PTEN Deletion in Prostate Cancer Cells Does Not Associate with Loss of RAD51 Function: Implications for Radiotherapy and Chemotherapy

Michael Fraser1,3, Helen Zhao1, Kaisa R. Luoto1,3, Cecilia Lundin4, Carla Coackley1,3, Norman Chan1,3, Anthony M. Joshua1, Tarek A. Bismar5, Andrew Evans1,2, Thomas Helleday4, and Robert G. Bristow1,3

Abstract

Purpose: PTEN deletions in prostate cancer are associated with tumor aggression and poor outcome. Recent studies have implicated PTEN as a determinant of homologous recombination (HR) through defective RAD51 function. Similar to BRCA1/2-defective tumor cells, PTEN-null prostate and other cancer cells have been reported to be sensitive to PARP inhibitors (PARPi). To date, no direct comparison between PTEN status and RAD51 expression in primary prostate tumors has been reported.

Experimental Design: Prostate cancer cell lines and xenografts with known PTEN status (22RV1-PTEN+/−, DU145-PTEN+/−, PC3-PTEN−/−) and H1299 and HCT116 cancer cells were used to evaluate how PTEN loss affects RAD51 expression and PARPi sensitivity. Primary prostate cancers with known PTEN status were analyzed for RAD51 expression.

Results: PTEN status is not associated with reduced RAD51 mRNA or protein expression in primary prostate cancers. Decreased PTEN expression did not reduce RAD51 expression or clonogenic survival following PARPi among prostate cancer cells that vary in TP53 and PTEN. PARPi sensitivity instead associated with a defect in MRE11 expression. PTEN-deficient cells had only mild PARPi sensitivity and no loss of HR or RAD51 recruitment. Clonogenic cell survival following a series of DNA damaging agents was variable: PTEN-deficient cells were sensitive to ionizing radiation, mitomycin-C, UV, H2O2, and methyl methanesulfonate but not to cisplatin, camptothecin, or paclitaxel.

Conclusions: These data suggest that the relationship between PTEN status and survival following DNA damage is indirect and complex. It is unlikely that PTEN status will be a direct biomarker for HR status or PARPi response in prostate cancer clinical trials. Clin Cancer Res; 18(4); 1015–27. ©2011 AACR.

Introduction

The PTEN gene encodes a dual specificity lipid/protein phosphatase which antagonizes the activation of the phosphatidylinositol-3′-OH-kinase (PI3K)/AKT pathway. Mono- and biallelic losses of the PTEN gene have been implicated in prostate cancer progression and inferior clinical outcome (1–6). In a number of models, the PTEN protein mediates its antitumorigenic effects via PI3K/AKT-dependent and PI3K/AKT-independent pathways (7), and PTEN-independent AKT signaling has been implicated in the MRE11-ATM DNA damage response (DDR) (8). Murine embryonic fibroblasts (MEF) lacking PTEN were recently observed to have high levels of genomic instability and increased endogenous DNA double-strand breaks (DSB) associated with a reduction in the expression of RAD51 [a key gene involved in homologous recombination (HR) repair of DSBs]. Restoration of PTEN in PTEN−/− MEFs restored RAD51 expression in a manner independent of its phosphatase activity (7). However, subsequent reports in human tumor cell lines have shown conflicting data as to whether PTEN loss is associated with a reduced expression of RAD51 (9, 10). To date, no information exists as to whether PTEN gene status determines RAD51 expression in primary prostate cancers in vivo.

A defined link between PTEN status and HR function in prostate and other human tumors would be important as it would support the treatment for PTEN-null tumors with
agents targeted against defects in DNA repair. An example of this approach is the use of inhibitors of PARP as single agents in germ line BRCA1/2-defective ovarian, breast, and prostate cancers that are HR defective (11). The PARP1 and PARP2 proteins are required for repair of DNA single-strand breaks (SSB) and in cells treated with small-molecule inhibitors of PARP (PARPi), unrepaired SSBs at replication forks are converted into DSBs which require HR-mediated repair to offset cell lethality. Tumor cells lacking HR function (e.g., deficient in BRCA1 or BRCA2 expression) are exquisitely sensitive to PARPi due to the inability to repair replication-associated DSBs; this results in “synthetic cell lethality” (12–15). The results of clinical trials with PARPi in germ line BRCA1- and BRCA2-deficient tumors are promising, but not perfect. It is now recognized that biomarkers that predict functional losses in DNA repair activity, in addition to mutations in DNA repair genes, will be required to more accurately predict clinical PARPi efficacy (11, 16, 17).

As a potentially important biomarker of DNA repair status, recent reports have suggested that tumor cells that lack PTEN have a marked reduction in RAD51-dependent HR and are therefore sensitive to PARPi in vitro and in vivo (18–20). This suggests that many sporadic tumors could be amenable to PARPi-specific treatments or other agents that are highly toxic to HR-deficient tumor cells such as mitomycin C (MMC), cis-platinum and ionizing radiation (IR; refs. 21–23). Novel trials using PARPi in prostate and other cancers could therefore stratify patients on the basis of intact or abrogated function of the HR, FA, DDR (MRE11-ATM) and now, PTEN pathways (24–26). On the basis of a recent prostate cancer–specific report, they may also be stratified by the presence or absence of aberrant signaling associated with a TMPRSS2:ERG fusion (27, 28). BRCA2-deficient prostate cancers are particularly aggressive and the use of PARPi in their treatment could help with overcoming castration resistance (29). Similarly, as PTEN loss and TMPRSS2:ERG fusions are common events in high-grade and castrate-resistant prostate cancers (CRPC; ref. 2), the additional use of PARPi in these tumors would be an important new therapeutic option (27, 28).

