Cervical Cancer Growth Is Regulated by a c-ABL–PLK1 Signaling Axis

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Abstract

The nonreceptor tyrosine kinase c-ABL controls cell growth but its contributions in solid tumors are not fully understood. Here we report that the Polo-like kinase PLK1, an essential mitotic kinase regulator, is an important downstream effector of c-ABL in regulating the growth of cervical cancer. c-ABL interacted with and phosphorylated PLK1. Phosphorylation of PLK1 by c-ABL inhibited PLK1 ubiquitination and degradation and enhanced its activity, leading to cell-cycle progression and tumor growth. Both c-ABL and PLK1 were overexpressed in cervical carcinoma. Notably, PLK1 tyrosine phosphorylation correlated with patient survival in cervical cancer. In a murine xenograft model of human cervical cancer, combination treatment with c-ABL and PLK1 inhibitors yielded additive effects on tumor growth inhibition. Our findings highlight the c-ABL–PLK1 axis as a novel prognostic marker and treatment target for human cervical cancers.

Introduction

The mammalian ABL1 gene encodes the ubiquitously expressed nonreceptor tyrosine kinase ABL (1). ABL kinase plays a fundamental role in the regulation of important cell functions, including cell migration, responses to oxidative stress and DNA damage, cell proliferation, and survival (2–5). The messages carried by c-ABL are carefully regulated because they carry important information for the whole organism. If these messages are corrupted, this balance is destroyed. Chronic myelogenous leukemia (CML) is an example of what can go wrong. In most cases, this leukemia is caused when a chromosomal translocation occurs, trading pieces between two chromosomes to form an oddly small chromosome termed the “Philadelphia chromosome” (6). The site where these chromosomes break and reform is in the middle of the c-ABL gene on one chromosome and the bcr gene on the other. The resultant oncogene, BCR/ABL, encodes a 210-kDa oncoprotein, BCR-ABL, which exhibits constitutive tyrosine kinase activity and is present in >90% of the patients (7). The BCR-ABL kinase inhibitor imatinib is regarded as standard frontline treatment in CML (8).

Although c-ABL is well known for driving leukemia development, their role in solid tumors has not been appreciated until recently. Accumulating evidence now indicates that c-ABL is activated in some solid tumor cell lines via unique mechanisms that do not involve gene mutation/translocation, and c-ABL activation regulates cell cycle, proliferation, tumorigenesis, and/or metastasis, depending on the tumor type (9–11). Furthermore, c-ABL inhibitors have been used in numerous solid tumor clinical trials (12–15). However, the downstream signaling pathways, survival molecules, and cell-cycle regulators contributing to ABL-induced functions remain poorly understood.

Paradoxically, some literature show loss of PLK1 is also associated with tumor formation (27, 28). PLK1 homozygous–null mice were embryonic lethal, and early PLK1 (−/) embryos failed to survive after the eight-cell stage. Immunocytochemistry studies revealed that PLK1-null embryos were arrested outside the mitotic phase, suggesting that PLK1 is important for proper cell-cycle progression. PLK1 heterozygotes were healthy at birth, the incidence of tumors in these animals was 3-fold greater than their wild-type counterparts, demonstrating that the loss of one PLK1 allele accelerates tumor formation (29).
The tumorigenesis induced by either overexpression or down-regulation of PLK1 suggests the level of PLK1 has to be tightly regulated, which reflects its critical role in cell growth. Although, the exact role of PLK1 in carcinogenesis still needs to be further confirmed. Multiple PLK1 inhibitors, including the specific ATP-competitive inhibitor BI2536, counteract cell-cycle progression and growth in neoplastic cells, and is sufficient to prompt tumor regression in mouse xenograft models (30). However, the side effects of these drugs and the buried mechanism should be carefully observed (31).

In this study, we found that c-ABL–mediated PLK1 tyrosine phosphorylation correlates with tumor progression and patient survival rate in cervical cancers. Mechanistically, PLK1 is an important downstream effector of c-ABL. c-ABL can directly phosphorylate PLK1 and activate PLK1. Our results suggest that c-ABL–PLK1 axis may represent a novel target in cervical cancers.

Materials and Methods
Plasmids construction and reagents
The coding sequences for c-ABL were PCR-cloned into Gateway-compatible entry vectors and transferred into HA-tagged (C terminus) expression vectors by using a LR recombination kit (Invitrogen). The catalytic inactive c-ABL (K570R) was constructed by site-directed mutagenesis using a QuikChange kit (Stratagene). PLK1 gene was subcloned into pCDH cloning and expression lentivirus with epitope-tagged in its N terminus. PLK1 and its mutations were cloned into pGEX-4T-2 for bacterial expression.

