Dual Disruption of DNA Repair and Telomere Maintenance for the Treatment of Head and Neck Cancer

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Abstract

Purpose: Poly(ADP-ribose) polymerases (PARP) and the Mre11, Rad50, and Nbs1 (MRN) complex are key regulators of DNA repair, and have been recently shown to independently regulate telomere length. Sensitivity of cancers to PARPi is largely dependent on the BRCAness of the cells. Unfortunately, the vast majority of cancers are BRCA-proficient. In this study, therefore, we investigated whether a targeted molecular “hit” on the MRN complex, which is upstream of BRCA, can effectively sensitize BRCA-proficient head and neck squamous cell carcinoma (HNSCC) to PARP inhibitor (PARPi).

Experimental Design: Human HNSCC cell lines and a mouse model with HNSCC xenografts were used in this study. In vitro and in vivo studies were conducted to evaluate the effects and underlying mechanisms of dual molecular disruption of PARP and the MRN complex, using a pharmacologic inhibitor and a dominant-negative Nbs1 expression vector, respectively.

Results: Our findings demonstrate that downregulation of the MRN complex disrupts homologous recombination, and, when combined with PARPi, leads to accumulation of lethal DNA double-strand breaks. Moreover, we show that PARPi and MRN complex disruption induces significantly shortening telomere length. Together, our results demonstrate that dual disruption of these pathways causes significant cell death in BRCA-proficient tumor cells both in vitro and in vivo.

Conclusion: Our study, for the first time, elucidates a novel mechanism for MRN complex and PARP inhibition beyond DNA repair, demonstrating the feasibility of a dual disruption approach that extends the utility of PARPi to the treatment of BRCA-proficient cancers.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with more than 400,000 new cases and 300,000 deaths annually (1). In the United States, HNSCC represents approximately 4% of all malignancies, and in 2013 it was estimated that more than 52,000 patients would be diagnosed with the disease, and approximately 11,500 people would die from their disease (2). Despite advances in medical and surgical management, 5-year survival has only slightly improved over the last two decades to around 65% for all stages across all subsites (2, 3). This has prompted the development of novel molecular therapeutic approaches that target tumor cells while preserving normal tissue function. Intratumoral (i.t.) injection of viral vectors is an attractive approach because HNSCC tumors are often accessible for direct injection of molecular therapy, localizing the therapy to the tumor and draining lymph nodes.

Human telomeres are composed of repetitive noncoding TTAGGG DNA sequences and telomere-associated proteins known as shelterin complex, which consists of six proteins that are collectively responsible for protecting and maintaining normal telomere structure and function (4, 5). In contrast to normal tissue, the majority of human cancers upregulate telomere maintenance to compensate for the telomere shortening that occurs during each replication cycle, allowing cancer cells to proliferate indefinitely. These mechanisms, including upregulation of telomerase [human telomerase reverse transcriptase (hTERT)] and alternative lengthening of telomeres (ALT) pathways, have been identified as potential therapeutic targets.

Synthetic lethality (SL) arises when a simultaneous deficiency in two pathways induces cell death, while disruption of either gene alone is nonlethal (6). This concept has been explored in the context of ovarian and breast cancer, and has
Translational Relevance

Previous preclinical and clinical studies have demonstrated that BRCA-mutated tumors are hypersensitive to poly(ADP-ribose) polymerase (PARP) inhibition. However, these types of tumors occur only in a small minority of patients. To expand the translational applicability of this approach, this proof-of-concept study looks to sensitize BRCA-proficient cancers to PARP inhibition through downregulation of the Mre11, Rad50, and Nbs1 (MRN) complex, which is both upstream of BRCA and has critical functions beyond DNA repair, including telomere maintenance. Our study demonstrates that the concurrent disruption of these two molecular pathways involved in homologous recombination (HR). However, molecular inhibition of the redundant pathways induces SL in these cells. The current understanding of SL is, therefore, centered on preexistent defects in DNA repair pathways, and its therapeutic use has been largely limited to the BRCA-mutated cancer cells (11, 12). In addition, recent studies challenge the current mechanism of SL as incomplete, and point to factors other than DNA repair as being involved (13, 14). To our knowledge, no prior studies have investigated whether it is possible to induce SL through dual disruption of two critical compensatory pathways in tumor cells with no preexistent mutations.