We previously reported that prostate cancer cells were defective in SSB, DSB, and BER gene expression and selected functional repair endpoints when compared with normal prostate epithelium or stromal cells (30). We therefore evaluated whether PTEN loss in human prostate cancer cells is associated with loss of RAD51 expression and HR and leads to altered clonogenic sensitivity. The current report represents, to our knowledge, the first systematic study of the relationship between PTEN status and RAD51 expression in primary prostate cancers and cell lines.

Materials and Methods

Cell culture

H1299 human lung carcinoma cells were cultured in α-minimum essential medium supplemented with 10 mmol/L HEPES. Prostate cancer cell lines with varying TP53 status (31) included DU145 (mutant TP53), 22RV-1 (wild-type TP53), and PC3 (null TP53); these were cultured as previously reported (30). These cell lines are all negative for the TMPRSS2:ERG fusion (32). HCT116-PTEN+/− and HCT116-PTEN−/− cells (a generous gift from Dr. Todd Waldman, Georgetown University, Washington DC) and U2OS cells (American Type Culture Collection) were cultured in McCoy’s 5A media. Ataxia telangiectasia-like disorder (ATLD; MRE11 deficient) fibroblasts were cultured as previously reported (8). All cell media were supplemented with 10% fetal calf serum, penicillin, streptomycin, and were grown at 5% CO2, 21% O2, and 37°C. The origin and correct status of all cell lines used for this study was confirmed by short tandem repeat DNA analyses as previously described (8, 33).

siRNA transfections and homologous recombination assay

Cells were seeded for 18 hours in 6-well plates such that their density on the day of transfection was approximately 35%. Cells were transfected with siRNA duplexes to RAD51 (0.25 nmol/L), PTEN (1 nmol/L), or control siRNA with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. HR-dependent DNA DSB repair was assessed by the DR-GFP/Ise-I assay, as previously described (34).

Western blot analysis

Cells were lysed and subjected to Western blot analysis as previously reported (34). Primary antibodies were as follows: rabbit anti-RAD51 (Santa Cruz Biotechnologies; 1:1,000), rabbit anti-PTEN (Cell Signaling Technologies; 1:1,000), rabbit anti-phospho-AKT (S473; Cell Signaling;

Translational Relevance

Biomarkers that reflect functional DNA repair deficiencies are urgently needed to personalize cancer treatment with synthetic lethality. PARP inhibitors (PARPi) preferentially kill tumor cells with defective homologous recombination (HR) and/or DNA damage response (DDR) during the repair of DNA single-strand breaks and double-strand breaks. The PTEN gene is lost in many prostate cancers and PTEN status has been reported to confer sensitivity to PARPi via RAD51 downregulation, which raises the possibility of individualizing treatment of PTEN-null tumors with PARPi. Using preclinical prostate cancer models and primary prostate cancer tissues, we show that PTEN loss does not predict HR function nor RAD51 expression as the basis for profound sensitivity to irradiation, chemotherapy, or PARPi. Instead, other DDR genes, including MRE11, may confer PARPi sensitivity. PTEN status alone is unlikely to be a direct and predictive biomarker of HR or RAD51 function in prostate cancer radiotherapy, chemotherapy, or PARPi clinical trials.
washed, and then allowed to dry overnight at 37°C. The cells were stained with methylene blue for 1 hour, and once colonies of more than 50 cells were observed, the cells were counted manually (using Trypan Blue exclusion to ensure seeding of viable cells) and then seeded in triplicate at a density of approximately 6,500 cells per cm² in 6-well plates in the presence of PARP inhibitor (PARPi; KU-0059436; Olaparib; PARPi) at a final concentration of 1 μmol/L, or in the presence of dimethyl sulfoxide (DMSO) as a vehicle control. Cells were harvested by trypsinization at 1, 2, 4, and 8 days postseeding, and cell number was assessed by manual counting with a hemocytometer. For cell-cycle analyses, cells were stained with propidium iodide and analyzed by a fluorescence-activated cell sorting Aria flow cytometer, as previously described (35).

ATLD fibroblasts (and isogenic fibroblasts expressing either wtMRE11 or an endonuclease-deficient mtMRE11; see ref. 8 for details) were counted manually (using Trypan Blue exclusion to ensure seeding of viable cells) and then seeded in triplicate at a density of approximately 6,500 cells per cm² in 6-well plates in the presence of PARP inhibitor (PARPi; KLI-0559436; Olaparib; PARPi) at a final concentration of 1 μmol/L, or in the presence of dimethyl sulfoxide (DMSO) as a vehicle control. Cells were harvested by trypsinization at 1, 2, 4, and 8 days postseeding, and cell number was assessed by manual counting with a hemocytometer. For cell-cycle analyses, cells were stained with propidium iodide and analyzed by a fluorescence-activated cell sorting Aria flow cytometer, as previously described (35).

