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Research Paper

A Nucleolar Stress–Specific p53–miR-101 Molecular Circuit Functions as an Intrinsic Tumor-Suppressor Network

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ABSTRACT

Background: Activation of intrinsic p53 tumor-suppressor (TS) pathways is an important principle underlying cancer chemotherapy. It is necessary to elucidate the precise regulatory mechanisms of these networks to create new treatment strategies.
Methods: Comprehensive analyses were carried out by microarray. Expression of miR-101 was analyzed by clinical samples of lung adenocarcinomas.
Findings: We discovered a functional link between p53 and miR-101, which form a molecular circuit in response to nucleolar stress. Inhibition of RNA polymerase I (Pol I) transcription resulted in the post-transcriptional activation of miR-101 in a p53-dependent manner. miR-101 induced G2 phase–specific feedback regulation of p53 through direct repression of its target, EG5, resulting in elevated phosphorylation of ATM. In lung cancer patients, low expression of miR-101 was associated with significantly poorer prognosis exclusively in p53 WT cases. miR-101 sensitized cancer cells to Pol I transcription inhibitors and strongly repressed xenograft growth in mice. Interestingly, the most downstream targets of this circuit included the inhibitor of apoptosis proteins (IAPs). Repression of cIAP1 by a selective inhibitor, birinapant, promoted activation of the apoptosis induced by Pol I transcription inhibitor in p53 WT cancer cells.
Interpretation: Our findings indicate that the p53–miR-101 circuit is a component of an intrinsic TS network formed by nucleolar stress, and that mimicking activation of this circuit represents a promising strategy for cancer therapy.
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1. Introduction

The p53 tumor-suppressor (TS) protein, encoded by the *TP53* gene, has been termed the "guardian of the genome" in recognition of its role in maintaining genome integrity in response to various oncogenic insults [1, 2]. *TP53* is mutated and/or inactivated in half of human cancers, and dysfunction of p53 makes a critical contribution to the onset of carcinogenesis [3, 4]. On the other hand, nearly half of all tumors retain wild-type (WT) p53 function, but the effector networks downstream of p53 are disrupted in many tumors due to mutations in regulatory genes. In the context of therapeutics, inactivation or reduced activation of the downstream networks of p53 is a more difficult to address than mutation in p53 itself. Many chemotherapeutic agents activate p53 through various mechanisms, resulting in induction of the appropriate downstream networks by selective activation of p53 target genes. Consequently, even after activation of p53, incomplete activation of downstream pathways can dramatically decrease the efficacy of chemotherapy.

MicroRNAs (miRNAs), a class of small non-coding RNAs, act as intrinsic mediators in intracellular networks by regulating gene expression at the post-transcriptional level [5]. miRNA expression is altered in almost all human cancers, strongly suggesting that miRNA dysfunction is associated with cancer pathogenesis [6–8]. In addition, miRNAs are globally downregulated in many types of human cancers, suggesting that they function as intrinsic TSs [9, 10]. Consistent with this idea, multiple miRNAs are involved in the regulation of p53 TS pathways [11].

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Research in context

Activation of p53 tumor-suppressor (TS) pathways is an important principle underlying chemotherapeutic strategies for treating p53–wild-type (WT) cancers. Here, we describe the p53–miR-101 circuit as a component of a TS network. miR-101 is upregulated in a p53-dependent manner after exposure of cells to Pol I inhibitors, and is involved in positive-feedback regulation of p53 *via* repression of EG5, resulting in induction of apoptosis. Moreover, reduced expression of miR-101 is associated with poor prognosis in p53 WT lung adenocarcinoma (LADC) patients. The most downstream targets of this circuit included the inhibitor of apoptosis proteins (IAPs). Combination treatment with inhibitors of IAP and Pol I represents a promising strategy for efficient elimination of p53 WT cancer cells.

Moreover, p53 itself regulates multiple miRNAs, many of which have tumor-suppressive functions, at the transcriptional and post-transcriptional levels. p53 selectively transactivates tumor-suppressive miRNAs according to the type of stress experienced by the cell [12, 13]. Thus, it is clear that precise activation of intrinsic p53 networks, as well as control of the degree and duration of pathway activation, is fine-tuned by multiple miRNAs. Comprehensively understanding the molecular connections between p53 downstream networks and miRNAs is key to elucidating TS networks, and detailed analyses of these networks are expected to reveal crucial molecules and facilitate the formulation of novel strategies for effective therapy.

In this study, we discovered that a p53-dependent TS network triggered by nucleolar stress is tuned by miR-101. Activation of this network, the p53–miR-101 circuit, enables induction of apoptosis in p53 WT cancer cells by G2 phase–specific positive-feedback regulation of p53 mediated by direct repression of EG5. The importance of this circuit is highlighted by the observation that, in lung adenocarcinoma (LADC) patients, reduced expression of miR-101 is associated with significantly worse prognosis exclusively in p53 WT cases. We identified the inhibitor of apoptosis proteins (IAPs) as the most downstream target of this circuit. Repression of cellular inhibitor of apoptosis protein 1 (cIAP1; also known as BIRC2) by the molecularly targeted drug birinapant, in combination with the polymerase I (Pol I) transcription inhibitor CX-5461, promoted induction of apoptosis in p53 WT cancer cells, implying that this combination therapy mimics activation of the p53–miR-101 circuit. Our data provide molecular insights that could facilitate development of strategies for treating p53 WT cancer.