For shRNA knockdown prior to in vitro or in vivo analysis, cells were transfected with individual clones of Mission shRNA lentiviral transduction particles (non-target control: SCH202V; c-ABL-1: NM_005157.3: TRCN0000039900; c-ABL-2: NM_005157.3: TRCN0000039901; PLK1: NM_005303.3: TRCN0000121072, all from Sigma-Aldrich). The mouse anti-PLK1 (F-8), rabbit anti-c-ABL (K-12) were from Santa Cruz Biotechnology, rabbit anti-CDC25C (4628), anti-phospho-CDC25C S198 (#5929), mouse anti-phospho-PLK1-T210 (#5472), mouse anti-p-Tyr (#9411), rabbit anti-Aurora A (#3092), and rabbit anti-phospho-Aurora A Thr288 (#3079) were from Cell Signaling Technology, rabbit γ-Tubulin (ab11317), rabbit anti-histone-H3 (ab1791), and anti-phospho-histone H3S10 (ab32107) were from Abcam.

RNAi target sequences
The following siRNA oligonucleotides (Shanghai GenePharma Co., Ltd) were used in this study: siPLK1 #1 (CACCAGAUGAAAUGGUAACACCA), and sic-ABL #1 (GACCAACTGTTCAAGGCGCTG), sic-ABL #2 (GAAGAGAGGGGAGAUGGC-UCU), Cells were transfected with siRNAs (100 nmol/L) using Oligofectamine (Invitrogen) according to the manufacturer’s protocol.

Cell culture and treatment
Human embryonic kidney 293 cells and human cervical epithelioid carcinoma HeLa cells were obtained in 2014 from the ATCC and maintained as recommended. Cells were used within 6 months of purchase, and authentication of cell line was performed in 2015 and the profile was compared with that in ATCC STR database.

HeLa and HEK293 cells were grown in DMEM supplemented with 10% FBS, 1% penicillin and streptomycin. HeLa/RFP-H2B cell line was maintained in selective media containing G418 (200 μg/mL, Sigma). Cells were treated with imatinib (Selleck), BI2536 (Selleck), MG132 (Sigma), cycloheximide (Sigma) as noted in the text.

Immunoprecipitation and GST pull-down assay
Cells were lysed with NETN buffer [20 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 1 mmol/L EDTA, and 0.5% NP-40] containing protease inhibitors on ice for 30 minutes. Following sonication, cell lysates were clarified by centrifugation and incubated with protein G or protein A agarose beads coupled with antibody against the indicated proteins for 8 hours at 4°C. Beads were then washed with NETN buffer three times and analyzed by Western blot analysis. For tagged protein IP, cell lysates were incubated with Anti-FLAG M2 Affinity beads (Sigma) for 3 hours at 4°C, EZview Red anti-HA affinity beads (Sigma). Precipitates were then washed and immunoblotted with the indicated antibodies. For the GST pull-down assay, GST fragments fusion proteins were expressed in E. coli. Purified fusion proteins were immobilized on glutathione Sepharose 4B beads and incubated with cell lysates at 4°C. The samples were separated by SDS-PAGE and analyzed by Western blot analysis.

Immunofluorescence staining
Immunofluorescence staining was conducted as described previously. Briefly, cells cultured on coverslips were arrest at G(S) phase, then released for the indicated times. After washing with PBS, cells were fixed with 3% paraformaldehyde for 15 minutes and permeabilized in 0.5% Triton X-100 solution for 5 minutes at room temperature. Cells were blocked with 5% goat serum and incubated with primary antibody for 60 minutes. Subsequently, samples were washed and incubated with secondary antibody for 60 minutes. DAPI staining was performed to visualize nuclear DNA. The coverslips were mounted onto glass slides with anti-fade solution and visualized using a Nikon ECLIPSE E800 fluorescence microscope.

Tandem affinity purification
HEK293 cells stably expressing SFB-c-ABL were used for tandem affinity purification. Cells stably expressing SFB-c-ABL were lysed with NETN buffer on ice for 20 minutes. After removal of cell debris by centrifugation, crude lysates were incubated with streptavidin Sepharose beads for 4 hours at 4°C. The bead-bound proteins were washed three times with NETN buffer and eluted twice with 2 mg/mL biotin (Sigma-Aldrich) for 1 hour at 4°C. The eluates were combined and then incubated with S-protein agarose (Novagen) for 4 hours at 4°C. The S-protein agarose beads were washed three times with NETN buffer. The proteins bound to S-protein agarose beads were separated by SDS-PAGE and visualized by Coomassie Blue staining.

