Synergistic Effect of Olaparib with Combination of Cisplatin on PTEN-Deficient Lung Cancer Cells

Daisuke Minami1, Nagio Takigawa2, Hiromasa Takeda1, Minoru Takata3, Nobuaki Ochi1, Eiki Ichihara1, Akiko Hisamoto1, Katsuyuki Hotta1, Mitsune Tanimoto1, and Katsuyuki Kiura1

Abstract

PARP enzyme plays a key role in the cellular machinery responsible for DNA damage repair. PTEN is a tumor-suppressor gene deactivating PI3K downstream of EGFR signaling. We hypothesize that PTEN-deficient lung cancer cells suppressed DNA damage signaling and that the absence of PTEN can sensitize these cells to a concurrent treatment of a DNA-damaging agent (cisplatin) and a PARP inhibitor (olaparib). To investigate the effect of olaparib and cisplatin on PTEN-deficient lung tumors, two EGFR-mutant (deletion in exon 19) non–small cell lung cancer (NSCLC) cell lines, PC-9 (PTEN wild-type) and H1650 (PTEN loss), were used. We transfected intact PTEN gene into H1650 cells (H1650PTEN+/−) and knocked down PTEN expression in the PC-9 cells (PC-9PTEN−/−) using short hairpin RNA (shRNA). Combination of cisplatin with olaparib showed a synergistic effect in vitro according to the combination index in H1650 cells. Restoration of PTEN in the H1650 cells decreased sensitivity to the combination. Ablation of PTEN in PC-9 cells increased sensitivity to olaparib and cisplatin. We also examined the effectiveness of cisplatin and olaparib in a xenograft model using H1650 and PC-9PTEN−/− cells. The combination of cisplatin with olaparib was more effective than each agent individually. This effect was not observed in a xenograft model using H1650PTEN+/− and PC-9 cells. Mechanistic investigations revealed that PTEN deficiency caused reductions in nuclear RAD51 and RPA focus formation and phosphorylated Chk1 and Mre11. Thus, genetic inactivation of PTEN led to the suppression of DNA repair. Mol Cancer Res; 11(2); 140–8. ©2012 AACR.

Introduction

PARP inhibitor is one of the most promising new therapeutic approaches to cancers, either as a single agent or in combination with other DNA-damaging agents including radiation therapy (1). When PARP is inhibited, single-strand breaks (SSB) degenerate to more lethal double-strand breaks (DSB) that require repair by homologous recombination (HR). Therefore, cells that are deficient in HR are highly susceptible to PARP inhibitors (2–4), and this finding has been clinically validated (5–7).

As many cancer chemotherapeutic drugs and radiation therapy cause DNA damage, tumor cells defective in DNA repair pathways are predicted to be sensitive to their effects (8). Indeed, cell lines deficient in BRCA1 and BRCA2 have been shown to be sensitive to the DNA crosslinking agents cisplatin and mitomycin C (9, 10), the topoisomerase inhibitor etoposide (11), and oxidative DNA damage (12).

PARP1 has been suggested to be involved in base excision repair and SSB repair (13). Moreover, PARP-1 was reported to bind to DNA damages induced by platinum compounds, suggesting a direct role of PARP-1 in the repair of such damages (14, 15). The exquisite sensitivity of these cells to the PARP inhibitor olaparib (AZD2281), alone or in combination with cisplatin, provides strong support for olaparib as a novel targeted therapeutic against BRCA-deficient cancers (16). Olaparib alone and in combination with carboplatin greatly inhibit growth in BRCA2-mutated ovarian serous carcinoma (17). The exquisite sensitivity of BRCA1- or BRCA2-mutant cells to PARP inhibitors forms the rationale behind clinical trials that are now assessing the potential of these agents (18). The preliminary results from these clinical trials are promising, with favorable toxicity and sustained tumor responses to the drug (3).

