NEOPLASIA
www.neoplasia.com

Volume 20 Number 11 November 2018 pp. 1135–1143 1135

Inactivation of PRIM1 Function Sensitizes Cancer Cells to ATR and CHK1 Inhibitors[1]

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Abstract

The phosphoinositide 3-kinase–related kinase ATR is a central regulator of the DNA damage response. Its chemical inhibition eliminates subsets of cancer cells in various tumor types. This effect is caused at least partly by the synthetically lethal relationship between *ATR* and certain DNA repair genes. In a previous screen using an siRNA library against DNA repair genes, we identified *PRIM1*, a part of the polymerase α-primase complex, as acting synthetically lethal with *ATR*. Applying a genetic *ATR* knock-in model of colorectal cancer cells, we confirmed that *PRIM1* depletion inhibited proliferation of *ATR*-deficient cells and excluded artifacts due to clonal variation using an *ATR* reexpressing cell clone. We expanded these data by demonstrating in different cell lines that also chemical inhibition of ATR or its main effector kinase CHK1 reduces proliferation upon depletion of *PRIM1*. Mechanistically, *PRIM1* depletion in *ATR*-deficient cells caused S-phase stasis in the absence of increased DNA damage followed by Wee1-mediated activation of caspase 8 and apoptosis. As *PRIM1* inactivation sensitizes cancer cells to ATR and CHK1 inhibitors, mutations in *PRIM1* or other components of the polymerase α-primase complex could represent novel targets for individualized tumor therapeutic approaches using ATR/CHK1 inhibitors, as has been previously demonstrated for *POLD1*, the catalytic subunit of polymerase δ.

Neoplasia (2018) 20, 1135–1143

Introduction

Synthetic lethality is defined as the interaction between two genes in which single mutations alone are not lethal but in combination are incompatible with cell survival. This mechanism could facilitate an individualized and targeted cancer therapy through pharmacological inhibition of proteins that interact synthetically lethal with tumor-specific gene mutations [1]. A prominent example is the treatment of patients harboring *BRCA1/2*-deficient cancers using PARP inhibitors [2,3]. ATR is a phosphoinositide 3-kinase–related kinase and acts as central regulator of the replication checkpoint during the DNA damage response [4]. Activated by the accumulation of single-stranded DNA at sites of replication stress or DNA damage, ATR initiates replication fork stabilization, cell cycle arrest, and DNA repair via homologous recombination [5,6]. ATR inhibitors are currently tested in clinical trials either as stand-alone therapy or in combination with DNA-damaging agents. However, the specific determinants of therapeutic response are not sufficiently defined, as only subsets of tumor cells appear to be effectively eliminated [7,8].

Abbreviations: 5-FU, 5-fluouracil; CRC, colorectal cancer; DSB, double-strand break; FBS, fetal bovine serum; ICL, interstrand-crosslinking; MMC, mitomycin C; MSI, microsatellite instability; NTC, non-transfected cells; PBS, phosphate buffered saline; pol, polymerase; RPMI 1640, Roswell Park Memorial Institute; TBS-T, TBS + 0.1% Tween 20.

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[1] Competing Interests: The authors declare that they have no competing interest.

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Received 2 July 2018; Revised 29 August 2018; Accepted 31 August 2018

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1476-5586
https://doi.org/10.1016/j.neo.2018.08.009

Due to the above function of ATR, this selectivity could at least in part be attributable to a synthetically lethal relationship between *ATR* and certain DNA repair genes. This hypothesis is supported by a systematic screening approach performed previously by us using an siRNA library targeting 288 DNA repair genes [9] in a well-defined *ATR* knock-in model [10].

In this screen, we identified six genes which may act synthetically lethal with *ATR*, including *PRIM1*. *PRIM1* encodes the catalytic subunit of primase of the polymerase (pol) α-primase complex, a major polymerase during replication, mediating the *de novo* and progressive synthesis of hybrid RNA-DNA primer as starting point for the replication of the leading and lagging strand [11,12]. However, the significance of this polα-primase complex as a potential target for cancer therapy remains enigmatic.

In the study presented here, we confirmed and characterized the synthetic lethal relationship between *ATR* and *PRIM1*. In addition, we investigated the underlying molecular mechanism through assessment of cell cycle progression, apoptosis, and DNA damage in *PRIM1*-depleted cells upon either genetic or chemical disruption of *ATR* function.

Material and Methods

Cell Lines and Culture Conditions

The human colorectal cancer (CRC) cell lines DLD-1, SW480, and RKO were purchased from the Leibniz Institut DSMZ (Braunschweig, Germany) or the American Type Culture Collection (LGC Standards, Wesel, Germany), respectively. The human pancreatic cancer cell line PaTu 8988t was kindly provided by Hans-Peter Elsässer (Philipps-University Marburg, Germany). $ATR^{s/s}$ cells were kindly provided by Fred Bunz (John Hopkins University, Baltimore, MD, USA) and have been characterized previously [7,10,13]. All cell lines and clones were maintained in Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C and 5% CO_2.