**PARP activity assay**

Cells growing in log phase were pretreated for 1 hour with 2.5 μmol/L PARPi (or DMSO as control). Cells were lysed in 1X PARP Lysis Buffer from the Universal Chemiluminescent PARP Assay Kit (Trevigen Inc.). Protein concentrations were determined and PARP activity was assessed using 30 μg of total protein per experimental group, according to the manufacturer’s instructions. Activated DNA was omitted from some reactions to obtain a measure of basal PARP activity in treated versus untreated cells.

**Primary prostate cancer and xenograft studies**

A tissue microarray (TMA) was constructed using donor cores from 142 radical prostatectomy (RP) specimens with usual acinar-type prostate carcinoma obtained at University Health Network (UHN) between 2001 and 2002. The RP slides from all 142 cases were reviewed to identify prostate cancer foci that would be suitable for sampling. Where possible, the TMA was constructed with up to six 0.6 mm donor cores from each RP specimen. In cases of multifocal and bilateral carcinoma, we attempted to include 3 donor cores from the largest foci in each lobe of the prostate. Distinct tumor foci were defined as those separated by a distance of 3 mm or more in a single donor block or 4 mm or more in adjacent donor blocks (above or below). Where possible, we also specifically sampled areas with different Gleason patterns within each focus of tumor. The resulting TMA comprised 733 donor cores distributed in 3 paraffin blocks. Standard 5 μm hematoxylin and eosin sections from each TMA block were stained with an Aperio ScanScope CS (Aperio). The digital slides were reviewed to confirm the presence of representative tumor in each donor core and, when applicable, to annotate specific Gleason patterns in each core. Standard clinical follow-up data, representing 7 to 9 years of follow-up, were compiled for each case represented on the TMA by a UHN RP clinical database. Prostate cancer cell line xenografts and human prostate cancer TMAs were stained with rabbit anti-RAD51 (Santa Cruz Biotechnologies) or rabbit monoclonal anti-phospho-AKT (Ser473; Cell Signaling), as previously described (33). In selected studies, the hypoxic biomarker EF5 was injected into tumor-bearing animals prior to sacrifice as previously described (Chan and colleagues; Can Res; 2010). RAD51 IHC signal was quantified with a custom algorithm that considers both immunohistochemistry (IHC) signal intensity and signal distribution, normalized for total area. Results are presented as arbitrary relative units.

**FISH**

Three color interphase FISH (for PTEN and TMPRSS2:ERG) was applied to formalin-fixed paraffin-embedded prostate cancer TMAs, as previously described (2). Analyses were done by an epifluorescence microscope (Zeiss Axio Imager) equipped with a triple band pass filter set (DAPI/green/orange), dual band pass filter set (green/orange) and single band pass filters (DAPI, green, red, and orange) (Chroma). Image capture was done with a digital ProgRes MF video camera (JENOPTIK) and the fluorescence image acquisition software ISIS (MetaSystems). PTEN genomic losses and TMPRSS2:ERG fusions were evaluated for each probe by counting signals in 100 nonoverlapped, intact interphase nuclei per tumor tissue. Nuclei with signals from each fluorochrome and complete 4’,6-diamidino-2-phenylindole (DAPI) nuclear staining were randomly selected for scoring from tumor regions.

**RNA isolation, reverse transcription PCR, and real-time quantitative PCR**

Total RNA was isolated with the RNAeasy isolation kit (QIAGEN). Sample RNA or human reference RNA (Stratagene) was treated with DNase I (Roche Diagnostics). Reverse transcription PCR (RT-PCR) was carried out by the TaqMan Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR analysis of RAD51 was carried out as previously described (34).

**UV laser microirradiation and immunofluorescence microscopy**

UV laser microirradiation (UVLM) was carried out as previously described (8). Briefly, cells to be subjected to UVLM were seeded on round #1.5 coverslips, pretreated with 5-bromo-2’-deoxyuridine (BrdU) for 24 hours and then transferred to a humidified, temperature-controlled live cell chamber for irradiation. Cells were fixed in...
paraformaldehyde 30 min postirradiation, and double stained with the indicated antibodies. Nuclei were visualized by staining with DAPI.

Stained cells were imaged as previously described (8), using an Olympus Spinning Disk Confocal microscope with a 100× Olympus objective lens, producing 0.16 × 0.16 μm pixels. Z-stacks were obtained at a resolution of 0.25 μm per slice, yielding 0.16 × 0.16 × 0.25 μm voxels. Images were subjected to 3D deconvolution, as previously described (8).

**Gene expression analyses**

Gene expression analyses in 54 primary castrate-resistant prostate cancers were conducted as previously reported (36), using Complementary DNA-mediated Annealing Selection and Ligation (DASL; Illumina).

**Statistical analyses**

Quantitative data are shown as the mean ± one SEM for at least 3 independent experiments. Comparative statistics used the paired Student t test, Mann–Whitney test, or 2-way ANOVA with Tukey posthoc tests to evaluate differences between experimental groups. P < 0.05 was considered statistically significant.

