Hypomethylation-Linked Activation of PLCE1 Impedes Autophagy and Promotes Tumorigenesis through MDM2-Mediated Ubiquitination and Destabilization of p53

Yunzhao Chen1,2, Huahua Xin1, Hao Peng1, Qi Shi1, Menglu Li1, Jie Yu2, Yanxia Tian1, Xueping Han1, Xi Chen1, Yi Zheng3, Jun Li4, Zhihao Yang1, Lan Yang1, Jianming Hu1, Xuan Huang5, Zheng Liu5, Xiaoxi Huang5, Hong Zhou6, Xiaobin Cui1, and Feng Li1,5

ABSTRACT

Esophageal squamous cell carcinoma (ESCC) is one of the deadliest malignant diseases. Multiple studies with large clinic-based cohorts have revealed that variations of phospholipase C epsilon 1 (PLCE1) correlate with esophageal cancer susceptibility. However, the causative role of PLCE1 in ESCC has remained elusive. Here, we observed that hypomethylation-mediated upregulation of PLCE1 expression was implicated in esophageal carcinogenesis and poor prognosis in ESCC cohorts. PLCE1 inhibited cell autophagy and suppressed the protein expression of p53 and various p53-targeted genes in ESCC. Moreover, PLCE1 decreased the half-life of p53 and promoted p53 ubiquitination, whereas it increased the half-life of mouse double minute 2 homolog (MDM2) and inhibited its ubiquitination, leading to MDM2 stabilization. Mechanistically, the function of PLCE1 correlated with its direct binding to both p53 and MDM2, which promoted MDM2-dependent ubiquitination of p53 and subsequent degradation in vitro. Consequently, knockdown of PLCE1 combined with transfection of a recombinant adenoviral vector encoding wild-type p53 resulted in significantly increased levels of autophagy and apoptosis of esophageal cancer in vivo. Clinically, the upregulation of PLCE1 and mutant p53 protein predicted poor overall survival of patients with ESCC, and PLCE1 was positively correlated with p53 in ESCC cohorts. Collectively, this work identified an essential role for PLCE1- and MDM2-mediated ubiquitination and degradation of p53 in inhibiting ESCC autophagy and indicates that targeting the PLCE1–MDM2–p53 axis may provide a novel therapeutic approach for ESCC.

Significance: These findings identify hypomethylation-mediated activation of PLCE1 as a potential oncogene that blocks cellular autophagy of esophageal carcinoma by facilitating the MDM2-dependent ubiquitination of p53 and subsequent degradation.

Graphical Abstract: http://cancerres.aacrjournals.org/content/canres/80/11/2175/F1.large.jpg.

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common cancers worldwide, with mortality ranking sixth and morbidity ranking seventh in China (1). Despite improvements in ESCC treatments, the outcomes of patients with ESCC are still dismal due to the limited knowledge about its molecular pathogenesis, the difficulty in detecting the disease at its early stages, and the lack of effective therapies (2, 3). Although studies have indicated that some oncogenes and tumor suppressor genes are involved in the progression of esophageal cancer, the molecular mechanisms remain unclear. Autophagy, or...
cellular self-digestion, is a regulated bulk degradation process in cells in which the degradation of damaged proteins and organelles is regulated by autophagy-related genes (4, 5). Several studies have indicated that esophageal cancer cells display inhibitory autophagy, and autophagy has been implicated in the resistance to cancer therapies (6, 7). Hence, it is of great clinical value to determine the mechanisms by which genes involved in ESCC tumorigenesis regulate the process of autophagy and influence the progression of esophageal cancer.

Recently, phospholipase C epsilon 1 (PLCE1) at chromosome 10q23 was shown to have a consistently strong association with ESCC risk in three independent genome-wide association studies in Chinese Han populations (8–10). PLCE1 is a member of the phospholipase C family (PLC) that converts polyphosphoinositides such as phosphatidylinositol 4,5-bisphosphate, generates secondary messengers, including 1,4,5 triphosphate (IP3) and diacylglycerol, regulates protein kinase C activity and calcium mobilization, and is physiologically involved in carcinogenesis and cancer progression (11). Accumulated evidence has shown that PLCE1 plays crucial roles in several cancer types, such as head and neck, bladder, gastric, skin, and prostate cancers (12). Although our previous investigation also showed that PLCE1 might play an important role in the oncogenesis and progression of ESCC (13, 14), the definitive mechanisms underlying how PLCE1 regulates autophagy in cancer has remained unknown.

Once autophagy is induced, the subcellular membrane structures undergo dynamic morphologic changes that lead to the degradation of cytosolic proteins and organelles by lysosomes where autophagic cargo undergoes protease-dependent degradation (15). Recent studies have suggested that autophagy dysfunction plays a major role in the development of cancer (16). P53 is a sequence-specific DNA-binding transcription factor that regulates many genes, including autophagy-related genes, such as IFN-stimulated 20 kDa exonuclease-like 1 (Ig20L1), unc-51-like autophagy activating kinase (ira1), and autophagy-related 7 (atg7; refs. 17, 18). The functional significance of p53-induced autophagy has been revealed by its stimulation of apoptosis in response to DNA damage and suppression of transformation by adenovirus early region 1A and the H-ras oncogene (17). Although two preliminary studies indicated that PLCE1 can suppress p53 expression in both lung and esophageal cancers (19, 20), the post-transcriptional mechanisms by which PLCE1 regulates p53 and autophagy have not been reported. Therefore, further research must be conducted to elucidate the specific mechanisms by which PLCE1 and p53 effects autophagy in ESCC.

Here, by performing phenotypic and mechanistic investigations, we elucidated the essential role of hypomethylation-mediated activation of PLCE1-regulated phenotypes in controlling ESCC autophagy and apoptosis through its interplay with mouse double minute 2 homolog (MDM2) and p53, and revealed that PLCE1 stabilizes MDM2 by inhibiting MDM2 polyubiquitination and proteasome-mediated degradation to promote p53 ubiquitination and destabilization through the MDM2-mediated proteasome pathway. The results of this study reveal an important role for PLCE1 in autophagy and tumorigenesis and provide a theoretical basis for new molecular therapies for ESCC.

Materials and Methods

Cell lines and cell culture

The human ESCC cell lines (Eca109, EC9706, TE-1, and Kyse-150) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM or RPMI1640 (Gibco) supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37°C under 5% CO2. All cell lines were obtained more than 1 year prior to experiments and were propagated for less than 6 months after thawing. Cellular experiments were performed within low passage (<10) number after thawing. All cell lines were authenticated by short tandem repeat profiling. Mycoplasma test was performed monthly according to PCR Mycoplasma Detection Kit (ABM Inc.).