In vitro kinase assay
For c-ABL in vitro kinase assays, 2.5 μg GST-PLK1 recombinant protein was incubated with 250 ng c-ABL recombinant protein, unless otherwise indicated, in a kinase buffer (20 mmol/L Hepes, pH 7.4, 10 mmol/L MgCl2, 5 mmol/L EGTA, 150 mmol/L NaCl, 20 mmol/L β-glycerol phosphate) at 45°C for 30 minutes. Phosphorylated proteins were separated by SDS/PAGE and analyzed by immunoblotting.

For Aurora A in vitro kinase assays, recombinant GST-PLK1 was purified from the HEK293 cell with indicated treatment. The
Aurora A protein was purified from E. coli, then Aurora A protein was incubated with 1 mg PLK1 in a kinase reaction buffer (100 μmol/L ATP, 20 mmol/L HEPES, pH 7.8, 15 mmol/L KCl, 10 mmol/L MgCl2, 1 mmol/L EGTA, 0.1 mg/mL BSA) for 60 minutes at room temperature.

Cell-cycle analysis
Cells were synchronized in G1–S using a thymidine block (2 mmol/L, Sigma) for 18 hours. Cells were then released from the block by washing three times with DMEM containing 10% FBS and refed with DMEM containing 10% FBS and thymidine (2 mmol/L) for another 15 hours. To arrest exponentially growing HeLa cells at prometaphase, nocodazole was added to a final concentration of 100 ng/mL for 12–24 hours as described previously (32). Cell-cycle distributions were confirmed by flow cytometry.

Time-lapse imaging
HeLa/RFP–H2B–stable cell lines were seeded in an eight-chambered cover glass (Lab-Tek Chambered no 1.0 Borosilicate Cover...
Figure 2.
c-ABL phosphorylates PLK1 at Y217, Y425, and Y445. A and B, HEK293 cells treated and immunoprecipitated as indicated, then analyzed with anti-phospho-Tyr antibody. C, c-ABL, phosphorylates PLK1 in vitro. Purified c-ABL kinase was incubated with equal amounts of GST, GST-PLK1 fusion proteins as indicated in Materials and Methods. D–F, PLK1 was phosphorylated by c-ABL in vitro as described in C, and the tyrosine phosphorylation site was identified by mass spectrometry. G, HEK293 cells were coexpressing with indicated plasmids. Cell lysates were incubated with anti-FLAG beads and immunoblotted with indicated antibodies. H, Bacterial-expressed WT PLK1 and the Y217F/Y425F/Y445F mutant were incubated with purified c-ABL in the presence of ATP. Phosphorylated proteins were examined by anti-tyrosine phosphorylation antibody.

Glass System, Nunc). CO₂-independent DMEM (Gibco). Images were collected every 1 hour using a 0.1-second exposure for 12 hours using a 40× (or 20×) lens objective on inverted fluorescence microscope (Nikon Eclipse Ti-E) with an UltraView spinning-disc confocal scanner unit (Perkin Elmer). The temperature of the imaging medium was kept at 37°C. Image sequences were viewed using Volocity software, and cell behavior was analyzed manually.
Mass spectrometry
FLAG-tagged PLK1 immunoprecipitates prepared from whole-cell lysates or gel-fractionated fractions were resolved by SDS-PAGE, and protein bands were excised. After adequate trypsinization, phosphopeptides were enriched with TiO₂ resin (GL Science Inc Japan). LC-electrospray ionization-MS/MS-resolved peptides were analyzed using an Orbitrap Fusion (Thermo Scientific), and the data were compared against SWISSPROT using the Mascot search engine (http://www.matrixscience.com) for phosphorylation.

Tumor xenografts
Nude mice [BALB/c; specific-pathogen-free grade; 5 to 6 weeks old] were injected subcutaneously with 200 μL (5 × 10⁵) cells. The tumor size was measured with a vernier caliper every 3 days. Tumor volumes were determined according to the following formula: A × B²/2, where A is the largest diameter and B is the perpendicular diameter. Mice were sacrificed at day 18 after cell injection, and tumors were taken for future use. All manipulations involving living mice were approved by the Animal Care and Use Committee of Beijing Friendship Hospital, Capital Medical University.

