Targeting ceramide synthase 6–dependent metastasis-prone phenotype in lung cancer cells

Motoshi Suzuki, … , Mamoru Kyogashima, Takashi Takahashi

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Sphingolipids make up a family of molecules associated with an array of biological functions, including cell death and migration. Sphingolipids are often altered in cancer, though how these alterations lead to tumor formation and progression is largely unknown. Here, we analyzed non–small-cell lung cancer (NSCLC) specimens and cell lines and determined that ceramide synthase 6 (CERS6) is markedly overexpressed compared with controls. Elevated CERS6 expression was due in part to reduction of microRNA-101 (miR-101) and was associated with increased invasion and poor prognosis. CERS6 knockdown in NSCLC cells altered the ceramide profile, resulting in decreased cell migration and invasion in vitro, and decreased the frequency of RAC1-positive lamellipodia formation while CERS6 overexpression promoted it. In murine models, CERS6 knockdown in transplanted NSCLC cells attenuated lung metastasis. Furthermore, combined treatment with 1-α-dimyristoylphosphatidylcholine liposome and the glucosylceramide synthase inhibitor D-PDMP induced cell death in association with ceramide accumulation and promoted cancer cell apoptosis and tumor regression in murine models. Together, these results indicate that CERS6-dependent ceramide synthesis and maintenance of ceramide in the cellular membrane are essential for lamellipodia formation and metastasis. Moreover, these results suggest that targeting this homeostasis has potential […]
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Motoshi Suzuki,1 Ke Cao,1 Seiichi Kato,2 Yuji Komizu,3 Naoki Mizutani,4 Kouji Tanaka,4 Chinatsu Arima,1 Mei Chee Tai,1 Kiyoshi Yanagisawa,1 Norie Togawa,1 Takahiro Shiraishi,1 Noriyasu Usami,5 Tetsuo Taniguchi,5 Takayuki Fukui,5 Kohei Yokoi,5 Keiko Wakahara,6 Yoshinori Hasegawa,6 Yukiko Mizutani,7 Yasuyuki Igarashi,7 Jin-ichi Inokuchi,7 Soichiro Iwaki,8 Satoshi Fujii,9 Akira Satou,9 Yoko Matsumoto,1 Ryuichi Ueoka,1 Keiko Tamiya-Koizumi,4 Takashi Murate,4 Mitsuhiro Nakamura,10 Mamoru Kyogashima,11 and Takashi Takahashi1

Sphingolipids make up a family of molecules associated with an array of biological functions, including cell death and migration. Sphingolipids are often altered in cancer, though how these alterations lead to tumor formation and progression is largely unknown. Here, we analyzed non–small-cell lung cancer (NSCLC) specimens and cell lines and determined that ceramide synthase 6 (CERS6) is markedly overexpressed compared with controls. Elevated CERS6 expression was due in part to reduction of microRNA-101 (miR-101) and was associated with increased invasion and poor prognosis. CERS6 knockdown in NSCLC cells altered the ceramide profile, resulting in decreased cell migration and invasion in vitro, and decreased the frequency of RAC1-positive lamellipodia formation while CERS6 overexpression promoted it. In murine models, CERS6 knockdown in transplanted NSCLC cells attenuated lung metastasis. Furthermore, combined treatment with L-α-dimyristoylphosphatidylcholine liposome and the glucosylceramide synthase inhibitor D-PDMP induced cell death in association with ceramide accumulation and promoted cancer cell apoptosis and tumor regression in murine models. Together, these results indicate that CERS6-dependent ceramide synthesis and maintenance of ceramide in the cellular membrane are essential for lamellipodia formation and metastasis. Moreover, these results suggest that targeting this homeostasis has potential as a therapeutic strategy for CERS6-overexpressing NSCLC.

