**Title:**

# Secondary mutations as a mechanism of cisplatin resistance in *BRCA2*-mutated cancers

**Authors:** Wataru Sakai[1, 2], Elizabeth M. Swisher[3, 6], Beth Y. Karlan[4], Mukesh K. Agarwal[5], Jake Higgins[6, 7], Cynthia Friedman[1], Emily Villegas[1, 2], Céline Jacquemont[1, 2], Daniel J. Farrugia[5], Fergus J. Couch[5], Nicole Urban[2], Toshiyasu Taniguchi[1, 2]

[1] Division of Human Biology
Fred Hutchinson Cancer Research Center, Seattle, WA 98109-1024, USA
[2] Division of Public Health Sciences
Fred Hutchinson Cancer Research Center, Seattle, WA 98109-1024, USA
[3] Department of Obstetrics and Gynecology, University of Washington, Seattle, WA 98195-7720, USA.
[4] Cedars-Sinai Medical Center, Department of Obstetrics and Gynecology, Los Angeles, CA 90048-1865, USA
[5] Department of Laboratory Medicine and Pathology, Mayo Clinic College of Medicine, Rochester, MN 55905, USA.
[6] Department of Medicine, University of Washington, Seattle, WA 98195-7720, USA
[7] Department of Genome Sciences, University of Washington, Seattle, WA 98195-7720, USA.

**Corresponding author:** Toshiyasu Taniguchi
Divisions of Human Biology and Public Health Sciences
Fred Hutchinson Cancer Research Center
1100 Fairview Ave. N., C1-015
Seattle, WA 98109-1024, USA
Phone: 206-667-7283
Fax: (206) 667-5815
email: ttaniguc@fhcrc.org

**Ovarian carcinomas with mutations in the tumor suppressor *BRCA2* are particularly sensitive to platinum compounds [1]. However, such carcinomas ultimately develop cisplatin resistance. The mechanism of that resistance is largely unknown [2]. Here we show that acquired resistance to cisplatin can be mediated by secondary intragenic mutations in *BRCA2* that restore the wild-type *BRCA2* reading frame. First, in a cisplatin-resistant *BRCA2*-mutated breast cancer cell line, HCC1428, a secondary genetic change in *BRCA2* rescued BRCA2 function. Second, cisplatin selection of a *BRCA2*-mutated pancreatic cancer cell line, Capan-1 [3,4], led to 5 different secondary mutations that restored the wild-type *BRCA2* reading frame. All clones with secondary mutations were resistant both to cisplatin and to a poly(ADP-ribose) polymerase (PARP) inhibitor (AG14361). Finally, we evaluated recurrent cancers from patients whose primary *BRCA2*-mutated ovarian carcinomas were treated with cisplatin. The recurrent tumor that acquired cisplatin resistance had undergone reversion of its *BRCA2* mutation. Our results suggest that secondary mutations that restore the wild-type *BRCA2* reading frame may be a major clinical mediator of acquired resistance to platinum-based chemotherapy.**

Women with ovarian cancer generally have a high initial response to platinum-based chemotherapy, but over time the majority of ovarian carcinomas become refractory, and most patients die with progressive chemoresistant disease [2]. The molecular basis of initial platinum sensitivity and acquired resistance remains largely unknown. Individuals with germline mutations in *BRCA1* or *BRCA2* (*BRCA1/2*) have an increased risk of developing cancer in the breast and ovary [5] as well as in some other organs including the pancreas and prostate [6]. *BRCA2* is identical to the Fanconi anemia

(FA) gene *FANCD1* [7]. The BRCA2 protein directly binds to and regulates RAD51, an essential protein for DNA repair through homologous recombination (HR) [8]. Tumors from *BRCA1/2* mutation carriers usually have deletion of the wild-type allele at the *BRCA1/2* locus and are BRCA1/2-deficient [9-11]. BRCA1/2-deficient cancer cells are hypersensitive to DNA-crosslinking agents including cisplatin [1, 12] . Therefore, cisplatin or its derivative, carboplatin, is a logical choice for the treatment of *BRCA1/2*-mutated tumors [13] and women with *BRCA1/2*-mutated ovarian carcinoma have a better prognosis than those without *BRCA1/2* mutation if they receive platinum-based therapy [14, 15]. However, even *BRCA1/2*-mutated tumors eventually develop platinum resistance.
In several genetic disorders such as FA, spontaneous genetic alterations compensating for inherited disease-causing mutations have been described [16]. These alterations in FA include secondary genetic changes of one of the mutated FA alleles, such as back-mutations to wild-type, compensatory mutations in *cis,* intragenic crossovers, and gene conversion [16-18]. We speculated that secondary mutations in *BRCA2*-mutated alleles might also occur in cancer cells during chemotherapy.