Cell proliferation assay
Cell proliferation was analyzed by MTS assay, as described previously (33). Briefly, a total of 2 × 10⁴ cells were seeded in 96-well plates, and MTS was added to each well. The absorbance was measured with a microplate reader (model 680; Bio-Rad) at 490 nm. The experiments were performed in triplicate.

Statistical analysis
The statistical data were from three independent experiments. Statistical analysis was performed by the Student t test for two groups and by ANOVA for multiple groups. P < 0.05 was considered significant.

Results
c-ABL interacts with PLK1
To examine the mechanism of c-ABL in cancer development, we overexpressed SFB-tagged c-ABL in HEK293 cells, and performed tandem affinity purifications to search for the functional partner(s) of c-ABL. We reproducibly found PLK1 as a major c-ABL-associated protein (Supplementary Table S1). PLK1 is a serine/threonine kinase that plays an important role in mitosis and cell-cycle progression in normal and cancer cells (16, 17, 20). PLK1 has recently been introduced as a potential therapeutic target in oncology (26, 34). Indeed, pharmacologic PLK1 inhibitors block cell-cycle progression and growth in neoplastic cells (35). One of these inhibitors is BI 2536, which has recently been tested in patients with advanced solid tumors in clinical trials (30, 36). However, it is not yet clear whether c-ABL and PLK1 function together in cells and in tumorigenesis.

The endogenous PLK1 and c-ABL coimmunoprecipitated together (Fig. 1A), suggesting a strong c-ABL–PLK1 interaction. A direct interaction between recombiant PLK1 and c-ABL was confirmed by a GST pull-down assay (Fig. 1B). To identify the regions of PLK1 that are responsible for the c-ABL–PLK1 interaction, we generated deletion mutants of PLK1 (Fig. 1C). We found that the PLK1 PBD domain is required for its interaction with c-ABL. Similarly, we generated deletion mutants of c-ABL (Fig. 1D). The PLK1-binding regions of c-ABL were mapped to the PTks domain and SH2/SH3 domain. A direct interaction between the PTks domain and SH2/SH3 domain of c-ABL and the PBD domain of PLK1 was confirmed by in vitro GST pull-down assay (Supplementary Fig. S1A–S1C). Furthermore, point mutation at the PBD domain (H538A, K540A and W414A) of PLK1 affected the c-ABL–PLK1 interaction (Fig. 1E; refs. 37, 38). Collectively, these findings indicate that the PLK1 PBD domain and the c-ABL SH2/SH3 and PTks domains are both necessary and sufficient for the interaction between c-ABL and PLK1.

c-ABL phosphorylates PLK1 at Y217, Y425, and Y445
As c-ABL is a human tyrosine kinase, and c-ABL interacts with PLK1, we asked whether c-ABL phosphorylates PLK1. FLAG-PLK1 was transiently expressed in HEK293 cells and by using a pan-anti-phospho-tyrosine antibody, we readily detected tyrosine phosphorylation of PLK1 (Fig. 2A). More importantly, expression of wild-type c-ABL, but not the c-ABL kinase-dead (c-ABL-KD) mutant, dramatically increased tyrosine phosphorylated PLK1. On the other hand, knockdown of c-ABL by shRNA decreased tyrosine phosphorylation of PLK1 (Fig. 2B). In vitro kinase assays also confirmed this result (Fig. 2C). Above results suggest that PLK1 is a substrate of c-ABL.

We sought to determine the residues of PLK1 that are phosphorylated by c-Ab. MS analysis of the product from in vitro kinase assay showed that Y217, Y425, and Y445 are candidate phosphorylation sites by c-ABL (Fig. 2D–F). To confirm these results, we generated mutants of the potential PLK1 tyrosine phosphorylation sites and checked whether c-ABL could regulate the tyrosine phosphorylation of these residues. As shown in Fig. 2G and Supplementary Fig. S2A, single-site mutants (Y217F, Y425F and Y445F) had little effect on the PLK1 tyrosine phosphorylation, while when all of these three sites were mutated, the phosphorylation level of PLK1 was dramatically decreased. Knockdown of c-Ab decreased the phosphorylation of PLK1 to a basal level, and overexpression of c-Ab increased the phosphorylation of wild-type PLK1, but had no effect on the 3F mutant PLK1. We also did the in vitro phosphorylation assay, as shown in Fig. 2H, these three sites are the major phosphorylation sites of PLK1 by c-ABL. Although the amino acid sequence around this site does not completely match with the consensus phosphorylation motif by c-ABL (I/V/LYXXP/F; ref. 39). Y217, Y245, and Y445 are conserved in mouse, dog, and other species (Supplementary Fig. S2B).

