Gain-of-Function Mutant p53 R273H Interacts with Replicating DNA and PARP1 in Breast Cancer

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ABSTRACT

Over 80% of triple-negative breast cancers (TNBC) express mutant p53 (mtp53) and some contain oncogenic gain-of-function (GOF) p53. We previously reported that GOF mtp53 R273H upregulates the chromatin association of mini chromosome maintenance (MCM) proteins MCM2-7 and PARP and named this the mtp53–PARP–MCM axis. In this study, we dissected the function and association between mtp53 and PARP using a number of different cell lines, patient-derived xenografts (PDX), tissue microarrays (TMA), and The Cancer Genome Atlas (TCGA) database. Endogenous mtp53 R273H and exogenously expressed R273H and R248W bound to nascent 5-ethynyl-2-deoxyuridine-labeled replicating DNA. Increased mtp53 R273H enhanced the association of mtp53 and PARP on replicating DNA. Blocking poly-ADP-ribose glycohydrolase also enhanced this association. Moreover, mtp53 R273H expression enhanced overall MCM2 levels, promoted cell proliferation, and improved the synergistic cytotoxicity of treatment with the alkylating agent temozolomide in combination with the PARP inhibitor (PARPi) talazoparib. Staining of p53 and PARP1 in different cell lines, patient-derived xenografts (PDX), tissue microarrays (TMA), and The Cancer Genome Atlas (TCGA) database indicated a higher double-positive signal in basal-like breast cancer than in luminal A or luminal B subtypes. Higher PARP1 protein levels and PAR proteins were detected in mtp53 R273H than in wild-type p53-expressing PDX samples. These results indicate that mtp53 R273H and PARP1 interact with replicating DNA and should be considered as dual biomarkers for identifying breast cancers that may respond to combination PARPi treatments.

Significance: p53 gain-of-function mutant 273H and PARP1 interact with replication forks and could serve as potential biomarkers for breast cancer sensitivity to PARP inhibitors.

Graphical Abstract: http://cancerres.aacrjournals.org/content/cancerres/80/3/394/F1.large.jpg.

Introduction

Breast cancer is the leading cause of cancer-related death in women worldwide (1). Biological markers used to categorize breast cancer types include estrogen receptor (ER), progesterone receptor (PR), and HER2. Patients with triple-negative breast cancer (TNBC) lack these three targetable markers and have a higher recurrence that results in a higher mortality (1). The Cancer Genome Atlas (TCGA) found that in 80% of TNBC, the p53 tumor suppressor gene (TP53) is mutated (2). The p53 protein is a transcription factor that regulates its downstream target genes that control cellular functions, including cell-cycle arrest, apoptosis, and cell senescence (3). Wild-type p53 has DNA replication functions that help to protect the genome during S phase, by enhancing the processivity of replication forks (4). The majority of missense mutations in p53 are located within the DNA-binding domain, including six frequent “hotspot” amino acid codons (R175, G245, R248, R249, R273, and R282; ref. 5). Some mutant p53 (mtp53) proteins have oncogenic gain-of-function (GOF) that promote tumor growth, genomic instability, invasion, chemoresistance, and altered diverse proteome and metabolic pathways (6). These GOF properties are known to share some properties while being distinct for others, and therefore it has been described that different GOF mtp53 proteins can have specific oncogenic properties (3). Therefore, it is of interest to study specific mtp53 variants for their generalized functions. We...
Previously determined that high levels of stable mtp53 R273H drive the association of DNA replication proteins MCM2-7 and PARP onto chromatin and improve the synergistic cytotoxicity of the PARP inhibitor talazoparib in combination with the DNA-damaging agent temozolomide (7, 8). Single-agent treatment in this context is not cytotoxic. The wtP53 protein works at replication forks to increase processivity, but very little information exists on the direct interaction of R273H mtp53 with replicating DNA (4). The GOF mtp53 proteins R175H and R273H have been shown to enhance transcription of genes that activate cell cycle and DNA replication, upregulating cyclin A expression at early S-phase and stabilizing replication forks by upregulating Chk1 expression (9). Many GOF mtp53 proteins also co-opt chromatin pathways to drive cancer proliferation (10). Both mtp53 R273H and R175H increase serine-threonine kinase Cell division cycle 7 (Cdc7) transcription and stimulate Cdc7-dependent DNA replication initiation (11). Cdc7 phosphorylates the N-terminus of MCM2/4/6 proteins and recruits Cdc45, GINS, and DNA polymerase e during replication (23, 24). To induce shRNA expression, cells were treated with 8 μg/mL doxycycline for time periods indicated, and fresh medium with doxycycline was supplemented every 48 hours. PDX lines were derived at Washington University (Seattle, WA) from patients with extensive stage breast cancer (25). A total of 2 × 10^6 viable cells/mouse were injected with a 1:1 mix of DMEM and Matrigel basement membrane (Corning) in a final volume of 100 μL subcutaneously into female NSG mice (NOD. Cg-Prkdcs-def2Il2rgtm1Wjl/Sj; The Jackson Laboratory). Mice were 6 to 8 weeks old at time of PDX injection/implantation. The animals were euthanized by CO_2(g) asphyxiation when tumor size reached to 1.0 cm^2 and tumors were removed and snap frozen. Protein extraction from frozen tumor was described previously (26). Proteins were extracted by homogenization of tissue with RIPA buffer [0.1% SDS, 1% IGEPA NP-40, 0.5% deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 50 mmol/L Tris-Cl pH 8.0, 1 mmol/L phenylmethylsulfonyl-fluoride (PMSF), 8.5 μg/mL aprotinin, 2 μg/mL leupeptin, and phosphatase inhibitor cocktail]. RIPA buffer was added at a ratio of 0.4 mL per 0.1 g tissue (1:4 w/v). Incubation was carried out on ice for 10 minutes and the sample was vortexed every 2 minutes. Additional sonication of the lysate (3 × on ice for 30 seconds on/off at 98% amplitude) was carried out after the incubation. Samples were centrifuged at 13,000 rpm for 10 minutes at 4°C. This study was given Institutional Animal Care and Use Committee approval from the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center (MSKCC, New York, NY). CRISPR of the mtp53 R273H in MDA-MB-468 cells was carried out using in situ assembled sgRNA and Cas9 enzyme plus a eGFP-Puro plasmid for selection introduced by Nucleofector at 1,700 V/20 ms/1 pulse.