**Results**

**Lack of PTEN expression or TMPRSS2:ERG fusion does not alter RAD51 expression in prostate cancer cell lines or primary human prostate tumors**

Several recent reports have shown that loss of PTEN is often associated with downregulation of RAD51 (7, 18, 19), a key regulator of HR-mediated DNA DSB repair. Because PTEN loss is common in human prostate cancer, we evaluated whether PTEN status is associated with differential RAD51 expression in prostate cancer cells. To that end, we first assessed PTEN and RAD51 protein levels in whole-cell lysates of 22RV1 (PTEN+/−), DU145 (PTEN+/−), and PC3 (PTEN−/−) prostate cancer cells that also vary in RAD51 expression with a 100 mM PTEN nullizygous status (Fig. 1A, left). In contrast, all of the cell lines expressed similar levels of RAD51, despite their disparate PTEN genotypes. Similar effects were observed in xenografts derived from these cell lines, whereas PTEN loss was associated with increased phospho-AKT immunostaining in these xenografts, there was no differential RAD51 expression in vivo among this xenograft panel (Fig. 1A, right).

Little data are available on RAD51 expression in vivo in relation to PTEN status in which the microenvironment can alter protein expression. As we had previously shown that hypoxia can downregulate RAD51 expression (33), we first determined the potential effects of hypoxia on RAD51 expression with PTEN-null or PTEN-intact status (34). As a positive control, culturing DU145 cells under hypoxia in 0.2% O2 for 72 hours markedly downregulated RAD51, as previously reported (34, 38); however, there was no concomitant effect of this hypoxic gassing on PTEN expression (Fig. 1B). We also observed that RAD51 staining was inversely correlated with hypoxia (as demarcated by EF5 staining) in the tested xenografts, independent of their PTEN status (Fig. 1C, bottom). Pretreatment with a PARPi did not affect either RAD51 or PTEN levels.

Although PTEN loss has been linked to suppression of RAD51 expression in vitro, to our knowledge no data exist directly comparing the expression of RAD51 in primary human cancers with differential PTEN status. To that end, we stained 29 PTEN+/− and 29 PTEN−/− human prostate tumors from a TMA of RP specimens that had been evaluated for PTEN status by FISH (Supplementary Fig. S1A). Quantification of the immunohistochemistry signal intensity and distribution revealed that PTEN loss was not associated with diminished RAD51 expression (Fig. 1D). Indeed, we observed a significant increase in RAD51 expression in prostate cancers with PTEN−/− status by FISH (Supplementary Fig. S1A).

### Table 1. Known genetic aberrations in cell lines used in this study

<table>
<thead>
<tr>
<th>22RV1</th>
<th>DU145</th>
<th>PC3</th>
<th>HCT116</th>
<th>H1299</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 Genotype</td>
<td>22RV1</td>
<td>DU145</td>
<td>PC3</td>
<td>HCT116</td>
</tr>
<tr>
<td>IR-induced p21WAF1</td>
<td>Yes</td>
<td>No</td>
<td>Null</td>
<td>Yes</td>
</tr>
<tr>
<td>IR-induced G1/S checkpoint</td>
<td>Minimal</td>
<td>No</td>
<td>No</td>
<td>Intact</td>
</tr>
<tr>
<td>PTEN</td>
<td>WT</td>
<td>Heterozygous</td>
<td>Null</td>
<td>WT</td>
</tr>
<tr>
<td>Known DNA repair genes</td>
<td>MMR</td>
<td>MMR (MLH1, PMS2, MSH2)</td>
<td>None</td>
<td>MMR (MLH1)</td>
</tr>
<tr>
<td>Other reported mutations (COSMIC)</td>
<td>PIK3CA</td>
<td>CDKN2A, RB1, STK11</td>
<td>PIK3CA, CDKN2A, CTNNB1, KRAS</td>
<td>NRAS</td>
</tr>
</tbody>
</table>

*http://www.sanger.ac.uk/perl/genetics/CGP/cosmic.*
expression in PTEN−/− tumors, relative to PTEN+/+ tumors (P < 0.01). RAD51 expression was also unrelated to TMPRSS2:ERG fusion status (Supplementary Fig. S1B). We also compared mRNA expression of PTEN and RAD51 using a public database from a prostate cancer cohort of 218 primary localized and metastatic tumors and xenografts following array CGH and RNA expression studies (5). In this cohort, we found no correlation between reduced PTEN and reduced RAD51 expression (Supplementary Fig. S2); indeed, in no single case was PTEN loss associated with reduced RAD51 expression. Finally, in a cohort of castrate-resistant prostate cancer, the expression of genes thought to
be synthetically lethal to PARPi (RAD51, ATM, PRKDC, BRCA1, BRCA2, MRE11, CDK6, and MSH2) was not correlated to PTEN or TMPRSS2:ERG status (Supplementary Fig. S1C).