Mutations in the phosphatase and tensin homolog (PTEN) gene and loss of PTEN expression have both been associated with a wide range of human tumors (19). Approximately, 2% to 9% of non–small cell lung cancer (NSCLC) tumors are considered to have PTEN loss. PTEN loss and EGFR receptor (EGFR) mutation co-occurred in 1 of 24 EGFR-mutant patients with lung adenocarcinoma (20, 21), and a recurrent gross mutation of the PTEN gene is identified in lung cancer with deficient DNA DSB repair. In
other studies, 24% of early NSCLC samples lacked PTEN expression, which correlated with PTEN promoter methylation (22) and PTEN protein expression was reduced or lost in 74% of lung tumors, and was associated with low or aberrant TP53 staining (23). In a later study, PTEN has novel nuclear functions, including transcriptional regulation of the RAD51 gene, whose product is essential for HR repair of DNA breaks (24, 25). McEllin and colleagues reported that loss of PTEN in astrocytes resulted in increased sensitivity to N-methyl-\(N\)-nitro-N-nitrosoguanidine, a functional analogue of temozolomide, and PARP inhibitor, due to inefficient repair (26).

We hypothesized that PTEN-deficient lung cancer cells suppressed DNA damage signaling and investigated whether the absence of PTEN could sensitize these cells to a concurrent treatment of cisplatin and olaparib.

**Materials and Methods**

**Cell lines**

Cells were cultured at 37°C with 5% CO\(_2\) in RPMI 1640 supplemented with 10% heat-inactivated FBS. H1650 is a lung adenocarcinoma cell line with co-occurrence of an EGFR mutation (in-frame deletion in exon 19) and homozygous deletion of PTEN. PC-9 is a lung adenocarcinoma cell line having the same in-frame deletion mutation of EGFR with wild-type PTEN. PTEN transfected into H1650 cells (H1650\(^{PTEN^+}\)) and PTEN expression knocked down in the PC-9 cells (PC-9\(^{PTEN^-}\)) using shRNA, were established in our laboratory (27). H1299 and A549 are NSCLC lines with wild-type EGFR and wild-type PTEN. PC-3 is a prostate cancer cell line with PTEN loss (Supplementary Fig. S1). PC-9 was obtained from Immuno-Biological Laboratories. H1650, H1299, A549, and PC-3 were purchased from the American Type Culture Collection. The Mre11 expression vector was kindly provided by Drs. Kenshi Komatsu and Junya Kobayashi (Kyoto University, Kyoto, Japan). The expression vector was transfected to H1650 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

**Sensitivity test**

Antiproliferative activity was determined by a modified 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay in terms of 50% inhibitory concentration (IC\(_{50}\)) values. Briefly, the cells were plated on 96-well plates at a density of 1,000 to 3000 cells per well, and continuously exposed to each drug for 144 hours. Each assay was conducted in triplicate or quadruplicate, and IC\(_{50}\) values were expressed as mean ± SD.

**Design for drug combination**

The constant-ratio design for the combination assay is highly recommended as it allows the most efficient data analysis (28). The multiple drug effect analysis of Chou and Talalay, based on the median-effect principle, was used to calculate the combined drug effect (29). H1650, H1650\(^{PTEN^+}\), PC-9, PC-9\(^{PTEN^-}\), H1299, A549, and PC-3 cells were seeded in triplicate in 96-well plates and were treated with cisplatin and olaparib at the indicated doses. After simultaneous exposure of the cells to 2 drugs for 144 hours, growth inhibition was determined using an MTT assay and the multiple drug effect analysis (Supplementary Method). Computer programs based on the median-effect plot parameters and combination index (CI) equation have been used for data analysis in the present study (30). Experiments were repeated in triplicate.

**Immunoblotting analysis**

Cells were exposed to cisplatin (10 μmol/L) or/and olaparib (20 μmol/L) for 6 hours. The cells at the point of 6 hours after irradiation at a dose of 10 Gy using a Hitachi MBR-1520-R irradiator (150 kV; 20 mA; filter: 0.5-mm aluminum and 0.1-mm copper) were also used. They were lysed in radioimmunoprecipitation assay buffer [1% Triton X-100, 0.1% SDS, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β-glycerophosphate, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate containing protease inhibitor tablets (Roche Applied Sciences GmbH)]. Proteins were separated by electrophoresis on polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with specific antibodies followed by detection with enhanced chemiluminescence plus (GE Healthcare Biosciences).