Introduction

Recent evidence has shown altered levels of biologically active sphingolipids and enzymes related to sphingolipid metabolism in cancer, indicating roles for these pathways in cancer pathogenesis and progression (1). Ceramides, the central molecules of sphingolipid metabolism, constitute a family of closely related molecules that function as stress coordinators in response to various stress stimuli, such as cytokines, ionizing radiation, and chemotherapeutic agents (2). In addition, they serve as intracellular mediators of apoptosis induced by TNF-α (3), with d18:1-C16:0 ceramide (C16:0 ceramide) identified as an important mediator of apoptosis in response to ionizing radiation (4) as well as other types of proapoptotic treatments (ref. 5 and references therein). It was also shown that endogenous ceramide levels were significantly elevated in the majority of human head and neck squamous cell carcinoma (HNSCC) tissues as compared with those in normal tissues (6), while ceramide synthase 2 (CERS2) and CERS6 expression levels were found to be increased in cancerous breast tissues as compared with those in normal breast tissues (7). Furthermore, the survival of some cancer cells is dependent on ceramide, as CERS6 downregulation produced ER stress that led to apoptosis of HNSCC cells (8). Together, these findings suggest that ceramide and ceramide synthase (CERS) family enzymes play various roles in cancer, though their functions leading to cancer pathogenesis in tumor formation and progression have not been well documented.

Lung cancer is the leading cause of cancer death in many industrialized countries; thus, a better understanding of the molecular basis of this fatal disease and development of treatment strategies focused on the intrinsic pathogenesis are greatly anticipated to reduce the intolerable death toll. To this end, gene expression profiling analysis has provided an approach to identify genes and pathways responsible for development of the disease (9, 10). For example, our previous analysis focused on 257 genomic stability–related genes revealed that pol δ is frequently downregulated in the POLD4 subunit in lung cancer and causes genomic instability in vitro (11, 12). Furthermore, attenuated expression levels were shown to be associated with poor prognosis...
and well correlated with clinical genomic instability; low POLD4 expression was significantly associated with 8p, 9q, and 13q deletions and 5p, 7p, 8q, and 14q amplifications that are frequently observed in lung cancer.

Here, we analyzed lung cancer-associated gene expression profiles of sphingolipid metabolic genes and found pivotal functions of CERS6 in invasion and metastasis. Moreover, we report evidence demonstrating that CERS6 overexpression in cancer cells may be targeted as a cancer treatment strategy we believe to be novel.

Results
CERS6 overexpressed in non-small-cell lung cancer and inversely correlated with clinical outcome. Comparison of non-small-cell lung cancer (NSCLC) tissues with normal lung tissues revealed altered expression of the ceramide metabolic pathway gene probes (Figure 1A and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI79775DS1). Among them, elevated CERS6 expression was significantly associated with apparent invasiveness in the surgical specimens (Figure 1B) as well as poor prognosis (Figure 1C). A similar association between expression levels and prognosis/invasiveness was also observed in other cancer data sets (Supplemental Figure 1). Accordingly, higher CERS6 mRNA and protein levels were observed in adenocarcinoma and squamous cell carcinoma specimens relative to those in normal tissues (Figure 1, D and E, and Supplemental Table 2). In contrast, CERS5, another gene with a substrate specificity similar to that of CERS6 (13), showed comparable expression between NSCLC and normal specimens and had no significant correlation with survival (Supplemental Figure 2).

Figure 1. CERS6 overexpressed in NSCLC and inversely correlated with clinical outcome. (A) Diagram of ceramide metabolic pathway genes. Red, blue, and black arrows indicate genes that were upregulated, downregulated, and neutrally regulated, respectively (see Supplemental Table 1). (B) Relationship between CERS6 expression and invasion status in human lung adenocarcinomas. Human lung adenocarcinomas with positive invasive growth (definite) as well as those with positive focal invasive growth and those with negligible invasive growth or without invasive growth (focal/none) were noted in 65 and 25 cases, respectively (9). P value, 2-tailed t test. (C) Kaplan-Meier analyses showing overall survival and relapse-free survival curves (9). The high and low groups were classified by CERS6 expression level relative to the median value. Cases lacking clinical information were omitted from the analysis. P value, log-rank test. (D) Box plot analysis showing mRNA expression levels of CERS6 in NSCLC and normal tissues (normal, 5 normal lung mixtures; NSCLC, 149 cases) (9). P values, 2-tailed t test. (B and D) In the box-and-whisker plots, horizontal bars indicate the medians, boxes indicate 25th to 75th percentiles, and whiskers indicate 1.5 times the interquartile range from the box. Dots outside of box plots represent outliers. (E) Representative images of CERS6 protein expression in lung squamous cell carcinoma (SCC), adenocarcinoma (AD), and normal lung tissue (Normal). NC, no primary antibody. Scale bar: 0.2 mm.
miR-101 is a direct target gene of CERS6. Given that microRNAs (miRNAs) are frequently deregulated in cancer (14), we used prediction algorithms to identify CERS6-targeting miRNAs to elucidate the mechanism of CERS6 expression. Among the top 6 miRNAs nominated, 5 were detected by real-time PCR analysis, leading us to postulate that they were expressed in all 79 lung cancer specimens examined (Supplemental Figure 3). As compared with normal tissues, lung cancer tissues exhibited a significantly lower expression of miR-101 (miR-101) and an obvious negative correlation with CERS6 (Figure 2A). Furthermore, except for the neuroblastoma cell line SHSY-5Y, the lung and other cancer cell lines showed similar or lower miR-101 expression levels as compared with those of the normal cell lines (Figure 2B). Other miRNAs did not show such patterns (Supplemental Figure 3B and Supplemental Figure 4).