Effects of PTEN loss on sensitivity to DNA damaging agents in prostate cancer cells

Previous reports have suggested that PTEN loss causes a defect in HR-mediated DNA DSB repair by attenuating RAD51 gene expression and recruitment to DNA DSB (9, 18). To test whether PTEN status is associated with diminished RAD51 recruitment to DSB in prostate cancer cells, we employed UV laser microirradiation, which produces a subnuclear track of DNA DSBs that can be visualized by indirect immunofluorescence against γH2AX, and to which RAD51 is recruited (39). RAD51 was present at UVLM-induced DSB in 22RV1, DU145, and PC3 cells, showing that altered PTEN status does not correlate with RAD51 recruitment to DSB in prostate cancer cells (Fig. 2A).

If PTEN loss produces a defect in HR-mediated DSB repair, it may be possible to treat patients whose tumors have mono- or biallelic loss of PTEN with agents that preferentially kill HR-defective cells, such as IR or MMC (34). However, PTEN status did not correlate with sensitivity to either agent (Fig. 2B). By contrast, experimental downregulation of RAD51 showed that PTEN status was associated with sensitivity to PARPi (18), as would be predicted if PTEN loss does, in fact, produce a defect in HR. However, we did not observe a correlation between PARPi sensitivity and PTEN status in prostate cancer cells, although experimental downregulation of RAD51 markedly sensitized DU145 cells to PARPi (Fig. 2D). Indeed, we found that PTEN wild-type
22RV1 cells were the most sensitive of the prostate cancer cell lines to PARPi.

Gottipati and colleagues recently showed that cells with defects in HR-mediated DSB repair have elevated PARP activity and are sensitive to PARPi (24). Because PTEN status has been linked to alterations in HR, and because we observed differential PTEN protein stability in prostate cancer cells, we next evaluated basal PARP activity in these cell lines in the absence or presence of PARPi; the assay was also carried out with and without activated DNA. Absolute basal PARP activity was highest in 22RV1 cells (Supplementary Fig. S3). Of note, PTEN-null PC3 cells did not show elevated basal PARP activity relative to 22RV1 or DU145 cells. In all cell lines, basal PARP activity was markedly attenuated by PARPi.

PTEN knockout or downregulation does not alter RAD51 expression

To further evaluate PTEN as a biomarker of chemosensitivity, null-TP53 H1299 cells were transfected with siRNA to RAD51 or PTEN, and expression of these proteins were evaluated by Western blotting. siRNA to RAD51 or PTEN effectively downregulated the respective protein, and downregulation of PTEN increased phospho-AKT content, showing the functional loss of PTEN (Fig. 3A, left). However, PTEN downregulation did not affect RAD51 expression, consistent with the results shown in Fig. 1 in prostate cancer cells. Similarly, we observed no change in RAD51 expression in H1296 colon cancer cells in which the PTEN gene has been knocked out (HCT116-PTEN<sup>−/−</sup>), relative to the parental cells (HCT116-PTEN<sup>+/+</sup>), despite an increase in phospho-AKT content (indicative of loss of PTEN function; Fig. 3A, right). Indeed, we observed no downregulation of RAD51 in 2 independent clones of HCT116-PTEN<sup>−/−</sup> (data not shown). Moreover, we did not observe any decrease in RAD51 mRNA content in either H1299 cells transfected with PTEN siRNA or HCT116-PTEN<sup>−/−</sup> cells, relative to the respective control (Supplementary Fig. S4A).

Shen and colleagues previously showed that PTEN promotes RAD51 expression in MEFs by promoting the binding of E2F1 to the RAD51 promoter (7). To examine whether PTEN downregulation or knockout has a similar effect in human cancer cells, we carried out chromatin immunoprecipitation in H1299 cells transfected with siRNA to RAD51 and in HCT116-PTEN<sup>−/−</sup> cells, with an antibody against E2F1 and primers directed against the RAD51 promoter. E2F1 was bound to the RAD51 promoter in H1299 cells, and this was not affected by downregulation of PTEN (Supplementary Fig. S4B). Similarly, knockout of PTEN in HCT116 cells did not markedly affect E2F1 binding to the RAD51 promoter (Supplementary Fig. S4B).

Mendes-Pereira and colleagues previously showed that PTEN loss reduced the formation of DSB-induced RAD51 foci (18), although previous reports have shown that RAD51 foci are induced normally in cells lacking PTEN (40). To assess whether downregulation of PTEN affects recruitment of RAD51 to DSB, we transfected U2OS osteosarcoma cells with siRNA to PTEN or RAD51, and treated these cells with IR (4 Gy), and then evaluated RAD51 recruitment to IR-induced DSB by immunofluorescence. As shown in Fig. 3B, and similar to the effects shown above in H1299 and HCT116 cells, downregulation of PTEN in U2OS cells did not affect RAD51 protein levels. Although IR induced the formation of RAD51 nuclear foci, and while this was completely abrogated by downregulation of RAD51, downregulation of PTEN did not significantly affect the formation of RAD51 foci in response to IR (Fig. 3B).

To further investigate whether PTEN affects RAD51 recruitment to DSB, HCT116-PTEN<sup>+/+</sup> and HCT116-PTEN<sup>−/−</sup> cells were subjected to UVLM, to monitor RAD51 recruitment to subnuclear DSB. As shown in Fig. 3C, RAD51 accumulation occurred in HCT116 cells lacking PTEN as well as in the parental cells.