Y425 phosphorylation inhibits ubiquitination and degradation of PLK1
We next examined whether c-ABL affects PLK1 level and function. Indeed, a dose-dependent increase of PLK1 was observed upon transfection of HEK293 or HeLa cells with c-ABL (Fig. 3A; Supplementary Fig. S3A). Depletion of c-ABL with two different shRNAs resulted in lower level of PLK1 (Fig. 3B; Supplementary Fig. S3B) without affecting mRNA level of PLK1 (Fig. 3A and B). In addition, when we treated cells with the c-ABL inhibitor imatinib, PLK1 protein level was decreased, but PLK1 mRNA level did not change significantly (Fig. 3C; Supplementary Fig. S3C). In accord with these observations, wild-type c-ABL, but not the kinase-dead (KD) mutant, significantly prolonged the half-life of PLK1 (Fig. 3D). Protein phosphorylation often serves as a signal prompting or blocking
Figure 3.
Y425 phosphorylation inhibits ubiquitination and degradation of PLK1 in proteasomes. A and B, Western blot and quantitative PCR analysis of PLK1 from HEK293 cells transfected with increasing amount of c-ABL expression plasmids or shRNAs against c-ABL (±SEM, n = 3). Ratios of PLK1 to actin are shown as relative values. C, PLK1 protein and mRNA levels in cells treated with imatinib (10 μmol/L) were analyzed (±SEM, n = 3). Ratios of PLK1 to actin are shown as relative values. D, Cells were cotransfected with HA-c-ABL or the control plasmid. After 48 hours post-transfection, cells were treated with 100 μg/mL cycloheximide (CHX) and cultured further for indicated times. E–G, HEK293 cells transfected with the indicated plasmids were treated with MG132 (10 μmol/L, 8 hours) or left untreated, and then analyzed by immunoblotting. H, HEK293 cells were transfected with indicated plasmids and the PLK1 level was checked by immunoblotting. I, FLAG-PLK1-WT or FLAG-PLK1 Y425F mutant transfected HEK293 cells were treated with cycloheximide for the indicated times and then the cell lysates were analyzed by immunoblotting. J, HEK293 cells transfected with indicated plasmids; the ubiquitination analysis was performed as in E.
Figure 4.
c-ABL-mediated PLK1 phosphorylation regulates PLK1 activity. A, HeLa cells transfected with the indicated plasmids were treated with or without nocodazole as indicated, then FLAG-PLK1 was immunoprecipitated and adjusted to equal. Phosphorylation of PLK1 at Thr 210 was assayed. B, PLK1 expression vector was transfected into c-ABL knockdown cells. Cell lysates were immunoprecipitated with anti-FLAG beads and immunoblotted as indicated. C, c-ABL coexpressed with PLK1 WT or PLK1 Y425F in HeLa cells. Twenty-four hours after transfection, cells were treated with nocodazole (100 ng/mL, 12 hours) before being harvested. Lysates were immunoprecipitated with FLAG beads and immunoblotted as indicated. D, Wild-type PLK1 or PLK1 Y425F mutant was purified from 293T cells, then cell lysates were incubated with Aurora A in kinase buffer, and PLK1 T210 phosphorylation was checked by Western blot analysis. E, HEK293T cells were transfected with HA-tagged c-ABL or Aurora A. (Continued on the following page.)
Regulation of PLK1 by c-ABL

ubiquitination and subsequent proteasome-mediated degradation of the substrate. Supporting this possibility, HEK293 cells transiently expressing the wild-type FLAG-PLK1 with or without c-ABL shRNA were treated with proteasome inhibitor, MG132 (10 μmol/L) for 8 hours (Fig. 3E). Again, knockdown of c-ABL decreased PLK1 level, and MG132 blocked this effect.