**Reagents and antibodies**

Olaparib and cisplatin were kindly provided by AstraZeneca and Nippon Kayaku Kogyo Co. Ltd., respectively. Rabbit antisera against Akt, phosphorylated (p)Akt (Ser473; D9E), PTEN, pChk1 (Ser345), Mre11 (31H4), and β-actin were purchased from Cell Signaling Technology. Rabbit antisera against RPA70 was purchased from Abcam. Mouse antisera against Chk1 (G-4) was purchased from Santa Cruz Biotechnology.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded tissue blocks from the samples were cut to a thickness of 5 μm, placed on glass slides, then deparaffinized in xylene and graded alcohol for 10 minutes. The antigen was incubated in 10 mmol/L sodium citrate buffer, pH 6.0, for 10 minutes in a 95°C water bath. The sections were then blocked for endogenous peroxidase with 0.3% hydrogen peroxide in methanol. The slides were rinsed with TBS containing 0.1% Tween 20 and the sections were blocked with goat normal serum for 60 minutes. The sections were incubated with 1:200 dilution of cleaved caspase-3 (Asp175; SA1E; Cell Signaling) antibody overnight at 4°C. The sections were amplified using biotinylated anti-rabbit antibodies and avidin–biotinylated horseradish peroxidase conjugate for 10 minutes (LSAB 2 Kit, Dako Cytomation) then reacted with 3,3′-diaminobenzidine. Finally, the sections were counterstained with hematoxylin. Cleaved caspase-3 expression was scored as positive if more than 10% of the tumor cells exhibited cytoplasmic staining (31).
Immunofluorescence staining

We fixed cells 8 hours after exposure to cisplatin (10 μmol/L), or/and olaparib (20 μmol/L), or treatment with 10 Gy. Cells were cultured on glass coverslips, fixed with 4% formaldehyde, and permeabilized in PBS-0.25% Triton X-100. For DNA damage and repair analyses, cells were stained with 1:50 dilution of rabbit polyclonal anti-RAD51 (Santa Cruz Biotechnology) and 1:500 dilution of mouse monoclonal anti-γH2AX (Millipore) for 2 hours at room temperature. Cells were washed with PBS and incubated for 30 minutes at room temperature with either Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen) secondary antibody for RAD51 or γH2AX, respectively. Nuclei were visualized by staining with 4’, 6-diamidino-2-phenylindole (DAPI). For quantification of RAD51 and γH2AX foci, at least 100 cells from each group were visually scored. Cells showing more than 5 foci were counted as positive for γ-H2AX or RAD51. These slides were examined under a fluorescence microscope (BIOR-EVO BZ-9000; Keyence).

Xenograft model

Female athymic mice at 7 weeks of age were purchased from Japan Charles River Co. All mice were provided with sterilized food and water and housed in a barrier facility under a 12-hour light/dark cycle. Cells (1 × 10⁶) were injected bilaterally subcutaneously into the backs of 7-week-old female athymic mice. At 10 days after injection, mice were randomly assigned to 4 groups (5 mice/group) that received either vehicle, 5 mg/kg/week of cisplatin, 50 mg/kg/day of olaparib, and 5 mg/kg/week of cisplatin plus 50 mg/kg/day of olaparib. Vehicle and olaparib were administered once a day, 5 times a week by intraperitoneal injection, and cisplatin was administered once a day, once a week by intraperitoneal injection. Tumor volume (width² × length/2) and body weight were determined periodically. Tumor volume was expressed as mean ± SD. After the completion of the treatment, all mice per group were sacrificed and the tumor specimens were obtained for analysis. All experiments involving animals were conducted under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research (Okayama, Japan).

Results

Olaparib synergizes cisplatin in PTEN-deficient lung cancer cells

Synergy between PARP inhibitors and platinum drugs was expected (14, 15) in triple-negative breast cancer and BRCA2 ovarian cancer cells (8, 17). We expected that the combination of cisplatin with olaparib showed a synergistic effect in PTEN-deficient lung cancer cell lines. Cells were treated either with 10 μmol/L cisplatin and 10 to 50 μmol/L olaparib or 20 μmol/L cisplatin and 20 to 100 μmol/L olaparib. Combination of cisplatin with olaparib showed a synergistic effect according to the combination index in the H1650 cells (Fig. 1A, 1B; Table 1). Combination indices were 0.23, 0.20, 0.57, and 0.29 when concentration ratios of cisplatin and olaparib were day of olaparib, and 5 mg/kg/week of cisplatin plus 50 mg/kg/day of olaparib. Vehicle and olaparib were administered once a day, 5 times a week by intraperitoneal injection, and cisplatin was administered once a day, once a week by intraperitoneal injection. Tumor volume (width² × length/2) and body weight were determined periodically. Tumor volume was expressed as mean ± SD. After the completion of the treatment, all mice per group were sacrificed and the tumor specimens were obtained for analysis. All experiments involving animals were conducted under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research (Okayama, Japan).

