycycline was then withdrawn to initiate tumor regression and the ratio of eGFP-labeled Cerk knockdown cells to mCherry-labeled control cells was determined in primary tumors, residual tumor cells 96 hours following HER2/neu downregulation, and residual tumor cells 28 days following HER2/neu downregulation by fluorescence microscopy performed on histologic sections (Fig. 3A).

Cerk shRNA-expressing cells were neither selected for, nor against, during the outgrowth of primary orthotopic tumors (Fig. 3B and C and Supplementary Fig. S2C), indicating that Cerk knockdown does not confer a selective advantage or disadvantage during primary tumorigenesis in the presence of HER2/neu expression. In contrast, eGFP-labeled Cerk knockdown cells were strongly selected against within 96 hours following doxycycline withdrawal and HER2/neu downregulation, as these cells comprised only 20% to 25% of the fluorescent tumor cells at this time point (Fig. 3B and C and Supplementary Fig. S2C). By 28 days following doxycycline withdrawal, Cerk knockdown cells comprised only 14% to 20% of fluorescent cells, indicating modest further negative selection against Cerk knockdown cells (Fig. 3B and C and Supplementary Fig. S2C). Tumors containing control mCherry-labeled cells and control eGFP-labeled cells maintained an approximately 1:1 ratio throughout tumor progression throughout these time points (Supplementary Fig. S2B). These findings indicate that cells in which Cerk has been knocked down are rapidly and persistently selected against following HER2/neu downregulation, but not in actively growing primary tumor cells expressing HER2/neu.

Cerk promotes tumor cell survival in vivo following HER2/neu downregulation

One mechanism by which Cerk-expressing cells could be selected for following HER2/neu downregulation would be if Cerk provides a cell-autonomous survival advantage in this context. To address this possibility, we generated mice bearing orthotopic tumors derived from MTB/TAN primary tumor cells overexpressing wild-type Cerk, kinase-dead G198D-Cerk, or a control vector. We then performed immunofluorescence staining for cleaved caspase-3 on histologic sections from primary tumors on doxycycline or in which HER2/neu had been acutely downregulated for 48 or 96 hours.

Primary tumors from control, Cerk, and G198D-Cerk–overexpressing cohorts exhibited comparable, low levels of cleaved caspase-3 staining in the presence of HER2/neu expression, and a marked increase in cleaved caspase-3 staining was observed 48 hours following doxycycline withdrawal (Fig. 3D). Strikingly, however, tumors overexpressing Cerk displayed a significantly blunted apoptotic response to HER2/neu downregulation at 48 hours after deinduction and cleaved caspase-3 levels remained lower in Cerk-overexpressing tumors compared with controls at 96 hours (Fig. 3D and E). In contrast, tumors overexpressing the kinase-dead–mutant G198D-Cerk did not blunt the apoptotic response and exhibited high levels of cleaved caspase-3 comparable with control tumors following HER2/neu downregulation (Fig. 3D and E). These findings indicate that Cerk upregulation protects tumor cells from apoptosis following HER2/neu pathway inhibition, suggesting a cellular mechanism for the observation that cells with Cerk knockdown are selected against following oncogene downregulation in vivo.

Cerk promotes mammary tumor recurrence in vivo

Cerk’s ability to inhibit apoptosis following HER2/neu downregulation in tumor cells in vivo and in vitro, coupled with its spontaneous upregulation in recurrent mammary tumors in multiple genetically engineered mouse models, suggested the possibility that Cerk might promote breast cancer recurrence by enhancing the survival of tumor cells following therapy. To test this hypothesis, we injected primary MTB/TAN tumor cells transduced with a Cerk expression vector or a control vector into the mammary fat pads of nu/nu mice maintained on doxycycline. Following primary tumor formation, mice were withdrawn from doxycycline to induce oncogene downregulation and tumor regression (Fig. 4A). All tumors regressed to a nonpalpable state, irrespective of Cerk expression status. Mice were then monitored for tumor recurrence.

Consistent with our hypothesis, Cerk expression in MTB/TAN tumor cells markedly accelerated tumor recurrence, with median time to recurrence decreasing from 178 days for control tumors to 130 days in Cerk-overexpressing tumors (Fig. 4B; HR, 4.44; P = 0.0006). However, the mean growth rate of recurrent tumors was not found to be different between control and Cerk-overexpressing cohorts, indicating that increased rates of proliferation are unlikely to be responsible for the observed difference in latency to tumor recurrence (Fig. 4C).

To determine whether the ability of Cerk to promote tumor recurrence was dependent upon Cerk kinase activity, we repeated this experiment with the addition of a cohort of mice injected with primary tumor cells expressing the G198D kinase-dead allele of Cerk. As before, tumors overexpressing wild-type Cerk exhibited significantly accelerated tumor recurrence with median latency at 121 days (Fig. 4D; HR, 2.61; P = 0.0054). However, in contrast to the effects of overexpressing wild-type Cerk, and consistent with our in vitro findings indicating that Cerk kinase activity is required for its ability to promote tumor cell survival following HER2/neu downregulation, overexpression of G198D-Cerk did not accelerate the rate of mammary tumor recurrence compared with control tumors, with a median time to recurrence of 154 days in each cohort.