Isolation of proteins on nascent DNA
Isolation of proteins on nascent DNA (iPOND) was performed as described previously (27) with modifications. A total of 1 × 10^6 cells were plated for each condition 1 day before 5-ethynyl-2'-deoxyuridine (EdU) incubation. Cells were incubated with 10 μmol/L EdU for 45 minutes. Cells were fixed in 4% formaldehyde in PBS for 20 minutes and quenched by adding 1 mL 1.25 mol/L glycine. Cells were permeabilized with 0.25% Triton X-100 in PBS for 30 minutes and subsequently underwent a click reaction. Click reaction was 2 mmol/L copper sulfate, 10 μmol/L biotin-azide, and 10 mmol/L sodium ascorbate added to PBS for 1.5 hours at room temperature with rotation. Cells were incubated in RIPA buffer on ice for 30 minutes, vortexing every 5 minutes. Additional sonication of the lysate (18 × on ice for 30 seconds on/off at 98% amplitude) was done after the incubation. Samples were centrifuged at 13,000 rpm for 30 minutes at 4°C. Biotin-EdU--labeled DNA was incubated with streptavidin--agarose beads at 4°C for 20 hours. The beads were washed with RIPA buffer three times and proteins bound to nascent DNA were eluted by incubating in 2 × 50 mM NaCl buffer containing 0.2 mol/L dithiothreitol (DTT) for 25 minutes at 95°C.

In situ proximity ligation assay and EdU proximity ligation assay
Cells were seeded at 2 × 10^5 per well in a 12-well glass bottom plate (MatTek). After removing media, cells were rinsed with ice-cold PBS three times, fixed in 4% formaldehyde for 15 minutes, and permeabilized in 0.5% Triton X-100 in PBS for 10 minutes at room temperature. After washing cells three times in PBS, proximity ligation assay (PLA) was carried out using Duolink In-Situ Red Kit (Sigma-Aldrich). Briefly, cells were incubated in blocking buffer for 30 minutes at 37°C in a humidified chamber and then incubated with primary antibodies
overnight at room temperature in a humidified chamber. The next day, cells were washed with Sigma buffers (catalog no. DU082049). First, Buffer A for 5 minutes three times and incubated with secondary antibody–conjugated oligonucleotides (PLA probes MINUS and PLUS) for 60 minutes at 37°C in a humidified chamber. This was followed by a 5-minute wash in Buffer A two times. The ligation reaction was carried out at 37°C for 30 minutes in a humidified chamber followed by 2× 2 minute wash in Buffer A. Cells were then incubated with the amplification mix for 100 minutes at 37°C in a darkened humidified chamber. After washing with 1× Buffer B for 10 minutes 2× and a 1-minute wash with 0.01× Buffer B, cells were mounted with mounting media containing 4′,6-diamidino-2-phenylindole (DAPI). PLA with EdU (SIRF) was performed as described previously (28–29). Cells were incubated with 125 μmol/L EdU in growth media for 15 minutes and fixed with 4% formaldehyde in PBS (pH 7.4) for 15 minutes at room temperature. For the thymidine chase experiment, EdU was removed and cells were washed two times with media before addition of media with 100 μmol/L thymidine before fixation. After fixation, cells were washed with PBS two times for 5 minutes each. Cells were permeabilized in 0.5% Triton X-100 in PBS for 15 minutes at room temperature then washed in PBS two times for 5 minutes each. Click reaction contained 2 mmol/L copper sulfate, 10 μmol/L biotin-azide, and 100 mmol/L sodium ascorbate added to PBS and incubated at room temperature for 1 hour. Cells were washed with PBS for 5 minutes and blocked for 1 hour at room temperature. Primary antibodies incubation was 4°C overnight in a humidified chamber. Cells were washed three times with wash buffer A for 5 minutes each. Duolink In-Situ PLA probes anti-mouse plus and anti-rabbit minus were diluted 1:5 and incubated for 1 hour at 37°C. Cells washed in Buffer A solution three times for 5 minutes each. Ligation reaction was incubated at 37°C for 30 minutes. Cells were washed in Buffer A 2× for 2 minutes each. Amplification reactions were incubated at 37°C for 100 minutes. Cells were washed in wash Buffer solution three times for 10 minutes each and one time in 0.01× diluted wash Buffer solution for 1 minute. EdU was detected by incubation with FITC–conjugated anti-mouse antibody for 1 hour at room temperature and washed with PBS three times for 5 minutes each and once in 0.01× diluted wash Buffer solution for 1 minute. Cells were mounted with mounting media containing DAPI. Z-stack images were taken using Nikon A1 confocal microscope with 60× objective oil immersion, acquired by Nikon elements.