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designed to be molar ratios of 1:1, 1:2, 1:3, and 1:5, respectively. Restoration of PTEN in the H1650 cells decreased sensitivity to olaparib and cisplatin (CI >1; Fig. 1C, 1D). Ablation of PTEN in PC-9 cells increased sensitivity to olaparib and cisplatin (CI <1), and PC-9 cells decreased sensitivity to olaparib and cisplatin (CI >1). Our results showed that PTEN-deficient lung cancer cell lines, H1650 and PC-9PTEN+/–, exhibited synergism for all combinations of olaparib doses, whereas H1650PTEN+/– and PC-9 cells exhibited antagonistic effects for most dose combinations. A synergistic effect was also shown in the PC-3 cells, whereas antagonistic effects at most dose combinations were shown in H1299 and A549 cells (Supplementary Table S1).

Sensitivity of cisplatin or olaparib monotherapy is shown in Table 2. IC50 values of olaparib in H1650 and H1650PTEN+/– cells were 15.47 ± 6.8 μmol/L and 50.83 ± 7.7 μmol/L, respectively. PTEN-restored H1650 that became resistant to olaparib (P < 0.05). On the other hand, the IC50 values of olaparib in PC-9 and PC-9PTEN+/– cells were 5.88 ± 1.4 μmol/L and 6.52 ± 6.7 μmol/L, respectively. PC-9PTEN+/– cells did not confer sensitization to olaparib alone. IC50 values of cisplatin or olaparib alone in PC-3, H1299, and A549 cells are shown in Supplementary Table S2.

**Table 1. Combination index**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Concentration ratio (molar) of cisplatin to olaparib</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:1</td>
</tr>
<tr>
<td>H1650</td>
<td>0.23</td>
</tr>
<tr>
<td>H1650PTEN+</td>
<td>1.76</td>
</tr>
<tr>
<td>PC-9</td>
<td>4.38</td>
</tr>
<tr>
<td>PC-9PTEN+</td>
<td>0.43</td>
</tr>
</tbody>
</table>

NOTE: Combination index according to various concentration ratios of cisplatin and olaparib in each cell line is described. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively.

**Table 2. Drug sensitivity**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cisplatin (μmol/L)</th>
<th>Olaparib (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1650</td>
<td>2.12 ± 0.72</td>
<td>15.47 ± 6.8 *1</td>
</tr>
<tr>
<td>H1650PTEN+</td>
<td>1.65 ± 0.97</td>
<td>50.83 ± 7.7</td>
</tr>
<tr>
<td>PC-9</td>
<td>0.21 ± 0.019</td>
<td>5.88 ± 1.4</td>
</tr>
<tr>
<td>PC-9PTEN+</td>
<td>0.42 ± 0.10</td>
<td>6.52 ± 6.7</td>
</tr>
</tbody>
</table>

NOTE: Values are presented as mean ± SD of 50% inhibitory concentration (IC50) of the drug. H1650PTEN+; PTEN-restored H1650; PC-9PTEN+/–; PTEN-ablated PC-9; *P < 0.05.