Antibodies and reagents

Antibodies used against the following proteins were: PLCE1 (Santa Cruz Biotechnology-sc-28404, 1:200 for Western blotting), PLCE1 (Sigma-Aldrich-HPA015598, 1:50 for IHC and immunofluorescence), Ub (Abcam-ab7780, 1:1,000 for Western blotting), p53 (Abcam-ab1101, 1:250 for WB, 1:400 for IHC), p21 (Beyotime-AB021, 1:1,000 for Western blotting), PUMA (Beyotime-AF0270, 1:500 for Western blotting), Beclin-1 (Abcam-ab51031, 1:200 for IHC, 1:1,000 for Western blotting), LC3A/B (Abcam-ab128025, 1:100 for IHC, 1:600 for Western blotting), MDM2 (Abcam-ab3110, 1:1,000 for Western blotting), PIRH2 (Boster-BM2227, 1:200 for Western blotting), ARFBP1 (Bioss-bs-12512R, 1:150 for Western blotting), NIFR (Bios-bs-6389R, 1:200 for Western blotting), Bax (Abcam-Ab32503, 1:1,000 for Western blotting), caspase 3 (Beyotime-AO333, 1:250 for Western blotting), caspase 7 (Boster-BA0088-1, 1:1,000 for Western blotting), cleaved PARP (Abcam-Ab32561, 1:3,000 for Western blotting), Bel-2 (Beyotime-AB112, 1:1,000 for Western blotting), Ki-67 (ZSGB-ZA6050, 1:200 for IHC and immunofluorescence), GAPDH (ZSGB-OT12D9, 1:600 for Western blotting), and β-actin (Solarbio-RG000120, 1:1,000 for Western blotting). Nutlin-3 was from Sigma-Aldrich (N6287). U73122 (112648-68-7) was purchased from MedChem Express. shR-PLCE1, control lentivirus, and transfection reagents were from Shanghai GeneChem Co. 3-methyladenine (3-M) was purchased from Sigma-Aldrich (m9281).

Transfections

Expression plasmids for short hairpin RNA (shRNA)-targeting PLCE1 were made in a pGLVH1/GFP-Puro vector (Shanghai GenePharma Co., Ltd). The targeting sequence was PLCE1 shRNA 5’-CAGGTCTTGGCCAGTGCAGTA-3’, and the negative control shRNA (sh-ctrl) sequence was 5’-TTCCTCGAAGGTTGCGACGT-3’. Stable shRNA transfectants were selected in medium containing 1 μg/mL puromycin (Sangon Biotech). Knockdown efficiency of shPLCE1 was validated by qRT-PCR (Supplementary Fig. S1A). The PLCE1 overexpressed lentiviral vector and relative negative scramble control were synthesized by Cyagen Biosciences. PLCE1 was fused with Puromycin by T2a and cloned into the Asci/BamHI site of a LV-EFS-MCS backbone. The lentiviral vector was packaged using pCD/NL-DDD packaging plasmid mix (Addgene) and transiently cotransfected into 293T cells to generate recombinant virus particles. Two siRNAs targeting MDM2 and p53 were purchased from Sangon Biotech Co., Ltd. The sense and antisense sequences of si-p53 areCUACUUCUGCAGAAAAAAGGdTdT and CGUUGUUUUCAG-GAAUGAgdTdT. The sense and antisense sequences of si-MDM2 are GUCUCCGAACAGGAGCC and GGGUCUCUUUGUC-GAAAG. Recombinant adeno viral p53 and Ad-LacZ as a vector control were purchased from Shanghai TranSheepBio Co., Ltd. In most experiments, cells were treated with sh-PLCE1 at a multiplicity of infection (MOI) of 15, MDM2-siRNA at 70 nmol/L, rad-p53 at an MOI of 25. Cell transfections were performed with polybrene (GeneChem, REVG0001) and enhanced infection solution (ENiLS; GeneChem, REVG0002). HiPerfect transfection reagent (Qiagen, 301705), or Lipofectamine 2000 reagent (Invitrogen, 11668-027) by following the manufacturer’s instructions.

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U73122 was also used to inhibit PLCE1, and our results indicated that U73122 exerted time-dependent and dose-dependent inhibition effects on PLCE1 in human esophageal cancer cells (Supplementary Fig. S1B). Subsequently, we used the U73122 with the concentration of 10 μmol/L for 48 hours in follow-up experiments. 3-MA was used to inhibit the autophagy level in cells. The stock solution of 3-MA was diluted with sterile PBS to a concentration of 40 mmol/L, for storage, aliquoted, and stored at −20°C. The concentration for transfection was 5 mmol/L.

Quantitative analysis of PLCE1 promoter DNA methylation by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry

In this assay, 145 samples, including 99 ESCC and 46 normal tissue samples from a Kazakh population were used for PLCE1 methylation detection. The Kazakhs, a major minority in the Xinjiang region living a traditional lifestyle, have a higher incidence and mortality of ESCC than other ethnic populations in Xinjiang (21). The patient ages were 60.44 ± 10.48 (mean ± SD) years for the cancer samples and 61.77 ± 8.18 years for the normal samples (P = 0.73). A total of 62 (62.6%) males and 37 (37.4%) females were selected for the case group and 26 (56.5%) males and 20 (43.5%) females were selected for the control group (P = 0.89). Primer sets for methylation analysis of the PLCE1 promoter were designed using EpiDesigner (http://epidesigner.com) as follows: tag-For: 5'-aggagaggaggGGAATATGGAGTTATGGAAGTTTTTT-3'; T7-Rev: 5'-cagtaatacgactcactatagggagaaggctATTAAA-CCCCATCAATATCCCAAC-3'. Matrix-assisted laser desorption/ionization—time-of-flight mass spectrometry (MALDI-TOF MS) was performed as described previously (13) to detect the methylation values of all CpGs units within the PLCE1 promoter.

Methylation-specific PCR

In this assay, 51 ESCC tissues and 51 normal esophageal tissues (NET) were used. Following the manufacturer's instructions, a DNA kit was utilized to obtain genomic DNA in prostate tissues and cell lines, and the bisulfite modification assay was performed by an EZ DNA Methylation-Gold Kit (ZYMO Research Co). To detect methylation of the PLCE1 gene in ESCC tissues and cell lines, methylated primers and unmethylated primers were used to amplify the modified DNA. The methylated primers included forward primer: 5'-TTAGGAGTTAAGGAGGAAGGC-3' and reverse primer: 5'-AACTC- AGACTACCTCCCAAGACCAAG-3'. The unmethylated primers consisted of forward primer: 5'-TTAGGAGTTAAGGAGGAAGGC-3' and reverse primer: 5'-AAATCAAACACTCCCTTAAACAAA-3'. The amplification system used in this experiment contained 20 μL, consisting of 10 μL of Taq PCR MasterMix, 7 μL of nuclease-free water, 1 μL of each upstream and downstream primer, and 1 μL of modified DNA. The amplification conditions involved in this reaction are as follows: 95°C for 5 minutes; 38 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 5 minutes, and 72°C for 7 minutes. The amplified product was separated on a 3% agarose gel and visualized under UV illumination.

