RAD51C-Deficient Cancer Cells Are Highly Sensitive to the PARP Inhibitor Olaparib

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Abstract
A PARP inhibitor is a rationally designed targeted therapy for cancers with impaired DNA repair abilities. RAD51C is a paralog of RAD51 that has an important role in the DNA damage response. We found that cell lines sensitive to a novel oral PARP inhibitor, olaparib, had low levels of RAD51C expression using microarray analysis, and we therefore hypothesized that low expression of RAD51C may hamper the DNA repair process, resulting in increased sensitivity to olaparib. Compared with the cells with normal RAD51C expression levels, RAD51C-deficient cancer cells were more sensitive to olaparib, and a higher proportion underwent cell death by inducing G2–M cell-cycle arrest and apoptosis. The restoration of RAD51C in a sensitive cell line caused attenuation of olaparib sensitivity. In contrast, silencing of RAD51C in a resistant cell line enhanced the sensitivity to olaparib, and the number of RAD51 foci decreased with ablated RAD51C expression. We also found the expression of RAD51C was downregulated in cancer cells due to epigenetic changes and RAD51C expression was low in some gastric cancer tissues. Furthermore, olaparib significantly suppressed RAD51C-deficient tumor growth in a xenograft model. In summary, RAD51C-deficient cancer cells are highly sensitive to olaparib and offer preclinical proof-of-principle that RAD51C deficiency may be considered a biomarker for predicting the antitumor effects of olaparib. Mol Cancer Ther; 12(6); 865–77. ©2013 AACR.

Introduction
The DNA repair system is critical for maintaining genomic integrity. Synthetic lethality is defined as the loss of cell viability when multiple genes lose their functions altogether, especially when compensatory genes are also defective. The concept of synthetic lethality has been shown using the novel PARP inhibitor in patients with breast and ovarian cancer harboring mutations in the BRCA1 or BRCA2 genes (1, 2). There is clinical evidence showing that olaparib (AZD2281; KU-0059436), a small-molecule inhibitor of PARP, has potential as a therapeutic agent alone and in combination with radiotherapy or chemotherapy to treat cancers with BRCA1 and BRCA2 mutations (3–5). PARP inhibitors block the repair of DNA single-strand breaks (SSB); unrepaired SSBs lead to the formation of DNA double-strand breaks (DSB). If DSBs cannot be repaired because of homologous recombination dysfunction, genomic instability or cell death can result (6). Therefore, PARP inhibitors may be effective against various human cancer cells with defective DNA repair genes. An example of that is CDK1 depletion increased PARP inhibitor sensitivity in cancer cells because the DSBs induced by PARP inhibition could not be repaired because of inactivation of homologous recombination–associated repair in CDK1-depleted cells (7). In addition, recent study suggests that nonhomologous end joining (NHEJ) plays an important role in the genomic instability and hypersensitivity to PARP inhibitors in homologous recombination–deficient cells (8).

RAD51C is a RAD51-like gene that has a key role in maintaining genomic stability (9–12). The functional role of RAD51C in DNA damage repair has also been examined (13). The results of these studies suggest that RAD51C acts sequentially with RAD51 at the DNA damage site to repair DNA damage. Therefore, RAD51C depletion leads to impaired RAD51 foci formation, resulting in impaired DNA repair (13, 14). In addition, some studies have shown that RAD51C is required for the checkpoint response to DNA damage (13, 15). Furthermore, recent studies have found that germline mutations of RAD51C are associated with cancers. In these
cancers, germline mutations of RAD51C impede RAD51 foci formation results in blocking the homologous recombination–mediated DNA repair. RAD51C-defective cancers can therefore potentially be treated with olaparib because the DNA damage induced by olaparib cannot be effectively repaired by homologous recombination, as RAD51C deficiency interferes with RAD51-mediated homologous recombination.

In the present study, we evaluated the antitumor activity of olaparib in cancer cell lines in vitro and observed that olaparib-sensitive cell lines had low levels of RAD51C expression using microarray analysis. Subsequently, we evaluated whether the silencing of RAD51C-sensitize olaparib sensitivity and restoration of RAD51C cause decreased sensitivity to olaparib. We also characterized the mechanisms of RAD51C silencing in human cancers. RAD51C expression using immunohistochemistry was evaluated in gastric cancer tissue with paired normal gastric mucosa. This is the first report to show that RAD51C-deficient cancer cells are selectively sensitive to olaparib or dimethyl sulfoxide (DMSO). The total RNA was extracted and hybridized to an Affymetrix GeneChip human Gene 1.0 ST array (Affymetrix, Inc.). The results were normalized to the robust multi-array average (RMA) and analyzed by DNALINK, Inc.. The microarray data were deposited in the ArrayExpress database (accession number: E-MTAB-1012).