To directly evaluate the effects of PTEN on HR-mediated DSB repair, we transfected H1299 cells expressing the DR-GFP plasmid HR reporter system (34) with siRNA to RAD51 or PTEN, and then induced DNA DSB by expression of I-SceI. Downregulation of RAD51 produced an approximately 90% reduction in HR-mediated repair, without a reduction in S- and G2-M phase cell-cycle distribution (Fig. 3D and Supplementary Fig. S4C). In contrast, downregulation of PTEN did not produce an HR defect, and instead produced a significant increase in HR-mediated repair in this reporter system.

Disruption of PTEN produces a complex pattern of sensitivities to DNA damaging agents

Although our data do not support the hypothesis that PTEN status affects RAD51 expression or recruitment to DSB, or HR-mediated DSB repair, PTEN loss could still be associated with increased sensitivity to PARPi or other DNA damaging agents. If true, this would suggest that tumors with loss of PTEN may be amenable to treatment with novel agents, similar to a strategy in BRCA1/2 breast and ovarian cancer (11, 18, 25). However, in experimental isogenic models of PTEN loss, knockdown or knockout of PTEN did not increase basal PARP activity (Supplementary Fig. S4D).

Consistent with the results of Mendes-Pereira and colleagues, PTEN knockout in HCT116 cells produced a small, but significant sensitization to PARPi (Fig. 4A, top, left). Similarly, transfection of PTEN siRNA in H1299 cells sensitized the cells to PARPi, albeit to a much lesser extent than the dramatic sensitization induced by downregulation of RAD51 (Fig. 4A, bottom, left).

These PTEN<sup>−/−</sup> cells have previously been shown to be sensitive to IR, relative to the parental cells (41). To validate this phenotype, we carried out clonogenic survival assays using HCT116-PTEN<sup>+/+</sup> and HCT116-PTEN<sup>−/−</sup> cells following irradiation with 0 to 6 Gy of IR. As shown in Fig. 4A (top, right), HCT116 cells lacking PTEN were sensitive to IR, relative to the parental cells, consistent with the original experiments by Lee and colleagues (41). We also observed...
that PTEN downregulation sensitized H1299 cells to UV irradiation and to MMC, H₂O₂, and methylmethanesulfonate (MMS), but did not affect sensitivity to cisplatin, camptothecin (CPT), or taxol (Fig. 4B). Underscoring the heterogeneity associated with PTEN status, we found that PTEN knockdown or knockout produced a highly variable pattern of mRNA expression of genes involved in the DDR (Supplementary Fig. S5 and Table S1) and did not produce a gene "signature" that could be used as a surrogate marker for either PTEN loss or sensitivity to specific DNA damaging agents.

Disruption of MRE11 sensitizes cells to PARP inhibition

Previous reports have suggested that PARP1 is required for recruitment of MRE11 to DSB and that PARP1 interacts with MRE11 to facilitate replication fork restart following DNA replication blocks (26, 42). These data suggest that MRE11 may be an important component of PARP1 biology. Moreover, a recent report showed that PTEN knockout HCT116 cells have a defect in MRE11 recruitment to DSB (43), consistent with our own findings (Fig. 5A). For these reasons, we assessed whether MRE11 recruitment to DNA
DSB is associated with altered PARPi sensitivity. We subjected 22RV1, DU145, or PC-3 cells to UVLM and monitored MRE11 and γH2AX by immunofluorescence. Although MRE11 was recruited to DSB in DU145 and PC-3 cells, we did not observe any recruitment of MRE11 in 22RV-1 cells (Fig. 5B), which were also the most PARPi sensitive of the prostate cancer cell lines we evaluated (Fig. 2). We confirmed that MRE11 is downregulated in 22RV1 cells at the protein and mRNA levels (Fig. 5C and D).

To evaluate whether MRE11 can specifically influence PARPi toxicity, we treated MRE11-deficient ATLD fibroblasts (ATLD2) with PARPi, and assessed cell proliferation over 8 days as clonogenic survival experiments were not possible on these cells due to poor plating efficiency. As a control, we carried out the same experiment in ATLD fibroblasts expressing either wild-type MRE11 (ATLD2-wtMRE11) or endonuclease-deficient MRE11 (ATLD2-mtMRE11). PARPi significantly attenuated proliferation in ATLD2 fibroblasts, relative to DMSO treatment, an effect that was completely abrogated by reconstitution of either wild-type or mutant MRE11 (Fig. 5E).