We have previously investigated multiple therapeutic targets focused on DNA repair pathways, including the sensitization of cancer cells to chemotherapy and radiotherapy through the molecular disruption of the Mre11, Rad50, and Nbs1 (MRN) complex (15–19), as well as through poly (ADP-ribose) polymerase (PARP) inhibition (20). The MRN complex, which plays a critical role in the repair of DNA double-strand breaks (DSB), has been recently implicated in telomere maintenance through interaction with the shelterin complex in both hTERT-positive and ALT cancer cells (21–24). Similarly, the PARPs, which are essential for the repair of single-strand breaks (SSB), have been shown to regulate telomere length and function through their interaction with and PARylation of shelterin complex protein telomere repeat-binding factor 1 (TRF1; refs. 25–27).

Given the intertwined roles of PARP and MRN in both DNA repair and telomere maintenance, we hypothesized that the concurrent disruption of these two molecular pathways would induce SL in hTERT-positive BRCA-proficient HNSCC cells, and would serve as a prototype for the use of this dual-disruption concept for the treatment of these cancers. In this study, we investigate this dual-disruption antitumor strategy for the treatment of HNSCC as an alternative to existing chemoradiation regimens.

Materials and Methods

Cell lines

Two human papillomavirus (HPV)–negative human HNSCC cell lines, JHU006 and JHU012, originally derived and genetically characterized at the Johns Hopkins University Head and Neck Laboratories (Baltimore, MD) from human tumor explants, were propagated and maintained in our laboratory for use in this study. They were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in a humidified 37°C incubator at 5% CO₂. Saos-2 and HeLa cells were obtained from the ATCC to be used as positive and negative controls for ALT status, respectively. HeLa cells were cultured in Eagle minimum essential medium supplemented with 10% FBS and 1% penicillin–streptomycin. Saos-2 cells were cultured in McCoy 5a medium modified and supplemented with 10% FBS and 1% penicillin–streptomycin. The cell lines used in this study were not authenticated.

PARP inhibitor

GPI-15427 [10-(4-Methyl-piperazin-1-ylmethyl)-2H-7-oxa-1,2-diazabenzol[d]-anthracen-3-one; MGI Pharma] was used as a PARP inhibitor (PARPi) that is selective and orally bioavailable. It was prepared on the day of use by diluting it in PBS and filtering. For all in vitro experiments, we used 8 μmol/L final concentration in RPMI-1640 full media. For in vivo experiments, the PARPi was administered at a dose of 100 mg/kg as previously described (20).

Adenoviral vector

A previously reported dominant-negative mutant Nbs1 recombinant adenoviral vector (Ad-Nbs1) containing the last 300 amino acids of the C-terminal of Nbs1 cDNA, preserving the Mre11 binding domain, and green fluorescent protein (GFP) under the control of two independent cytomegalovirus (CMV) promoters was used in the experiments (see Supplementary Fig. S1; ref. 17). An adenoviral vector containing only GFP (Ad-GFP) was used as a control. The transduction efficiency of the adenoviral vectors was confirmed by the GFP expression in both cell lines at a multiplicity of infection (MOI) of 10. In this study, 48 hours after treatment, time point was chosen on the basis of preliminary experiments, which demonstrated high levels of gene transduction as determined by GFP expression in vitro (data not shown). Furthermore, this time point allowed for the effective evaluation of the mechanism of action of our proposed strategy.

Western blot analysis

Whole-cell protein was collected from both cell lines 48 hours after treatment, and it was run on a NuPAGE 4% to 12% Bis–Tris gel (Invitrogen) followed by its transfer onto a Hybond polyvinylidenefluoride (PVDF) membrane (GE...
Healthcare). Rabbit polyclonal anti-PAR (BD Pharmingen), anti-PARP and anti-Mre11 (Cell Signaling Technology), anti-Nbs1 (NB-100-143), anti-Rad50 (Santa Cruz Biotechnology) antibodies, polyclonal anti-hTERT (Rockland), anti-BRCA1 (684; Genetex), and mouse monoclonal anti-β actin (Novus Biologicals) antibody were used as primary antibodies.

Alkaline and neutral comet assay

Alkaline and neutral comet assays (Trevigen) were performed as previously described (13, 15, 20) to quantify the DNA damage 48 hours after treatment. Fluorescent images were captured at ×40 magnification using a Nikon 80i coupled with a SPOT2 CCD camera (Diagnostic Instruments) and IPLab 3.71 software (BD Biosciences). Comet measurements and quantitative analysis were performed using Scion Imaging software (28). The mean tail moment (MTM), which directly correlates with the quantity of SSBs under alkaline condition and the quantity of DSBs under neutral condition, was derived from 50 to 100 randomly selected cells per treatment group.