IHC analysis

Surgically resected primary ESCC tissues from 147 patients from Xinjiang, China, were collected for this study. The 147 formalin-fixed esophageal cancer tissues and 90 normal tissues were collected between 1998 and 2015 at the First Affiliated Hospital of Shihezi University (Xinjiang, China). Our research protocol was approved by the Medical Ethics and Human Clinical Trial Committee of the Shihezi University School of Medicine, and all recruited subjects were enrolled with written informed consent. All of the operative specimens were used as residual specimens after diagnostic sampling. The deadline for follow-up for all patients with ESCC was December 2016, which was 1–10 years after radical resection. None of the patients received chemotherapy or radiotherapy treatment before surgery, and patients who survived less than 1 month after surgery were excluded. IHC staining was performed as described previously (13).

RNA extraction and qRT-PCR

RNA was extracted using an RNA Extraction Kit (Qiagen) according to the standard protocol and quantified by a NanoDrop ND-2000 (NanoDrop Technologies). A One Step PrimeScript mRNA cDNA Synthesis Kit (Qiagen) was then used for cDNA generation. For qPCR analysis, the miScript SYBR Green PCR Kit (Qiagen) was used. The target genes were amplified in an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The experiments were replicated three times in two independent experiments. The sequence of primers used was as follows: human GAPDH sense 5'-GAGTCAACGGATT-TGTTTG-3', and antisense 5'-GACAAGCTCCGTTCTCA-3', human PLCE1 sense 5'-CTCTGGCAATACAGCATCAACG-3', and antisense 5'-GTCTTGAGGATCGAAGACACCTC-3'.

In vivo xenograft mouse model

Athymic nude mice (BALB/c, female, 4–6 weeks old, weight 15–22 g) were purchased from the Institute of Experimental Animals, Chinese Academy of Medical Sciences (Beijing, China). Animals were maintained under "specific pathogen-free" conditions, and all of the animal studies were conducted according to the guidelines approved by the University of Shihezi Institutional Animal Care and Use Committee. Eca109 cells were steadily infected with LV-R-PLCE1-RNAi (sh-PLCE1) and negative scramble control CON053 (sh-sc) lentiviral plasmids after selection with polybrene. Then the cells were injected subcutaneously into the left axillary areas of each mouse. When the diameter of the tumor was 0.2–0.4 cm, recombinant human adenosine p53 (rAd-p53) was injected into the tumor. The animals were sacrificed and pictured on day 31, and tumors were detected by an IVIS Imaging System (Caliper Life Sciences). All the tumor tissues were evaluated by IHC staining, Western blot analysis, and immunofluorescence. All animal studies were approved by the Animal Experimental Ethical Inspection of First Affiliated Hospital, Shihezi University, Xinjiang, China.

Statistical analysis

Data were assessed using the SPSS (version 17.0) statistical software package (SPSS Inc.). R programming language and GraphPad Prism 5.0 (GraphPad Software Inc.) were used to visualize the data. A Mann–Whitney U test was used to compare methylation between groups. A bivariate correlation was used to evaluate correlations. Survival curves were plotted using the Kaplan–Meier method and compared with a log-rank test. Differences were statistically significant at P < 0.05 (two-sided). All data are presented as means ± SD. Additional methods can be found in the Supplementary Materials and Methods.

Results

Knockdown of PLCE1 inhibits cell proliferation and induces apoptosis

We suppressed PLCE1 expression by using shRNA or U73122 in ESCC cell lines Eca109 and EC9706. Knockdown efficiency of shPLCE1 was validated by qRT-PCR (Supplementary Fig. S1A) and...
U73122 exerted time-dependent and dose-dependent inhibition effects on PLCE1 in human esophageal cancer cells (Supplementary Fig. S1B). The results of CCK-8 cell viability assays, colony formation assays, and the EdU proliferation showed that PLCE1 knockdown cells were markedly inhibited (Fig. 1A–C; Supplementary Fig. S2A). In addition, we also performed rescue experiments by transfecting sh-resistant PLCE1 in the depleted cells. We used an overexpressing lentiviral vector to transfect PLCE1 into ESCC cell line TE-1. Results showed that overexpression of PLCE1 partially rescued the negative effect of shPLCE1 on cell proliferation (Supplementary Fig. S2B). Accordingly, PLCE1 knockdown significantly enhanced the percentage of cells in the G2/M-phase (Supplementary Fig. S2C). In addition, flow cytometry analyses showed that the inhibition of PLCE1 by shPLCE1 increased the number of apoptotic cells compared with their respective controls (Fig. 1D; Supplementary Fig. S2D). Compared with shPLCE1 treatment alone, apoptosis in cells treated with shPLCE1 and PLCE1 lentiviral vector was lower (Fig. 1E). Consistent with prior reports, the red-to-green fluorescence ratio increased by sh-PLCE1 or the PLC inhibitor U73122 (Fig. 1F), indicating that PLCE1 inhibited depolarization of the mitochondrial membrane. Treatment with sh-PLCE1 or U73122 increased the number of terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)-positive cells (Supplementary Fig. S2E), whereas overexpression of PLCE1 could partially decrease the number of TUNEL-positive cells induced by PLCE1 knockdown (Fig. 1G). Levels of proapoptotic proteins were increased by PLCE1 knockdown or decreased by overexpressed PLCE1 (Fig. 1H). Together, these results demonstrated that apoptosis and cell proliferation are involved in the response of ESCC to PLCE1.

**Knockdown of PLCE1 induces autophagy**

As autophagy plays an important role in the apoptosis of tumor cells, we next determined if PLCE1 plays a role in the regulation of autophagy by morphologic and biochemical characterization. As shown in Fig. 1I, GeneChip Array Analysis showed that inhibition of PLCE1 significantly activated autophagy-related genes. In addition, compared with the control group exhibiting diffuse LC3-associated green fluorescence, a punctate pattern of LC3 fluorescence in the anti-PLCE1 treatment group was clearly observed, indicating the recruitment of LC3 to autophagosomes and the formation of autophagic vacuoles (Fig. 1J). Acidine orange (AO) and monodansylcadaverine (MDC)-labeled cells were visualized using a fluorescent microscope after anti-PLCE1 treatment, and the results showed an increase in acidic vesicular organelles after knockdown of PLCE1 (Fig. 1K). To further confirm the inhibitory effects of PLCE1 on the autophagy of ESCC cells, the cells were treated with 3-MA and/or shPLCE1. Compared with the 3-MA group, the fluorescence level of the 3-MA/shPLCE1 cotreatment group was partially rescued (Fig. 1L). JC-1 and CCR-8were used to detect the survival curve of ESCC cells, which was found to be higher in the control group than in the shPLCE1 group (Fig. 1I; Supplementary Fig. S3A and S3B). However, PLCE1 significantly attenuated the autophagy capacity of PLCE1-knockdown TE-1 cells (Fig. 1M). Moreover, Western blot analysis showed that induction of autophagy by PLCE1 knockdown was confirmed by the well-established measurements of autophagy: enhancement of Beclin-1, a component of the class III PI3K complex essential for autophagosome formation (Fig. 1N). The gene set enrichment analysis (GSEA) showed that expression of PLCE1 was significantly and negatively correlated with the regulation of autophagy (Fig. 1O).