Establishment of an ATR Reexpressing Cell Clone

$ATR^{s/s}$ cells were co-transfected with vectors pcDNA3-ATR WT (Addgene plasmid #31611, conferring neomycin resistance), kindly donated by Aziz Sancar [14], and pLKO-U6-Tet-on-shNT5E-965 (conferring puromycin resistance), kindly provided by Stephan A. Hahn (Laboratory of Molecular Oncology, University Bochum, Germany), in a ratio of 10:1, as $ATR^{s/s}$ cells already harbor a neomycin resistance [10]. After transfection, the cells were maintained in RPMI 1640 containing 1 μg/ml puromycin (InvivoGen, San Diego, CA). After 3 weeks of selection, single puromycin-resistant cell clones were seeded and grown in 96-well plates and consecutively screened by immunoblotting for high expression of ATR as compared to $ATR^{s/s}$ cells. The clone with the highest expression of ATR was chosen for consecutive experiments (termed ATR^{resc}).

Drugs

AZD6738 and VE-822 were purchased from MedKoo Biosciences (Morrisville, NC), MK-8776 and LY2603618 from Selleckchem (Munich, Germany), and mitomycin C (MMC) and 5-fluouracil (5-FU) from Sigma-Aldrich (Hamburg, Germany). Oxaliplatin was kindly donated from the cytostatic drug department of the University Hospital Marburg.

Transfection

Reverse transfection was used for transfection experiments. siRNA targeting *PRIM1* (AACCACAGATCAAATACTTCA) (QIAGEN, Hilden, Germany) at a final concentration of 10 nM was incubated with HiPerFect from QIAGEN in RPMI 1640 medium free of FBS for 20 minutes at room temperature and then added to freshly seeded cells.

Cell Proliferations Assays

Cell proliferation assays were performed over a broad range of concentrations covering 100% to 0% cell survival. Either 600-800 cells of DLD-1 $ATR^{+/+}$, $ATR^{s/s}$, and ATR^{resc} were plated and transfected for 144 hours in 96-well plates to reach a final confluence of 50%-70%, or 60,000–100,000 cells of DLD-1, SW480, RKO, or PaTu 8988 t were plated and transfected for 96 hours in 6-well plates. Eight hundred to 2000 of DLD-1, SW480, RKO, or PaTu 8988t cells were then transferred to 96-well plates to reach a final confluence of 50%-70% and allowed to adhere overnight before being treated with various drugs at multiple concentrations for 120 hours. Following incubation, the cells were washed and lysed in 100 μl H_2O, and 0.2% SYBR Green (Lonza, Cologne, Germany) was added. Fluorescence was measured using a Victor^3 V plate reader (PerkinElmer, Waltham, MA), and growth inhibition was calculated as compared to the untreated control samples.

Immunoblotting

Cells were lysed and protein extracts boiled and loaded on 10% or 15% polyacrylamide gels. After electrophoretic separation, the proteins were transferred to PVDF membranes, which were blocked with 5% milk powder in TBS + 0.1% Tween 20 (TBS-T) for 1 hour. Incubation of the primary antibody in TBS-T was performed at 4°C overnight. Membranes were then washed and stained with secondary antibody. Chemiluminescence was elicited using Western Lightning Ultra from PerkinElmer or Clarity Western ECL Substrate from Bio-Rad Laboratories (Munich, Germany), respectively, according to the manufacturers' instructions. The following primary antibodies were used: anti-Caspase 3, anti-cleaved Caspase 3 (Asp175), anti-Caspase 8 (1C12), anti-PARP, anti-pCHK1 (Ser345) (133D3), and anti-PRIM1 (8G10) from Cell Signaling Technology (Cambridge, UK); anti-ATR (N-19), anti-Caspase 9 (H-170), anti-Cdc25A (5H51), anti-CHK1 (G4), anti-Cyclin A (H-432), and anti-Wee1 (B-11) from Santa Cruz Biotechnology (Dallas, TX); and anti-β-Actin (AC-15) from Sigma-Aldrich. HRP-conjugated anti-goat, anti-rat, anti-mouse, and anti-rabbit antibodies from Santa Cruz Biotechnology were used as secondary antibodies.

γH2AX Immunofluorescence

Cells were seeded and transfected on coverslips in 6-well plates to reach a final confluence of 50%-70%. One hundred twenty hours later, cells were washed with phosphate-buffered saline (PBS) and fixed in 3.7% formaldehyde for 10 minutes. After a short incubation in ice-cold methanol, the cells were washed in PBS and then permeabilized in TBS + 0.5% Triton X-100 for 10 minutes and incubated in blocking solution (TBS + 0.5%Triton X-100 + 2% BSA) for 30 minutes. Primary antibody targeting γH2AX (20E3) (Cell Signaling Technology) was diluted in the blocking solution and applied at 4°C overnight. Cells were washed and incubated with the secondary antibody conjugated with Alexa Fluor 488 (Life Technologies, Carlsbad, CA) for 2 hours. Then, slides were washed

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