**Quantification of mtpt3 R273H/EdU foci**

Images acquired through Nikon NIS Elements were processed using ImageJ. Maximum intensity projections were generated by taking two representative z-stack slices for the determination of S-phase progression. Look-up tables for each resulting image were automatically applied by ImageJ to DAPI, EdU, and mtpt3R273H/EdU channels. For each cell line, a pipeline was constructed in CellProfiler for the identification of cells positive for EdU, followed by identification and quantification of foci per cell. S-phase progression (early, mid, and late) was manually scored according to EdU staining (30). Graphpad Prism 8 was used for statistical analysis.

**siRNA transfection plus aphidicolin treatment**

siRNA electroporation was performed with the Neon Transfection System (MPK5000). MDA-MB-468 cells were grown to 70% confluence on 100-mm tissue culture dishes and rinsed with PBS without Ca²⁺ and Mg²⁺. This was followed by addition of 1× Trypsin-EDTA and incubated for 2 minutes at 37°C. After neutralizing the reaction with supplemented DMEM plus 10% FBS, the cells were resuspended and counted by hemocytometer. Cells were pelleted by centrifugation at 1,100 rpm for 5 minutes and resuspended in Resuspension Buffer R (Neon 100 μL kit, Invitrogen) to a final concentration of 1× 10⁶ cells/mL. A total of 1.5 × 10⁵ cells were transferred to a sterile 1.5 mL microcentrifuge tube and mixed with 10 μL of 20 μmol/L siRNA (Dharmacon; PARP, L-006656-03-0005; Nontargeting Pool, D-001810-10-05). Electroporation was then carried out with 100 μL of cells plus siRNA at 1,100 volts/30 milliseconds/2 pulses. Cells were seeded in 6-well cell culture plate with 2 mL of prewarmed supplemented DMEM plus 10% FBS without antibiotics for 48 hours and treated with 5 μmol/L aphidicolin for an additional 24 hours.

**Cell proliferation assay**

Twenty-thousand cells per well were seeded in 6-well plates and grown for 1, 3, and 4 days, respectively. At each time point, cells were trypsinized and cells were counted by hemocytometer.

**Comunoprecipitation assays**

Cells were lysed in NP-40 buffer [50 mmol/L Tris pH 8.0, 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% NP40, 1 mmol/L PMSF, 8.5 μg/mL aprotinin, 2 μg/mL leupeptin and phosphatase inhibitor cocktail (Sigma-Aldrich)] for 30 minutes at 4°C with rotation. After every 10 minutes, a 21G 1/2 needle was used to homogenize the sample 10 times. Cell lysates were centrifuged at 13,000 rpm for 15 minutes at 4°C. One milligram total protein was precleared with 1 μg mouse IgG (2.5 μL), 30 μL A/G PLUS-agarose (Santa Cruz Biotechnology), balanced with wash buffer [50 mmol/L Tris 8.0, 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L PMSF, 8.5 μg/mL aprotinin, 2 μg/mL leupeptin and phosphatase inhibitor cocktail (Sigma-Aldrich)] to 1 mL and rotated for 30 minutes at 4°C. Thirty-five microliters of anti-p53 DO1-AC antibody beads (Santa Cruz) or mouse IgG-AC beads were added to precleared lysates and incubated at 4°C for 2 hours with rotation. The beads were washed with 1 mL wash buffer four times for 4 minutes each at 4°C. Bound proteins were eluted by incubating in 2× SDS Laemmli sample buffer containing 0.2 mol/L DTT at 95°C for 10 minutes and loaded onto SDS-PAGE.

**Protein extraction**

Cells were harvested at 1,100 rpm for 5 minutes at 4°C on Sorvall benchtop centrifuge. Cells were washed three times with ice-cold PBS. Cells then were resuspended in RIPA buffer. The cell suspension was incubated on ice for 30 minutes, vortexing every 5 minutes. Additional sonication of lysate (three times on ice, 30 seconds on/off at 98% amplitude) was done after the incubation. Samples were centrifuged at 13,000 rpm for 30 minutes at 4°C and supernatant was collected.

**Immunoblotting assay**

Cell extracts were run on 10% SDS-PAGE followed by electrotransfer onto polyvinylidene fluoride membrane (GE). The membrane was blocked with 5% nonfat milk (Bio-Rad) in either 1× PBS-0.1% Tween-20 or 1× TBS-0.1% Tween-20 followed by incubation with primary antibody overnight at 4°C. The membrane was washed with either 1× PBS-0.1% Tween-20 or 1× TBS-0.1% Tween-20 and incubated with secondary antibody for 1 hour at room temperature. The signal was detected by chemiluminescence with Super Signal West (Pierce) and autoradiography with Hyblot CL films (Denville Scientific) or Typhoon FLA 7000 laser scanner (GE Healthcare).