Next, we examined whether CERS6 is directly targeted by miR-101. Knockdown of miR-101 by antisense RNA moderately increased CERS6 expression in the cell line BEAS-2B, which has high expression of miR-101 (miR-101-high) and low expression of CERS6 (CERS6-low) (Figure 2, B and C). In addition, reporter assays with luciferase constructs (Figure 2C) showed suppression of luciferase activity with the wild-type 3′ UTR, but not the mutant 3′ UTR, while suppression by the wild-type 3′ UTR was canceled by antisense miR-101 (Figure 2C). Furthermore, the miR-101-low and CERS6-high cell line NCI-H460-LNM35 (LNM35) consistently exhibited an inverse pattern, clearly demonstrating that miR-101 directly targets CERS6 mRNA.

CERS6 may promote lung cancer metastasis. To further investigate the role of CERS6 in lung cancer, LNM35 cells were treated with siRNAs as well as with shRNAs against CERS6. Tested RNAs markedly reduced migration activity in motility and scratch assays (Figure 3, A and B) as well as invasion activity (Supplemental Figure 5A). Conversely, migration activity increased when CERS6 was overexpressed in a lung cancer cell line that expresses a relatively low level of CERS6, RERF-LC-AI (Figure 3C). Similarly, knockdown resulted in decreased cell migratory activity.
activities in ACC-LC-319 and A549 cells, while overexpression resulted in increased cell migratory activities in SK-LC-5 cells (Supplemental Figure 5, B and C). Additionally, downregulation of CERS6 by miR-101 expression in LNM35 cells markedly attenuated cell motility (Figure 3D). Furthermore, LNM35 and A549 cells treated with shRNA against CERS6 (shCERS6) and siRNA against CERS6 (siCERS6) exhibited a markedly decreased metastatic potential in the mouse models (Figure 3, E and F, and Supplemental Figure 5D). Taken together, these results implicate CERS6 as a metastasis-promoting protein, while siCERS6 treatment of LNM35 or ACC-LC-319 cells showed only marginal or no effects on cell growth in vitro (Supplemental Figure 6). Therefore, the metastatic functions of CERS6, if any, may not simply be the result of increased proliferation or cell survival.

**CERS6 and ceramide-dependent lamellipodia formation.** CERS6 catalyzes C16:0 ceramide synthesis. Accordingly, CERS6 silencing reduced C16:0 ceramide and increased ceramides with longer acyl chains (Figure 4A). To examine whether ceramide synthesis is required for migration activity, we treated LNM35 cells with the serine palmitoyltransferase inhibitor myriocin, which suppressed migration activity (Figure 4B). Furthermore, the migration activity of siCERS6-treated LNM35 cells was partially recovered by
were serum stimulated, approximately 34% of cells were found to have RAC1-positive lamellipodia/ruffling structures, a frequency which was reduced by approximately one-third by CERS6 knock-down (Figure 4, D and E). Reciprocally, in RERF-LC-AI cells, CERS6 overexpression stimulated RAC1-positive lamellipodia formation/membrane ruffling (Figure 4, F and G). Lamellipodia formation/membrane ruffling may be dependent on C16:0 ceramide, addition of C16:0 ceramide (Figure 4C). These results suggest that CERS6 stimulates migration activity via C16:0 ceramide.