**Hypomethylation-mediated upregulation of PLCE1 in ESCC is associated with poor prognosis**

The expression levels of PLCE1 were detected in 147 ESCC tissues and 90 normal NETs. As shown in Fig. 2A, PLCE1 protein was mainly localized in the cytoplasm of cancer cells. A total of 124 of 147 (84.35%) ESCC tissues showed high-PLCE1 expression; however, only 35 of 90 (38.9%) NETs exhibited high PLCE1 expression. As shown in Fig. 2B, Kaplan–Meier survival analysis showed that patients with ESCC with higher PLCE1 expression had a significantly worse prognosis than patients with ESCC with low or no expression ($P = 0.015$). In addition, the prognostic value of PLCE1 in available ESCC dataset was also identified and the analysis showed that there was no statistically significant difference between PLCE1 expression and the overall survival time of patients with ESCC ($P > 0.05$); however, patients with high-PLCE1 expression tended to live shorter than patients with low expression of PLCE1 (Supplementary Fig. S4A–S4C). To clarify the underlying mechanism of PLCE1 gene overexpression in ESCC, we analyzed the methylation status and the protein expression of PLCE1 in 51 ESCC tissues and 51 paired adjacent normal tissues using methylation-specific PCR (MSP). As shown in Fig. 2C and D, methylation of PLCE1 was observed in most adjacent normal tissues (92.2%, 47/51), whereas methylated DNA was detected in ESCC tissues (54.9%, 28/51; $P = 0.0001$). Among the 23 unmethylated tumor tissues, PLCE1 protein expression was markedly upregulated in 19 samples (82.6%; Fig. 2E), and there was a significant inverse association between PLCE1 methylation and its expression in ESCC tissues ($P = 0.028$). Furthermore, Kaplan–Meier analysis showed that there was no significant relationship between methylation levels and overall survival time of patients with ESCC ($P = 0.120$); however, patients with fully methylated PLCE1 promoters tended to outlive than those with nonmethylated PLCE1 promoters (Fig. 2F), indicating a need to collect more samples and conduct follow-up work. We further evaluated the relationship between the methylation of PLCE1 and the clinicopathologic parameters of 51 Kazakh patients with ESCC (Supplementary Table S1). Low-PLCE1 methylation was correlated with a greater likelihood of lymph node metastasis ($\chi^2 = 7.242; P = 0.027$) and tumor–node–metastasis stage ($\chi^2 = 7.883; P = 0.019$). However, the methylation level had no significant relationship with gender and age of patients, tumor size, or postoperative survival time.

MALDI-TOF MS was used to acquire accurate CpG site methylation data of the PLCE1 promoter in all samples collected from Kazakh patients with ESCC ($n = 99$) and NETs ($n = 46$). We used an unsupervised hierarchical clustering analysis, which provides an equi-solid view of the relationships between ESCC and CpG units (Fig. 2G). CpG methylation levels of the samples were identified on the basis of color (which varied from blue to red, indicating a methylation range of 0%–100%) for each PLCE1 CpG unit in each sample. The patterns observed in the cluster analysis indicated that the methylation status of ESCC tissues was notably different from that of NETs. The methylation levels of every CpG unit within the PLCE1 promoter were also analyzed by GraphPad Prism 5 and were exhibited by scatter plot (Fig. 2H) and boxplot (Fig. 2I). The results showed that normal samples tended to be characterized by high methylation levels, and ESCC samples displayed less variable methylation patterns (Fig. 2J). We further analyzed the methylation rates of PLCE1 promoter, whereas Eca109 and EC9706 with no
PLCE1 deletion suppresses ESCC cell growth and induces apoptosis and autophagy. 

Figure 1.

A-C, Knockdown of PLCE1 by shRNA or U73122 suppresses the proliferation of Eca109 and EC9706 cells as revealed by the CKK-8 assay (A), colony formation assay (B), and EDU assay (C). **P < 0.01; ***P < 0.001 versus scramble control (Student t test).

D-F, Effects of knockdown of PLCE1 or overexpressed PLCE1 on the apoptosis of ESCC cell lines as revealed by flow cytometry (D and E) and JC-1 analysis (F).

G, Overexpression of PLCE1 partially decreased the number of TUNEL-positive cells induced by the PLCE1 knockdown.

H, Western blot analysis of apoptosis-related proteins in Eca109 and EC9706 cells with PLCE1 knockdown and in TE-1 cells with stably expressing PLCE1.

I, GeneChip Arrays analysis demonstrating a relationship between PLCE1 expression and the autophagy-related genes. Colors represent intensity of PLCE1 shRNA vector versus control as calculated by log2 transformation.

J, Representative images of AO, MDC staining, and LC3 immunofluorescence staining of Eca109 and EC9706 cells upon PLCE1 inhibition for the indicated times. In AO staining, the red intensity shows acidic vesicular organelles representing autophagolysosomes. In MDC and LC3 staining, a punctate pattern of the fluorescence represents the density of autophagolysosomes.

K, Representative images of AO, MDC staining, and LC3 immunofluorescence staining of Eca109 and EC9706 cells with or without 3-MA (± 3-MA) for the indicated times.

L, JC-1 analysis of apoptotic Eca109 and EC9706 cells upon PLCE1 inhibition with or without 3-MA (± 3-MA) for 48 hours.

M, PLCE1 significantly attenuated the autophagy capacity of PLCE1-knockdown TE-1 cells. Representative images of AO, MDC staining, and LC3 immunofluorescence staining of TE-1 cells after transfection with sh-PLCE1 and/or PLCE1 lentiviral vector.

N, Western blot analysis of apoptosis- and autophagy-related proteins following PLCE1 inhibition with or without 3-MA (± 3-MA) for 48 hours.

O, GSEA revealed that PLCE1 was significantly and negatively correlated with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of autophagy regulation.
Figure 2. Hypomethylation-mediated PLCE1 overexpression in ESCC is associated with poor prognosis. 

A, IHC staining of PLCE1 in 147 ESCC tissues and 90 NETs. Representative images are shown. The area marked by the red circle represents the ESCC tissues and the area marked by the blue circle represents the normal tissues. 

B, Kaplan-Meier survival curves show that patients with high-PLCE1 expression had a shorter survival period (PLCE1, *P* = 0.015). 

C, PLCE1 methylation status was determined by MSP-PCR in 51 ESCC tissues and 51 NETs. 

D, Methylation status of the PLCE1 gene promoter in ESCC tissues and NETs. 

E, Relationship between PLCE1 gene promoter methylation and protein expression in ESCC. 

F, Kaplan-Meier survival curves show a relationship between PLCE1 promoter methylation status and patient prognosis. 

G, Genomic structure and distribution of PLCE1 CpG dinucleotides over the transcription start site, and hierarchical cluster analysis of CpG unit methylation profiles of the PLCE1 promoter region in ESCC (*n* = 100) and NETs (*n* = 69). Each vertex indicates an individual CpG site. The positions and orientation of the MassARRAY primers are indicated by horizontal black bars. Each row represents a sample. Each column displays the clustering of CpG units, which are a single CpG site or a combination of CpG sites. The color gradient between blue and red indicates methylation of each PLCE1 CpG unit in each sample ranging from 0% to 100%. Gray represents technically inadequate or missing data. 