Rad51 foci

Cells were evaluated for HR of spontaneous and PARPi-induced DSBs using the Rad51 foci assay. Briefly, cells were seeded on glass coverslips in 6-well plates. Forty-eight hours after treatment, cells were fixed and permeabilized with 100% acetone for 10 minutes at −20°C, followed by blocking with 1% BSA and incubation with anti-Rad51 (H-92; Santa Cruz Biotechnology) rabbit polyclonal antibody overnight at 4°C. Cells were washed and incubated with Alexa Fluor 594 goat anti-rabbit (Molecular Probes) for 1 hour at room temperature. Mounting was performed with ProLong Gold anti-fade solution (Molecular Probes). Fluorescent images were captured at ×40 magnification as described above. The foci of at least 100 cells per group were quantified using ImageJ 1.44 (NIH, Bethesda, MD).

Telomere length

Analysis of the telomere length was conducted at 48 hours after corresponding treatments, which corresponds to an average of approximately 1.5 population doublings for these cell lines. Culture medium was supplemented with 10 μg/mL Colcemid (Gibco) for 1.5 hours to arrest cells in M-phase. Cells were treated with 60 mmol/L KCl, pelleted by centrifugation, and resuspended in fixative (3:1, methanol:acetic acid). To prepare M-phase chromosome slides, the suspensions were applied to slides and allowed to dry overnight at room temperature. Telomere fluorescent in situ hybridization (Telomere-FISH) was performed using the Telomere PNA FISH Kit (Dako) according to the manufacturer’s instructions. Slides were mounted with Pro-Long Gold anti-fade solution containing 0.2 μg/mL DAPI (Molecular Probes). Fluorescent images were captured, and at least eight metaphase spreads from randomly selected high-powered fields (100× oil lens) on each slide were analyzed. Overlaid images were autothresholded and the average fluorescence intensity of chromosomal ends corresponding to telomere-specific staining intensity was quantified using IPLab 3.71 software (BD Biosciences).

Telomerase activity

Telomerase activity was determined with the TRAPEze Telomerase Detection Kit (Millipore) per the manufacturer’s instructions using the nonisotopic detection option. Briefly, whole-cell protein was extracted using 1× CHAPS buffer containing RNase inhibitor (100 U/mL) for 30 minutes on ice, followed by centrifugation at 12,000 × g for 20 minutes at 4°C. Protein concentration was quantified using the BCA protocol (Pierce, Thermo Scientific), equalized using 1× CHAPS buffer, and stored at −80°C until. On the following day, samples were combined with a Master Mix containing TRAP primer mix and reaction buffer, dNTP mix, TS primers, Taq DNA polymerase recombinant (Invitrogen), and water. As a positive control, HeLa whole-cell protein was included in the experiment. For the heat-inactivation controls, samples were heated at 85°C for 10 minutes before combining with the master mix. Samples were incubated for 30 minutes at 30°C, followed by 30 cycles of PCR with the following conditions: 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute for 30 cycles. Samples were then run on a 12% non-denaturing PAGE gel with 0.5× TBE buffer for 1.5 hours at 250 V. The gel was stained with SYBR Green I (Invitrogen) and visualized with UV transillumination using a Carestream Gel Imaging System (Kodak).

C-circle assay

The ALT status was determined using the C-Circle assay as previously described (29, 30). Briefly, genomic DNA from cell lines was isolated from cells using the DNeasy Blood and Tissue Kit (Qiagen), quantified by UV spectroscopy using the NanoDrop 1000 (Thermo Scientific) and digested HinfI and RsaI restriction enzymes (NEB) and RNase (DNase-free; Roche). The rolling circle amplification of C-circles was performed on 30 ng of DNA with and without Φ29 DNA polymerase (NEB) for 8 hours at 30°C, followed by 20 minutes at 65°C. The C-circles were detected using dot-blotting with an end-labeled 32P-(CCTAA)6 telomeric oligonucleotide probe. Image quantification was performed using ImageJ (NIH) where C-circles were determined by calculating the fold increase in telomeric signal (optical density) in samples with and without Φ29 DNA polymerase (Φ29+ and Φ29−) and are represented as arbitrary units (A.U.)