**Materials and Methods**

**Reagents**

Olaparib was kindly provided by AstraZeneca Ltd.. The chemical structure of olaparib is shown in Fig. 1A. 5-Aza-2’-deoxycytidine (5-aza-dc) was purchased from Sigma-Aldrich.

**Cell lines and cell culture**

Human gastric cancer cells (SNU-1, -5, -16, -484, -601, -620, -638, -668, -719, and KATO-III) were purchased from the Korean Cell Line Bank; the identities of the cell lines were authenticated by DNA fingerprinting analysis (16). Human breast cancer cells (BT-549 and MCF-7) authenticated using short tandem repeat analysis were purchased from the American Type Culture Collection. All cell lines were stored in liquid nitrogen, passaged for less than 6 months before use in this study, and cultured in RPMI-1640 supplemented with 10% FBS and 10 μg/mL gentamicin at 37°C in a 5% CO2 atmosphere.

**Cell growth inhibition assay**

Cells were seeded in 96-well plates and exposed to increasing concentrations of olaparib (doses ranged from 1–10 μmol/L) for 5 days. After drug treatment, the absorbance of MTT dye was measured at 540 nm with a VersaMax microplate reader (Molecular Devices). The absorbance and IC50 of olaparib were analyzed using SigmaPlot software [Statistical Package for the Social Sciences, Inc. (SPSS)].

For the colony formation assay (CFA), the cells were seeded in 6-well plates and treated with various concentrations (5, 1, 0.1, 0.01, and 0.001 μmol/L) of olaparib for 5 days and were cultured until colonies formed (14 days). The cell colonies were stained with 0.1% Coomassie blue solution (Sigma-Aldrich) and counted using Gel-Doc (Bid-Rad); the cell survival rate and IC50 of olaparib were determined using SigmaPlot software.

**cDNA microarray analysis**

The SNU-601 and SNU-668 cells were treated for different periods of time (24, 48, and 72 hours) with 1 μmol/L olaparib or dimethyl sulfoxide (DMSO). The total RNA was extracted and hybridized to an Affymetrix GeneChip human Gene 1.0 ST array (Affymetrix, Inc.). The results were normalized to the robust multi-array average (RMA) and analyzed by DNALINK, Inc.. The microarray data were deposited in the ArrayExpress database (accession number: E-MTAB-1012).

**BRCA1 and BRCA2 mutation analysis**

Genomic DNA (gDNA) was extracted from gastric cancer cell lines using an Accuprep Genomic DNA Extraction Kit (Bioneer) according to the manufacturer’s protocol. The BRCA1 and BRCA2 mutations were analyzed using fluorescent-conformation sensitive gel electrophoresis (F-CSGE), as described previously (17).

**Reverse transcription PCR and real-time PCR**

Total RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH). cDNA was synthesized by conducting reverse transcription PCR (RT-PCR) with ImFrom-II reverse transcriptase (Promega) and amplified using AmpliTaq Golf DNA polymerase (Applied Biosystems) with gene-specific primers. Quantitative real-time PCR was conducted using an iCycler iQ detection system (Bio-Rad Laboratories, Inc.) with SYBR Green. All data from all samples were normalized to the actin cDNA levels. The sequences of the primers used for the RT-PCR and qRT-PCR are listed in Supplementary Table S1. cDNA was synthesized at least 3 times from 3 independent sets of samples, and all PCR analyses were conducted in triplicate.

**Western blot analysis**

Proteins were extracted and equal amount of proteins were separated on 8% to 15% SDS-PAGE as previously described (18). The resolved proteins were transferred onto nitrocellulose membranes, the blots were probed overnight at 4°C with appropriate primary antibodies [RAD51C and XRCC3 (Novus Biologicals), RAD51 and RAD51D (Santa Cruz Biotechnology), caspase-3, cyclin B1, p21, phosphorylated p53, cdc2, and PTEN (Cell Signaling Technology), phosphorylated histone H2AX (Millipore), RAD51B (Abcam), PARP (BD Biosciences), and α-tubulin (Sigma-Aldrich)]. Antibody binding was detected using an enhanced chemiluminescence system according to the manufacturer’s protocol (Amersham Biosciences).

**Cell-cycle analysis**

The cells treated with olaparib and/or radiation were harvested, fixed with cold 70% ethanol, and then stored at −20°C for at least 24 hours. The cells were washed in...
PBS and incubated with 10 μg/mL RNase A (Sigma-Aldrich) at 37°C for 20 minutes. Next, the cells were stained with 20 μg/mL propidium iodide (Sigma-Aldrich), and the DNA content of the cells (10,000 cells per experimental group) was quantified using a FACS Calibur flow cytometer (BD Biosciences).