**PTEN inactivation suppresses DNA damage signaling**

Oncogenic activation of Akt frequently results from loss of PTEN expression or function leads to suppression of DNA damage signaling (32). In this study, immunoblotting assay revealed that H1650 and PC-9PTEN+/– cells exhibited much higher levels of pAkt than H1650PTEN+/– and PC-9 cells, respectively (Fig. 2A). In addition, pChk1 was not overexpressed in both H1650 and PC-9PTEN+/– cells despite drug treatment. pChk1 was expressed after irradiation; however, the increase was less in PTEN-deficient lung cancer cells compared with their counterparts (Fig. 2B). Meanwhile, PTEN-deficient lung cancer cells exhibited lower levels of Mre11 compared with their counterparts (Fig. 2C). To address how lower expression levels of Mre11 affects the synergism between cisplatin and olaparib, Mre11 expression

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**Figure 2.** PTEN inactivation and DNA damage signaling. A, PTEN loss activated Akt and suppressed pChk1. pChk1 was not overexpressed in both H1650 and PC-9PTEN+ cells (PTEN-deficient lung cancer cells) despite drug treatment. B, pChk1 was expressed after irradiation; however, the increase was less in PTEN-deficient lung cancer cells compared with their counterparts. C, PTEN-deficient lung cancer cells exhibited lower levels of Mre11 compared with their counterparts.
vector was transfected into H1650 cells, and a stable transformant in which Mre11 was overexpressed was obtained (designated H1650Mre11– cells; Supplementary Fig. S2). In this cell line, combination indices were 0.76, 0.91, 0.97, and 0.77 when concentration ratios of cisplatin and olaparib were designed to be molar ratios of 1:1, 1:2, 1:3, and 1:5, respectively in H1650Mre11– cells (Supplementary Table S3). Although these combination index values were somewhat elevated compared with those observed in original H1650 cells, these results indicated that lower levels of Mre11 alone could not be the sole reason for this synergism.

On the other hand, PTEN has other nuclear functions, including transcriptional regulation of the RAD51 gene, whose product is essential for HR repair of DNA breaks (24, 25). Replication protein A (RPA) is displaced from single-stranded DNA by RAD51 to initiate HR (32). In this study, we investigated whether the formation of RAD51 and RPA foci was reduced in PTEN–deficient lung cancer cell lines. Subcellular localization of RAD51, γ-H2AX, and RPA is shown in Fig. 3A and in Supplementary Fig. S3A. PTEN deficiency resulted in significant reduction in RAD51 and RPA focus formation after drug exposure or γ-irradiation compared with H1650PTEN+ cells (P < 0.05); although γ-H2AX was similarly increased in both cells (Fig. 3B and Supplementary Fig. S3B).

**Effectiveness of the cisplatin with olaparib in a xenograft model**

We examined xenograft tumors to determine the effectiveness of the cisplatin with olaparib in PTEN–deficient lung cancer cells in vivo. H1650 and H1650PTEN+ xenograft tumors grew at almost same rate. The immunostaining of cleaved caspase-3 is shown in Fig. 4A. The combination of cisplatin and olaparib induced significant higher positive cells than other groups in H1650 xenografts (P < 0.05). The positive cells were 43 ± 3% for cisplatin plus olaparib, 10.6 ± 2.6% for cisplatin alone, 16 ± 3.6% for olaparib alone, and 3 ± 2% for vehicle. However, the combination did not display synergistic effect in H1650PTEN+ xenografts. The positive cells were 10 ± 2.6% for cisplatin plus olaparib, 17.3 ± 2.0% for cisplatin alone, 6.6 ± 0.5% for olaparib alone, and 4.6 ± 0.5% for vehicle (Fig. 4A).

Treatment with cisplatin plus olaparib significantly suppressed growth of the H1650 tumors compared with

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**Figure 3.** PTEN inactivation and expression of RAD51 and γ-H2AX. A, subcellular localization of RAD51 and γ-H2AX in H1650 or H1650PTEN+ cells after exposure of olaparib (20 μmol/L) or irradiation (10 Gy). B, PTEN deficiency resulted in significant reduction in RAD51 focus formation after exposure of cisplatin (10 μmol/L) and/or olaparib (20 μmol/L), or irradiation (10 Gy) compared with H1650PTEN+ cells (P < 0.05); although γ-H2AX was similarly increased in both cells.
cisplatin alone, olaparib alone, and the untreated controls (Fig. 4B). The tumor sizes (mm$^3$) at day 22 were 124.8 ± 33.1, 351.8 ± 150.9, 413.1 ± 66.0, and 847.8 ± 98.5, respectively. H1650$^{PTEN^+}$ xenograft tumors did not show significant response to the combination of cisplatin and olaparib compared with cisplatin alone, olaparib alone, and the untreated controls. The tumor sizes (mm$^3$) at day 22 were 467.8 ± 103.0, 373.9 ± 113, 524.5 ± 145.7, and 801.4 ± 113.2, respectively.