Telomere genomic instability

The telomere genomic instability was assessed through the use of Telomere-FISH, which was performed as described in the Materials and Methods. Metaphase chromosome spreads were analyzed for the loss of telomere fluorescence signal at the ends of chromatids.

Molecular imaging

Three days after treating a JHU006 tumor xenograft with i.t. injection of Ad-Nbs1 as described in the Materials and
Methods, the mouse was re-anesthetized and placed in the imaging chamber of the Bruker FX-PRO Molecular Imaging system (Bruker). Black and white (BW), X-Ray, and GFP (ex/em 480/535) images were recorded using the appropriate filter sets, and merged for evaluation of GFP expression.

**Flow cytometry analysis**

Seven days after treatment of JHU006 tumor xenografts with i.t. injection of either PBS (Control) or Ad-Nbs1, mice were re-anesthetized and the tumors were exposed and resected off the flanks. The tumors were placed in ice-cold sterile PBS. Next, tumors were cut in small pieces (1–2 mm) using a scalpel with sterile blade, followed by washing with PBS in duplicate. Samples were digested using TrypLE Express Enzyme (Life Technologies), and passed through a 100-µm filter. Ten minutes before analysis, DAPI (0.5 µg/mL final concentration) was added as viability marker.

Samples were analyzed at the Flow Cytometry & Cell Sorting Core Facility at the University of Pennsylvania (Philadelphia, PA) using a BD LSR II Flow Cytometer System (BD Biosciences) coupled with the DiVa Software. Violet (405) and blue (488) lasers were used for the detection of DAPI and GFP fluorescence in at least 10,000 cells per sample. Only singlets and live cells were included in the analysis, which was performed using FlowJo software (FlowJo). The log GFP fluorescence intensities of the samples (labeled as FITC) were plotted on a histogram for interpretation.

**In vitro cell growth**

Growth was evaluated for using a soluble tetrazolium salt MTT assay as previously described (15, 16, 20). Briefly, cells were seeded into 96-well plates and treated. At the desired time points, cells were incubated with of the MTT solution and analyzed for the formation of formazan using a MRX II microplate reader (Dynex Technologies).

**In vitro apoptosis analysis**

JHU006 and JHU012 cells were also evaluated for apoptosis after 24, 48, and 72 hours using an Annexin V–AF647 staining kit (eBiosciences) following the manufacturer’s instructions. Briefly, cells were seeded into dishes and treated. At the indicated time points, cells were trypsinized, counted, and resuspended in Annexin staining buffer in 5 mL FACS tubes. Annexin-V-FA647 and DAPI (0.5 µg/mL final concentration) were added and cells were incubated for 15 minutes at room temperature in the dark and analyzed within 1 hour. Samples were analyzed at the Flow Cytometry & Cell Sorting Core Facility at the University of Pennsylvania using a BD LSR II Flow Cytometer System (BD Biosciences) coupled with the DiVa Software. Violet (405), blue (488), and red (635) lasers were used for the detection of DAPI, GFP, and AF-647 fluorescence, respectively, in at least 10,000 to 20,000 cells per sample. Singlets and GFP-positive cells (in Ad-GFP or Ad-Nbs1 groups) were included in the analysis, which was performed using FlowJo software (FlowJo). Cells positive for early [DAPI (−)/AnnexinV-AF647 (+)] and late [DAPI (+)/AnnexinV-AF647 (+)] apoptosis were considered positive for the apoptosis analysis. Apoptosis rates relative to controls (Control for PARPi; Ad-GFP for Ad-Nbs1 and Ad-Nbs1+Pi) were plotted. Two independent experiments were performed.

**In vivo tumor growth**

Experiments were performed on 6-week-old athymic female BALB/c nude mice maintained in an animal facility. Animals were cared for and used in accordance with protocols approved by the University of Pennsylvania School of Medicine Institutional Animal Care and Use Committee. The animals were randomly divided into the following treatment groups: saline control, i.t. Ad-Nbs1, PARPi, Ad-GFP with PARPi, and Ad-Nbs1 with PARPi, with each group consisting of 6 to 10 mice. A total of 1 × 10⁶ JHU006 or JHU012 cells were injected into the dorsal flank. When tumors reached an average volume of 80 to 100 mm³, the mice were anesthetized by intraperitoneal injection of tribromoethanol, skin flaps were raised, and the internal tumor volume was measured at the time of treatment and at the end of the experiment (7–10 days after treatment). A single i.t. injection of virus (50 µl, 2 × 10¹⁰ PFU/mL) or equal volume of saline was performed using a Hamilton syringe as previously described (15, 18). The PARPi was administered orally as a single dose on the same day after the mice recovered from anesthesia. For the dynamic tumor volume experiment, external tumor diameter was measured using digital calipers, and tumor volume was calculated using the formula A² × B × 0.536, where A represents the smallest diameter and B equals the largest diameter as previously described (15).