Immunofluorescence assay (RAD51 foci formation)

SNU-668 cells were plated on 0.01% poly-L-lysine (Sigma-Aldrich)–coated coverslips, transfected with RAD51C-specific or nonspecific control siRNA, and treated with 1 μmol/L olaparib. After 2 days, the cells were exposed to 10 Gy of radiation for 2 hours. Afterward, the coverslips were rinsed once in PBS (37°C), fixed in 3.7% paraformaldehyde for 10 minutes, permeabilized with PBS-T (0.5% Triton X-100 in PBS) for 5 minutes, and incubated with primary antibody for 24 hours at 4°C. The primary antibodies used in this study were rabbit polyclonal anti-RAD51 (H-92; Santa Cruz Biotechnology) and mouse monoclonal anti-phosphorylated histone H2AX (clone JBW301; Millipore) at a dilution of 1:100. The coverslips were rinsed 3 times for 10 minutes in PBS, followed by incubation with the appropriate fluorophore-conjugated secondary antibody (Invitrogen). The coverslips were mounted on slides using Faramount aqueous mounting medium (DAKO). Immunofluorescence was visualized using a Zeiss LSM 510 laser scanning microscope.
**Comet assays**

SNU-601, SNU-668, and RAD51C-specific or nonspecific control siRNA-transfected SNU-668 cells were treated with 1 μmol/L olaparib or 10 Gy of radiation. After treatment, cells were trypsinized and subjected to an alkaline comet assay using the Trevigen Comet Assay Kit (Trevigen) following the manufacturer’s protocol. Tail lengths were measured with Comet assay IV program.

**Plasmid and siRNA transfection**

The pcDNA3-RAD51C expression plasmid (14) was kindly provided by Dr. Masson (Laval University Cancer Research Center, Québec, Canada). siRNAs specific for RAD51C and PTEN were obtained from Qiagen. SNU-668 cells were transfected with siRNA at a final concentration of 80 nmol/L or 4 μg of RAD51C plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 48 hours, the cells were harvested and subjected to Western blot analysis. The sequence of the RAD51C-specific siRNA was 5'-CACCTCTGGTAC-CACTAGA-3', the sequence of the PTEN-specific siRNA was 5'-AAGCGGTATACAGAAACAATA-3', the sequence of the XRCC3-specific siRNA was 5'-CACAGAAT-TATTGCTGCAAATTA-3', and the sequence of the Ezh2-specific siRNA was 5'-AAGACTCTGAATGCAGTTGCT-3'. The sequence of the control (nonspecific) siRNA was 5'-AATTCTCCGAACGTGTCACG-3'.

**Stable overexpression of RAD51C**

The pcDNA3 and pcDNA3-RAD51C plasmids (14) were used to transfect SNU-601 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The cells were treated with 300 μg/mL G418 (Cellgro) to select cells that had stably integrated the plasmid. After selection in G418, RAD51C expression was measured in the clones by Western blot analysis.

**Bisulfite modification and genomic sequencing**

gDNA was isolated using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s instructions and dissolved in H2O to a final volume of 20 μL. Bisulfite gDNA modification was conducted using an EpiTect Bisulfite kit (Qiagen) according to the manufacturer’s protocol. The modified gDNA was amplified with RAD51C-specific primers. These primers were designed to amplify the CpG island of RAD51C. The PCR products were loaded onto a 1.5% agarose gel (Lonza) and separated at 100 V for 10 minutes in 1 × tris-borate EDTA (TBE). DNA was extracted from the gel and sequenced. The DNA sequencing was conducted by Bionics, Inc.

**RAD51C expression in cancer tissue**

Thirteen gastric tumor tissue and matched adjacent normal tissue samples were obtained from Seoul National University Hospital [Seoul, Republic of Korea; Institutional Review Board (IRB) number: H-1004-078-316]. Each sample was collected immediately after surgery and divided into 2 portions. One portion was stored in liquid nitrogen and then used for RNA and protein extraction, and the other portion was embedded in paraffin. The RAD51C expression in the normal and cancer tissues was analyzed via immunohistochemical (IHC) staining using the anti-mouse monoclonal antibody against RAD51C (Novus Biologicals) at a dilution of 1:50. The quantification of the IHC slides was conducted in a blinded fashion, and 3 staining patterns were distinguished relative to the number of positive cells and the staining intensity: the scores are negative (negative; no staining to weak staining in ≤10% tumor cells), 1+ (weak staining in >10% of tumor cells), 2+ (moderate staining in >10% of tumor cells), and 3+ (strong staining in >10% of tumor cells).

