Tumor Growth Inhibition by Olaparib in BRCA2 Germline-Mutated Patient-Derived Ovarian Cancer Tissue Xenografts

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Abstract

Purpose: Most patients with ovarian carcinomas succumb to their disease and there is a critical need for improved therapeutic approaches. Carcinomas arising in BRCA mutation carriers display defective DNA double-strand break repair that can be therapeutically exploited by inhibition of PARP-1, a key enzyme in the repair of DNA single-strand breaks, creating synthetic lethality in tumor cells.

Experimental Design: To investigate synthetic lethality in vivo, we established a BRCA2 germline-mutated xenograft model that was developed directly from human ovarian cancer tissue, treated with the PARP inhibitor olaparib (AZD2281) alone and in combination with carboplatin.

Results: We show that olaparib alone and in combination with carboplatin greatly inhibit growth in BRCA2-mutated ovarian serous carcinoma. This effect was not observed in a serous carcinoma with normal BRCA function, showing a specific antitumor effect of olaparib in mutation carriers. Immunohistochemistry (cleaved caspase-3 and Ki-67 stains) of remnant tissue after olaparib treatment revealed significantly decreased proliferation and increased apoptotic indices in these tumors compared with untreated controls. Furthermore, olaparib-treated tumors showed highly reduced PARP-1 activity that correlated with olaparib levels.

Conclusions: We established a BRCA2-mutated human ovarian cancer xenograft model suitable for experimental drug testing. The demonstrated in vivo efficacy of olaparib extends on the preclinical rationale for further clinical trials targeting ovarian cancer patients with BRCA mutations. Clin Cancer Res; 17(4); 783–91. © 2010 AACR.

Introduction

Ovarian cancer is the fifth leading cause of cancer death in North American women and the most fatal gynecological cancer (1). Approximately 10% of all ovarian cancers are hereditary and of these, more than 90% are associated with BRCA1 or BRCA2 germline mutations (2). In addition, a smaller number of nonhereditary ovarian cancers show somatic BRCA mutations, and functional loss of BRCA1 through promoter methylation is commonly observed. These changes together result in the BRCA deficiency associated with high-grade serous ovarian carcinoma (HGSC), the most frequent histologic subtype in epithelial ovarian cancer (3, 4). Patients with BRCA germline mutations treated with platinum-based therapy regimens have a survival advantage compared with nonhereditary ovarian carcinoma of HGSC subtype (5). Regardless, the majority of patients with HGSC relapse and ultimately die from their disease, therefore development of effective first-line therapies is critical.

BRCA1 and BRCA2 encode tumor suppressor proteins that are part of a multicomponent complex (RAD51 complex) involved in the repair of DNA double-strand breaks (DSBs) and collapsed replication forks by the process of homologous recombination, and are therefore essential for maintaining genomic stability (6). This genetic defect in the DNA repair pathway of affected tumors can be exploited by using poly[ADP-ribose] polymerase (PARP) inhibitors to induce selective tumor cytotoxicity while sparing normal cells. The most abundant member of the PARP family, PARP-1, plays a crucial role in the repair of DNA
Translational Relevance

The efficacy of conventional platinum-based chemotherapy for high-grade serous carcinoma is limited; most patients show an initial response to treatment but upon relapse, response rates to platinum progressively diminish and they ultimately die of progressive disease. By capitalizing on the genetic defect in DNA double-strand repair in serous carcinomas with BRCA loss, through targeting of DNA single-strand repair with the poly(ADP)-ribose polymerase (PARP)-inhibitor olaparib, selective tumor cytotoxicity can be induced while sparing normal cells. We show that olaparib therapy results in tumor cell death in a human ovarian carcinoma xenograft with a BRCA2 mutation, but not in a xenograft with normal BRCA1 and BRCA2, and the effects of olaparib are increased with concomitant carboplatin treatment. This study describes a clinically relevant model of serous ovarian carcinoma for drug testing, and provides a rationale for trials of combined PARP-inhibitor/platinum chemotherapy for serous carcinomas with BRCA loss.