**Immunohistochemistry**

Immunohistochemistry for Mre11 was performed using VECTASTAIN Elite ABC kit (Vector Laboratories) as previously described to evaluate the MRN expression following i.t. injection with Ad-Nbs1. Procedures were followed by the manufacturer’s protocol. Human-specific rabbit polyclonal anti-Mre11 antibody (Cell Signaling Technology) was used at a dilution of 1:250. DAB peroxidase substrate kit (Vector Laboratories) was used for visualization. At least 10 randomly selected low-power fields (×10) per tumor sample were digitally recorded. The Mre11 expression was measured using Immunoratio, where percent (%) represents the labeling index (percentage of DAB-stained area out of the total nuclear area; ref. 31).

**In vivo apoptosis analysis**

The ApopTag Peroxidase Kit (Chemicon) detects early DNA fragmentation associated with apoptosis in tumors extracted from treated mice, using the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay. Tumors samples were harvested from each mouse and were fixed in 4% paraformaldehyde and embedded in paraffin using standard procedures. Tissue samples were cut in 4-µm thick sections, and mounted on slides. Ten to 15 randomly selected fields (×20 magnification) were acquired for analysis using the imaging system described above. The number of TUNEL-positive cells stained...
(brown) from each image was counted and the results plotted as relative to the Control treatment group.

Statistical analysis

The SigmaStat version 2.03 was used for the statistical analysis. The Student t test was used to evaluate statistical significant differences between groups in all experiments, except the MTT assay in which the Mann–Whitney U test was used. A P value of less than 0.05 was considered statistically significant.

Results

PARP inhibition induces SSBs in HNSCC cell lines

As the first step in our study, we wanted to evaluate whether the PARPi, CPI-15427, could inhibit the PARP activity in JHU006 and JHU012 HNSCC cell lines. We performed Western blot analysis to detect the poly(ADP-ribose) (PAR) levels in these cells, and as shown in Fig. 1A, treatment with PARPi for 48 hours significantly reduced the PAR levels by more than 57% in JHU006 and more than 47% in JHU012 cells. Next, we evaluated the effects of PARP inhibition on the ability of cancer cells to repair DNA SSBs, which are the most common type of DNA damage and are primarily repaired by base excision repair, a process catalyzed by PARP-1 through the formation of (PAR) chains (32–35). It has previously been shown that the use of PARP inhibition leads to the accumulation of SSBs (12, 36). We looked to evaluate whether the use of PARPi would lead to an increase in SSBs in JHU006 and JHU012 HNSCC cell lines. As shown in Fig. 1B, as compared with control, SSBs were significantly increased in cells exposed to the PARPi represented by an increase in the relative MTM in the alkaline comet assay.

MRN disruption affects HR, and in combination with PARPi leads to persistent DNA damage in BRCA wild-type HNSCC cell lines

We have previously demonstrated that HNSCC cell lines treated with a dominant-negative adenoviral construct expressing a truncated (C-terminal only) Nbs1 protein, mutant Nbs1 (Ad-Nbs1), results in downregulation of all three components of the MRN complex (18). As shown in Fig. 1C and D, Ad-Nbs1 significantly downregulated the endogenous expression levels of MRN proteins compared with control and viral control (Ad-GFP) treatment groups in JHU006 and JHU012 HNSCC cell lines. PARP inhibition did not have a significant effect on wild-type MRN complex protein expression compared with control.