**In vivo study**

The animal experiments were carried out at the animal facility of Seoul National University (Seoul, Republic of Korea) according to the institutional guidelines with prior approval from the Institutional Animal Care and Use Committee (IRB number: SNU-100816-2). A total of 16 female Balb/c athymic nude mice of ages 4- to 6-week were purchased from Central Lab Animal Inc.. The mice were injected subcutaneously in the right flank with 1 × 10^9 of SNU-601 cells in 100 μL of PBS. After implantation of the tumor cells, the sizes of the resulting tumors were measured every other day using calipers; the body weight of each mouse was also determined twice per week. The tumor volume was calculated using the following formula: (width^2 × height)/2. When the tumor volume reached 150 to 200 mm^3, the mice were randomly divided into 2 groups (8 mice per group). One group of mice was treated daily with 50 mg/kg olaparib for 28 consecutive days via oral gavages. The control group was treated with a 10% 2-hydroxyl-propyl-β-cyclodextrine/PBS solution alone. The mice were euthanized with CO₂ when the tumor volume reached 1,500 mm^3. The tumors were excised and stored in liquid nitrogen until further analysis.

**Immunohistochemistry**

The histologic sections from individual paraffin-embedded xenograft tumor tissues were deparaffinized and dehydrated. IHC detection of proliferating cells was conducted using the anti-rabbit polyclonal antibody against Ki-67 (GeneTex) at a dilution of 1:100. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was conducted for IHC detection of apoptosis using ApopTag In situ Apoptosis Detection Kit (Chemicon International), in accordance with manufacturer’s protocol.

**Statistical analysis**

Statistical analyses were conducted using SigmaPlot version 9.0. A two-sided Student t test was used when appropriate. The results are expressed as the mean ± SE or ± SD. A P value less than 0.05 was considered to be statistically significant.
Results

Olaparib selectively targets RAD51C-deficient human cancer cells

We examined olaparib sensitivity in gastric and breast cancer cells using CFA. Several cell lines (SNU-601, KATO-III, and BT-549) were highly sensitive to olaparib compared with other cell lines (Supplementary Fig. S1A). We chose SNU-601 and BT-549 as the sensitive cell lines and SNU-668 and MCF-7 as the resistant cell lines for further study. The SNU-601 and BT-549 cells were highly sensitive to olaparib compared with the SNU-668 and MCF-7 cells (Supplementary Fig. S1B). To identify the predictive markers of olaparib sensitivity, we conducted a microarray analysis of the sensitive SNU-601 and resistant SNU-668 cells. We found that RAD51C expression was much lower in the SNU-601 cells. RAD51C protein expression was also low in olaparib-sensitive SNU-601 and BT-549 cells (Supplementary Fig. S2A). Next, the mRNA expression of BRCA1/2, RAD51, and MRE11 was measured. No significant differences were observed between the sensitive and resistant cell lines (Supplementary Fig. S2B). In addition, mutational analysis of BRCA1 and BRCA2 indicated that olaparib sensitivity was not associated with BRCA mutations, at least in these cell lines (Supplementary Table S2). Because the BT-549 cells were characterized by a PTEN deficiency, we determined whether PTEN overexpression affected olaparib sensitivity in the BT-549 cells. Our data showed that PTEN expression did not affect olaparib sensitivity in the BT-549 cells (Supplementary Fig. S3). We therefore hypothesized that RAD51C deficiency may be linked with the synthetic lethality associated with olaparib; thus, a lack of RAD51C expression could be a marker of olaparib sensitivity.

Olaparib sensitivity is mediated by the absence of RAD51C expression

To determine whether olaparib sensitivity was a direct result of RAD51C deficiency, we measured the viability of SNU-668 cells treated with olaparib. These cells were transfected with control or RAD51C-specific siRNA. Cell viability was measured in the presence of olaparib with a CFA. RAD51C knockdown enhanced olaparib sensitivity to a level comparable with that of olaparib-sensitive SNU-601 cells (Fig. 1B and Supplementary Fig. S4A). The successful knockdown of RAD51C expression was validated by Western blot analysis (Fig. 1B). RAD51C-deficient SNU-601 cells were transfected with an empty pcDNA3 vector or pcDNA3-RAD51C plasmid to overexpress RAD51C, after which stable clones were selected using G418. CFA was conducted with these stable clones to measure the growth inhibitory activity of olaparib after RAD51C overexpression. Two SNU-601 clones that stably overexpressed RAD51C had attenuated olaparib sensitivity compared with SNU-601 clones transfected with empty pcDNA3 vector (Fig. 1C and Supplementary Fig. S4B). To further validate our findings, we transiently overexpressed RAD51C in BT-549 cells, which are sensitive to olaparib (IC_{50}, 0.44 ± 0.05 μmol/L by MTT). Exogenous RAD51C overexpression was confirmed by Western blot analysis (Fig. 1C and Supplementary Fig. S5B). BT-549 cells overexpressing RAD51C were less sensitive to olaparib compared with those transfected with the empty vector (Supplementary Fig. S5A). These findings suggest that RAD51C deficiency plays a role in the growth inhibitory activity of olaparib.