To date, ceramides have been implicated in lamellipodia formation/membrane ruffling by activating the atypical PKC, PKCζ, and the associated complex formation with RAC1 (reviewed in ref. 15). Accordingly, a PKCζ pseudosubstrate inhibited the migration activity (Supplemental Figure 7A). Furthermore, when LNM35 cells were serum stimulated, approximately 34% of cells were found to have RAC1-positive lamellipodia/ruffling structures, a frequency which was reduced by approximately one-third by CERS6 knock-down (Figure 4, D and E). Reciprocally, in RERF-LC-AI cells, CERS6 overexpression stimulated RAC1-positive lamellipodia formation/membrane ruffling (Figure 4, F and G). Lamellipodia formation/membrane ruffling may be dependent on C16:0 ceramide,
because supplemented ceramide rescued siCERS6-induced suppression (Figure 5, A and B). In line with previous reports (15–18), at the lamellipodia/ruffling region, PKCζ and its phosphorylated form, as well as ceramides, were found to be colocalized (Figure 4D; Figure 5, A and C; and Supplemental Figure 7B). These results strongly suggest that CERS6 promotes cell migration through formation of a RAC1-positive lamellipodia/ruffling structure.

**L-α-dimyristoylphosphatidylcholine liposome induces cancer-specific apoptosis.** Ceramides function as intracellular mediators of apoptosis (3); thus, CERS6 overexpression may sensitize cells to anticancer drug treatments. However, most of these drugs have intrinsic cytotoxic effects. For example, cisplatin induces elevation of cellular ceramide concentration via modification of membrane structures (19, 20), while it also targets chromosomes and causes DNA damage. Combined targeting of lipid membranes and specific CERS6 overexpression may represent a highly selective strategy to treat lung cancer.

Toward this end, we used L-α-dimyristoylphosphatidylcholine (DMPC) liposome (21, 22), which is composed of precursor molecules of the CERS6 substrate. The fibroblast lines FB227 and TIG112 and immortalized epithelial cell line BEAS-2B remained unaffected (Figure 6A and Supplemental Figure 8A), whereas most of the lung cancer cells died within 48 hours after treatment with 200 μM DMPC liposome (Supplemental Figure 8A). The CERS6-high cell lines LNM35, A549, and ACC-LC-319 were more sensitive to DMPC liposome treatment than the CERS6-low and -moderate cell lines SK-LC-5 and RERF-LC-AI, respectively. That effect was considered to be not dependent on cell proliferation rate (Figure 6A). Treatment of LNM35 cells with myriocin or the CERS family inhibitor fumonisin B1 (FB1) suppressed DMPC liposome–induced apoptosis (Figure 6B). Furthermore, a 2- to 3-fold increase in C16:0 ceramide was detected in LNM35 cells but not in TIG112 cells (Figure 6C and Supplemental Figure 8B), suggesting that ceramide accumulation is crucial for DMPC liposome–dependent apoptosis.