H, Average methylation levels of 10 CpG units in the PLCE1 promoter between ESCC and NETs. Green, CpG methylation rate distribution of NETs; red, CpG methylation rate distribution of ESCC. The middle line indicates the average rate of methylation, and the horizontal lines on both sides of the middle indicate the SE of the average methylation rate. The bottom of the graph shows the bar plot of quantitative methylation results of 10 CpG units within the PLCE1 promoter between ESCC and NETs. 

I, The methylation levels of every CpG unit within the PLCE1 promoter are exhibited by the box plot. 

J, Comparison of overall average methylation statuses of the PLCE1 promoter between control and ESCC subjects. 

K, Methylation status and expression level of PLCE1 in four ESCC lines, Eca109, EC9706, TE-1, and Kyse150. 

L, Effect of the methyltransferase inhibitor 5-aza-2'-deoxycytidine on methylation status and protein expression of PLCE1 in ESCC cell lines.
methylation had high expression of the PLCE1 protein (Fig. 2K). However, TE-1 and Kysel50 cell lines treated with the methylation inhibitor 5-aza-2’-deoxycytidine had increased expression of PLCE1 protein due to the decreased methylation (Fig. 2L).

PLCE1 regulates p53 cellular distribution and protein stability
As p53 is a major antioncogene in most cancer types and is involved in various biological processes, we next determined whether the carcinogenic effects of PLCE1 could affect p53 in ESCC cells. As shown in Fig. 3A, GeneChip Array Analysis showed that inhibition of PLCE1 significantly activated p53 downstream genes. The specific biological role of PLCE1 in ESCC was further analyzed by GSEA and the result showed that expression of PLCE1 was significantly and negatively correlated with p53 signaling pathway (Fig. 3B). Consistent with this result, the expression of PLCE1 and p53 showed an opposite trend in ESCC cell lines (Supplementary Fig. S5), and an increase in p53 levels in Eca109 and EC9706 cells after PLCE1 knockdown was observed (Fig. 3C). Furthermore, an increase in expression of the p53

Figure 3.
PLCE1 regulates the stability and distribution of p53. A, GeneChip Arrays Analysis demonstrating a relationship between PLCE1 expression and the p53 signaling pathway. Color represents intensity scale for PLCE1 shRNA vector versus control as calculated by log2 transformation. B, GSEA revealed that PLCE1 was significantly and negatively correlated with the KEGG p53 signaling pathway. C, PLCE1 depletion increased p53 protein levels in Eca109 and EC9706 cell lines. Eca109 and EC9706 cells were transfected with control (sh-ctrl) or shPLCE1. After 48 hours, p53 and PLCE1 levels were determined by Western blot analysis. GAPDH was used as the internal control. D, Knockdown of PLCE1 promoted the expression of p53 and its downstream target genes. The overexpression of PLCE1 downregulated the expression of p53 and its downstream target genes in TE-1 cell lines. TE-1 cells stably integrated PLCE1 lentiviral vector or negative control. E, Effects of PLCE1 depletion on the expression of p53 target genes. F, PLCE1 regulated cellular p53 distribution. Cellular fractions were blotted with the indicated antibodies. C, cytoplasmic; N, nuclear. H, PLCE1 depletion did not further increase the stability of p53 in the presence of the proteasome inhibitor MG132. I and J, PLCE1 depletion increased p53 protein stability in Eca109 and EC9706 cells (I) and PLCE1 overexpression decreased p53 protein stability in TE-1 cells (J). Eca109 and EC9706 cells were transfected with control (sh-ctrl) or shPLCE1. TE-1 cells were transfected with PLCE1 lentiviral vector or negative control. After 48 hours, cells were treated with a protein biosynthesis inhibitor (100 μmol/L, cycloheximide) for different times before whole-protein extraction. The levels of PLCE1, p53, and the internal control GAPDH were determined by Western blot analysis. The bar chart shows quantified p53 band density, followed by a normalization of p53 levels, with the level at timepoint zero set as 1. *P < 0.05; **P < 0.01; ***P < 0.001.
target genes, p21 (CDKN1A), Bax, and Puma, was observed (Fig. 3D). However, a decrease of p53 levels and its target genes p21 (CDKN1A), Bax, and Puma in TE-1 cells after PLCE1 overexpression was observed (Fig. 3E). Even though p53 protein levels were increased upon PLCE1 knockdown, the p53 mRNA levels remained unchanged (Fig. 3F), suggesting that PLCE1 directly regulates p53 protein levels. To understand the underlying molecular mechanisms, the cellular distribution of p53 was examined in the cytosol and nucleus in PLCE1-knockdown cell lines (Fig. 3G) with antibodies against p53 and PLCE1. The results showed that p53 accumulated in the nucleus of cells in which PLCE1 was knocked down. Furthermore, when cells were treated with the proteasome inhibitor MG132, there was no further increase of p53 in PLCE1-depleted cells, supporting the notion that PLCE1 regulates p53 stability by proteasome-mediated mechanisms (Fig. 3H). Finally, PLCE1 depletion significantly increased the half-life of endogenous p53, indicating that PLCE1 negatively affected p53 stability in the ESCC cells. Eca109 and EC9706 (Fig. 3I). In addition, consistently, ectopic expression of PLCE1 significantly reduced the half-life of endogenous p53 in TE-1(Fig. 3I). Together, these data suggest that PLCE1 regulates the stability, protein levels, and cellular distribution of p53.

**PLCE1 associates with the p53/MDM2 complex and increases p53 polyubiquitination in an MDM2-dependent manner**

Next, we investigated the molecular mechanisms by which PLCE1 promotes p53 degradation. Previous studies have revealed that p53 could be regulated by MDM2 by escorting p53 from the nucleus to the cytoplasm, and then poly-ubiquitinating p53 (22, 23). To determine how ubiquitination may work in regulating the role of PLCE1, we explored whether PLCE1 could affect ubiquitin-ligating enzymes, including PIHR2, MDM2, AREFBP1, and NIFR. Knockdown of PLCE1 significantly reduced the expression levels of these enzymes (Fig. 4A). Next, we determined whether shPLCE1 and/or siMDM2 affected the intracellular distribution of p53 protein in Eca109 and EC9706 cells. The results showed that in the shPLCE1 group, p53 expression in the nucleus was much higher and expression in the cytoplasm was much lower than those in the control group (Fig. 4B). In the siMDM2 treatment group, the cells showed similar expression levels in the cytoplasm and nucleus compared with the control group (Fig. 4B). Therefore, we speculated that PLCE1-mediated p53 protein nucleation was dependent on MDM2. In the MG132 treatment group, the protein levels of p53 in the cytoplasm were increased in both the treatment and control groups (Fig. 4B). We further explored the interactions among p53, MDM2, and PLCE1 in Eca109 and EC9706 cells using the appropriate antibodies. Coimmunoprecipitation results suggested that PLCE1 associated with the p53/MDM2 complex in Eca109 and EC9706 cells (Fig. 4C). Figure 4D shows that PLCE1 still interacted with p53 in the presence of Nutlin-3, which blocked the interaction between p53 and MDM2, whereas association of PLCE1 with MDM2 was partially blocked by Nutlin-3. These data suggest that PLCE1 associated with p53 independently of the p53–MDM2 interaction, whereas the association of PLCE1 with MDM2 depended, at least in part, on the p53–MDM2 interaction. Eca109 and EC9706 cells were used to investigate the effect of PLCE1 on the p53/MDM2 complex. As shown in Fig. 4E and F, MDM2-mediated p53-polyubiquitination was increased by PLCE1. Interestingly, in the presence of Nutlin-3, PLCE1 depletion did not increase p53 protein levels (Fig. 4G). Thus, MDM2 may have an important role in mediating the PLCE1-mediated effect on p53 protein stability.