Olaparib sensitivity in RAD51C-deficient cells is associated with G2–M cell-cycle arrest and apoptosis

RAD51C deficiency leads to G2–M cell-cycle arrest and subsequent genomic instability (9, 12). We therefore investigated whether olaparib promotes G2–M cell-cycle arrest and/or apoptosis in RAD51C-deficient cells by conducting a fluorescence-activated cell sorting (FACS) analysis. SNU-601 and SNU-668 cells were exposed to increasing doses of olaparib for 4 days, the DNA content was measured with propidium iodide staining. The G2–M and sub-G1 populations were significantly increased in a dose-dependent manner in the RAD51C-deficient SNU-601 cells (Fig. 2A). In the SNU-601 cells, PARP cleavage was detected in a dose-dependent manner (Fig. 2A). The G2–M population also increased in the BT-549 cells (Supplementary Fig. S6). In addition, the SNU-668 cells were subjected to siRNA transfection to reduce RAD51C expression and treated with increasing concentrations of olaparib for 96 hours. We then analyzed cell-cycle progression using FACS analysis. The siRNA-mediated knockdown of RAD51C increased the olaparib-induced apoptosis and G2–M cell-cycle arrest compared with the control siRNA transfection (Fig. 2B). The olaparib-induced apoptosis in RAD51C-depleted SNU-668 cells was also confirmed by detecting the PARP cleavage (Fig. 2B). Conversely, SNU-601 cells that stably overexpressed RAD51C failed to undergo cell-cycle arrest and apoptosis when treated with olaparib. Populations in the G2–M and sub-G1 phases did not increase with olaparib treatment in the SNU-601 cells overexpressing RAD51C (Fig. 2C). In addition, PARP cleavage associated with apoptosis did not change (Fig. 2C). Our results indicate that olaparib-induced G2–M arrest and apoptosis are a result of RAD51C deficiency.

Olaparib sensitizes cancer cells to ionizing radiation

Olaparib has also been reported to enhance the effects of conventional cytotoxic agents in homologous recombination-defective cells (19–21). The suppression of homologous recombinational repair due to RAD51C deficiency is associated with disturbed RAD51 foci formation at irradiation-induced DSB sites (14). Thus, olaparib may increase the cytotoxic effects of irradiation in RAD51C-deficient cells, especially during the G2–M phase, due to the presence of unrepaired DSBs. To address whether olaparib administered in combination with radiation can increase the percentage of RAD51C-depleted cells in the G2–M phase, we conducted FACS analysis on SNU-601, SNU-668, and RAD51C-depleted SNU-668 cells treated with olaparib, radiation, or both. The numbers of SNU-601...
Figure 2. Olaparib induces G2–M cell-cycle arrest and apoptosis in RAD51C-deficient cell lines. The cells were treated with the indicated concentrations of olaparib for 96 hours, and the percentage of cells in the G2–M phase and those undergoing apoptosis are determined using FACS analysis. The columns represent the mean of 3 independent experiments and are shown with error bars (±SE). A, a, the proportion of the SNU-601 and SNU-668 cells undergoing G2–M phase and apoptosis are presented in the bar graphs (top). a, P = 0.038; b, P = 0.032; and c, P = 0.005. The total cellular proteins were extracted and Western blotting was conducted with the indicated antibodies. PARP cleavage was observed in the olaparib-sensitive cells in a dose-dependent manner (bottom). B, SNU-668 cells transfected with nonspecific control or RAD51C-specific siRNA were harvested and the percentages of cells in the G2–M phase and undergoing apoptosis were calculated and are shown in the bar graph (top). a, P = 0.013; b, P = 0.0001; c, P < 0.0001; d, P < 0.0001; and e, P < 0.0001. Western blot analysis showed that PARP cleavage was observed after olaparib treatment in the RAD51C-depleted SNU-668 cells in a dose-dependent manner (bottom). C, the pcDNA3 or pcDNA3-RAD51C plasmids were stably transfected to the SNU-601 cells and the cell-cycle distribution was analyzed. The percentages of cells in the G2–M phase and those undergoing apoptosis are presented in the bar graphs (top). a, P = 0.0028; b, P < 0.0001; c, P < 0.0001; and d, P < 0.0001. Western blot analysis of SNU-601 cells overexpressing RAD51C revealed that RAD51C expression can lead to evasion of apoptosis (bottom).
and SNU-668 cells transfected with RAD51C-specific siRNA in the G2-M and sub-G1 phases were increased compared with untreated SNU-668 cells and SNU-668 cells transfected with nonspecific control siRNA (Fig. 3A). Olaparib significantly enhanced the cell-cycle arrest and apoptosis induced by irradiation, indicating that there is synergy between olaparib and radiation. Increased PARP cleavage was clearly detected in RAD51C-deficient cells following radiation exposure combined with 1 μmol/L olaparib (Fig. 3B). Overall, our data indicate that olaparib sensitized the RAD51C-deficient cancer cells to radiation.