Given that C16:0 ceramide is synthesized de novo by CERS5 and/or CERS6 (13), we next determined which CERS is required for DMPC liposome–induced apoptosis. While CERS6 knockdown as...
Figure 6. DMPC liposome induces CERS6-dependent apoptosis. (A) MTT assays were performed for determining the viability of a panel of cell lines after treatment with 100 μM DMPC liposome (mean ± SD; \( n = 4–8 \)). In the presence of 100 μM DMPC liposome, cell viability was plotted against cell doubling time. (B) Effects of 100 nM myriocin, 50 μM FB1, or control vehicle on LNM35 cells treated with 100 μM DMPC liposome (mean ± SD). (C) Quantification of ceramides in LNM35 cells treated with 200 μM DMPC liposome for 8 hours (mean ± SD; \( n = 3 \)). The experiments were replicated, with similar results obtained. (D) Effects of CERS6 silencing by siCERS6-1 in LNM35 cells and of CERS6 overexpression in BEAS-2B cells (mean ± SD; \( n = 3 \)). Cells were treated with 100 μM DMPC liposome. The experiments were replicated, with similar results obtained. (E) Effects of siCERS6-1 and/or 200 μM DMPC liposome on ceramide composition in LNM35 cells (mean ± SD; \( n = 3 \)). The experiments were replicated, with similar results obtained. (F) LNM35 cells were incubated with 200 μM NBDPC containing liposome for 60 minutes and 4 μM ER-Tracker Red Dye for 30 minutes. Scale bar: 10 μm. The experiments were replicated, with similar results obtained. (G) Illustration of hypothetical pathway between deuterated DMPC and C16:0 ceramide. The relative amounts of deuterated or nondeuterated acyl and sphingoid chains in C16:0 ceramide were quantified in the presence or absence of deuterated DMPC using LC/MS/MS analysis (mean ± SD).
analyzed cellular ceramide composition using a liquid chromatography–tandem mass spectrometry (LC/MS/MS). As shown in Figure 6G, both the acyl and sphingoid chains were markedly deuterated in DMPC liposome–treated cells. These results showed that DMPC liposome is converted, at least in part, into C16:0 ceramide to induce apoptosis.

CERS6 is a key regulator of DMPC liposome–induced apoptosis. The above data suggest that CERS6 and the ceramide synthesis pathway may be used for cancer therapy. In order to extend current findings, and to construct better therapeutic models, we further explored the potential synergistic cytotoxicity that occurs when DMPC liposome is combined with ceramide pathway inhibitors (Supplemental Figure 11A). Among the candidates tested, the glucosylceramide synthase inhibitor d-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) clearly showed a synergistic effect on LNM35 cells, but not TIG112 fibroblast cells, as determined by isobologram analysis (Figure 7, A and B). Synergistic effects were also found with the D-PDMP derivatives D-PMMP and D-PPMP (ref. 24 and Supplemental Figure 11, B and C), with similar results observed with the other tested cell lines (Supplemental Figure 11D), while other inhibitors showed no obvious effects (Supplemental Figures 12 and 13). To clarify whether CERS6 is required for apoptosis induction, we knocked down CERS6 and found that the apoptotic phenotype was rescued (Figure 7C). Mass spectrometry analysis further revealed that the combined treatment increased cellular C16:0 ceramide to a greater extent than DMPC liposome or D-PDMP alone (Figure 7D).

DMPC liposome induced apoptosis and tumor regression in mice. In order to know whether our approach induces apoptosis in vivo, we implanted LNM35 cells in mice and treated them with DMPC liposome and/or D-PDMP. Importantly, that treatment significantly decreased tumor weight (Figure 8, A–C), irrespective of the timing of drug administration (Figure 8D). Pathological findings showed that tumor development was associated with an increased frequency of apoptotic areas as well as decreased Ki-67–positive cells (Figure 8, C, E, and F), while mouse body weights were largely unaffected (Supplemental Figure 14). Col-
in patients with lung cancer. Recent studies have noted that miR-101 expression is downregulated in cancer by genetic deletion (27, 28). Additionally, miR-101 has been shown to inhibit cell proliferation and invasion of tumor cells (28), and its knockdown led to radiosensitization of NSCLC cells (29). Our findings are consistent with those of these studies and also suggest what we believe is a novel mechanism, whereby miR-101 exerts tumor-suppressive functions via CERS6 suppression (Figure 9).

In addition, our study provides evidence that CERS6 promotes RAC1-positive lamellipodia formation/membrane ruffling (Figure 4, D–G, and Figure 5). Since cell migration driven by RAC1-positive lamellipodia formation/membrane ruffling is a critical step in tumor invasion and metastasis (reviewed in ref. 15), we speculated that CERS6-high cancer cells show a phenotype via the activity of C16:0 ceramides to activate the local PKCζ/RAC1 complex. However, other possibilities cannot be denied, such as the contribution of downstream effector sphingolipids to the phenotype. We also note that CERS6 somewhat inhibited migration in some cell lines, suggesting the existence of alternative pathways that have effects on cancer cell phenotypes (30).