**PLCE1 stabilizes MDM2 by inhibiting MDM2 polyubiquitination and proteasome-mediated degradation**

PLCE1 can bind to both p53 and MDM2 to form a ternary complex, suggesting that PLCE1 might also be a regulator of MDM2. Thus, we determined whether PLCE1 could regulate MDM2 stability. To examine how MDM2 mediates PLCE1 regulation of p53 stability, we determined the effects of PLCE1 on MDM2. PLCE1 knockdown decreased MDM2 protein levels in Eca109 and EC9706 cells; however, in the presence of MG132, PLCE1 did not further increase MDM2 protein levels (Fig. 5A). Furthermore, PLCE1 stabilized the MDM2 protein. The half-life of MDM2 was compared in shPLCE1-infected Eca109 and EC9706 cells, and the results showed that the half-life of MDM2 was shorter in PLCE1-knockdown cells (Fig. 5B). Next, we examined the ubiquitination of MDM2 in PLCE1-knockdown cells, and the results showed increased polyubiquitination of MDM2 upon depletion of PLCE1 (Fig. 5C), whereas overexpressed PLCE1 decreased the polyubiquitination of MDM2 (Fig. 5D). These data suggest that PLCE1 stabilizes the MDM2 protein by inhibiting MDM2 polyubiquitination and proteasome-mediated degradation.

**Inhibition of p53 significantly attenuates the PLCE1-knockdown–induced apoptosis and autophagy in vitro**

To explore the biological significance of p53 in the tumor-promoting function of PLCE1, we examined the effects of p53 knockdown or p53 overexpression on tumorigenicity in PLCE1-shRNA lentivirus-stable-transfected ESCC cells. As shown in Fig. 6A–C, p53 knockdown significantly attenuated the hypo-proliferative phenotype and clone-forming capacity of PLCE1-knockdown ESCC cells. However, p53 overexpression enhanced the capacity of PLCE1 knockdown to inhibit the proliferation of tumor cells (Supplementary Fig. S6A–S6C). TUNEL staining experiments showed that additional p53 knockdown accelerated the impeded in vitro tumor growth of PLCE1-knockdown cancer cells (Fig. 6D). TUNEL staining of shPLCE1-transfected cells was markedly enhanced in the presence of rAd-p53 (Supplementary Fig. S6D). After treatment with si-p53 followed by PLCE1 knockdown, the proliferation rate of ESCC cells was significantly higher compared with PLCE1 knockdown alone as determined by the EdU assay (Fig. 6E), whereas the proliferation rates of tumor cells were significantly lower after treatment with rAd-p53 followed by PLCE1 knockdown (Supplementary Fig. S6E). In addition, pretreatment of cells with si-p53 decreased the PLCE1-knockdown-induced depolarization of the mitochondrial membrane (Fig. 6F), whereas the cells with rAd-p53 enhanced the PLCE1-knockdown-induced apoptosis (Fig. 6G). MDC, AO, and LC3 staining showed that silencing of PLCE1 increased the number of autophagosomes, but the numbers of autophagosomes were lower when treated with si-p53 along with shPLCE1 (Fig. 6H). However, PLCE1 knockdown or increased p53 levels using rAd-p53 increased the number of autophagosomes, and the number of autophagic vesicles changed the most with the combined treatment of lentivirus shPLCE1 and rAd-p53 (Fig. 6I). Furthermore, the expression levels of Bax, PUMA, and Beclin-1 increased with the treatments by control, shR-PLCE1/rAd-p53, and shPLCE1+rAd-p53, whereas the negative apoptosis–related molecules Bcl-2 and ubiquitin-binding protein p62 gradually decreased (Fig. 6J). However, the pretreatment with si-p53 and shPLCE1 partially decreased the level of apoptosis and autophagy-related proteins compared with shPLCE1 treatment alone (Fig. 6K). Taken together, PLCE1 silencing can promote autophagy and subsequently inhibit tumor cell proliferation at least partially through p53.
PLCE1 associates with the p53/MDM2 complex and increases p53 polyubiquitination and degradation in an MDM2-dependent manner. A, PLCE1 significantly upregulated MDM2. B, PLCE1 regulated the intracellular distribution of p53 protein depending on MDM2. Eca109 and EC9706 cells were treated with vehicle (DMSO), si-MDM2, or MG132. C, Coimmunoprecipitation assays revealed the association between endogenous PLCE1 and the p53/MDM2 complex in Eca109 and EC9706 cells. IgG was used as the control. D, Nutlin-3 did not affect the association between PLCE1 and p53. Eca109 and EC9706 cells were treated with vehicle or Nutlin-3 for 4 hours. Coimmunoprecipitation was performed using antibodies as indicated. IgG was used as a negative control for immunoprecipitation (IP), and immunoprecipitation of MDM2 was used as a control for Nutlin-3 function. E, Knockdown of PLCE1 significantly attenuated the p53 polyubiquitination. Eca109 and EC9706 cells were transfected with shPLCE1 combined with siMDM2 for 44 hours, then MG132 (10 μmol/L) was added. After 4 hours, whole-cell extracts were prepared for Western blot analysis. F, Overexpression of PLCE1 enhanced p53 polyubiquitination and degradation in an MDM2-dependent manner. TE-1 cells were transfected with PLCE1 lentiviral vector combined with siMDM2, and after 44 hours MG132 (10 μmol/L) was added. After 4 hours, whole-cell extracts were prepared for Western blot analysis. G, The effects of PLCE1 on p53 stability were dependent on the interaction between p53 and MDM2. Nutlin-3 (10 μmol/L) was added to PLCE1 knockdown Eca109 and EC9706 cells for 24 hours. **, *P < 0.01; ***, *P < 0.001.
Silencing of the PLCE1 gene combined with rAd-p53 can promote autophagy and apoptosis of esophageal cancer in vivo