**RAD51C silencing impairs the efficiency of homologous recombinational DSB repair**

We hypothesize that olaparib sensitivity results from reduced homologous recombinational repair efficiency of olaparib-induced DSBs in cells lacking RAD51C. We conducted an immunofluorescence study to examine RAD51 foci formation, indicative of DNA repair activity, in RAD51C-deficient SNU-668 cells exposed to ionizing radiation. RAD51C protein expression was depleted by siRNA (Fig. 4A). Control and RAD51C siRNA-transfected SNU-668 cells were treated with 10 Gy of radiation and stained with antibodies against RAD51 (a marker of homologous recombinational repair) and phosphorylated H2AX (a marker of DNA damage). The number of RAD51 foci in the RAD51C knockdown cells was significantly reduced at the sites of DNA damage even when the degree of damage was comparable with that of the controls (Fig. 4B and C). In addition, accumulation of DNA damage in individual cells was detected using a comet assay. SNU-601 and RAD51C-depleted SNU-668 cells showed accumulation of DNA DSBs when treated with olaparib and 10 Gy of radiation (Supplementary Fig. S7A and S7B). The knockdown of RAD51C expression was confirmed by Western blot analysis (Supplementary Fig. S7C). These data indicate that RAD51C downregulation leads to the accumulation of DNA damage due to homologous recombination inactivation. Our results support a role for RAD51C in the repair of DSBs in human cancer cells.

**RAD51C is epigenetically repressed in human cancer**

Our previous experiments showed that there was an inverse relationship between RAD51C protein expression and olaparib sensitivity in cancer cell lines. Next, we asked whether we can find the tumors with RAD51C deficiency using human cancer tissues for future use in clinic as a predictive marker and what mechanism might underlie RAD51C silencing in human cancers. RAD51C protein expression was significantly downregulated in 4 of 11 primary gastric tumor tissues (36%) compared with the corresponding normal tissues (Supplementary Fig. S8A). For future clinical application, an IHC analysis was conducted to examine the RAD51C protein expression in paraffin-embedded tissues. We assigned scores between negative and 3+ according to the intensity and extent of RAD51C protein expression in the tissues (Supplementary Fig. S8B). Similar to the Western blotting results, the IHC analysis showed that 5 of 13 tumors (38%) were IHC 1+ and that 2 tumors (15.3%) were IHC 2+, in contrast to the normal tissues, which were 3+ according to the IHC analysis. We next explored DNA methylation as a potential mechanism underlying the loss of RAD51C expression in cancer cells. To determine whether the absence of RAD51C is due to DNA methylation in the SNU-601 cells, we analyzed the induction of RAD51C expression in SNU-601 and SNU-668 cells treated with 5-aza-dc, an inhibitor of DNA methylation. The 5-aza-dc increased RAD51C expression in the SNU-601 cells more than 160-fold (Supplementary Fig. S9A). We also observed that the RAD51C gene was densely methylated in the SNU-601 and KATO-III cells using bisulfite-modified sequencing (Fig. 5A). To further investigate whether the low expression levels of RAD51C in gastric tumor tissues are caused by DNA methylation, we conducted DNA sequencing with bisulfite modification. Interestingly, almost every tumor tissue sample with reduced RAD51C expression showed a level of RAD51C methylation ranging from 19% to 56% (Supplementary Fig. S9B). These data suggest that the loss of RAD51C mediated by DNA methylation may frequently occur in cancer. Unlike the gastric cancer cell lines, SNU-601 and KATO-III, RAD51C expression was absent in the BT-549 cells regardless of the DNA methylation status (Fig. 5B). Thus, we speculated that histone modification might also be responsible for controlling RAD51C downregulation. Ezh2 is a component of the polycomb group protein that is essential for the transcriptional regulation of a number of genes involved in the DNA repair process. Recently, increased Ezh2 expression was found to be correlated with the epigenetic repression of DNA repair genes, including RAD51 (22–24). We therefore evaluated the possible effect of Ezh2 on RAD51C expression. First, we conducted real-time PCR to quantify the basal expression levels of RAD51C and Ezh2 to determine whether RAD51C expression was inversely correlated with Ezh2 expression in BT-549 and MCF-7 breast cancer cells. Cells overexpressing Ezh2 had downregulated RAD51C expression (Fig. 5C). Ezh2 depletion in BT-549 cells resulted in markedly increased RAD51C expression (Fig. 5D). Thus, the depletion of RAD51C mediated by epigenetic silencing resulted in the attenuated formation of RAD51C foci and increased sensitivity to DNA damage in cancer cells.