An approach to apply these findings is the development of low-molecular-weight compounds that inhibit CERS6 and, thereby, metastasis. In this study, we took an approach to target the miR-101/CERS6 pathway and disrupt ceramide homeostasis, which consequently induced cancer-specific apoptosis (Figure 9).

Discussion

Altered expressions of ceramides and CERS family proteins have been reported in malignant tumors (6, 7, 25, 26), though their functions leading to tumor formation and/or progression remain poorly understood. In the present study, we showed that the miR-101/CERS6 pathway found in cancer cells is closely correlated with invasion- and/or metastasis-prone phenotypes...
We used a DMPC liposome that induces cancer cell apoptosis (21, 22). DMPC liposomes fuse and accumulate in tumor cell membranes, after which their apoptosis signals pass through caspase-9, -3, and -8 (22). However, the signals that initially activate these apoptotic pathways have yet to be elucidated. We hypothesized that cellular DMPC uptake may force synthesis of the downstream molecule of C14:0 acyl-CoA, which is further elongated into C16:0 acyl-CoA and used for C16:0 ceramide synthesis by the sequential actions of enzymes, including serine palmitoyl transferase and CERS6 (Figure 6G). Our results showed that the DMPC liposome was converted, at least in part, into C16:0 ceramide to induce apoptosis. Interestingly, the DMPC-derived fatty acid was incorporated into a fatty acid of C16:0 ceramide more efficiently than sphingosine of C16:0 ceramide, which was in accordance with our findings showing that the expression level of the acyl chain transferase CERS6, but not of the sphingoid chain transferase subunits SPTLC1 or SPTLC2, is significantly associated with prognosis (Supplemental Table 1).

In order to develop a promising synthetic lethal strategy for taking advantage of CERS6 overexpression, we screened for inhibitors that induce cancer apoptosis in a synergistic manner with DMPC. Among the ceramide catabolic pathway inhibitors, we examined D-PDMP, which inhibits GlcCer synthesis uncompetitively with UDP-glucose and by mixed competition with ceramide (31). Interestingly, other compounds did not show such synergism (Figure 7 and Supplemental Figures 11–13). Generally, the sphingomyelin pathway plays a major role to maintain apoptosis (21, 22). DMPC liposome induces cancer cell-specific killing. Moreover, targeting the miR-101/CERS6 pathway is advantageous, because normal cells do not show obvious CERS6 expression (Figure 1, D and E), thus demonstrating resistance to apoptosis (Figure 7A and Figure 8A). Therefore, induction of synthetic lethality with CERS6 overexpression may become a viable therapeutic option in NSCLC in the future.

**Methods**

**Cell lines and materials.** The LNM35 and ACC-LC-176 cell lines have been described previously (34). Cells were maintained in RPMI 1640 supplemented with 10% FBS (Gibco), while the immortalized lung epithelial cell line BEAS-2B was cultured as previously described (35). All were tested and confirmed to be free from mycoplasma contamination. Anti-CERS6 antibodies were purchased from Abnova (clone 5H7); anti-α-tubulin was purchased from Sigma-Aldrich; anti-RAC1 was purchased from Millipore (clone 23A8); anti-PKCζ and anti-p-PKCζ were purchased from Santa Cruz (SC216 and SC12894-R); anti-ceramide was purchased from GlycoTech (S58-9); and anti-mouse IgG and anti-rabbit IgG conjugated with HRP were purchased from Cell Signaling Technology. Anti-mouse IgG and anti-rabbit IgG conjugated with Alexa 488 or 568 were from Invitrogen. DMPC liposome refers to a hybrid liposome consisting of 90 mol% DMPC (NOF) and 10 mol% polyoxyethylene(n) dodecyl ether [C12(EO)20 or C12(EO)25] (Sigma-Aldrich) in a 5% glucose solution (21, 36). NBPC containing liposome and D-PDMP derivatives has been described previously (37, 38). It has been reported that DMPC liposome and D-PDMP do not show any obvious toxicity in terms of weights and blood examination tests (39, 40). Other inhibitors were purchased, including D-MAPP (Matreya LLC), NVP-231 (Merck), D609 (Santa Cruz Biotech), Sphingosine Kinase Inhibitor (Calbiochem), FB3 (Cayman Chemical), and myriocin (Sigma-Aldrich). Sequences of the oligonucleotide primers used for PCR and sequencing details are provided in Supplemental Table 3.