Materials and Methods

Transplantable patient-derived ovarian cancer tissue lines

Ovarian tumor tissue specimens were obtained, with informed consent, from patients undergoing surgical staging for primary ovarian cancer at Vancouver General Hospital, Vancouver, BC, Canada. Fresh tumor tissue was cut into small pieces and grafted under the renal capsule of female NOD-SCID mice for subsequent serial transplantation and characterization as previously described (19). Animal care and experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care, Ottawa, ON, Canada and human tissue specimens managed per protocol approved by the University of British Columbia Clinical Research Ethics Board, Vancouver, BC, Canada. Treatment response (olaparib, carboplatin, and combination) was tested in LTL247, a tumor line derived from a patient with a known BRCA2 germline mutation (exon 11 c4848–4849 delAA). Pathology review of the original tumor specimen confirmed HGSC subtype. LTL258 (BRCA wild type) developed from the HGSC tumor of a different patient was selected as a paired control.

BRCA mutation analysis

BRCA2 coding sequence mutations were confirmed by whole transcriptome RNA sequencing (RNA-seq) as previously described (20) and direct sequencing (ABI, 3130xl Genetic Analyzer) in original patient tumor and in the later passages of tumor line LTL247 (primer sequences exon 11: BRCA2 forward aaagaccctaaagtacagagagg, BRCA2 reverse cgccgcggccccggccgctattcttctcttccttgaggagttacc). Wild-type status of BRCA 1 and 2 in LTL 258 were confirmed using reference primers as described before (4).

Affymetrix SNP 6.0 arrays were used to determine the copy number of chromosome 13q harboring the BRCA2 locus for the original patient tumor and a later generation xenograft of LTL247. The arrays were normalized using CRMAv2 (21) and default settings for performing allelic-crosstalk calibration, probe sequence effects normalization, probe-level summarization, and PCR fragment length normalization. Log ratios were then computed by normalizing against a reference generated using a normal dataset of 270 HapMap samples obtained from Affymetrix. Segmentation was performed using a modified 11-state hidden Markov model (http://compbio.bccrc.ca/?page_id = 401) derived from CNA-HMMer (20). Segment calls were generated according to 5 somatic states (homozygous deletion, hemizygous deletion, gain, amplification, and high-level amplification), 5 analogous germline states, and neutral copy number.

Olaparib efficacy studies

The structure and activity of olaparib (AZD2281, KU-0059436) has been previously described (9, 14, 17, 18). Olaparib was solubilized in DMSO and diluted to 5 mg/mL with PBS containing 10% (w/v) 2-hydroxy-propyl-beta-cyclodextrin (Sigma).
After 2 tumor doubling times (DT for LTL247: 10 days and DT for LTL258: 16 days), reaching a calculated average volume of 16.6 mm³, the animals were randomized into 4 treatment groups (4–6 mice/group; 2 grafts/kidney): (1) olaparib alone, (2) carboplatin alone, (3) both agents, and (4) vehicle control. LTL247 mice were treated for 4 weeks and LTL258 mice were treated for 5 weeks. Olaparib was dosed i.p. (50 mg/kg) once daily for 5 days × 4 weeks for LTL247 and ×5 weeks for LTL258 (17, 18); carboplatin (Novopharm Ltd.) was dosed i.p. (50 mg/kg) once weekly × 4 weeks for LTL247 and ×5 weeks for LTL258 (18). Mice were provided with food and water ad libitum and monitored daily for changes in general health, including body weight loss, diarrhea, food–water intake, appearance, and behavior. Tumors were harvested on day 29 and 35 for LTL247 and LTL258, respectively, 2 hours after last dosing.

Tumor burden was determined at necropsy as previously described (19). Mean tumor volume (mm³) ± (SD) was calculated and growth inhibition (GI) was reported as the percentage decrease of tumor volume compared with the vehicle control. Individual group comparisons were performed using independent student t-tests and overall group comparisons were performed using one-way ANOVA. Results with P < 0.05 were reported as significant.

Immunohistochemistry (proliferation and apoptotic indices)

Immunohistochemistry was performed on 5 μm paraffin-embedded tissue sections using TUNEL assays (Apop-Tag Apoptosis Detection Kit, Chemicon) and a monoclonal MIB1 antibody (Dako, dilution 1:50) directed against the Ki-67 antigen as previously described (19).

For cleaved caspase-3 staining, 4 μm thick sections were cut and immunostained on a Ventana Discovery XT staining system (Ventana Medical Systems). Sections were deparaffinized in xylene, dehydrated through 3 alcohol changes, and transferred to Ventana Wash solution. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide. Antigen retrieval was performed in cell conditioner 1 and slides were incubated with anti-cleaved caspase-3 rabbit monoclonal antibody (1:100 dilutions, clone 5A1E, Cell Signaling Technology) for 60 minutes. Finally, sections were incubated with the UltraMap anti-rabbit HRP-conjugate and ChromoMap detection system (Ventana Medical Systems), counterstained with hematoxylin, dehydrated, cleared, and mounted.