To study the mechanism of acquired cisplatin resistance of *BRCA2*-mutated cancer, first we screened 12 human breast cancer cell lines for alterations in BRCA2 protein expression (Fig. 1a). A pancreatic cancer cell line, Capan-1 [3, 4], with a truncated BRCA2 protein, was used as a control.

From breast cancer cell line, HCC1428 [19], BRCA2 protein was undetectable by western blotting using anti-BRCA2 (Ab-1), which recognizes the middle part of BRCA2 (Fig. 1a) but was detectable with anti-BRCA2 (Ab-2) that recognizes the BRCA2 C-terminus. The small size of HCC1428 BRCA2 identified by Ab-2 suggested that the

protein might have an internal in-frame deletion. Constitutional DNA from a lymphoblast cell line (HCC1428BL) from the same patient had one wild-type *BRCA2* allele and one mutant allele with a frameshift mutation, 6174delT, which is common in the Ashkenazi Jewish population (Figs. 1b and S1) [20].

Sequence of genomic DNA from HCC1428 cancer cells revealed no wild-type allele and a mutant allele with a 2135-basepair deletion surrounding the original 6174delT mutation and the exon 11/intron 11 junction (Figs. 1c and S1). Sequence of cDNA indicated that this deletion activated two cryptic splice donor sites in exon 11, resulting in expression of two transcripts with in-frame deletions (Figs. 1d, and S1). HCC1428 transcript 1 had a 2640-basepair deletion and encoded a protein lacking amino acids 1401 to 2281. Transcript 2 had a 2187-basepair deletion and encoded a protein lacking amino acids 1552 to 2281 (Figs. 1d, 1e and S1). Both HCC1428 BRCA2 proteins retain the single-strand DNA (ssDNA) binding domain and the C-terminus nuclear localization signals (NLS), whereas these domains are lost in *BRCA2.6174delT* (Fig. 1e). A recent report that only 1 BRC repeat plus ssDNA binding domain and NLS are sufficient for BRCA2 function [21] suggests that the novel BRCA2 proteins in HCC1428 might be functional. Indeed, HCC1428 cells were resistant to cisplatin (Fig. 1g). Furthermore, depletion of these novel BRCA2 proteins with siRNA restored sensitivity of HCC1428 cells to cisplatin (Figs. 1f and 1g).

HCC1428 was derived after chemotherapy from the pleural effusion of a 49 year-old woman with stage IV breast carcinoma who died 6 months later [19]. We speculate that the patient's chemotherapy selected *in vivo* for secondary mutations in *BRCA2*-mutated breast tumor cells.

To test this hypothesis, we selected *in vitro* for cisplatin-resistant clones from the cisplatin-sensitive *BRCA2*-mutated pancreatic cancer cell line Capan-1. Capan-1 has the mutant allele *BRCA2.6174delT,* but no wild-type allele (Fig. S5a) [3, 4]. Fluorescence in situ hybridization (FISH) revealed that Capan-1 harbors at least two copies of the *BRCA2* gene indicating duplication of the chromosome 13 with the mutant *BRCA2* gene (Fig. S2).

After selection in cisplatin, we obtained 14 cisplatin-resistant Capan-1 clones out of 12 million cells (Fig. S3 and Table S1). Importantly, in 7 of these 14 clones, BRCA2 expression, at close to the length of the wild-type protein, was now detectable (Fig. 2). The other 7 cisplatin-resistant clones still lacked BRCA2 protein expression.

In all 7 cisplatin-resistant clones with restored, nearly full-length BRCA2 protein expression, we identified additional *BRCA2* mutations that corrected the frameshift caused by the 6174delT mutation (Figs. 2b, S4, and S5, and Table S1). These secondary genetic changes included a small deletion, insertion, and deletion/insertion at sites close to the original mutation, and in-frame deletions surrounding the original mutation site. Interestingly, in each clone we observed both the original mutant *BRCA2.6174delT* sequence and the 6174delT sequence with additional mutations (Figs. S4 and S5) indicating that the secondary mutations occurred on only one of the duplicated mutant *BRCA2* copies (Fig. S5g). None of the 7 cisplatin-resistant clones lacking BRCA2 protein expression harbored additional mutations in *BRCA2.*

Next, we assessed the function of the restored BRCA2 proteins in the 14 cisplatin-resistant Capan-1 clones by evaluating RAD51 foci formation after exposure to ionizing radiation (IR) (Fig. 3a). IR-induced RAD51 foci formation was impaired in

parental Capan-1 [1]. In contrast, in 6 of the 7 cisplatin-resistant Capan-1 clones with restored BRCA2 expression, IR-induced RAD51 foci formation was significantly improved, suggesting that these novel BRCA2 isoforms are functional.