**Subcellular fractionation**

Cells were harvested, and subcellular fractionation was performed using the Stillman protocol (31). After removing the media, cells were rinsed with ice-cold PBS twice, scraped from plates, and pelleted by
centrifugation at 1,100 rpm for 5 minutes. Cell pellets were suspended in buffer A [10 mM Heps, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 mol/L sucrose, 10% (vol/vol) glycerol, 1 mM DTT, 0.5 mM PMSF, 2 μg/mL leupeptin, 8.5 μg/mL aprotinin] with 0.1% Triton X-100. After 5-minute incubation on ice, cells were spun down at 3,600 rpm for 5 minutes at 4°C. The supernatant was spun down for an additional 5 minutes at 13,000 rpm at 4°C (cytoplasmic fraction). Pellets were washed twice with Buffer A by centrifugation at 3,600 rpm for 5 minutes at 4°C. Resuspended nuclear pellet in Buffer B (3 mM EDTA, 0.2 mM EGTA, 0.5 mM PMSF, 2 μg/mL leupeptin, 8.5 μg/mL aprotinin) was incubated on ice for 30 minutes with vigorous vortexing every 5 minutes and spun down at 4,000 rpm for 5 minutes at 4°C. The supernatant was nuclear-soluble proteins, and the pellet, enriched in chromatin, was washed two times with Buffer B, resuspended in Buffer B, and sonicated three times on ice for 30 seconds on/off at 98% amplitude.

**MTT**

Cells were seeded at 1.25 × 10^5 cells per well in 12-well plates. MTT solution [5 mg/mL 3-(3,4-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] was added to the cells (10% vol/vol) and incubated at 37°C in 5% CO2 for 1 hour. The cells were resuspended in 0.04 N hydrochloric acid diluted in isopropanol. Samples were incubated for 5 minutes at room temperature. The absorbance was quantified by measuring the absorbance at 550 nm subtracted from the absorbance at 620 nm.

**Live-cell imaging**

Cells were seeded at 2 × 10^4 per well in a 12-well glass bottom plate one day before imaging (MatTek). Cells were incubated with 500 mM PARPi-FL for 20 minutes at 37°C. PARPi-FL was removed and cells were incubated in fresh medium for 10 minutes, washed twice with PBS at room temperature, captured the nuclear DNA with 1 μg/mL Hoechst 33342 (Thermo Fisher Scientific) in PBS for 5 minutes, and z-stack images of stained cells were taken by confocal microscopy using a Nikon A1 confocal microscope with 40× objective.

**Tissue microarray p53 and PARPi IHC**

Tissue microarrays were constructed from FFPE tumor samples (tumor and adjacent normal). Cores were precisely arrayed into a new recipient paraffin block using an automated arrayer (ATA-27, Beecher Instruments) with the method described by Kononen and colleagues (32). Documentation for each tissue core’s location was done on a ready-made grid using the program Microsoft Excel. MCF-7 and Hela cells were arrayed into a tissue microarray (TMA) block using an automated arrayer (ATA-27, Beecher Instruments). Documentation for each tissue core’s location was done on a ready-made grid using the program Microsoft Excel. MCF-7 and Hela cells were arrayed into a tissue microarray (TMA) block using an automated arrayer (ATA-27, Beecher Instruments). Documentation for each tissue core’s location was done on a ready-made grid using the program Microsoft Excel. MCF-7 and Hela cells were arrayed into a tissue microarray (TMA) block using an automated arrayer (ATA-27, Beecher Instruments).

**IHC and evaluation of p53 and PARPi**

The 4-μm-thick TMA tissue sections were deparaffinized in xylene and rehydrated through a graded alcohol series to distilled water. Slides were cooled to room temperature and washed in Tris-buffered saline. Antigen retrieval was performed in a vegetable steamer in Universal Decloaker target retrieval solution (BioCare Medical catalog no. UD1000 M) and then the slides were left to cool in solution for 15 minutes. Slides were then incubated in Peroxidase target retrieval solution (BioCare Medical catalog no. PX968) for 10 minutes, followed by incubation for 15 minutes in Background Sniper (BioCare Medical catalog no. BS966L). IHC assays were performed using the following antibodies: p53 (DO-1) antibody (Santa Cruz Biotechnology, dilution 1:100) and PARP-1 (F-2; Santa Cruz Biotechnology, dilution 1:250). The BioCare Medical Polymer Detection Kit (catalog no. M3M530L) was used as a detection system for the Anti-P53 MACH 3 Probe (10 minutes), ready to use MACH 3 HRP-Polymer (10 minutes). Slides were treated for 5 minutes with 3–3′-diaminobenzidine (DAB) chromogen (BioCare Medical catalog no. BDB2004 H), counterstained with hematoxylin, and coveredslipped. For the IHC analysis, Allred scores were determined semiquantitatively by two observers as described (33). Scoring was based on intensity and percentage of positive stained cells. Final scores (range 0–8) were converted to a scale from 0 to 3 (0–1 considered as negative; 2–3 considered as weakly positive-1; 4–5 considered as moderately positive-2; 6–8 considered as highly positive-3). All discrepancies were resolved by a second examination by two observers simultaneously using a microscope.