During HR, wild-type Nbs1 binds to γH2AX through the BRCT/FHA binding domain, and subsequently recruits the other components of the MRN complex through the C-terminal Mre11-binding domain (37). Subsequently, Nbs1 recruits and activates the ataxia–telangectasia mutated (ATM) kinase, ultimately resulting in the amplification of the DNA-damage response (38). Because our Ad-Nbs1 construct lacks the BRCT/FHA domain, we hypothesized that cells treated with Ad-Nbs1 would be unable to repair both spontaneous and PARPi-induced DSBs. To ensure that our findings were not due to BRCA1 deficiency that has previously been shown to increase sensitivity to PARP inhibition (13), we confirmed that both JHU006 and JHU012 cells expressed wild-type BRCA1 protein (Supplementary Fig. S2A). Rad51, a recombinase that drives the HR process and recruits and forms foci at the DNA DSB sites, is an established specific cellular marker for HR activity (39, 40). In addition, PARP inhibition induces Rad51 recruitment and foci formation, reflecting an increased assembly of HR repair complexes to repair persistent DNA lesions (41). As shown in Fig. 2A and B, PARP inhibition significantly increased Rad51 foci formation as compared with control in both JHU006 and JHU012 cell lines, suggesting a potential increase in DSBs and their subsequent repair. In contrast, we found that Ad-Nbs1 treatment significantly attenuated Rad51 foci formation, as compared with all groups in both cell lines, supporting our hypothesis that the downregulation of endogenous Nbs1 leads to a decrease in HR. Furthermore, PARP inhibition in cells treated with Ad-Nbs1 showed a significant decrease in Rad51 foci formation as compared with PARPi alone or Ad-GFP with PARPi, indicating that cells with downregulated endogenous Nbs1 are unable to mount an appropriate HR response to PARP inhibition. Overall, these data illustrate the essential role of an intact MRN complex in repairing DSBs induced by PARP inhibition.

Replicating cells are repeatedly exposed to endogenous and exogenous genotoxic stress, which has been estimated to cause tens of thousands of SSBs per day (33). Cancer cells undergo replication at a remarkably high rate as compared with normal cells and are potentially exposed to even higher levels of genotoxic stress. During replication, unrepaired SSBs are converted into DSBs that can then be repaired through highly evolved HR mechanisms (42). We hypothesized that in cells with downregulated Nbs1, both spontaneous and PARPi-induced DSBs would persist due to a deficiency in the HR repair pathway. In both JHU006 and JHU012 cell lines, we found that Ad-Nbs1 treatment led to a significant increase in DSBs when compared with control groups, as measured by the relative MTM in the neutral comet assay, illustrated in Fig. 2C and D. Moreover, when PARP was inhibited in Ad-Nbs1-infected cells, there was a greater accumulation of DSBs as compared with all groups. Taken together, our data suggest that if highly proliferative cancer cells deficient in HR repair proteins are exposed to an inhibitor of base excision repair, such as a PARPi, there is an accumulation of lethal DNA DSBs.

Disruption of PARP and the MRN complex leads to telomere shortening and instability in telomerase-positive cancer cells

It has been shown that both Nbs1 and PARP, independently, play critical roles in the maintenance of telomeres through their interaction with the shelterin complex. However, it is not known if these mechanisms act in sync to regulate telomere length in hTERT-positive cancer...
cells. In its nonphosphorylated, non-PARSylated form, TRF1 acts mainly as a negative regulator of telomere length preventing hTERT access to the ends of telomeres (4, 22, 25, 26, 43). When TRF1 is phosphorylated (pTRF1) through MRN complex mediated ATM activation, it no longer shields the telomeric DNA, and telomerase is able to elongate the telomere (22). Similarly, PARsylation of TRF1 has been shown to decrease its binding to the telomeres (25, 26), allowing hTERT to extend the ends. Therefore, we hypothesized that cells with downregulated Nbs1 and those exposed to PARP inhibition, independently, would undergo a reduction in telomere length in HNSCC cells, which express wild-type hTERT and have ALT-negative status (Supplementary Fig. S2). Furthermore, we hypothesized that the combination of MRN disruption and PARP inhibition would lead to an enhanced reduction in telomere length. Although telomere shortening needs DNA replication, it has been shown that human
Figure 2. Disruption of HR leads to persistent accumulation of DSBs in HNSCC cell lines. A, representative images of Rad51 foci formation was used as a surrogate for HR in both JHU006 and JHU012 tumor cells (original magnification, >40). B, quantified Rad51 foci data for both cell lines 48 hours after various treatments. Mean percentage of cells with more than 5 foci ± SEM is shown. JHU006 and JHU012 cells treated with PARP-1i had a significant 15% (P < 0.001) and 17% (P < 0.002) increase in Rad51 foci, respectively, as compared with control groups. In contrast, Ad-Nbs1 treatment led to a significant reduction in the ability of the cancer cells to form Rad51 foci compared with all groups in both JHU006 (P < 0.002) and JHU012 (P < 0.02) cell lines. Furthermore, cells infected with Ad-Nbs1, when treated with PARPi, showed a significant decrease in Rad51 foci formation as compared with PARPi and Ad-GFP + PARPi treatment groups in both cell lines (P < 0.001), leading to a potential accumulation of lethal DNA DSBs. C, Ad-Nbs1 treatment led to 3.8- and 2.5-fold increases in DSBs in JHU006 (P < 0.001) and JHU012 (P < 0.01), respectively, compared with control groups. Importantly, the combination of Ad-Nbs1 and PARPi resulted in a much greater increase in DSBs compared with all groups in JHU006 (P < 0.003) and JHU012 (P < 0.001). D, representative "comets" produced by the neutral comet assay representing DNA DSBs in JHU012 cancer cells 48 hours after treatment (scale bar, 10 μm; original magnification, >40).
hTERT-positive cancer cells, HeLa cells, are susceptible to rapid changes in telomere length after a short treatment with PARPi (44), and therefore this provides the rationale for the early evaluation of telomere length in this study. The results of our Telomere-FISH experiments performed 48 hours after treatment, as shown in Fig. 3, indicate that when both JHU006 and JHU012 tumor cells were exposed to either PARPi or Ad-Nbs1 individually, there was a significant reduction in telomere length as compared with control. Importantly, when cells were exposed to PARPi and Ad-Nbs1 in combination, they experienced a markedly greater telomere shortening compared with control or to either treatment used alone. Similarly, treatment of both cell lines with PARPi resulted in significant telomere instability as measured by the increase in loss of telomere signal (Supplementary Fig. S3). Interestingly, combination of Ad-Nbs1 with PARPi resulted in even greater instability (Supplementary Fig. S3) than either therapy alone. Together, these findings illustrate that this dual-disruption approach has a significant effect on the telomere maintenance of hTERT-positive cancer cells, elucidating a novel mechanism for SL.