**Oligonucleotides and transfection.** LNM35 cells were transfected with 1 nM of the Pre-miRNA Precursor Molecule of miR-101 and Negative Control #2 (AM17111) (both from Applied Biosystems), a 10-nM siRNA duplex (Sigma-Aldrich) targeting CERS6 (siCERS6-1, -2, or -3), CERS5 (siCERS5-1 or -2), and the corresponding concentrations of negative control (Mission siRNA Universal Negative Control #2 [SICO02], Sigma-Aldrich) using Lipofectamine RNAiMax (Invitrogen). BEAS-2B cells were transfected with 20 nM locked nucleic acid (LNA) antisense oligonucleotide (Ambion) against miR-101 using Lipofectamine RNAiMax (Invitrogen). miR-20a scramble LNA (41) was used as the control oligonucleotide.

**Plasmid construction and isolation of stable clones.** BEAS-2B cells were transfected with pcDNA3-HA-CERS6 (13) and then selected with 1 mM G418 to establish bulk stable clones. For RERF-LC-AI and SK-LC-5 cells, the CERS6 open reading frame was cloned into the pLenti 7.3/V5-DEST Gateway vector (Invitrogen). Two days of thal dose inhibited migration activity. This suppression effect may have been induced via a pathway independent of ceramide accumulation, which should be examined in a future study.

We believe that the strategy outlined in the present study for anticancer action is novel and quite distinct from conventional molecular targeting, which is usually aimed at inhibition of survival kinases (32, 33), while CERS6 activity is not inhibited but rather used to induce cancer cell–specific killing. Moreover, targeting the miR-101/CERS6 pathway is advantageous, because normal cells do not show obvious CERS6 expression (Figure 1, D and E), thus demonstrating resistance to apoptosis (Figure 7A and Figure 8A). Therefore, induction of synthetic lethality with CERS6 overexpression may become a viable therapeutic option in NSCLC in the future.

Figure 9. Illustration of relationship of miR-101 and CERS6. In cancer cells, miR-101 downregulation leads to CERS6 upregulation and facilitates cell metastasis. CERS6 overexpression may be used for induction of cancer-specific apoptosis.
after lentiviral infection, green fluorescent protein-positive cells were isolated using FACS FACS/ria II (BD). In some experiments, 2 independent shRNAs against CERS6 (shCERS6-2 and shCERS6-3, SuppTable 3) were used.

*In vitro motility and scratch and invasion assays.* In vitro motility and invasion assays were performed using Cell Culture Inserts (Transparent PET Membrane, 24-well plate, 8-μm pore size, BD Falcon). After culturing cells in RPMI supplemented with 5% FBS overnight, the medium was replaced with RPMI supplemented with N2 Supplement (GIBCO-BRL) and 20 ng/ml EGF to minimize ceramide uptake from the medium. Two days later, cells were collected using 5 mM EDTA in PBS and then resuspended in RPMI supplemented with 0.1% FBS, seeded at a cell density of 1 × 10^5 into the upper chambers, and incubated for 16 to 24 hours. For the scratch assay, LNM35 cells were plated in 6-well plates and a single linear wound was created with a 200-μl pipette tip.

**Immunofluorescence and immunohistochemistry.** After cells were cultured in RPMI supplemented with 5% FBS overnight, the medium was replaced with RPMI containing N2 Supplement (GIBCO-BRL). Two days later, the medium was replaced with RPMI supplemented with 5% FBS and left for 12 to 16 hours. Cells were fixed and stained as previously described (42, 43). For immunohistochemistry, after antigen retrieval following microwave oven heating treatment, formalin-fixed paraffin sections were subjected to immunoperoxidase assays using an avidin-biotin peroxidase complex method.

**Cell viability assays.** Cells were plated at a density of 2 × 10^4 cells/ml and cultured overnight. After treatment with siRNAs, cells were further cultured in RPMI supplemented with N2 and 20 ng/ml EGF for 48 hours, followed by incubation in RPMI medium supplemented with 10% FBS containing various drug concentrations for 48 hours. Viable cells were measured in triplicate using TetraColor One (Seikagaku), with reference to the viability of mock-treated cells.