The Ki-67 proliferation index (PI) was determined on a minimum of two randomly selected high-power fields (400× magnification) containing representative sections of tumor tissue and calculated as the percentage of positively stained tumor cells to total tumor cells. The apoptotic index (AI) using cleaved caspase-3 and TUNEL stains was calculated as the percentage of positively stained apoptotic tumor cells to total tumor cells by counting at least three high-power fields (at 400× magnification). Areas with extensive necrosis were avoided.

Compound level quantification and PARP-1 activity determination in BRCA2−/− tumor tissues

To show the specific PARP inhibitory effect of olaparib therapy, we determined the ex vivo PARP-1 activity in relation to the drug compound levels in 5 BRCA2−/− xenografts of LTL247 after 4 weeks of treatment and six untreated controls.

Olaparib levels were analyzed by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). Tumors were weighed, homogenized in 3 volumes of PBS, and samples extracted using solid phase extraction. Chromatographic separation was by gradient elution using an Acquity BEH Phenyl (5.0 × 2.1 mm, 1.7 μm) column coupled to a Sciex API 2000 with electrospray ionization (ESI) in positive ion mode using multiple reaction monitoring of ions 435.32 to 281.10 m/z. Calibration standards (10–5,000 ng/mL) and quality control samples were prepared in mouse plasma and tumor homogenate.

PARP-1 activity within tumor samples was analyzed using an ex vivo activation assay as previously described (9).

Results

Model characterization

We selected a tumor line originating from a patient with known heterozygous BRCA2 germline mutation. This mutation could be characterized as a 2 base pair deletion frameshift mutation in BRCA2 exon 11 using the patients constitutional DNA (peripheral blood; Fig. 1). Copy number analysis of the patient tumor and the late generation xenograft revealed a hemizygous deletion of the BRCA2 locus with loss of heterozygosity. Accordingly, direct sequencing showed a homozygous state of the frameshift mutation in the primary and xenograft tumors. RNA-seq confirmed exclusive expression of the mutated allele that was supported by 2 reads (data not shown). In summary, this homozygous frameshift mutation can be considered as a loss of function mutation. In contrast, tumor line LTL258 did not show BRCA1 or BRCA2 mutations (data not shown).

Olaparib inhibits growth of BRCA2−/− ovarian cancer tissue xenografts

All 39 mice included in the study were available for evaluation and measurement of tumors on harvest day. On macroscopic inspection, LTL247BRCA2−/− xenografts treated for 4 weeks with vehicle showed, on average, markedly enlarged tumor masses. This was confirmed on microscopic evaluation, with H&E staining identifying viable cancer cells (Fig. 2, middle). In contrast, olaparib-treated tumors showed much smaller tumor masses, lower overall cell numbers, increased numbers of dead cells, and more cells with enlarged cellular and nuclear structures than controls (Fig. 2, right). Morphometric measurements revealed significant differences in tumor mass between treatment groups tested with simultaneous comparison of means (ANOVA; P = 9.78e-14). Individual group comparisons showed that treatment with olaparib alone markedly inhibited growth of the LTL247BRCA2−/− HGSC line.
compared with the untreated controls (19.7 mm$^3$ ± 25.0 mm$^3$ vs. 97.3 mm$^3$ ± 72.6 mm$^3$; GI 79.8%; $P = 3.1 \times 10^{-5}$), although the tumor volume on average was still minimally larger than the calculated volume on the first day of treatment. Treatment with carboplatin alone also resulted in significant GI (4.4 mm$^3$ ± 7.4 mm$^3$ vs. 97.3 mm$^3$ ± 72.6 mm$^3$; GI 95.5%; $P = 2.1 \times 10^{-6}$), whereas combination of olaparib and carboplatin showed the best treatment response (1.2 mm$^3$ ± 1.4 mm$^3$ vs. 97.3 mm$^3$ ± 72.6 mm$^3$; GI 98.8%; $P = 1.29 \times 10^{-6}$; Fig. 3A, left). Carboplatin and carboplatin + olaparib-treated tumors resulted in very small residual explant tissue consisting mostly of scar tissue.
After regrafting the 2 largest residual tumors of the olaparib alone and the combination treatment group as well as 2 tumors of the control group, different latency times were observed before regrowth: both control tumors reconstituted to palpable size after 6 weeks while one of the olaparib alone treated tumors reconstituted after 12 months. In contrast, the second olaparib-treated tumor and the combination-treated tumors never expanded in size until the mice had to be euthanized after 14–18 months.