Figure 5.
c-ABL-mediated PLK1 phosphorylation regulates cell mitotic entry. A, HeLa cells were transfected with indicated plasmids or shRNAs, synchronized at the G1–S transition as described in Materials and Methods, and subsequently released to enter mitosis with nocodazole. The p-H3-positive cells were used to determine the percentage of mitotic cells at the indicated time points. Data are shown as mean ± SEM. (n = 500). B, HeLa cells stably transfected with PLK1 shRNA were transfected with PLK1 shRNA-resistant expression construct, including PLK1 WT or its mutant Y425F, and synchronized by a method as in A. The percentage of p-H3S10 mitotic cells was determined by flow cytometry. C, HeLa/RFP-H2B cells were cotransfected with PLK1 shRNA together with GFP-Plk1 or Y425F mutant and synchronized by a thymidine block. After 4 hours following release to nocodazole, the cells through mitosis were imaged by fluorescence time-lapse microscopy. Data are shown as mean ± SEM. (n = 30). D, Selected frames from time-lapse movies of representative HeLa/RFP-H2B cells transfected with indicated plasmids. The time on the images is in hours. Scale bar, 10 μm. Levels of histone p-H3S10 were determined by immunoblot at indicated times.
c-ABL-mediated PLK1 tyrosine phosphorylation correlates with tumor progression and patient survival rate in cervical cancers. A, The mRNA level of c-ABL and PLK1 from normal cervical tissues (n = 7) or cervical cancer patient tissues (n = 7) was detected by quantitative PCR. Error bars, SEM. B, The c-ABL protein level of normal or cervical cancer patient tissues was checked by Western blotting. T, tumor; N, normal cervical tissue. Numbers 1–7 indicate patient number. C, The tyrosine phosphorylation of PLK1 was determined from normal or cervical cancer patient tissues. (Continued on the following page.)
Regulation of PLK1 by c-ABL

decrease. Ubiquitination of PLK1 was significantly increased in c-ABL–depleted cells (Fig. 3F). Consistent with this result, expression of wild-type c-ABL, but not c-ABL-KD mutant, dramatically decreased the ubiquitination level of PLK1 (Fig. 3G). Taken together, c-ABL regulates PLK1 turn over.

Next, we investigated whether c-ABL–mediated PLK1 phosphorylation regulates PLK1 stability. Because point mutation at the PBD domain (H538A, K540A, and W414A) of PLK1 greatly diminished the c-ABL–PLK1 interaction, we checked whether c-ABL–PLK1 interaction is essential for PLK1 stability. As shown in Fig. 3H, overexpression of c-ABL increased the protein level of wild-type PLK1, but not the PBD domain mutant of PLK1. This result confirmed that the c-ABL–PLK1 interaction regulates PLK1 stability. c-ABL phosphorylates PLK1 on three tyrosine residues, we checked which site is important for PLK1 stability control. As shown in Supplementary Fig. S3D–S3F, there are total 23 tyrosine sites in PLK1, when we mutated all of these 23 tyrosine sites, we found that the PLK1 23F mutant stability was dramatically decreased. These results suggest that the tyrosine phosphorylation of PLK1 regulates its turn over. Surprisingly, When we mutated F217, F425, or F445 back to Y, we found that reinstatement of Y425 restored PLK1 stability. On the other hand, when we mutated PLK1 Y425 to F, the stability of PLK1 was decreased (Fig. 3I). All of these results clearly indicated that Y425 is the major site of PLK1 regulating its turn over. Consistent with this result, PLK1 Y425SF ubiquitination level was higher than that of wild-type PLK1 in cells overexpressing c-ABL (Fig. 3J). The above results indicate that c-ABL–mediated PLK1 Y425 phosphorylation regulates PLK1 ubiquitination and stability.

c-ABL–mediated PLK1 phosphorylation regulates PLK1 activity

PLK1 plays a central role in regulating mitotic entry (16). Activation of PLK1 requires phosphorylation of a conserved threonine residue (Thr210) in the T-loop of the kinase domain (22). Aurora-A kinase directly phosphorylates PLK1 on Thr210, and the activity of Aurora-A toward PLK1 is greatly enhanced by BORA (16, 23). We have shown that among the 3 Tyr phosphorylation of PLK1 by c-ABL, Y425 is important for the regulation of PLK1 stability. We asked whether c-ABL–mediated PLK1 tyrosine phosphorylation regulates PLK1 activity. To evaluate the activity of PLK1, PLK1 was immunoprecipitated and adjusted to equal. As shown in Fig. 4A and B, overexpression of c-ABL increased Thr210 phosphorylation of PLK1, and knockdown of c-ABL decreased Thr210 phosphorylation of PLK1 and downstream CDC25C phosphorylation. More importantly, wild-type (WT) PLK1 Thr210 phosphorylation was higher than PLK1 Y425SF mutant in M-phase (Fig. 4G), and PLK1 Y425SF inhibited Aurora-A kinase–mediated PLK1 Thr210 phosphorylation in vivo (Fig. 4D). Above results clearly indicate that c-ABL–mediated PLK1 Y425 phosphorylation also regulates PLK1 activity. Mechanistically, we found that c-ABL did not interact with Aurora A (Fig. 4E), did not affect Aurora A activity (T288 phosphorylation; Fig. 4F), but knockdown of c-ABL dramatically inhibited Aurora A–PLK1 interaction (Fig. 4F). c-ABL inhibitor (imatinib) treatment also inhibited Aurora A–mediated PLK1 phosphorylation (Fig. 4G). c-ABL–regulated Aurora A–PLK1 interaction is dependent on c-ABL–mediated PLK1 Y425 phosphorylation, because c-ABL can regulate wild-type PLK1–Aurora interaction, but had no effect on the interaction between PLK1 Y425F and Aurora A (Fig. 4H). So we deduced that c-ABL phosphorylated PLK1 at Y425 site, then induced its conformation change, affected Aurora A–PLK1 interaction, and regulated Aurora A–mediated PLK1 T210 phosphorylation.