**Olaparib impedes the growth of RAD51C-defective cells in an in vivo mouse model**

Olaparib showed significant antitumor activity in a SNU-601 gastric cancer xenograft model. Olaparib significantly delayed tumor growth not only during treatment but also after treatment was ceased (Fig. 6A). Tumor tissues from mice treated with olaparib showed lower Ki-67 expression, which suggests lower proliferation ability compared with the tumor tissue from mice treated with vehicle control and it was associated with increased apoptosis by TUNEL assay (Fig. 6B). There were no signs of...
Figure 3. Olaparib sensitizes RAD51C-deficient cancer cells to radiation-induced G2–M arrest and apoptosis. A, the wild-type SNU-668 and SNU-601 cells along with SNU-668 cells transfected with nonspecific control or RAD51C-specific siRNA were treated with or without 1 μmol/L olaparib. The cells were either irradiated (10 Gy) or not 48 hours after the treatment. The cells were allowed to recover and grow for another 48 hours regardless of treatment with olaparib. Next, the percentages of cells in the G2–M phase and those undergoing apoptosis were measured and are presented in the bar graph. Column, the mean of 3 independent experiments; bars, /SE; a, P = 0.0003; b, P < 0.0001; c, P = 0.014; d, P = 0.0066; e, P = 0.009; f, P = 0.0002; g, P = 0.0006; and h, P < 0.0001. B, SNU-601, SNU-668, and nonspecific or RAD51C-specific siRNA-transfected SNU-668 cells were exposed to 1 μmol/L olaparib, irradiation, or left untreated. The cells were treated with olaparib for 48 hours, followed by irradiation (10 Gy) for 48 hours. The total cellular proteins were subjected to Western blot analysis with the indicated antibodies.
toxicity in mice undergoing extended treatment (Fig. 6C). This study showed the antitumor effect of olaparib in a RAD51C-deficient gastric cancer xenograft model.

Discussion

The use of a PARP inhibitor is a promising new strategy for treating cancers using the concept of synthetic lethality. There is preclinical and clinical evidence showing that olaparib (AZD2281; KU-0059436), a small-molecule inhibitor of PARP, has potential as a therapeutic agent to treat cancers with BRCA1 and BRCA2 mutations that have an homologous recombination deficiency (1–5, 25, 26). In addition to the BRCA mutations, a recently published phase II trial showed that 24% of high-grade ovarian carcinoma or triple-negative breast cancers without a BRCA mutation also respond to olaparib (4, 5). The effects of several proteins, including the deficiency of RAD51, involved in homologous recombination on the sensitivity to PARP inhibition was also reported (1), but the role of RAD51C deficiency on the sensitivity to the PARP inhibitor in cancer cells has not yet been reported. Importantly, models of sensitivity to PARP inhibition suggest that the key ingredient is a deficiency in homologous recombination, indicating that this approach may be more widely applicable in the treatment of sporadic cancers sharing homologous recombination impairments. In the present study, we first reported that RAD51C expression was low in olaparib-sensitive cancer cells. We also found that olaparib was able to enhance the cytotoxic effects of irradiation-induced DNA damage in RAD51C-deficient cells. This effect subsequently led to the accumulation of unrepaired DSBs due to dysfunctional RAD51 foci.
Figure 5. RAD51C deficiency is caused by epigenetic silencing or the overexpression of a transcriptional repressor. A, methylation levels of the RAD51C gene in the SNU-601 and SNU-668 cells was analyzed by genomic sequencing of bisulfiite-modified DNA (left). The methylation levels in the KATO-III and MKN-45 cell lines were determined using bisulfite-modified sequencing (right). B, hypomethylation of the RAD51C gene in the BT-549 cells was determined using bisulfite-modified sequencing. C, the basal expressions of Ezh2 and RAD51C in the BT-549 and MCF-7 cells were analyzed by quantitative real-time PCR. The results were normalized to actin expression and are presented in the bar graph. Columns, the mean of 3 independent experiments; bars, ±SE. D, Ezh2 negatively regulates RAD51C expression. The siRNA-mediated depletion of Ezh2 induced RAD51C mRNA expression. The expression levels of RAD51C and Ezh2 were determined by quantitative real-time PCR in BT-549 cells transfected with nonspecific control or Ezh2-specific siRNA.
formation along with increased cell death and G2-M arrest. Moreover, olaparib had significant in vivo antitumor effects in a RAD51 normal and BRCA wild-type RAD51C-deficient SNU-601 xenograft model. Overall, our findings indicate that sensitivity to olaparib relies on RAD51C inactivation.