MRN complex disruption together with PARP inhibition significantly suppresses HNSCC cell proliferation in vitro and leads to regression of BRCA-proficient human HNSCC xenografts

From the above findings, we hypothesized that our combination treatment would lead to a significant reduction in cell proliferation in vitro. Our MTT assay data, shown in Fig. 4A and B, clearly demonstrate the anti-proliferative effect of a single combination treatment with Ad-Nbs1 and PARPi as compared with control or either treatment alone, in both cell lines. Next, we wanted to evaluate whether this effect was due to apoptosis. Annexin V staining data demonstrated significant apoptotic rates in JHU006 and JHU012 cells treated with PARPi as compared with control, as shown in Fig. 4C and D. Similarly, Ad-Nbs1 resulted in significantly increased apoptotic rates compared with Ad-GFP. As expected, treatment with Ad-Nbs1 and PARPi in combination resulted in a significantly greater apoptotic rates as compared with Ad-GFP and to either treatment alone.

These significant findings led us to further evaluate if this effect would translate to in vivo experiments in mice.
harboring JHU006 or JHU012 xenografts. In agreement with our in vitro results, mice that received a single combined treatment with Ad-Nbs1 and PARPi experienced a significantly greater antitumor effect, as compared with all other groups, as illustrated in Fig. 5A and B, suggesting that the SL effect observed in vitro can be extended to a biologic in vivo model of HNSCC.

First, we evaluated the gene transduction and effect of i.t. injection with Ad-Nbs1 on the expression of the MRN complex in vivo. As shown in Supplementary Fig. S4, we observed GFP expression at day 3 after i.t. injection using a molecular imaging, which indicated successful gene transduction. Seven days later, we performed flow cytometry analysis, which showed that more than 20% of...
Dual Disruption of Head and Neck Cancer

Breaks, as well as telomere shortening, ultimately trigger molecular "hit" on the MRN complex, would sensitize apoptotic cells when Ad-Nbs1 and PARPi were used in combination, illustrating that the cellular lethality of this combination treatment is, at least, partially mediated by apoptosis. Altogether, these results provide support that, in a xenograft model of HNSCC, combination treatment directed toward impairment of the MRN and PARP pathways induces an accumulation of irreparable DNA-strand breaks, as well as telomere shortening, ultimately triggering apoptosis.