**Discussion**

Mono- and biallelic deletions of the PTEN gene are among the most frequently observed molecular aberrations in human cancer. Although the vast majority of data surrounding the tumor suppressor role of PTEN pertain to its ability to antagonize the oncogenic PI3K/AKT pathway, recent reports have suggested that disruption of the PTEN gene is associated with reduction in the expression of the homologous recombination factor RAD51 (7, 18). This suggested that PTEN-mediated tumorigenesis may, in part, be secondary to RAD51-associated genomic instability (7, 18). These findings are potentially of immense clinical importance because of the high frequency of PTEN loss in human tumors and because they suggest that PTEN loss could potentially be used a biomarker for tumors that may...
be amenable to treatment with agents that preferentially target HR-deficient cells, including PARPi, IR, MMC, or cisplatinum. Recent studies have suggested that PTEN deletion is a fairly late event during prostate cancer development (5), suggesting that genomic alterations in these tumors may occur in a PTEN-independent manner. It is likely that PTEN loss promotes cell-cycle progression late in the development of prostate cancer, likely through AKT-dependent inhibition of CHK1, as previously reported (44). This may contribute to overall genomic instability because CHK1 inhibition has been associated with the induction of DNA DSB (45).

PTEN loss was originally associated with downregulation of RAD51 by Shen and colleagues, who suggested that PTEN promotes tumorigenesis by suppressing E2F1-dependent RAD51 gene transcription, leading to suppressed HR and genomic instability. Two subsequent reports showed that loss of PTEN is associated with RAD51 downregulation and PARPi sensitivity (18, 19). However, several other studies are not in agreement with these findings. In particular, Gupta and colleagues (10) failed to observe an association between PTEN loss and reduced RAD51 expression, using the same cells as both Shen and Mendes-Pereira. Moreover, our laboratory previously reported that expression of RAD51 mRNA and protein in 2 PTEN-null prostate cancer cell lines (PC-3 and DU145) were not reduced, relative to normal prostate epithelial cells (30). Similar IR-induced upregulation of RAD51 was observed in xenografts derived from PC3 and DU145 cells. These data are consistent with those shown here in Fig. 1 and suggest that RAD51 expression is independent of PTEN status. In addition, independent studies from both Gospodinov and Wakasugi found that RAD51 is expressed in PC3 cells, Cummings and colleagues showed the presence of

![Figure 5. MRE11 deficiency increases sensitivity to PARP inhibition. A, HCT116-PTEN+/+ and HCT116-PTEN−/− cells were subjected to UVLM and stained for γH2AX (red) and MRE11 (green). DNA was stained with DAPI (blue). B, PC3, DU145, and 22RV1 cells were subjected to UVLM as in A, C, whole-cell lysates of DU145, PC3, and 22RV1 cells were analyzed by Western blot using antibodies against MRE11, RAD50, or actin. D, MRE11 mRNA levels were analyzed by quantitative RT-PCR. E, MRE11-deficient ATLD2 fibroblasts were treated with 1 μmol/L PARPi (or DMSO), and cell proliferation was assessed over 8 days. ATLD2 fibroblasts expressing wild-type MRE11 (ATLD2-wtMRE11) or endonuclease-deficient MRE11 (ATLD2-mtMRE11) were also analyzed.](./image)
cisplatin-induced RAD51 foci in PC3 cells (40, 46, 47), and Russell found normal RAD51 expression in PTEN-null U251 glioblastoma cells (48). In this study, we observed no correlation between PTEN status and RAD51 expression or biological function in prostate cancer cell lines or xenografts. Likewise, our isogenic experimental models of PTEN loss (with siRNA or gene knockout) revealed no correlation between PTEN and RAD51 expression. These results contrast with those reported by both Shen and Mendes-Pereira and colleagues (7, 41), McEllin and colleagues (9) likewise showed no difference in RAD51 mRNA or protein expression in PTEN-null astrocytes versus wild type but did observe a significant decrease in mRNA expression of the RAD51 paralogs RAD51B, C, and D, reduced HR-mediated repair, and sensitivity to N-methyl-N-nitro-N-nitrosoguanidine, camptothecin, and a PARP inhibitor. They furthermore observed that PTEN-null astrocytes were resistant to IR, though it is not immediately clear how this can be reconciled with an HR-deficient phenotype, which would be expected to increase radiosensitivity, as we observed in Fig. 2C. In contrast, we failed to observe CPT or PARPi sensitivity in our models of PTEN loss. As such, the data presented herein are not consistent with those of Shen, Mendes-Pereira, or McEllin (7, 9, 18), with respect to the effect of PTEN loss on RAD51 expression and/or HR capacity, but are consistent with both Gupta and colleagues (10) and with the broader previous literature that has failed to show a correlation between PTEN loss and RAD51 expression. It is possible that the observed disparity results from a complex interaction between PTEN and various DDR and repair pathways, which may be cell- and/or tissue-type specific. Nevertheless, it is clear that PTEN status cannot be used universally as a biomarker of either RAD51 expression, HR capacity, or PARPi sensitivity.

In agreement with this, we observed that PTEN status de novo is not a biomarker of RAD51 expression in patients specimens based on FISH and protein expression in 48 patients with localized disease and RNA expression in 218 patients from a recently published prostate cancer outcomes cohort (5). RAD51 expression (as well as that of other genes previously implicated in the response to PARPi) was also not correlated with PTEN or TMPRSS2:ERG status in a cohort of castrate-resistant prostate cancers. This represents, to our knowledge, the first detailed examination of the expression of RAD51 in primary human prostate cancers with disparate PTEN status. Taken together, these data strongly argue against an association of PTEN or TMPRSS2:ERG status and RAD51 expression in primary prostate cancers.