RAD51C maintains genome integrity by participating in branch migration and Holliday junction resolution (12, 13, 27). Germline mutations in RAD51C confer an increased risk of breast and ovarian cancer (28–30). According to the previous studies, the role of RAD51C in homologous recombination activation via the regulation of RAD51 recruitment was understood. Here, we first determined that inactivation of homologous recombination-mediated by RAD51 increases DNA damage in RAD51C downregulated cancer cells, resulting in hypersensitivity to PARP inhibition. Our findings suggest that RAD51C plays an essential role in DNA damage response as a homologous recombination component, and that can promote synthetic lethality between PARP inhibition and RAD51C loss in cancer.

Some previous reports that have described the interaction between RAD51C and XRCC3, and the role of RAD51C in the resolution of homologous recombination intermediates have also been evaluated (15, 31). In addition, some studies have suggested that RAD51C expression can affect XRCC3 expression. We also observed that the levels of XRCC3 expression were proportional to the expression levels of RAD51C in the olaparib-sensitive cancer cell lines. In the present study,
we examined the potential physical interaction between RAD51C and XRCC3 with an IP assay and found that depletion of RAD51C expression reduced XRCC3 expression (data not shown). Although we found that RAD51C expression can affect XRCC3 protein levels, the mechanism through which XRCC3 expression may be regulated by RAD51C is not clearly understood. Elucidating the mechanism responsible for the regulation of XRCC3 expression by RAD51C and the effect of this regulation on the homologous recombination process would be very interesting and worthy of further study.

The downregulation of genes associated with the epigenetic mechanism involved in the DNA repair pathway prompts tumorigenesis (24, 32–35). We observed RAD51C downregulation in gastric and breast cancer cell lines and gastric tumor tissue samples. Interestingly, RAD51C expression was significantly decreased in cancer compared with the adjacent normal tissue, and the lack of RAD51C was attributed to DNA methylation and histone modification. Gene silencing mediated by DNA methylation has been well characterized (36, 37). Similar to BRCA1/2 mutations, BRCA1/2 hypermethylation is associated with the sensitivity to treatment with PARP inhibitors (32–35). Thus, the epigenetic inactivation of DNA repair genes by CpG island hypermethylation could be a marker that may be used to improve the response to cancer treatment and provide more personalized therapies with PARP inhibitors. We found that RAD51C was densely methylated in cancer cell lines and tumor tissue samples, resulting in RAD51C silencing. Moreover, our findings showed that the loss of RAD51C expression due to epigenetic silencing leads to olaparib sensitivity in cancer cells.

Conventional therapies induce drug resistance due to decreased drug accumulation, increased drug degradation, and consolidation of DNA repair pathways. PARP inhibition could overcome these problems by impairing the DNA repair pathway. In our study, we found that the combined use of radiation and olaparib enhanced irradiation-induced cytotoxicity by inducing cell-cycle arrest at the G2–M phase. Thus, olaparib can be potentially used as a therapeutic agent alone and in combination with radiation or with other DNA damaging agents.

In summary, this report is the first to show that epigenetically silenced RAD51C-deficient cancer cells are highly sensitive to the PARP inhibitor olaparib in vitro and in vivo. Moreover, RAD51C expression was downregulated in some gastric cancer tissues compared with adjacent normal tissues via DNA methylation. Using a novel synthetic lethal approach with a PARP inhibitor, we determined that RAD51C participated in the DNA repair pathway. Our findings have potential clinical implications for treating cancers with RAD51C deficiencies. Furthermore, RAD51C may serve as a novel biomarker for identifying tumors that are sensitive to olaparib, thereby allowing physicians to select patient populations who would receive the maximal benefit from olaparib treatment.

Disclosure of Potential Conflicts of Interest

S.-A. Im has commercial research grant from AstraZeneca through fellowship program and is a consultant/advisory board member of AstraZeneca. W.-H. Kim has commercial research grant from AstraZeneca and receives travel expense from AstraZeneca. Y.-J. Bang has commercial research grant, honoraria from speakers bureau, and is a consultant/advisory board member of AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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