**Dual-luciferase reporter assay.** The 520-bp region of the CERS6 3′UTR segment was amplified by PCR using primers with the Xba1/Apa1 site (SuppTable 3) and ligated into a pGL3 vector (Promega). Using a site-directed mutagenesis method, mutations were introduced to the putative miR-101–binding site (SuppTable 3). Cell cultures, transfection, and luciferase reporter assays were performed as previously described (44).

**Mouse models.** Experimental metastasis assays were performed using cancer cell lines. A volume of 3 × 10^5 or 4 × 10^5 LNM35 cells or 1 × 10^5 A549 cells in 0.2 ml of RPMI-1640 medium was injected into the tail veins of 6-week-old male nude mice (n = 8–14). At 5 to 6 weeks (LNM35) or 3 weeks (A549) after injection, the mice were euthanized and lung metastasis foci were assessed, though mice in poor condition were euthanized earlier. As a therapeutic model, 1 day after subcutaneous implantation of 1 × 10^5 LNM35 cells in NOD/SCID mice, 200 μl each of 50 mM DMPC liposome and/or 1 mM D-PDMP was locally injected daily for 10 consecutive days. Thereafter, the mice were euthanized and tumor weights were measured. Mice that died of unrelated causes were excluded from analyses. In some experiments, drug administration was started 7 days after LNM35 implantation at a volume of 1 × 10^5 cells in NOD/SCID mice for 9 consecutive days, with 50 mM DMPC liposome and/or 2 mM D-PDMP. Tumor mass was estimated by using a formula, L × W^2 (where L stands for length and W stands for width).

**Mass spectrometric analyses.** After extracting the lipid fraction from cells by the Bligh-Dyer extraction method, ceramide analyses were performed using ion trap/time-of-flight mass spectrometry coupled with high-performance liquid chromatography (LCMS-IT-TOF), with a 320 LC/MS/MS triple-quadrupole mass spectrometer (Agilent Technologies) or with Acquity UltraPerformance LC (Waters) and 4000 QTRAP LC/MS/MS (ABSciex) devices. Mass spectrometry was performed with an electrospray ionization source in positive ion mode. d18:1-C17:0 ceramide (Avanti Polar Lipids) was added prior to lipid extraction as an internal standard. For LCMS-IT-TOF and LC/MS/MS analyses, chromatographic separations were done in gradient mode using conventional ODS columns (Cadenza CD-C18, 2.0 i.d. × 100 mm, 5-μm particle size, and Cadenza CW-C18, 150 × 2 mm; Imtakt Corp.), respectively (45). Analysis of heterogeneously deuterated C16:0 ceramide was performed in MRM mode, in which expected precursor-product pairs were monitored during LC elution. The precursor-product pairs are shown in Supplemental Table 4.

**Statistics.** Statistics used in the experiments are indicated in figure legends. In brief, P values lower than 0.05 are considered significant. Two-tailed t and Fischer’s exact tests are used as indicated. Log-rank test are used for Kaplan-Meier analyses.

**Study approval.** Prior to obtaining patient samples, requisite approval from the review board of Nagoya University Graduate School of Medicine and written informed consent from the patients were obtained. The mouse experiments were also approved by the review board of Nagoya University Graduate School of Medicine.

**Author contributions**

MS, TM, and T Takahashi designed research studies. MS, KC, SK, YK, NM, KT, CA, MCT, K Yanagisawa, NT, TS, SI, SF, AS, KTK, MN, and MK conducted experiments and acquired and analyzed data. Y Mizutani, YI, JI, Y Matsumoto, and RU provided reagents. NU, T Taniguchi, TF, K Yoko, KW, and YH coordinated and provided clinical samples. MS, KC, TM, and T Takahashi wrote the manuscript.

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Address correspondence to: Motoshi Suzuki, Division of Molecular Carcinogenesis, Nagoya University Graduate School of Medicine, Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Phone: 81.52.744.2456; E-mail: msuzuki@med.nagoya-u.ac.jp.

Ke Cao’s present address is: Department of Oncology, The Third Xiangya Hospital of Central South University, Hunan, China.