2. Materials and Methods

2.1. Cell Culture and Transfection

The colon cancer cell lines HCT116 and RKO and the lung cancer cell lines A549 and A427 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). All cell lines were grown at 37 °C in humidified air in 5% CO_2. HCT116 *p53*$^{-/-}$ cells were kindly provided by Dr. Bert Vogelstein (The Johns Hopkins University, Baltimore, MD, USA). miRNAs and siRNAs used in this study are listed in Supplementary Table S1. miRNAs and siRNAs were introduced into cells at the indicated concentrations, individually or in combination, using the Lipofectamine RNAiMAX reagent (Life Technologies/Thermo Fisher Scientific, Waltham, MA, USA; Cat# 13778150).

2.2. Antibodies and Reagents

Anti-PARP (Cat# 9542; RRID: AB_2160739), anti-phospho-p53 (Ser15) (Cat# 9284; RRID: AB_331464), anti-phospho-Histone H3 (Ser10) (Cat# 3377; RRID: AB_1549592), anti-Histone H3 (Cat# 9715; RRID: AB_331563), anti-phospho-cdc2/CDK1 (Tyr15) (Cat# 9111S; RRID: AB_331460), anti-cdc2/CDK1 (Cat# 9112S; RRID: AB_10693432), anti-cyclin A2 (Cat# 4656S; RRID: AB_10691320), anti-p27 Kip1 (Cat# 3686S; RRID: AB_2077850), and anti-p21 (Cat# 2947; RRID: AB_823586) were purchased from Cell Signaling Technologies (Danvers, MA, USA). Anti-ATM (Cat# 1549-1; RRID: AB_725574), anti-phospho-ATM (Ser1981) (Cat# 2152-1; RRID: AB_991678), anti-DNA-PKcs (Cat# 3922-1), anti-stathmin1 (Cat# 1972-1; RRID: AB_991829), and anti-EG5 (Cat# S1765; RRID: AB_10640358) were purchased from Epitomics (Burlingame, CA, USA). Other antibodies included anti-ATR (Bethyl Laboratories, Montgomery, TX, USA; Cat# A300 – 138A; RRID: AB_2063318) and anti-NEK4 (Bethyl Laboratories, Cat# A302-673A; RRID: AB_10568794), anti-p53 (DO-1, Santa Cruz Biotechnology, Dallas, TX, USA; Cat# sc-126; RRID: AB_628082), anti-GAPDH (Chemicon/EMD Millipore, Burlington, MA, USA; Cat# MAB374; RRID: AB_2107445), anti-α-tubulin (Sigma-Aldrich, St. Louis, MO, USA; Cat# T6074; RRID: AB_477582), anti-EZH2 (BD Biosciences, San Jose, CA, USA; Cat# 612666; RRID: AB_2102429), anti-cyclin B (BD Biosciences, Cat# 610219; RRID: AB_397616), and anti-human Ciap-1/hiap-2 (R&D Systems, Minneapolis, MN, USA; Cat# AF8181, RRID: AB_2259001).

Actinomycin D (ActD; Cat# A9415; CAS: 50-76-0), 5-Fluorouracil (5-FU; Cat# F6627; CAS: 51-21-8), K858 (EG5 inhibitor) (Cat# K3644; CAS: 72926-24-0), and paclitaxel (PTX; Cat# T7402; CAS: 33069-62-4) were purchased from Sigma-Aldrich. CX-5461 (Cat# A11065; CAS: 1138549-36-6) was purchased from AdooQ BioScience (Irvine, CA, USA). ML-60218 (Cat# 557403; CAS: 577784-91-9) and RO-3306 (CDK1 inhibitor) (Cat# 217699; CAS: 872573-93-8) were from Calbiochem (San Diego, CA, USA). These reagents were dissolved in DMSO. Adriamycin (ADR; Sigma-Aldrich, Cat# D1515; CAS: 25316-40-9) and doxycycline (Dox; Sigma, Cat# D9891; CAS: 24390-14-5) and α-Amanitin (Wako Pure Chemical, Osaka, Japan; Cat# 010-229611; CAS: 23109-05-9) were dissolved in water. All reagents were used at the indicated concentrations.

2.3. Cell Proliferation Assay

Cell proliferation was measured by quantification of formazan product using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA; Cat# G3580). Cells transfected with either miRNA mimic or negative control were lysed in MTS tetrazolium solution and incubated for 1–4 h at 37 °C in a humidified 5% CO_2 atmosphere. Absorbance at 490 nm was measured on an ARVO plate reader (PerkinElmer, Waltham, MA, USA).

2.4. Clinical Samples

Surgical specimens of human LADC were obtained from patients treated at the National Cancer Center Hospital (NCC cohort) or recruited through the NCI-MD case–control study OH98CN027 (NCI cohort). In the NCC cohort, a total of 200 LADC cases were selected from 608 consecutive cases, and samples were subjected to multi-omics analysis [14]. Data from 76 of 200 LADC cases for which clinical data and genomic, transcriptomic, and miRNA profiles were available were used. None of the patients had received prior treatment with chemotherapy before surgery. Patients in the NCI cohort (n = 82) were recruited from seven hospitals. All were histologically diagnosed as having non-small cell lung cancer (NSCLC), and were recruited within 24 months after diagnosis. Only patients with LADC were included in this study. At the time of surgery, a portion of the tumor specimen and noninvolved adjacent lung tissue were flash-frozen and stored at −80 °C until use. Documented informed consent was obtained in each case. Gene expression and miRNA expression analyses of NCC cohort samples were carried out on the Agilent platform [15]. miRNA expression profiling of NCI cohort samples was performed using NanoString nCounter [16]. *TP53* mutation was confirmed by exome sequencing (NCC cohort) or targeted sequencing of *TP53* (NCI cohort), targeting exons and