Previous studies have evaluated the effects of PTEN on sensitivity to numerous agents with mixed results. For instance, Kao and colleagues showed that inducible reconstitution of PTEN in PTEN-null U251 cells attenuated DNA DSB repair and sensitized the cells to IR (49), suggesting that PTEN promotes radiosensitivity. By contrast, we observed that depletion of PTEN in PTEN+/− H1299 or HCT116 cells resulted in increased sensitivity to IR, consistent with data previously reported by Lee (41). One possible explanation for these discrepant results is that cancer cells that have developed within the context of a PTEN-null genotype may function differently than cells in which PTEN has been removed experimentally, perhaps due to secondary genetic aberrations that develop due to the absence of PTEN during tumorigenesis. Our data lend support to the hypothesis that experimental downregulation of PTEN does not phenocopy PTEN loss that occurs within the context of tumorigenesis. If this is indeed the case, it would have important ramifications for the study of PTEN biology because it would imply that experimental models of PTEN deficiency need to be chosen very carefully to properly relate the findings to the potential clinical use of PTEN loss as a biomarker to stratify patients to disparate therapies.

Consistent with this hypothesis, sensitivity to IR, MMC, or PARPi in prostate cancer cells was not correlated with PTEN status, whereas experimental downregulation of PTEN resulted in sensitization to all 3 agents. Similarly, PTEN knockout in HCT116 cells attenuated MRE11 accumulation at DNA DSB, consistent with a recent report (43), whereas PTEN-null PC3 cells showed no MRE11 defect. These results strongly suggest that the phenotype of prostate cancer and other cells that lack PTEN is complex and cell specific. Moreover, these results suggest that the clinical utility of PARPi in PTEN-null tumors is likely to be highly context specific and suggest that further studies are needed to more completely elucidate the complex relationship between PTEN status and sensitivity to PARPi and other agents, and to identify other genes and pathways that, when altered in tumors, may confer sensitivity to PARPi.

To that end, MRE11 recruitment to DSBs was correlated to PARPi sensitivity in both prostate cancer cells and in HCT116 cells, while experimental downregulation of MRE11 sensitized cells to PARPi. PARPi has recently been shown to interact with MRE11 (42), while MRE11 is required for activation of the ATM kinase in response to DSB, suggesting that the sensitivity of cells with suppressed MRE11 may be secondary to a diminished ATM response, given the previously reported synthetic lethality between loss or inhibition of PARP and ATM (50). Our finding that restoration of either wild-type MRE11 or endonuclease-deficient MRE11, both of which restore MRE11-dependent ATM activation (8), supports this hypothesis and the use of MRE11 function as a potential biomarker of PARPi sensitivity. Indeed, we have recently shown that AKT can bind to DSBs following MRE11-ATM activation in a PTEN-independent manner (8). As such, combining biomarkers that reflect MRE11, ATM, BRCA1/2, and AKT status in tumors may provide additional utility to individualize patient treatment strategies based on defective germ line or somatic DNA repair in tumors.

Taken together, our data suggest that in prostate cancer, PTEN status is not a biomarker of RAD51 expression or biological activity, or of sensitivity to PARPi, and further suggest that PTEN loss promotes a complex pattern of sensitivity to DNA damaging agents. These findings suggest...
that further studies will be required to identify novel cellular and molecular biomarkers that predict clinical response to PARPi (e.g., MRE11).

Disclosure of Potential Conflicts of Interest

The views expressed do not necessarily represent those of the Ontario Ministry of Health and Long Term Care. R.G. Bristow is a Canadian Cancer Society Research Scientist. The other authors disclosed no potential conflicts of interest.

Acknowledgments

The authors thank Mark O’Connor, Brad Wouters, Vuk Stambolic, and Gaetano Zalana for helpful comments and discussion. KU-0059436 was a gift of Astrazeneca.

References

Correction: PTEN Deletion in Prostate Cancer Cells Does Not Associate with Loss of RAD51 Function: Implications for Radiotherapy and Chemotherapy

In the Introduction section of this article (Clin Cancer Res 2012;18:1015–27), which was published in the February 15, 2012, issue of Clinical Cancer Research (1), the author incorrectly cited reference 6. This incorrect citation has now been replaced with the correct citation, reference 7, so that the corrected sentence reads as follows:

Restoration of PTEN in PTEN−/− MEFS restored RAD51 expression in a manner independent of its phosphatase activity (7).

The online version has been corrected and no longer matches the print version. The author regrets this error.

Reference

Clinical Cancer Research

**PTEN Deletion in Prostate Cancer Cells Does Not Associate with Loss of RAD51 Function: Implications for Radiotherapy and Chemotherapy**

Michael Fraser, Helen Zhao, Kaisa R. Luoto, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-2189

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2011/11/23/1078-0432.CCR-11-2189.DC1
http://clincancerres.aacrjournals.org/content/suppl/2012/02/15/1078-0432.CCR-11-2189.DC2

Cited articles
This article cites 50 articles, 20 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/18/4/1015.full.html#ref-list-1

Citing articles
This article has been cited by 14 HighWire-hosted articles. Access the articles at:
/content/18/4/1015.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.