**Results**

**Mutant p53 R273H and PARPi interact with replicating DNA**

We previously identified that mtp53 R273H increases the chromatin association of the DNA repair protein PARP and essential DNA replication protein complex mini chromosome maintenance (MCM2–7), and showed mtp53 (but not wtp53) directly associates with MCM2 and MCM4 (7–8). To investigate whether mtp53 plays a direct role in regulating DNA replication, here we examined the association of mtp53 R273H in MDA-MB-468 breast cancer cells and Panc-1 pancreatic cancer cells on replicating DNA by using the isolation of proteins on nascent DNA (iPOND) assay (Fig. 1A). In MDA-MB-468 cells, the influence of mtp53 R273H was compared in miR-30 expressing control MDA-MB-468 cells, miR30-based dicer cleavage-inducible shRNA isogenic knockdown in MDA-MB-468 cells, and increased expression that was shRNA-resistant in MDA-MB-468.shp53 cells, and increased expression that was shRNA-resistant in MDA-MB-468.shp53 cells (68.6.shp53+R273H; ref. 24). After thyminidine analogue EdU pulse labeling and covalent linkage to a biotin-azide using click chemistry, proteins bound to newly replicated DNA were analyzed. We detected significant mtp53 R273H on the replicating DNA in MDA-MB-468.shp53+R273H cells (Fig. 1A, MDA-MB-468). The exogenous expression of mtp53+R273H (Fig. 1A, MDA-MB-468 see input lane 4) increased the mtp53 R273H on replicating DNA (Fig. 1A MDA-MB-468, compare lane 8 to lanes 6 and 7). Furthermore, PARPi association on replicating DNA was increased when mtp53 R273H was overexpressed (Fig. 1A MDA-MB-468, compare lane 8 to lanes 6 and 7) and knockdown of mtp53 R273H reduced the mtp53 R273H and PARPi positive-2; 6 considered as weakly positive-1; 4–5 considered as moderately positive-2; 6–8 considered as highly positive-3). All discrepancies were resolved by a second examination by two observers simultaneously using a microscope.

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Mutant p53 R273H interacts with newly replicated DNA in association with PARP. A, iPOND analysis of newly replicated DNA association with mtp53 R273H and PARP in MDA-MB-468 vector, MDA-MB-468.shp53 cells, and mtp53R273H-overexpressing MDA-MB-468.shp53+R273H cells (left) and PANC-1 cells (right). A total of 1 x 10^6 cells were labeled with 10 μmol/L EdU for 45 minutes. The protein–DNA complexes were cross-linked, nascent DNA was conjugated to biotin using click chemistry, and protein–DNA complexes were purified. The eluted proteins were analyzed using Western blot analysis. A sample that did not include biotin-azide was used as a negative control. Representative of two independent experiments. B, Single-cell in situ PLA with pulse EdU labeling showed mtp53 R273H associated with newly replicated DNA throughout S-phase. Cells were labeled with 125 μmol/L EdU for 15 minutes. The protein–DNA complexes were cross-linked and nascent DNA was conjugated to biotin using click chemistry. Representative maximum intensity projection images of two central slices for S-phase progression of MDA-MB-468 (top left) and PANC-1 (top right) are shown; thickness, 2 μm. PLA of mtp53 R273H/EdU (red) was performed using antibiotin and anti-p53 antibodies. Anti-biotin IF (green) indicates cells undergoing DNA synthesis. DNA was counterstained with DAPI (blue). Analysis of mtp53/EdU foci per nuclei by CellProfiler and S-phase progression for each nucleus was manually grouped into early, mid, or late S-phase using GraphPad Prism 8. Statistical analysis for MDA-MB-468 and PANC-1: *, P < 0.05; **, P < 0.01; ***, P < 0.0001; ns, not significant. The P-value was determined by two-tailed Student t test. MDA-MB-468: early, n = 86; mid, n = 49; late, n = 69; PANC-1: early, n = 51; mid, n = 23; late, n = 44. Representative of three independent experiments, with two technical replicates each. C, The z-stack maximum intensity projection images showed mtp53 R273H and PARP associated with newly replicated DNA in MDA-MB-468 cells. Cells were labeled with 125 μmol/L EdU for 15 minutes with or without 100 μmol/L thymidine chases for 60 minutes. PLA with EdU (red) was performed as in Fig. 1B. Three independent experiments were performed. D, PLA with or without 20-minute incubation of 10 μmol/L PARGi and 10 μmol/L EdU labeling. Maximum intensity projection images are shown. Representative of two technical replicates.
newly replicated DNA as evident by red foci. This supports our earlier report that mtp53 directly associates with MCM2 and MCM4 (8). In addition, we detected that another mtp53 hotspot mutant, R248W, when exogenously expressed was also associated with replicating DNA (Supplementary Fig. S2). The observed PLA signal between biotinylated EdU and mtp53 R273H decreased after a 1-hour thymidine chase following the 15-minute EdU pulse. Endogenous PAR is detected during S-phase at sites of DNA replication (21) and p53 is PARylated by PARP1 and this activates the interaction between PARP1 and wild-type p53 (34). To investigate how increased PARylation influences replication-associated mtp53 and endogenous PARP in the absence of DNA damage, we carried out PLA with short incubation with the PAR glycohydrolase inhibitor (PARGi; Supplementary Fig. S3D). The increased mtp53 R273H was found in association with newly replicated DNA in PARGi-treated cells versus vehicle-treated cells. Moreover, increased mtp53 R273H and PARP were coassociated in PARGi-treated MDA-MB-468 and PANC-1 cells (Supplementary Fig. S3A and S3B). We also observed an endogenous protein–protein interaction between PARP and MCM2 and mtp53 R273H by communoprecipitation (co-IP) with anti-PARP (Supplementary Fig. S3C). No mtp53 R273H was detected on replicating DNA when Okazaki fragment formation was suppressed by talazoparib in combination with the DNA-damaging agent temozolomide (8). We hypothesized that overexpression of mtp53 R273H would further sensitize cells to the combination treatment. The cell viability was slightly reduced in mtp53 exogenously expressing 468.shp53 cells versus vehicle-treated cells. Moreover, increased mtp53 R273H and lower expression levels of MCM2 (Supplementary Fig. S3A). The level and decrease in cell number was detected when mtp53 was knocked down and a 17.6% increase in cell number was observed when mtp53 R273H was exogenously expressed. Our results demonstrate that mtp53 R273H increases MCM2 and inhibits cell proliferation, and that the level of PARP does not influence mtp53 levels but PARP is needed to increase progression into S-phase.