To explore the effects of PLCE1 and p53 on esophageal cancer growth and autophagy in vivo, several SCID mice were subcutaneously injected with Eca109 cells transfected with empty vector or low PLCE1 expression vector. Normal saline and adenovirus p53 were injected into the tumors for the corresponding groups when the tumor grew to 0.2–0.4 cm. IVIS spectrum in vivo imaging and intensity analysis demonstrated that sh-Con-Eca109 + N.S, sh-PLCE1-Eca109 + N.S, sh-Con-Eca109 + rAd-p53, and sh-PLCE1-Eca109 + Ad-p53 groups showed gradually diminished luciferase signals (Fig. 7A) as along with tumor size (Fig. 7B and C). There were no significant differences in body weights among nude mice (Fig. 7D). After 31 days of inoculation, mice were killed, and the IHC staining results showed that the expression of PLCE1 in the experimental groups (sh-PLCE1-Eca109 + N.S. and sh-PLCE1-Eca109 + rAd-p53) was significantly lower than those in the control group (sh-Con-Eca109 + N.S. and sh-Con-Eca109 + rAd-p53). The expression of Beclin-1, LC3, and Bax gradually increased in the sh-Con-Eca109 + N.S., sh-PLCE1-Eca109 + N.S., sh-Con-Eca109 + rAd-p53, and sh-PLCE1-Eca109 + Ad-p53 groups. There was no difference in p53 expression among the groups, and the expression of Ki-67 gradually declined (Fig. 7E). The immunofluorescence results showed that the fluorescence intensity of LC3 and TUNEL was gradually increased in the sh-Con-Eca109 + N.S., sh-PLCE1-Eca109 + N.S., sh-Con-Eca109 + Ad-p53, and sh-PLCE1-Eca109 + Ad-p53 groups. Western blot analyses of PLCE1, Bax, Beclin-1, and LC3 expression were consistent with the IHC staining and immunofluorescence results (Fig. 7F; Supplementary Fig. S7). Western blot analyses showed that p53 and caspase-3 expression gradually increased in the sh-Con-Eca109 + N.S., sh-PLCE1-Eca109 + N.S., sh-Con-Eca109 + rAd-p53, and sh-PLCE1-Eca109 + Ad-p53 groups (Fig. 7G). Together, these tumor xenograft experiments showed that additional p53 promoted the side effects of PLCE1 knockdown on in vivo tumor growth.

Figure 5.
PLCE1 stabilizes MDM2 and inhibits proteasome-mediated MDM2 degradation. A, PLCE1 inhibited proteasome-mediated MDM2 degradation. ESCC cells were transfected with control (sh-ctrl) or shPLCE1. After 48 hours, cells were treated with 10 μmol/L MG132/vehicle. B, PLCE1 increased the MDM2 half-life in Eca109 and EC9706 cells. Eca109 and EC9706 cells were transfected with control (sh-ctrl) or shPLCE1. After 48 hours, cells were treated with 100 μmol/L cycloheximide/vehicle for the indicated times. Cell lysates were prepared for Western blot analysis. C, Knockdown of PLCE1 promoted MDM2 polyubiquitination in Eca109 and EC9706 cells. D, The overexpression of PLCE1 stabilized MDM2 and inhibited proteasome-mediated MDM2 degradation. TE-1 cells were transfected with PLCE1 lentiviral vector or negative control. After 44 hours, cells were treated with 10 μmol/L MG132/vehicle for 4 hours. IP, immunoprecipitation.
Clinical relevance of PLCE1-induced p53 inactivation in ESCC

We performed IHC staining to detect PLCE1 and p53 expression in 147 ESCC tissues and 90 NETs. As it is well known, after the wild-type p53 is mutated, its preventative effect on cell proliferation is lost so that cells continue to proliferate and eventually lead to malignant transformation. In this research, we used a p53 antibody to detect mutant p53 protein expression, because the half-life of the mutant p53 is significantly prolonged, while the wild-type p53 protein has a very short half-life and cannot be detected by IHC methods. The mutant p53 protein was mainly localized in the nucleus and showed much higher expression in ESCC tissues (54.42%, 80/147) than in NETs (17.78%, 16/90; Fig. 7H). The immunoreactivity scores of p53 in all of the samples were significantly higher in ESCC than in NETs (Fig. 7I).

As shown in Fig. 7J, Kaplan–Meier survival analysis showed that patients with ESCC with higher mutant p53 protein expression had significantly worse prognosis than those with little or no expression ($P = 0.017$). We used Spearman correlation analysis to evaluate the correlation between PLCE1 and p53. The results showed a positive
Figure 7.
PLCE1/p53 signaling axis is implicated in esophageal carcinogenesis. A, Tumors grew to 0.2–0.4 cm as revealed by IVIS spectrum in vivo imaging and intensity analysis. B–D, p53 overexpression accelerated the impeded in vivo tumorigenicity of cancer cells stably expressing sh-PLCE1. Tumor weight, tumor size, and tumor volume are represented as means ± SD from 10 mice in each group. **P < 0.01 represents levels of significant differences among nude mice. E, Thirty-one days after inoculation, the mice were sacrificed, followed by IHC staining of Beclin-1, LC3, Bax, p53, and Ki-67. F, TUNEL analysis of apoptosis in tumor xenograft tissues. G, Western blot analysis of apoptosis- and autophagy-related proteins following PLCE1 inhibition with or without p53 overexpression in vivo. H, IHC staining of mutant p53 protein in 147 ESCC tissues and 90 NETs. Representative images (magnification, ×100 and ×400) are shown. Scale bar, 50 μm. I, Box plot showing the range of p53 immunoreactivity scores in ESCC tissues and NETs. ***P < 0.001 represents significant difference between the ESCC and NETs. J, Kaplan–Meier survival curves show that patients with mutant p53 expression had shorter survival periods (p53, P = 0.017 × 10−3). K, Spearman correlation analysis showed a positive relationship between PLCE1 and mutant p53. L, Expression level (in transcripts per million) of PLCE1 and TP53 showed a significantly negative correlation (P = 1.2e-23). M, Model of PLCE1 regulation of autophagy and apoptosis of ESCC by promoting the MDM2-dependent ubiquitination of p53. H&E, hematoxylin and eosin; wt, wild-type. N.S, normal saline.
PLCE1 Impedes Autophagy via MDM2–p53 Axis in ESCC

Discussion

In this study, the role of PLCE1 knockdown in the proliferation, apoptosis, and autophagy of ESCC cells was examined in vitro and in vivo. In addition, we demonstrated that PLCE1 associates with the MDM2/p53 complex. As the principle antagonist, MDM2 maintains p53 at low levels in cells so that the survival and growth of cells are maintained steadily. Therefore, investigations into the mechanisms underlying the tight regulation of MDM2 are of great importance for understanding p53 biology. In this study, we discovered that PLCE1 is essential for the maintenance of MDM2 stability. PLCE1 enhanced MDM2-dependent p53 polyubiquitination and degradation and regulated cell proliferation, DNA damage–induced apoptosis, and tumorigenesis via the inhibition of p53. Furthermore, PLCE1 depletion induced the p53 signaling pathway, and cell-cycle arrest, and facilitated apoptosis and autophagy, making it an interesting therapeutic target. The results of this study present novel insights into the clinical role of PLCE1 in the treatment of ESCC (Fig. 7F).