Phosphorylation of PLK1 Y425 regulates cell mitotic entry

Given the known role of PLK1 in normal M-phase entry, c-ABL knockdown cells may have delayed cell-cycle progression due to reduced PLK1 levels and activity. To test this hypothesis, we first examined cell-cycle distribution in cells knocking down c-ABL. We treated HeLa cells with double thymidine block, then released them. As shown in Supplementary Fig. S4A, knockdown of c-ABL affected G1–S transition. To further determine whether c-ABL–PLK1 axis functions in mitotic entry, we synchronized cells with double thymidine treatment and then released into the fresh medium containing nocodazole. We examined M-phase cells by the mitotic marker phospho-histone H3 (P-H3) staining. Cells with reduced c-ABL function were markedly impaired in mitotic entry, compared with control-treated cells (Supplementary Fig. S4B). We next investigated whether Y425 phosphorylation of PLK1 is important for mitotic entry. We reintroduced shRNA-resistant WT PLK1 or PLK1-Y425F to cells stably transfected with PLK1 shRNA. As expected, compared with the wild-type cells, cells expressing the PLK1 Y425F mutant showed a delay in entering into M-phase (Fig. 5A and B; Supplementary Fig. S4C and S4D). We also checked mitotic progression by time-lapse microscopy. As shown in Fig. 5C and D, cells expressing PLK1 Y425F showed a delay in mitotic entry, suggesting that c-ABL–mediated PLK1 Y425F phosphorylation regulates mitotic entry. We also checked PLK1 localization during M-phase. As shown in Supplementary Fig. S5A and S5B, knockdown of c-ABL or c-ABL inhibitor imatinib treatment did not change PLK1 localization during M-phase. Overall, our results suggest that c-ABL–mediated PLK1 phosphorylation plays an important role in cell-cycle progression.

c-ABL–mediated PLK1 tyrosine phosphorylation correlates with tumor progression and patient survival rate in cervical cancers

Because c-ABL–mediated PLK1 phosphorylation promotes cell-cycle progression and cell growth, it is possible that c-ABL–PLK1 axis may be involved in tumorigenesis. To test this hypothesis, we checked the expression levels of c-ABL and PLK1 in cervical carcinoma patient tissues compared with normal cervical tissue. Quantitative mass spectrometry analysis of the identified PLK1Y425 phosphorylation peptides from normal or cervical cancer patient tissues. E, Association between tyrosine phosphorylation of PLK1 and overall survival rate analyzed by the Kaplan-Meier method. F, Evaluation of tumor growth for xenograft mouse models of cervical cancer. Mice were inoculated subcutaneously in the right flank with 0.1 ml Matrigel containing 5 × 10^6 HeLa cells. After implantation for 7 days, tumor volume measurement began and was performed every 4 days. n = 5, **P < 0.05. Error bars, SEM. Representative images were captured at the end of 3 weeks. G, HeLa cells were transfected with the indicated plasmids and then treated with imatinib and BI2536 alone or in combination. Dose-dependent effects of different treatments on cellular viability were tested by MTS assay at 72 hours (the mean ± SD of triplicates; *, P < 0.05).
tissues. These were fresh frozen samples obtained during surgical procedures. Figure 6A and B showed that both of the mRNA and protein expression levels of c-ABL and PLK1 were significantly higher in cervical tumor tissue than in normal cervical tissue. More importantly, higher levels of PLK1 tyrosine phosphorylation ratio were found in cervical cancer samples by Western blot and MASS SPEC analysis (Fig. 6C and D). These results implied that c-ABL–PLK1 axis might have functions in tumorigenesis. To test this possibility, we evaluated the role of PLK1 tyrosine phosphorylation on tumor growth in vivo. As shown in Fig. 6E, patients with higher PLK1 tyrosine phosphorylation show a poor 5-year survival rate. Furthermore, the PLK1 mutant Y425 partially inhibited tumor growth in nude mice (Fig. 6F). Overall, our results suggest that hyperactivation of PLK1 caused by the overexpression of c-ABL might contribute to tumor progression and may function as a new prognostic marker for human cervical cancers. Interestingly, we also found that combination treatment with imatinib and BI2536 exerted significantly greater growth inhibition than either drug alone in cervical cancer cells (Fig. 6G), suggesting that the combination of c-ABL and PLK1 inhibitors may have a potential application for cervical cancer treatment.