**Overexpression of mutant p53 R273H increases PARP1 chromatin interaction and synergistic PARPi plus temozolomide treatment sensitivity**

We carried out the PLA assay to identify whether mtp53 R273H interacts with PARP1 on chromatin (Fig. 3A). The detection of PARP1 and mtp53 in close proximity showed an average (1.085 ± 0.1305, n = 233) fluorescent foci per cell in MDA-MB-468 cells. In contrast, detection of PARP and wtp53 in MCF7 cells showed a lower average (1.085 ± 0.1305, n = 117) fluorescent foci per cell (Fig. 3B). A higher p53 protein level was detected in MDA-MB-468 cells compared with MCF7 cells (Fig. 3C) and this could account for the increased detectable interaction. The interaction of endogenous mtp53 R273H with PARP1 was also detected with the co-IP assay (Fig. 3D). Importantly, in MDA-MB-468 cells, the endogenous mtp53 R273H and MCM7 were also detected in a protein–protein interaction (Fig. 3D).

We previously reported that while single-agent treatment was not very cytotoxic, there was a synergistic activation of apoptosis in MDA-MB-468 cells treated with the PARP inhibitor/trapping agent (PARPi) talazoparib in combination with the DNA-damaging agent temozolomide (8). We hypothesized that overexpression of mtp53 R273H would further sensitize cells to the combination treatment. The cell viability was slightly reduced in mtp53 exogenously expressing 468.shp53–R273H cells compared with knockdown 468.shp53 cells (Fig. 3E). The increased synergistic sensitivity to the PARPi (talazoparib) with DNA damaging agent (temozolomide) in mtp53 R273H-overexpressing cells supports the inference that the increased interaction of mtp53 and PARP sensitizes the cells. Interestingly, when more mtp53 R273H was exogenously expressed, there was a reduction in the amount of the DNA repair protein 53BP1 localized to the chromatin (Fig. 3F). It has been shown that the inhibition of PARP blocks 53BP1 foci (38), and mtp53 R273H increasing this inhibition could be part of the improved killing mechanism in the presence of high mtp53 R273H.

**Mutant p53 R273H, MCM2, and PARP promote cell proliferation**

An increased frequency of origin firing has been implicated in genomic abnormalities (35). Replication origins are licensed in the G1 phase by the binding of MCM helicase complexes (36). Phosphorylation of MCMs by Cdc7 promotes subsequent assembly of replisome members and initiates DNA synthesis in the S-phase (12). The MCM2 amino acid residue serine 108 is a Cdc7 phosphorylation site (37). We examined the levels of MCM2 over the cell cycle in MDA-MB-468 control cells and a CRISPR mtp53-targeted clone that expressed extremely low levels of mtp53 R273H (Fig. 2A). The level and phosphorylation of MCM2 varied depending on the cell-cycle stage, and lower expression levels of mtp53 R273H correlated with lower levels of MCM2 (Fig. 2A). This was consistent with our previous results for total MCM2 protein reduction when mtp53 R273H was depleted by shRNA knockdown methods (8). Moreover, when we used siRNA targeting PARP (see Fig. 2B), we reduced the ability of the MDA-MB-468 cells to enter S-phase even in the absence of changes in mtp53 recruitment.

To investigate whether mtp53 R273H increased cell proliferation, we performed the MTT and cell counting assay in MDA-MB-468 miR-30 expressing, 468.shp53 knockdown, and 468.shp53–R273H increased mtp53-expressing cells (Fig. 2C–E). The mtp53 R273H protein was reduced in 468.shp53 cells and was higher in 468.shp53–R273H cells (Supplementary Fig. S1). Mitochondrial activity was reduced by 28.4% in response to knockdown of mtp53 and a 33.5% increase was observed when mtp53 R273H was exogenously expressed. At day 4 in the growth curve, a 33.5% increase was observed when mtp53 was knocked down and a 17.6% increase in cell number was detected when mtp53 R273H was exogenously expressed. Our results demonstrate that mtp53 R273H increases MCM2 and induces cell proliferation, and that
A higher average IHC score of p53 was present in basal-like breast cancer samples than luminal A or luminal B subtypes (Fig. 4B, top). Among 20 basal-like tumor samples, we found in the p53-high group (n = 10), nuclear PARP1 intensity was higher than in the p53-low group (n = 10; Fig. 4B, bottom). Because of the small sample size of basal-like tumor samples, the protein intensity of p53 and PARP1 was not statistically significant. However, in 62 luminal A tumor samples, the protein intensity of p53 and PARP1 showed a statistically significant positive correlation (Pearson r: 0.2745; P = 0.0308). Taken together, these data indicate that high mtp53 expression often correlates with high PARP1 in different breast cancer subtypes.

