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Cyclin-dependent kinase 1-mediated phosphorylation of YES links mitotic arrest and apoptosis during antitubulin chemotherapy

Zhan Wang[a,b], Xingcheng Chen[b], Mei-Zuo Zhong[a], Shuping Yang[c], Jiuli Zhou[b], David L. Klinkebiel[d], Adam R. Karpf[b], Yuanhong Chen[b], Jixin Dong[b,*]

[a] Department of Oncology, Xiangya Hospital, Central South University, Changsha 410008, PR China
[b] Eppley Institute for Research in Cancer & Allied Diseases, Fred & Pamela Buffett Cancer Center, University of Nebraska Medical Center, Omaha, NE 68198, United States
[c] Department of Oncology, Shandong Provincial Hospital Affiliated to Shandong University, Shandong 250021, PR China
[d] Department of Biochemistry and Molecular Biology, Fred & Pamela Buffett Cancer Center, University of Nebraska Medical Center, Omaha, NE 68198, United States

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ABSTRACT

YES is a member of the SRC family kinase (SFK) group of non-receptor tyrosine kinases, which are implicated in multiple key cellular processes involved in oncogenesis. Antitubulin agents have been widely used as chemotherapeutics for cancer patients and these drugs arrest cells in mitosis, leading to subsequent cell death. In the present study, we define a mechanism for phospho-regulation of YES that is critical for its role in response to antitubulin agents. Specifically, we found that YES is phosphorylated at multiple sites on its N-terminal unique domain by the cell cycle kinase CDK1 during antitubulin drug-induced mitotic arrest. Phosphorylation of YES occurs during normal mitosis. Deletion of YES causes arrest in prometaphase and polyploidy in a p53-independent manner. We further show that YES regulates antitubulin chemosensitivity. Importantly, mitotic phosphorylation is essential for these effects. In support of our findings, we found that YES expression is high in recurrent ovarian cancer patients. Finally, through expression profiling, we documented that YES phosphorylation affects expression of multiple cell cycle regulators. Collectively, our results reveal a previously unrecognized mechanism for controlling the activity of YES during antitubulin chemotherapeutic treatment and suggest YES as a potential target for the treatment of antitubulin-resistant cancer.

1. Introduction

Antitubulin drugs, such as paclitaxel (taxol) and vinblastine, are widely used for various malignancies, including ovarian, breast, and non-small cell lung cancers [1–3]. These drugs induce mitotic arrest through activation of the spindle checkpoint and subsequent apoptotic cell death. However, despite their wide use in cancer treatment, patient response is highly variable, with drug resistance remaining a major clinical issue [3,4]. Thus, identification of new regulators and/or signaling pathways that are triggered by antitubulin agents may provide useful information on mechanisms underlying chemoresistance and lead to the development of novel prognostic and/or therapeutic approaches related to antitubulin chemotherapeutics.

The SFKs are implicated in multiple signaling processes in oncogenesis [5]. The SFKs include nine members: SRC, YES, LYN, FYN, FGR, HCK, LCK, BLK, and YRK [5]. SRC, YES, LYN, and FYN are expressed in various cell types, while the expression of other members is restricted to specific tissues [5,6]. The catalytic activity of SFKs is mainly regulated at two highly conserved tyrosine residues: the autophosphorylation site Y419 (numbering in SRC) in the activation loop and Y530 at the extreme C-terminus regulatory tail. While phosphorylation of Y419 promotes kinase activity, increased Y530 phosphorylation inhibits SRC catalytic ability [7]. Previous reports showed that YES is oncogenic in malignant mesothelioma [8], melanoma [9], and colorectal cancer cells [10]. A recent study also showed that aberrant YES expression affects cell cycle progression and apoptosis in ovarian cancer cells [11]. Despite the oncogenic function of YES, its role and regulation in response to antitubulin chemotherapeutics have not been defined.