DNA hypomethylation is a key switch that controls gene expression. We previously found that miR-34a (24) and miR-203 (25) inactivation are correlated with CpG hypermethylation in Kazakh patients with ESCC. Similarly, we detected hypomethylated PLCE1 in ESCC cell lines and ESCC tissues, which may be due to CpG_4 hypomethylation in the PLCE1 promoter region. What is more, the downregulation of PLCE1 expression was knocked down, apoptosis and autophagy of ESCC cells was examined in vitro and in vivo. In addition, we hypothesized that PLCE1 may serve as a novel regulator of p53. It is important to note that we demonstrated that PLCE1 could promote esophageal tumorigenesis through the downregulation of p53 protein and suppression of p53 downstream signals. More importantly, this study showed that although full rescue was not observed, p53 significantly attenuated the tumor-promoting effects of PLCE1. We believe that p53 is at least partially responsible for the function of PLCE1 in esophageal cancer, but we do not exclude other targets of PLCE1 in this intricate biological process.

We addressed how PLCE1 modulates p53 and observed that PLCE1 knockdown inhibited p53 activation by promoting p53 promoter methylation and inhibiting the expression of p53 in esophageal cancer cells (20). In this study, PLCE1 was shown to physically bind to p53 and enhance the ubiquitination of p53, which led to a significant decrease in the half-life of p53 protein. However, PLCE1 gene silencing by shRNA resulted in prolonged p53 stability, suggesting that PLCE1 plays an important role in p53 stability and degradation. Many studies have shown that MDM2 is the major regulator of p53, and both are labile proteins. The human homolog of MDM2 is a well-known E3 ubiquitin ligase that promotes p53 degradation (22). Furthermore, previous findings have shown that tumor-related molecules, such as SPARC, RNF2, and XIAP, regulate p53 by MDM2–p53 signaling (30–32). Further research is required to determine whether PLCE1 inactivates p53 through MDM2–p53 signaling. When examining the interaction among PLCE1, p53, and MDM2, we found that PLCE1 can bind to both p53 and MDM2 to form a ternary complex. Therefore, we further determined whether PLCE1 might have a role in the regulation of MDM2 stability. Our results showed that PLCE1 could increase the half-life of MDM2 and decrease MDM2 ubiquitination. These observations combined with our data suggest that it is very likely that PLCE1 signaling downregulates p53 by activating MDM2-mediated ubiquitination and degradation of p53. However, despite the evidence presented for the role of PLCE1 in p53 activation, we could not completely elucidate the specific binding sites and ubiquitination sites for p53 that were found to be regulated by PLCE1 knockdown.

Considering the significant role of MDM2 in restraining p53 activity, the complex regulatory network of MDM2 is subjected to intricate regulation by various cellular factors. Several ubiquitin E3 ligases are reportedly involved in the rapid degradation of MDM2 such as PCAF, XIAP, and NAT10 (32–34). Unlike these ubiquitin E3 ligases with MDM2–destabilizing function, in this study, PLCE1 stabilized the MDM2 protein by inhibiting MDM2 polyubiquitination and proteasome-mediated degradation, which blocked MDM2 autoubiquitination and degradation, thereby leading to MDM2 stabilization. These findings reveal a novel, unexpected molecular mechanism by which MDM2 stability is regulated and indicate the complexity of the regulatory mechanisms involved. Because ubiquitination is a reversible

relationship between PLCE1 and mutant p53 protein ($r = 0.182; P = 0.025$; Fig. 7K). However, the expression level (in transcripts per million) of PLCE1 and TP53 showed a significantly negative correlation ($R = -0.47; P = 1.2e-25$) in ESCC (Fig. 7L). These data suggest that PLCE1 gene promotes p53 ubiquitination through the MDM2-mediated proteasome pathway and may inhibit autophagy and cause poor clinical outcomes in ESCC (Fig. 7M).
process, deubiquitinases are assumed to have important roles in regulating p53. Several USPs, including USP7, USP2a, and USP10, reportedly regulate p53 and/or MDM2 (35, 36). We determined that the novel molecule PLCE1 regulates p53 activity and expression in this study. Specifically, PLCE1 negatively regulated p53, and not only directly bound to p53 to activate its related signaling pathways but also deubiquitinated p53, possibly promoting the activity or expression of MDM2 in the p53 regulatory loop. Altered levels of PLCE1 influenced the stability and levels of MDM2 and further changed the ubiquitination level of p53.

Our study showed PLCE1 exerts inhibitory effects on p53 and its downstream signals, thereby promoting tumor growth and progression. It has been proven by different research groups that restoration of p53 function leads to marked regression of established lymphomas, sarcomas, and liver carcinomas in vivo (37). The observation that p53 is controlled largely by its master regulator MDM2 makes the inhibition of MDM2-mediated p53 degradation an attractive approach for reactivating p53 in p53 wild-type tumors. Nutlin-3, a small compound that blocks the interaction of MDM2 with p53, has shown promise in animal studies (38). Several Nutlin-3 analogs, such as RG7112 and RG7388, are currently in clinical trials for the treatment of human cancer (39). These studies have reinforced the concept that selective and nongenotoxic p53 activation via either direct or indirect MDM2 suppression might represent an alternative to current cytotoxic chemotherapy. Given the important role of PLCE1 in regulating the MDM2-p53 pathway and pharmacologic interventions in the interaction between PLCE1 and p53, PLCE1 may be a potential target for cancer therapy.

In conclusion, we report PLCE1 is a novel interaction partner of MDM2 that regulates p53 and autophagy in ESCC. Via a direct interaction between both MDM2 and p53, hypomethylation-mediated upregulation of PLCE1 inhibits the autoubiquitination and degradation of MDM2, thus increasing its protein stability with respect to the proteasome pathway. Furthermore, p53 and its downstream targets can be triggered after knockdown of PLCE1, and PLCE1 promotes the ubiquitination of p53, possibly through an MDM2-mediated proteasome pathway. Thus, we revealed a novel mechanism by which PLCE1 regulates p53 stability via an MDM2-mediated ubiquitination process (Fig. 7F). Functionally, PLCE1 regulates cell autophagy, proliferation, apoptosis, and tumorigenesis via the MDM2-p53 axis. Autophagy plays an antimtor role in ESCC. Collectively, these results reveal that PLCE1 is a critical regulator of MDM2 and p53 and define an important function of PLCE1 in the regulation of the MDM2-p53 pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: X. Cui, F. Li Development of methodology: Y. Chen, Q. Shi, X. Han, J. Li, L. Yang, F. Li Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Chen, H. Peng, Y. Zheng, J. Hu, X. Cui, F. Li Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Xin, H. Peng, M. Li, J. Yu, Y. Tian, X. Han, X. Chen, L. Yang, L. Yang, X. Huang, X. Huang, F. Li Writing, review, and/or revision of the manuscript: Y. Chen, H. Xin, H. Peng, Y. Tian, H. Zhou, X. Cui, F. Li Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Zheng, J. Li, L. Yang, F. Li Study supervision: Z. Liu, X. Cui, F. Li

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