Discussion

The utilization of the SL concept for the treatment of cancer has been pioneered in the clinical setting using PARPi in BRCA-mutated breast and ovarian cancers (7–10). However, the requirement of a preexisting mutation has limited the clinical utility of this approach, and chemoradiation still remains standard of care for advanced HNSCC (45). In this study, we evaluated whether a targeted molecular "hit" on the MRN complex, would sensitize BRCA-proficient tumors, such as HNSCC, to PARPi. Because HNSCC tumors are usually confined to locoregional spread and are clinically accessible, i.t. injection of molecular therapy has been used in multiple clinical trials with promising results (46–50). With this in mind, in this proof-of-concept preclinical study, we looked to demonstrate that SL can be induced in BRCA wild-type cancers through our "dual-hit" approach using a clinically established model of i.t.-based gene delivery combined with a systemically delivered drug. Although at the moment, our treatment strategy is not intrinsically designed to discriminate normal with cancer cells, we did not observe weight loss or any side effects or complications from these procedures within the course of the treatment. Nonetheless, we strongly believe that as novel targeted viral and nonviral molecular therapy approaches evolve, they will greatly improve our ability to precisely induce SL in the desired cancer cell population.

Here, we demonstrate a significant antitumor effect of concurrent MRN complex disruption and PARP inhibition on HNSCC cells. Furthermore, our results suggest two mechanisms that may contribute to this lethal effect: DNA repair disruption and telomere shortening (see Fig. 6). Our findings suggest that Ad-Nbs1 downregulates wild-type Nbs1, disrupts HR, and, when used in combination with PARPi, leads to a significant accumulation of DSBs. These results support the notion that dual disruption of HR and SSB repair leads to irrecoverable DNA damage. Moreover, our results demonstrate that PARP and the MRN complex, together, play an important role in the telomere maintenance of hTERT-positive cancer cells, as dual disruption of these pathways caused significant telomere shortening. Although the present experimental design makes it difficult to establish a direct causal relationship, we and others are actively pursuing this line of research, and hope to find answers to these important mechanistic questions in future studies.

In conclusion, this proof-of-concept study demonstrates that the SL approach can be used in BRCA-proficient cancer cells both in vitro and in vivo, through a dual molecular disruption. Our findings expand the present understanding of SL beyond the currently used paradigm of drug-induced DNA repair impairment in the setting of preexisting deficiencies in DNA repair genes. Indeed, in HNSCC cells that do not harbor well-characterized deficiencies in DNA repair, this true SL effect can be induced by targeting two interrelated DNA repair and telomere maintenance pathways. These findings should prompt the investigation of other critical molecular pathways as potential mediators of SL and, consequently, as targets for cancer treatment.
Importantly, with the development and improvement of novel molecular inhibitors of DNA repair pathways, such as the MRN complex Mirin (51), as well as other PARPis, we believe our study opens the door to many more molecular therapy approaches using targeted systemic delivery. Finally, as clinical samples from patients with HNSCC undergoing clinical trials with PARPis become available, it is critical to identify biomarkers that may predict response to these inhibitors, and therefore serve as tools to better stratify patients who may benefit from them.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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Figure 6. Overview diagram of the dual-disruption approach. A, in dividing cells, DNA SSBs are rapidly repaired by PARP-mediated SSB-repair pathways to ensure genomic integrity. When cells are treated with PARPi, these SSBs persist and collapse the replication fork, generating DSBs. In turn, these are repaired by the DSB-repair machinery, which depend on the MRN complex for damage recognition and amplification of the response. However, in the setting of disrupted DSB-repair pathways, PARP-inhibition leads to accumulation of DSBs, inducing apoptosis. B, the upregulation of telomerase (hTERT) is a hallmark of tumor cell immortalization in more than 85% of cancers. It has been demonstrated that TRF1, a key member of the shelterin complex, acts a negative regulator of telomere length by blocking hTERT’s access to the telomere. MRN-dependent phosphorylation, as well as PARsylation of TRF1 have been shown to independently modulate TRF1’s function by affecting its binding to the telomere and, therefore, allowing for continuous hTERT-dependent elongation of the telomere. We propose that combined inhibition of phosphorylation and PARsylation of TRF1 through MRN disruption and PARP inhibition, can enhance TRF1’s function by preventing hTERT’s access. Upon each subsequent cell division, telomeres would become critically short, leading to apoptosis. Altogether, these mechanisms highlight two independent but critical roles of PARP and MRN in cancer cells: enhanced DNA repair and telomere maintenance, and illustrate a dual-disruption approach that extends the current use and understanding of SL of PARPis for the treatment of BRCA-proficient cancers.
Dual Disruption of Head and Neck Cancer

References


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## Dual Disruption of DNA Repair and Telomere Maintenance for the Treatment of Head and Neck Cancer

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