Here we present that YES is hyperphosphorylated during antitubulin drug-induced mitotic arrest and dysregulation of YES influences antitubulin chemosensitivity. We further characterized the phosphorylation sites, the corresponding kinase, and the functional significance

Abbreviations: CDK1, cyclin-dependent kinase 1; CDKN1A (p21), cyclin-dependent kinase inhibitor 1A; CRISPR, clustered regularly-interspaced short palindromic repeats; KO, knockout; Plk1, Polo-like kinase 1; RBL1, RB-like protein 1; SFK, Src family non-receptor tyrosine kinase; YAP, Yes-associated protein

* Corresponding author.
E-mail address: dongj@unmc.edu (J. Dong).

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of the phosphorylation. Our data reveal a new layer of regulation for YES activity in response to antitubulin chemotherapeutics, suggesting YES as a potential target for the treatment of antitubulin drug-resistant cancer patients.

2. Materials and methods

2.1. Expression constructs, cell culture and transfection

Full-length human YES cDNA was a gift from William Hahn (Addgene 23,938, pDONR223-YES1). To make the lentiviral-mediated Flag-tagged YES expression construct, the abovementioned cDNA was cloned into pSIN4-Flag-IRES vector [12]. Point mutations were generated by the QuikChange Site-Directed PCR Mutagenesis Kit (Stratagene, CA, USA) and verified by sequencing.

HEK293T, HeLa, U2OS, and OVCAR8 cell lines were purchased from American Type Culture Collection (ATCC, VA, USA) and were maintained in DMEM media supplemented with 10% FBS and 1% antibiotics. The HEK293T, HeLa, U2OS, and OVCAR8 cell lines were authenticated at ATCC and used at low passages. HCT116 and HCT116-p53 −/− cell lines were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University) and were cultured with McCoy's 5A medium. Attractene (Qiagen, MD, USA) was used for transient overexpression following the manufacturer's instructions. Nocodazole (100 ng/ml for 16–20 h) and taxol (0.1 μM for 16 h) were used to arrest cells in G2/M phase unless otherwise indicated. The VX680 (Aurora-A, −B, −C inhibitor), ZM447439 (Aurora-B, −C inhibitor), and BI2536 (Plk1 inhibitor) were from Selleck Chemicals (TX, USA). The MK5108 (Aurora-A inhibitor) was from Merck (NJ, USA), and RO3306 (CDK1 inhibitor) and Purvalanol A (CDK1/2/5 inhibitor) were from ENZO Life Sciences (NY, USA). All other chemicals were either from Sigma (MO, USA) or Thermo Fisher (MA, USA).

2.2. Cell line establishment

Stable overexpression and re-expression of YES (wild type and mutants) in YES-knockout (KO) cells were achieved by lentivirus-mediated infection. Lentivirus packaging, infection, and subsequent selection were done as we have described previously [13]. Gene knockout was achieved by a CRISPR-mediated method. CRISPR/double nickase knockout plasmids were purchased from Santa Cruz Biotechnology (SC-400261-NIC-2) (TX, USA) and transfection and clone selection were done as instructed by the manufacturer.

2.3. Phos-tag and Western blot analysis

Phos-tag™ was purchased from Wako Pure Chemical Industries, Ltd. (304–93,521) (VA, USA) and used at 10 or 20 μM (with 100 μM $MnCl_2$) in 8% SDS-acrylamide gels as we described previously [14]. Western blotting, immunoprecipitation, and lambda phosphatase treatment assays were done as described [13].

2.4. Recombinant protein purification and In vitro kinase assay

YES cDNA (encodes amino acids 1–300 of YES) was cloned into the pGEX-5 × -1 vector. The glutathione S-transferase (GST)-tagged proteins were bacterially expressed and purified on GSTrap FF affinity columns (GE Healthcare, IL, USA) following the manufacturer's instructions. GST-YES or GST-YES-5A (0.5–1 μg each) was incubated with 10 U recombinant CDK1/cyclin B complex (New England Biolabs, MA, USA) in kinase buffer [15] in the presence of 5 μCi γ-^{32}P-ATP (3000 Ci/mmol, PerkinElmer, MA, USA). The samples were resolved by SDS-PAGE, transferred onto PVDF (Millipore, MA, USA) and visualized by autoradiography followed by Western blotting or detected by phospho-specific antibodies.