BRCA wild-type LTL258

BRCA1+/+, BRCA2+/+ xenografts did not show significant GI with olaparib compared with vehicle-treated mice (90.1 mm³ ± 66.4 mm³ vs. 112.9 mm³ ± 90.2 mm³; GI 20.2%; P = 0.403), whereas treatment with carboplatin (14.9 mm³ ± 12.7 mm³ vs. 112.9 mm³ ± 90.2 mm³; GI 86.8%; P = 0.0008) and combination therapy of carboplatin and olaparib (34.0 mm³ ± 25.6 mm³ vs. 112.9 mm³ ± 90.2 mm³; GI 69.9%; P = 0.004) significantly reduced tumor size (Fig. 3A, right).

Toxicity evaluation

Only 4 of all the treated animals showed substantial loss of body weight (>10%), 3 mice bearing tumor LTL258, and 1 bearing LTL247. Two of the mice received vehicle only, one carboplatin, and one the combination treatment. No gross changes in food–water intake, appearance, or behavior were observed, indicating that the treatments were well tolerated. Particularly, the addition of olaparib to carboplatin did not significantly increase weight loss compared with carboplatin single agent treatment in the LTL247BRCA2−/− tumor line (carboplatin average weight gain 0.4% vs. combination average weight loss 1.6%; P = 0.5). Similarly, no statistically significant increase of weight loss was observed in the BRCA wild-type line LTL258 (carboplatin average weight loss 5.7% vs. combination average weight loss 6.6%, P = 0.9).

Olaparib-treated tumors exhibit decreased proliferation and increased apoptosis

Immunohistochemical analysis of Ki-67-stained tissue sections from 4 randomly selected LTL247BRCA2−/− xenografts, treated for 4 weeks with olaparib (Fig. 4A, right), showed a significantly lower number of proliferating cells compared with 4 untreated LTL247BRCA2−/− control xenografts (Fig. 4A, left). On average, the percentage of proliferating cells per high-power field was 1.9-fold lower in the olaparib-treated tumors [29.5% ± 14.1% (SD) vs. 55.7% ± 10.3% (SD); P = 0.0006]. In contrast, olaparib-treated xenografts of the BRCA competent tumor line did not show significant reduction of proliferating cells compared with untreated control xenografts [19.1% ± 6.4% (SD) vs. 20.4% ± 5.7% (SD); P = 0.7; Fig. 4D, right and left).

Cleaved caspase-3 stains of 3 representative olaparib-treated LTL247BRCA2−/− tissue sections (Fig. 4C, right) displayed a significantly higher number of apoptotic cells than untreated control xenografts (Fig. 4C, left). On an average, the AI per high-power field was 3.8-fold increased in the olaparib-treated tumors [3.1% ± 1.8% (SD) vs. 0.8% ± 0.2% (SD); P = 0.0008].
In the BRCA competent tumor line, no significant difference in the AI between olaparib treated and untreated xenografts could be observed [0.7% ± 0.6% (SD) vs. 0.8% ± 0.9% (SD); P = 0.8; Fig. 4D, right and left].

In agreement with the caspase-3 assay, TUNEL stains of 6 representative olaparib-treated LTL247 BRCA2/C0 tissue sections (Supplementary Figs. 1A and B) equally displayed a significantly higher number of apoptotic cells compared with untreated control xenografts (Supplementary Figs. 1C and D). On an average, the AI per high-power field was 2.3-fold increased in the olaparib-treated tumors [5.4% ± 1.35% (SD) vs. 2.3% ± 1.0% (SD); P = 7.6e-16].

H&E staining of residual tumor tissues after treatment with carboplatin alone and in combination with olaparib revealed only scar tissue but no tumor cells. Accordingly, no further immunohistochemistry could be performed for those treatment groups.