Next, we examined whether the combination of cisplatin with olaparib in PC-9 and PC-9$^{PTEN^+}$ xenografts was effective or not. Cleaved caspase-3 expressions in PC-9$^{PTEN^+}$ xenografs treated with the combination displayed significantly higher number of positive cells than those of other groups (44.3 ± 4.0% for the combination, 7.3 ± 3.2% for cisplatin, 15.3 ± 1.5% for olaparib, 1.0 ± 1.7% for vehicle). In PC-9 xenografts, there were no differences among 4 groups: 10.6 ± 2.0% for the combination, 7.3 ± 1.5% for cisplatin, 8.3 ± 1.5% for olaparib, 4.0 ± 1.7% for vehicle (Fig. 4A). Western blotting also indicated that the combination of cisplatin with olaparib seemed to induce more cleaved caspase-3 expressions than other treatments in H1650 and PC-9$^{PTEN^+}$ xenografts, but not in PC-9 and H1650$^{PTEN^+}$ xenografts (Supplementary Fig. S4).
In the PC-9\textsuperscript{PTEN}\textsuperscript{−} xenograft model, cisplatin plus olaparib inhibited tumor growth than other treatment (152.6 ± 8.06 mm\(^3\) for the combination, 336.6 ± 45.7 mm\(^3\) for cisplatin, 411.2 ± 67.2 mm\(^3\) for olaparib, 774.3 ± 95.8 mm\(^3\) for vehicle). PC-9 xenograft tumors did not show significant response to the combination compared with cisplatin alone, olaparib alone, and the untreated controls (279.1 ± 69.5 mm\(^3\), 254.2 ± 42.2 mm\(^3\), 296.0 ± 57.5 mm\(^3\), and 642.3 ± 133.2 mm\(^3\), respectively; Fig. 4B).

In toxicity evaluation, all the treatment animals did not show substantial loss of body weight (>10%) and the addition of olaparib to cisplatin did not significantly increase weight loss compared with cisplatin single agent treatment (data not shown).

**Discussion**

We here showed that PTEN-deficient lung cancer cell lines suppressed DNA damage signaling, and were sensitive to the combination of olaparib with cisplatin. Synergy between PARP inhibitor and platinum drugs was expected in triple-negative breast cancer and BRCA2 ovarian cancer cells (8, 17). In other study, addition of PARP inhibitor after alkylating agent, demethyl sulfate, treatment increased SSB levels indicating ongoing repair even at this late time-point (33). Recently, evidence suggested that PTEN was important for the maintenance of genome stability (24, 25). The HR impairment caused by PTEN deficiency sensitized tumor cells to potent inhibitors of the DNA repair enzyme, both in vitro and in vivo (34). Our results were in agreement with their studies. We showed that xenograft tumors bearing PTEN-deficient lung cancer cells were sensitive to the combination of cisplatin with olaparib, although this effect was not observed in a xenograft model using PTEN wild-type cells (Fig. 4B). However, it is possible that the results may be cell specific or have a different effect of PTEN loss on HR capacity because PTEN-deficient prostate cancer cells had only mild PARP inhibitor and DNA damaging agent’s sensitivity (35).