2.5. Antibodies

Anti-YES antibodies from BD Biosciences (Cat. No. 610375, CA, USA) were used for Western blotting throughout the study. Rabbit polyclonal phospho-specific antibodies against YES S11, T21, S40, T60, and T69 were generated and purified by AbMart (Shanghai, China). The peptides for generating the phospho-antibodies are as follows: SKENK-pS-PAIKY (pS11); VRPEN-pT-PEPVS (pT21); EPTTV-pS-PCPSS (pS40); SSLSM-pT-PFGGS (pT60); and GSSGV-pT-PFGGA (pT69). The corresponding non-phospho-peptides were also synthesized for antibody purification. Anti-β-actin, anti-cyclin B, and anti-CDC27 antibodies were from Santa Cruz Biotechnology. Anti-GST, -Aurora-A, -Lats1, and -Lats2 antibodies were from Bethyl Laboratories (TX, USA). Anti-p-T288/T232/T198 Aurora-A/B/C, -p-S10 H3, -p-S642 Wee1, -Wee1, -p-Y15 CDK1, -CDK1, -p-T160 CDK2, -CDK2, -CDC20, -p21, -p53, -p-S127 YAP, -p-S397 YAP, -YAP, -p-Lats1/2 T1079/T1041, -p-Y419 SRC, -p-Y530 SRC, -LYN, -FYN, -SRC, and -HCK antibodies were from Cell Signaling Technology (MA, USA). Anti-Flag and anti-β-tubulin (for immunofluorescence staining) antibodies were from Sigma.

2.6. Immunofluorescence staining and confocal microscopy

Cell fixation, permeabilization, fluorescence staining, and microscopy were done as previously described [16]. The stained cells were mounted with Fluoromount (Vector Laboratories, CA, USA) and visualized on an LSM710 or LSM 800 Zeiss fluorescence microscope (Carl Zeiss, NY, USA). The Slidebook 4.2 software (Intelligent Imaging Innovations, CO, USA) was used for analyzing and processing all immunofluorescence images.

2.7. Quantitative real time PCR

Total RNA isolation, RNA reverse transcription and quantitative real time-PCR were done as we have described previously [13]. The primer sequences were as follows: 5′-AAGACCATGTGGACCTGTCACTG (p21-Forward), 5′-AGGGCTTCCTCTTGGAGAAGATC (p21-Reverse), 5′-GAA CCACCAAAGTTACCACGA (RBL1-Forward), and 5′-ATTAAACAGATCC TTAACACTGCAAG (RBL1-Reverse). The RT^2 cell cycle arrays (Qiagen) were used, and experiments and data analysis were performed following manufacturer's instructions.

2.8. Flow cytometry

DNA contents among cell lines were quantified *via* propidium iodide (PI) staining followed by flow cytometry analysis. Briefly, 1 × 10^6 cells were suspended in 100 μl PBS buffer and fixed by adding 1 ml 70% ethanol in −20 °C for an overnight. The cells were then mixed with 0.5 ml FxCycle™ PI/RNase Staining Solution (Invitrogen, F10797, MA, USA) and incubated for 30 min at room temperature in dark. The samples were analyzed by a FACSCalibur flow cytometer (Excitation at 488 nm/Emission at 530 nm). Profiles of cell cycle and polyploidy were examined by a standard method.

2.9. Annexin V/PI staining

The Dead Cell Apoptosis Kit with Annexin V Alexa Fluor™ 488 and propidium iodide (Invitrogen, V13241) was used for examination of apoptosis. Cells were seeded in 6-well plates at a density of 70–80% in duplicate. HeLa cells were treated with taxol at 50 nM for 24 h and OVCAR8 cells were treated with taxol at 20 nM for 24 h. Cell suspension preparation and staining was followed by procedures described in the manufacturer's manual. Stained cells were analyzed by flow cytometry with fluorescence emission at 530 and 575 nm. Both early (Annexin V positive/PI negative) and late (Annexin V positive/PI positive) apoptotic proportions for each population were included.