Discussion

Cervical cancer is a major gynecologic cancer that involves uncontrolled cell division and tissue invasiveness of the female uterine cervix. Identification of new biomarkers for cervical cancer is important for disease diagnosis and treatment (40). We have identified the c-ABL–PLK1 axis as an important determinant of cervical cancer cell growth. Both of c-ABL and PLK1 are overexpressed in cervical carcinoma. Moreover, c-ABL–mediated PLK1 tyrosine phosphorylation correlates with tumor progression and patient survival rate in cervical cancers. Mechanistically, PLK1 is an important downstream effector of c-ABL. c-ABL interacts with and phosphorylates PLK1. The phosphorylation of PLK1 by the c-ABL kinase inhibits PLK1 ubiquitination and degradation, enhances its activity, leading to cell-cycle progression and cell growth (Fig. 7). Our investigations will establish more useful biomarkers for accurate detection and management of gynecologic cancers especially cervical cancer. c-ABL is distributed in the nucleus and the cytoplasm of proliferating cells (41). In the nucleus, c-ABL activity is negatively regulated by the retinoblastoma protein (RB) and positively regulated by DNA damage signals (42). Activation of the c-ABL kinase by DNA damage requires the function of ATM, which regulates cell-cycle checkpoint, DNA repair and apoptosis in response to DNA damage (43). Cells lacking c-ABL can activate cell-cycle checkpoints and DNA repair, but show defects in apoptosis (44). However, the detailed mechanism of how c-ABL functions in cell-cycle control is not clear. In this study, we found that c-ABL phosphorylated PLK1 and regulates PLK1 stability and activity. Mechanistically, our data showed that c-ABL did not interact with Aurora A and did not affect Aurora A activity. However, c-ABL–mediated phosphorylation of PLK1 regulates its interaction with Aurora A, which means that c-ABL directly regulates PLK1 activity and its biological functions. PLK1 is considered an imperative regulator to tightly control the cell-cycle network, especially in M-phase. Therefore, this crosstalk represents a new paradigm for cell-cycle control.

Some previous data suggested that c-ABL expression was significantly increased in brain, lung, and other cancers (45–47). In our studies, we found that both of c-ABL mRNA level and protein level were significantly increased in human cervical carcinoma tissues. Moreover, the downstream PLK1 tyrosine phosphorylation and protein level was also upregulated, implying that c-ABL mediated PLK1 phosphorylation may contribute to PLK1 stability and high expression. And the hyperactivation of PLK1 is linked to patient survival rate in cervical cancers. However, the causal role of hyperactivation of PLK1 caused by c-ABL in tumorigenesis remains to be determined.

c-ABL inhibitor imatinib has been well used in CML and several solid tumors. However, if imatinib can be expanded to cervical cancer is not clear. In the current study, we found that PLK1 is a downstream effector of c-ABL. The combination effects of two kinase inhibitors (PLK1 inhibitor BI2536 and c-ABL inhibitor imatinib) are better than either drug alone in cervical cancer cells. The IC_{50} of both BI2536 and imatinib are low, suggesting that the concentration applied in our assays is not likely to cause unspecific hematotoxicity. Further study could be undertaken to study whether the Y425 phosphorylation functions in cervical cancer.

In summary, our findings will have a significant impact on the dissection of components in the pathway controlling PLK1 activity and cell-cycle progression. Furthermore, as dysregulation of the PLK1 pathway is frequently linked to cancer predisposition and poor prognosis, our findings may also have important implications for cervical cancer etiology and therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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