Although suggestive, the above experiments could not rule out the possibility that the increased rate of relapse observed for wild-type Cerk-overexpressing tumors was due to effects of Cerk expression during primary tumor formation. That is, if
Cerk expression resulted in the formation of primary tumors with different properties than control tumors, this could explain the reduced time to recurrence, rather than effects of Cerk following HER2/neu downregulation. To address the potential confounding effects of Cerk expression during primary tumor formation, we modified the orthotopic recurrence assay to eliminate primary tumor formation, thereby isolating the effects of Cerk on tumor cell survival and recurrence.

Recipient mice pretreated with doxycycline were injected with control or Cerk-overexpressing cells and then withdrawn from doxycycline after 48 hours, before primary tumors had formed (Fig. 4E). Consistent with our prior results, Cerk overexpression accelerated the rate of mammary tumor recurrence and reduced the median time to recurrence from 304 days in controls to 185 days in the presence of Cerk expression (Fig. 4F; HR, 3.64; \( P = 0.002 \)). Again, the mean growth rate was not found
Figure 4. Cerk promotes mammary tumor recurrence in the MTB/TAN mouse model. A, schematic of recurrence assay and timing of doxycycline (Dox) treatment. B, recurrence-free survival of nu/nu mice harboring primary orthotopic tumors from MTB/TAN primary cell lines expressing empty vector or Cerk induced to regress by doxycycline withdrawal. C, mean growth rate of control and Cerk-overexpressing recurrent tumors. D, recurrence-free survival of nu/nu mice harboring primary orthotopic tumors from MTB/TAN cell lines expressing empty vector, wild-type Cerk, or the kinase-dead–mutant G198D-Cerk induced to regress by doxycycline withdrawal. E, schematic of modified recurrence assay with no primary tumor formation. F, recurrence-free survival of nu/nu mice harboring MTB/TAN primary cell lines expressing empty vector, Cerk, or G198D-Cerk withdrawn from doxycycline 48 hours after injection. G, mean growth rate of control and Cerk-overexpressing recurrent tumors.
Cerk Promotes Tumor Recurrence

Because ER-negative, PR-negative, HER2-positive, basal-like, and high-grade tumors are each associated with a poor prognosis, we wished to determine whether the association between CERK expression and relapse-free survival in patients with breast cancer was independent of the association between CERK expression and these aggressive tumor subsets. To address this, we performed multivariate Cox proportional hazards regression in combination with meta-analyses and adjusted for each of these variables individually. In each case, we found that the association between elevated CERK expression and decreased recurrence-free survival remained significant after adjusting for ER status, PR status, HER2 status, molecular subtype, or tumor grade (Supplementary Fig. S4A–S4E). Influence analysis performed on each significant result obtained by meta-analysis confirmed that these results were independent of any single dataset (Supplementary Fig. S5).

In aggregate, our results indicate that CERK expression in human breast cancer is associated with an increased risk of recurrence within 5 years, and is independent of the association between CERK expression and aggressive subtypes of human breast cancer.

Discussion

The importance of ceramide metabolism in cancer has become of increasing interest in recent years (23, 26, 27). However, relatively little functional data have emerged regarding ceramide, its metabolites, and the metabolic enzymes responsible for their generation in tumorigenesis and tumor progression.

In a number of tumor cell lines, Cerk has been reported to exert cytoprotective effects in a variety of in vitro contexts, including serum starvation, TNFα signaling, and UV irradiation. To date, however, no evidence has existed for a functional role for Cerk in tumor cell survival in vivo, tumor progression, or tumor recurrence. Using genetically engineered mouse models, human breast cancer cell lines, and expression data from patients with breast cancer, we have now identified a functional role for Cerk as a regulator of tumor cell survival and breast cancer recurrence following HER2/neu downregulation.

We found that Cerk is spontaneously upregulated during the process of tumor recurrence in mammary tumors driven by four different oncogenic pathways. Notably, Cerk was upregulated as early as 96 hours following oncogene downregulation in mice bearing HER2/neu-driven mammary tumors and, consistent with this, human HER2/neu–amplified breast cancer cell lines also rapidly upregulated Cerk following HER2/neu pathway inhibition. Indicative of a role for Cerk in the context of HER2/neu inhibition, tumor cells in which Cerk had been knocked down were strongly selected against within 96 hours following HER2/neu downregulation, with further selection continuing into the dormant phase of tumor regression at 28 days after deinduction. Whether tumors comprised solely of Cerk knockdown cells would similarly be selected against will require further study. That this is likely to be the case, however, is suggested by additional in vivo and in vitro analyses, which revealed that enforced Cerk expression inhibited apoptosis, whereas Cerk knockdown enhanced apoptosis, following

to be different between control and Cerk-overexpressing cohorts (Fig. 4G). These results indicate that Cerk can promote mammary tumor recurrence following HER2/neu downregulation and that this effect is unlikely to be due to Cerk-induced alterations in primary tumor formation.