Drug interaction between cisplatin and olaparib in PTEN-deficient lung cancer cells has not been elucidated. Our investigation revealed that PTEN deficiency caused a reduction of pChk1 (Fig. 2A) and decreased drug or radiation-induced nuclear RAD51 and RPA focus (Fig. 3 and Supplementary Fig. S3). Oncogenic activation of Akt frequently resulted from loss of PTEN expression or function (36). How PTEN loss affects DNA damage signaling should be clarified. H1650 and PC-9\textsuperscript{PTEN}\textsuperscript{−} cells exhibited much higher levels of pAkt than H1650\textsuperscript{PTEN}\textsuperscript{+} and PC-9 cells, respectively. Activation of Chk1 after irradiation was attenuated in PTEN-deficient cells (Fig. 2B). Chk1 has a critical role in maintaining genomic stability by delaying S- and G\(_2\) phase progression of cells containing DNA damage to allow time for repair before mitosis and, the DSBs that arise when Chk1 is inhibited are apparently related to a specific S-phase role whereby Chk1 suppresses aberrant initiation of DNA replication that wound generate DNA lesions (37). Chk1 is reportedly required for HR repair and Chk1-depleted cells failed to form RAD51 nuclear foci after exposure to hydroxyurea (38). McEllin and colleagues showed a significant decrease in mRNA expression on RAD51B, C, and D, and reduced HR-mediated repair in PTEN-null astrocytes (26). Meanwhile, Xu and colleagues observed PTEN knockdown in HCT116 cells attenuated Mre11, which was a key gene in HR repair of DSBs (32). In addition, Fraser and colleagues reported PARP inhibitor sensitivity associated with a defect in Mre11 expression (35). Our observation that PTEN-deficient lung cancer cells exhibited lower levels of Mre11 compared with their counterparts was in agreement with their studies. To investigate the effect of Mre11 itself on the synergy, we examined the combination effect of olaparib with cisplatin using H1650\textsuperscript{Mre11\textsuperscript{−}} cells. Unexpectedly, restored levels of Mre11 did not suppress the phenotypes observed in PTEN-deficient cells in this study (Table 1 and Supplementary Table S3). Further investigations should be required to clarify whether and to what extent the molecular events including Chk1, Mre11, and RAD51 are responsible for the synergistic effect. Interestingly, Shen and colleagues recently showed that PTEN is important for maintaining basal levels of transcription of the RAD51 gene in mouse embryonic fibroblasts (24). Although there were no significant differences of RAD51 and RPA levels by Western blotting (Supplementary Fig. S5), the formation of RAD51 and RPA foci was reduced in PTEN-deficient lung cancer cells (Fig. 3 and Supplementary Fig. S3). As RPA binds to single-stranded DNA, the RPA focus could be a marker for end resection at the double-stranded DNA ends (32). Thus, inactivation of PTEN might lead to suppression of DNA damage signaling, leading to the lower levels of end resection and, hence, less RPA focus formation. As shown above, reduced levels of Mre11 alone could not provide a sufficient explanation for this, though Mre11 is involved in the molecular mechanisms of the end resection (39).

A number of clinical trials to treat triple-negative breast cancer, metastatic melanoma, malignant glioma, advanced colorectal cancer, ovarian cancer, and lung cancer are now underway to test the efficacy of PARP inhibitors or PARP inhibitors in combination with DNA-damaging agents (40). Later, phase II studies using olaparib established proof-of-concept of selectively killing of HR-deficient breast cancer and ovarian cancer cells with BRCA1 or BRCA2 mutations, resulting in a substantial clinical benefit with minimal toxicity (6, 7, 41). DNA repair biomarkers from multiple DNA repair pathways on treatment response and cancer survival offers opportunity to evaluate patient tumor samples and determine their status of DNA repair pathways before and during therapy for individual patients. In recent years, our understanding of how to treat NSCLCs has undergone a paradigm shift by the identification of EGFR mutations (42, 43) and EML4–ALK translocation (44). In BRCA1-deficient lung cancer, PARP inhibition induced BAX/BAK-independent synthetic lethality (45). Knowledge of the status of multiple DNA repair profiling of patients and may discriminate patients with likelihood to respond to PARP inhibitors.
We hypothesized that tumor cell with HR deficiency (such as PTEN-mutated cancer cells) were hypersensitive to PARP inhibitors in combination with cisplatin, resulting in killing of tumor cells based on the synthetic lethality principle. A major important solution to these barriers is to build biomarker testing into patient tumor identification, and to use the biomarker panels during treatment. The combination of cisplatin with olaparib in PTEN-deficient lung tumors might be further pursued in clinical trials.

Disclosure of Potential Conflicts of Interest
N. Takigawa has a honoraria from speakers’ bureau from AstaZeneca. K. Kiura has a honoraria from speakers’ bureau from AstaZeneca. No potential conflicts of interest were disclosed by other authors.

Authors’ Contributions
Conception and design: D. Minami, N. Takigawa, E. Ichihara, K. Kiura
Development of methodology: D. Minami, N. Takigawa, N. Ochi, E. Ichihara, K. Kiura
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Minami, N. Takigawa, K. Kiura

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