**PARP-1 activity is strongly inhibited in olaparib-treated BRCA2−/− tumors and correlates with compound levels**

Pharmacodynamic activity assays showed that all olaparib-treated tumors had greatly reduced PARP-1 activity compared with the average of vehicle-treated controls. On average, PARP-1 activity in olaparib-treated tumors was reduced to 16.1% (range 8.16%–33.75%; SD 10.1%) of untreated controls (100%). Untreated control tumors did not show detectable compound levels, whereas in olaparib-treated tumors, compound concentration ran-
ged from 198 to 3870 ng/mL with compound levels correlating well with PARP-1 activity (Pearson correlation 0.89; Fig. 3B).

Discussion

Using a patient-derived ovarian cancer xenograft model, we have demonstrated that BRCA2 germline-mutated tumors are sensitive to single agent treatment with the PARP inhibitor olaparib. Although carboplatin showed better single agent efficacy, olaparib treatment also induced a clear tumor growth inhibition. This was in contrast to BRCA wild-type tumors, supporting findings in preclinical studies and early clinical trials that BRCA deficient tumors are susceptible to a synthetic lethal therapeutic approach (14–16). Furthermore, in most of the
cases, combining olaparib with carboplatin either eradicated the tumors completely or left very small tumors which showed only scar tissue on microscopical inspection.

Notably, the addition of the PARP-inhibitor to carboplatin did not increase toxicity in mice.

Our results are in agreement with other studies that have tested olaparib in BRCA1<sup>−/−</sup>-<p53<sup>−/−</sup> and BRCA2<sup>−/−</sup>-<p53<sup>−/−</sup> genetically altered mouse models (17, 18). In these studies, the authors observed in vivo efficacy of olaparib (AZD2281) against BRCA-associated murine breast cancers with improvements in both recurrence-free and overall survival. We show, for the first time, that olaparib confers antitumor activity and improved survival in an experimental model utilizing human ovarian tumor tissue xenografts. Obvious differences between these models include variation of the genetic backgrounds across species, altered host immune responses, and the tissues studied. However, the comparability of the results further supports the efficacy of olaparib in these models independent of these parameters.

PARP inhibitors are currently being tested in various phase I/II clinical trials (7) and another PARP-inhibitor compound (BSI-201) is being investigated in a phase III trial of triple negative breast cancer (www.clinicaltrials.gov NC100938652). In the case of olaparib, Fong and coworkers have recently reported on the pharmacokinetics and pharmacodynamics of this drug in a phase I clinical trial involving patients with cancers associated with BRCA mutations (including 16 patients with ovarian cancer; ref. 14). The authors observed antitumor activity only in BRCA mutation carriers and concluded that olaparib was effective in this subgroup with few side effects compared with conventional chemotherapy. Subsequent phase II trials of olaparib in BRCA mutation carriers with chemoresistant breast or ovarian cancer have been completed and preliminary results presented show high activity in those patients and an acceptable tolerability profile (22, 23). The suggestion has been made by others that combination therapy with PARP inhibitors amplifies cytotoxic effects of drugs such as carboplatin (17). In our study, the addition of olaparib to carboplatin in the BRCA2 deficient tumor line did result in smaller tumors than single agent therapy, but the difference did not reach statistical significance (t-test P-value 0.07).

Furthermore, tumor relapse could be delayed in a mouse model by continuous treatment with olaparib alone after carboplatin chemotherapy, suggesting a role for olaparib in maintenance therapy during remissions (17, 18). It is possible that development of resistance to PARP inhibitors will hamper further clinical progress. Therefore, model systems that allow for the study of drug resistance would be useful. Here, we showed that a human ovarian cancer tissue explant xenograft mouse model has utility for the testing of experimental drugs in BRCA-mutated tumors. In the future, development of additional tumor tissue lines, including those that progressed under treatment, has the potential to elucidate the underlying molecular mechanisms of olaparib drug resistance.

In conclusion, this study shows the applicability of a xenograft model for experimental drug testing in human BRCA<sup>−/−</sup> ovarian tumors. To our knowledge, we are the first to develop a model for BRCA2 deficiency directly derived from a patient’s tumor. The efficacy of olaparib as a single agent or in combination with carboplatin warrants further investigation in clinical trials.

Disclosure of Potential Conflicts of Interest

A. Lau and M.J. O’Connor report being employees of AstraZeneca. A.N. Cranston as well as S. Tully and S. Shahzad report having been employed by KuDOS Pharmaceuticals at the time experiments were performed. No other potential conflict of interest relevant to this article was reported.

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