PARPi-FL imaging agents are extremely promising for detecting oral cancers with high PARP expression (41). We carried out live-cell imaging with PARPi-FL and detected PARPi-FL stains TNBC cells MDA-MB-468 more intensely than ERþ cells MCF7 and this staining is nuclear (Fig. 4C). Furthermore, we tested PARP1 protein level and PARylated proteins in PDX samples to determine whether mtp53 expression correlated with PARP detection (Fig. 4D). PDX models maintain the histologic and molecular heterogeneity of the progenitor...
and can be used to test cytotoxic drug responsiveness (42). We detected more PARP protein and significantly higher levels of PARylated proteins in WHIM25 expressing mtp53 R273H than in WHIM6, which expresses wtp53. In conclusion, our data provide strong evidence that the increased coexpression of mtp53 and PARP proteins can be biomarkers for companion diagnostics to identify TNBCs that have elevated expression of mtp53 and PARP thereby giving a potential proxy for cancers that are candidates for combined talazoparib and temozolomide treatment.

Discussion

DNA replication and repair are two essential biological processes that ensure accurate duplication of the genome. We previously showed a coassociation of mtp53 and PARP proteins in WHIM25 expressing mtp53 R273H than in WHIM6, which expresses wtp53. In conclusion, our data provide strong evidence that the increased coexpression of mtp53 and PARP proteins can be biomarkers for companion diagnostics to identify TNBCs that have elevated expression of mtp53 and PARP thereby giving a potential proxy for cancers that are candidates for combined talazoparib and temozolomide treatment.

DNA replication and repair are two essential biological processes that ensure accurate duplication of the genome. We previously showed a coassociation of mtp53 and DNA replication proteins (8). Here we demonstrated that mtp53 and PARP1 are associated with replicating DNA in TNBC cells. Furthermore, mtp53 enhanced PARP1 association with nascent DNA in TNBC and enhanced cell proliferation. We observed a positive association of increased p53 expression and PARP1 in analysis of cancer samples from the TCGA and TMA analysis for all subtypes of patients with breast cancer, suggesting that scoring mtp53 and PARP expression as a prognostic marker can act as a proxy for potential TNBCs that would benefit from combination talazoparib–temozolomide treatment. Our findings prompt us to propose a model (see Fig. 5A) in which mtp53 (shown in green) associates with replicating DNA and recruits MCM proteins (shown in purple) and PARP1 (shown in red) on stressed replicating DNA allowing replication to proceed even in the presence of DNA damage, thereby promoting tumorigenesis. Talazoparib has recently been granted FDA approval for metastatic breast cancers with BRCA mutations (43). We previously showed that mtp53 R273H increases the ability of talazoparib to trap PARP1 on the chromatin (7, 8). We propose that mtp53

Figure 3.

Mutant p53 R273H associates with PARP and MCM proteins and increases synergistic PARPi plus temozolomide treatment sensitivity. A, Analysis of p53/PARP complexes (red) by immunofluorescence microscopy in combination with in situ PLA in MDA-MB-468 and MCF7 cells. DNA was counterstained with DAPI (blue). The z-stack maximum intensity projection images are shown. Two independent experiments were performed. B, Analysis of fluorescent foci per cell by CellProfiler software and demonstrated by scatter plot using Prism7 GraphPad. ****, P < 0.0001. The P value was determined by two-tailed Student t test. Two independent experiments were performed. C, p53 protein level in MCF7 and MDA-MB-468 cells. Two independent experiments were performed. D, Co-IP of PARP and MCM7 with mtp53 in MDA-MB-468 cells. Two independent experiments were performed. E, MTT assay showed reduction of mitochondrial activity after combination treatment of 1 mmol/L temozolomide plus 10 μmol/L talazoparib for 24 hours in cells with mtp53 R273H overexpression compared with cells with endogenous mtp53. Cells were seeded at 1.25 x 10⁵ cells per well in 12-well plates and attached overnight. Cells were treated with either 10 μmol/L talazoparib or 1 mmol/L temozolomide, or both for 24 hours. The absorbance was quantified by measuring the absorbance at 550 nm subtracted from the absorbance at 620 nm. All MTT data are represented as mitochondrial dehydrogenase activity as percentage of a dimethyl sulfoxide (DMSO) vehicle-treated control. Three independent experiments were performed with two technical replicates. *, P < 0.05; NS, not significant. F, Chromatin protein levels of mtp53, 53BP1, and fibrillarin were determined by Western blot analysis with or without combination treatment of temozolomide plus talazoparib in MDA-MB-468 mtp53 R273H overexpression compared with cells with endogenous mtp53. Fifty micrograms of chromatin protein was loaded on 10% SDSPAGE gel. Two independent experiments were performed.
R273H expression in TNBC allows the PARPi agent talazoparib in combination with temozolomide to induce cell death because of increased PARP trapping (shown in yellow; see Fig. 5B).

DNA replication is regulated by various protein complexes that regulate origin licensing and firing (licensing and initiating factors), unwinding (helicases), and relaxation (topoisomerases). GOF mtp53 increases DNA replication origin firing (9) and mutant p53 transactivates Cdc7 (11). In addition, mtp53 R273H has been shown to facilitate the interaction of TopBP1 with Treslin, which is induced by Cdk2 under normal conditions (13). We previously identified that mtp53 directly interacts with MCM replication licensing factors in multiple breast cancer cell lines and mice tumors with the analogous human
damage occurs, they help facilitate aberrant repair (ii), allowing cells to survive, promoting tumorigenesis (iii). MCM2-7 on replicating DNA implies a role in tumorigenesis. Model for mutant R273H p53, PARP1, and MCM2–7 on replicating DNA promoting tumorigenesis versus cell death. The coordination of mtp53 R273H, PARP, and MCM2–7 on replicating DNA implies a role in tumorigenesis. A, MCM2–7 (purple), mtp53 R273H (green), and PARP (red) interact with replicating DNA (i); when damage occurs, they help facilitate aberrant repair (ii), allowing cells to survive, promoting tumorigenesis (iii). B, When high PARP and mtp53-expressing cells (i) are treated with talazoparib (yellow) in combination with temozolomide, PARP is trapped on replicating chromatin; this increases unrepaired DNA damage (brick; ii), promoting cell death (iii).