Elevated CERK expression is associated with an increased risk of recurrence in women with breast cancer

A prior report suggested that elevated CERK expression is associated with poor prognosis in patients with ER-negative breast cancer (37). To confirm and extend this analysis, we interrogated publicly available human breast cancer expression datasets corresponding to 2,224 patients with tumors of mixed ER status to evaluate the association of CERK expression with recurrence-free survival in a meta-analysis. Using a Cox proportional hazards model, we found that women with primary tumors expressing high levels of CERK exhibited an increased risk of tumor recurrence within 5 years of diagnosis (Fig. 5; HR: 1.32; 95% CI: 1.17–1.49).

We next asked whether CERK expression is associated with aggressive subtypes of human breast cancer. Association studies revealed that CERK is expressed at higher levels in ER-negative compared with ER-positive, and PR-negative compared with PR-positive, tumors. CERK expression was also associated with HER2-positive status as assessed by immunohistochemistry (Supplementary Fig. S3A–S3C). Evaluation of CERK expression as a function of molecular subtype revealed that CERK expression is higher in the basal and ErbB2 subtypes compared with the luminal A and luminal B subtypes (Supplementary Fig. S3D). CERK expression was also associated with high tumor grade (Supplementary Fig. S3E), but not with tumor size or lymph node status (data not shown).

We were interested in determining whether CERK expression and increased risk of recurrence within 5 years of diagnosis (Fig. 5; HR: 1.32; 95% CI: 1.17–1.49).

Overall P-value (fixed-effect): 9.3e–06

<table>
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<th>Study</th>
<th>n</th>
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<th>HR [95% CI]</th>
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<td>1.29</td>
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<td>Hess</td>
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<td>1.90</td>
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<td>Overall</td>
<td></td>
<td>1.32</td>
<td>[1.17–1.49]</td>
</tr>
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</table>

Figure 5. High CERK expression predicts decreased relapse-free survival in women with breast cancer. Forest plot representation of 5-year survival estimates and HRs for relapse-free survival of individual datasets of human patients with breast cancer.
HER2/neu downregulation in the absence of a competing population of cells.

In accord with the antiapoptotic effects of Cerk in tumor cells following HER2/neu downregulation, Cerk expression in orthotopic primary mammary tumor cells promoted tumor recurrence, indicating a functional role for Cerk in this stage of tumor progression. In aggregate, our findings indicate that Cerk is required for tumor cell survival following HER2/neu downregulation. Ostensibly, this may result in a larger pool of surviving residual tumor cells that can potentially give rise to recurrent tumors. These findings therefore suggest a therapeutic opportunity whereby inhibition of Cerk in concert with treatment with targeted therapies may enhance tumor cell death, reduce the reservoir of residual tumor cells, and thereby prevent tumor recurrence.

In agreement with its functional role in mammary tumor recurrence elucidated in mice, elevated Cerk expression was associated with increased risk of recurrence in human patients with breast cancer. Cerk expression was associated with aggressive subtypes of breast cancer, including ER-negative, PR-negative, HER2-positive, high grade, and ErbB2 and basal-like tumors. However, the association between Cerk and relapse-free survival was independent of its association with these aggressive subsets of tumors. As such, while Cerk was previously reported to be associated with decreased recurrence-free survival in patients with ER-negative breast cancer (37), our findings extend this analysis by demonstrating that Cerk is associated with poor outcome in all patients with breast cancer and does so independently of its association with individual aggressive prognostic factors.

Of note, we did not observe a selective effect against Cerk knockdown cells in primary tumors in an orthotopic competition assay, suggesting that Cerk expression in actively proliferating HER2/neu–driven tumor cells may not be rate limiting for growth and/or survival. This, in turn, suggests that rate-limiting pathways regulating tumor cell growth and survival may differ depending upon the stage of tumor progression. As such, pharmacologic agents that may be effective in treating primary breast cancers may have little effect in women with minimal residual disease or recurrent breast cancer. As such, the identification of pharmacologic targets unique to these stages of tumor progression will be essential for improving long-term survival in this disease. Consequently, the inhibition of pathways that allow cells to survive therapy and eventually recur may be key to preventing the persistence of DTCs and subsequent relapse. In aggregate, our findings identify one such pathway for cell survival in breast cancer cells, as well as define a subset of human breast cancers with a high likelihood of relapse. In addition, our data underscore the importance of ceramide metabolism and sphingolipids in cancer drug resistance and relapse.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A.W. Payne, L.A. Chodosh
Development of methodology: A.W. Payne
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.W. Payne
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.W. Payne, D.K. Pant, T.-C. Pan
Writing, review, and/or revision of the manuscript: A.W. Payne, D.K. Pant, L.A. Chodosh
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.-C. Pan
Study supervision: L.A. Chodosh

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References

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