R175H knockin mutation (Trp53R172H/R172H; ref. 8). The overexpression of MCMs is strongly associated with shorter survival in patients with breast cancer (44). Upregulation of Cdc7 expression during mammary tumorigenesis is linked to accelerating cell-cycle progression, arresting tumor differentiation, increasing genomic instability, and reducing disease-free survival (45). Cdc7-mediated phosphorylation triggers the assembly of the initiation complex to fire the replication origins (12). Thus, regulation of Cdc7 kinase to preformed pre-replication complexes is a major determinant for replication timing control.

The DNA fiber assay showed that activated oncogenes induce an increase in the fraction of terminated forks and fork asymmetry, along with a decrease in replication fork speed and increased origin firing (35). Oncogenes induce the firing of novel replication origins, unlike the constitutive origins, which are intragenic and give rise to replication forks that are prone to collapse (46). The collapse of forks initiating from intragenic, oncogene-induced origins can be attributed to replication–transcription conflicts, which is increased in cells with shortened G1 (46). Remarkably, genome-wide isolation and sequencing of Okazaki fragments (OK-Seq) revealed the comprehensive landscape of human genome replication (47). Replication initiation zones are mostly nontranscribed, enriched in open chromatin and with replication fork progression significantly co-oriented with transcription initiation (47). More work is required to elucidate the role of GOF mtp53 on replicating DNA.

PARP inhibitors impair the enzymatic activity of the ADP-ribose addition to substrates, which in turn suppresses the function of PARP in base excision repair, single-strand break repair and DNA damage sensing (48). Many PARP inhibitors also cause the protein to be trapped on chromatin thus causing more DNA damage and killing of cells via the formation of toxic DNA–PARP complexes (49). PARP inhibitors are used on familial and sporadic breast and ovarian cancers with biallelic mutations in the HR repair genes BRCA1, BRCA2, or PALB2 (49). The inhibition of PARP1 function in rapidly dividing cancer cells results in an accumulation of single-strand breaks leading to broken replication forks that are essentially double strand breaks (DSB; ref. 50). These DSBs that normally would be repaired by HR are not repaired in BRCA1-deficient cells. In this study, we demonstrated that PARP1 associated with mtp53 R273H on chromatin and enhanced PARylation also increased this association. Furthermore, higher levels of PARylation were detected in a R273H mtp53–expressing breast cancer PDX model than in a wtp53 expressing PDX model. As PARP activity is associated with the levels of PARylation, the detection of low levels of PAR may indicate an intrinsic low activity of PARP with a limited PARP inhibitor toxicity in tissue that bears wtp53. Figure 4A shows a positive correlation between p53 and PARP in TNBC samples (see red dots). Interestingly ERþ breast cancer samples show a positive correlation for some, but not all, of the patients examined (see green dots). High levels of p53 are associated with stabilized mtp53 and this is found more often in TNBC than in ERþ breast cancer. A possible molecular mechanism for the stabilization of PARP and mtp53 in the samples could be due to mtp53 being stabilized during replication stress, resulting in PARP activation and therefore increased recruitment. Replication stress may be a key facilitator of the activation of pathways that result in stabilization of both mtp53 and PARP. However, there remains the possibility that mtp53 changes the transcription of PARP. Our data demonstrate the complexity of the cross-talk between mtp53 R273H and PARP and suggest that in cancer cells, the increased levels of PARP and mtp53 can be used as a proxy for cell survival in the presence of replication stress. It will be interesting to determine whether talazoparib in combination with the DNA-damaging agent temozolomide suppresses organoid growth in 3D culture and possibly xenograft (and/or PDX) growth in vivo. Our results suggest that the detection of coexpression of mtp53

Figure 5. Model for mutant R273H p53, PARP1, and MCM2–7 on replicating DNA promoting tumorigenesis versus cell death. The coordination of mtp53 R273H, PARP, and MCM2–7 on replicating DNA implies a role in tumorigenesis. A, MCM2–7 (purple), mtp53 R273H (green), and PARP (red) interact with replicating DNA (i); when damage occurs, they help facilitate aberrant repair (ii), allowing cells to survive, promoting tumorigenesis (iii). B, When high PARP and mtp53-expressing cells (i) are treated with talazoparib (yellow) in combination with temozolomide, PARP is trapped on replicating chromatin; this increases unrepaired DNA damage (brick; ii), promoting cell death (iii).
(which is mutated in more than 80% of TNBC) and high expression of PARP may be a good combined biomarker to precisely identify the cancer patient populations that will benefit from combined PARPi talazoparib with temozolomide treatment.

Disclosure of Potential Conflicts of Interest
T. Reiner is a consultant and scientific advisory board member at Theragnostics and a founder and scientific advisory board member at Summit Biomedical Imaging, reports receiving a commercial research grant from Theragnostics and Summit Biomedical Imaging, has received other commercial research support from Pfizer, and has ownership interest (including patents) in Summit Biomedical Imaging. No potential conflicts of interest were disclosed by the other authors.

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Gain-of-Function Mutant p53 R273H Interacts with Replicating DNA and PARP1 in Breast Cancer

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