In most of the cisplatin-resistant Capan-1 clones without secondary mutations, IR-induced RAD51 foci formation remained impaired (Fig. 3a), suggesting that these clones acquired cisplatin resistance through mechanisms other than the restoration of the BRCA2-RAD51 pathway.

Next, we analyzed the homologous recombination (HR)-based DNA double-strand-break (DSB) repair function of some of the novel BRCA2 proteins using an I-SceI-dependent DR-GFP reporter assay in BRCA2-deficient VC-8 Chinese hamster cells [22]. The proportions of GFP-positive cells arising through the repair of I-SceI-induced DSB by HR after transfection of various mutant *BRCA2* constructs were compared (Figs. 3b and S6, Table S2). Transfection of a wild-type *BRCA2* construct resulted in about 5-fold more GFP-positive cells compared to transfection of vector control or the *BRCA2.6174delT* mutant. Transfection of constructs with any of the secondary *BRCA2* mutations resulted in GFP-positive frequencies equal to or greater than that of the construct with wild-type *BRCA2*. These results indicate that the novel BRCA2 proteins efficiently promote HR.

Poly(ADP-ribose) polymerase (PARP) inhibitors selectively kill BRCA1/2-deficient tumor cells [23, 24] and are expected to become a therapeutic option for patients with *BRCA1/2*-mutated cancers [13]. However, it remains unclear whether *BRCA1/2*-mutated tumors with acquired cisplatin resistance are sensitive to PARP inhibitors. We tested the sensitivity of cisplatin-resistant Capan-1 clones to a PARP

inhibitor (AG14361) (Fig. 3c). Both parental Capan-1 and cisplatin resistant clones without secondary mutations were sensitive to the PARP inhibitor. In contrast, Capan-1 clones with secondary *BRCA2* mutations were resistant, consistent with the restoration of functional BRCA2 in these clones, although the differences of the sensitivity between parental Capan-1 and these clones were relatively small.

Finally, we analyzed tissues of 5 patients with *BRCA2* mutations and recurrent ovarian carcinomas previously treated with platinum. Three recurrent tumors were clinically refractory to platinum and two were sensitive (Table S3). Platinum-refractory tumor UW3548 revealed genetic reversion of *BRCA2.6174delT* (Fig. 4). Evidence for genetic reversion is as follows. Constitutional DNA of this patient was heterozygous for *BRCA2.6174delT* and at two single nucleotide polymorphisms (SNPs). As expected, the microdissected specimen of the recurrent tumor was hemizygous at each SNP, reflecting loss of heterozygosity of *BRCA2* in the tumor. However, in the same tumor sample, both sequences of *BRCA2.6174delT* and wild-type *BRCA2* were detected, suggesting that the recurrent tumor had acquired wild-type *BRCA2* by genetic reversion, or back mutation to wild-type. We speculate that the appearance of both mutant and wild-type sequences resulted from duplication of the mutant *BRCA2* followed by genetic reversion of one of the duplicates, similar to the situation occurring in Capan-1 clones with secondary mutations (Figs. S4 and S5). A larger clinical study is warranted to determine the prevalence and clinical significance of *in vivo* secondary mutations in *BRCA2* in human tumors.

The second platinum-refractory relapsed tumor (CS2) presented a complex profile. This patient carried germline mutations in both *BRCA1* and *BRCA2* (Fig. S7).

The primary ovarian tumor was sensitive to platinum and showed loss of the wild-type *BRCA1* allele and heterozygosity of *BRCA*2, indicating that carcinogenesis was primarily driven by the *BRCA1* mutation. Importantly, the recurrent platinum-refractory tumor had lost the mutant *BRCA2* allele. We speculate that loss of the mutant *BRCA2* allele was the result of selection by chemotherapy and contributed to platinum resistance.

The tumor of a third patient (UW174) recurred 5 months after her primary chemotherapy, so her tumor is defined as clinically refractory to platinum. No secondary mutation in *BRCA2* was detectable in the tumor specimen of this patient. Recurrent tumors of two other patients (CS9 and CS15) were sensitive to cisplatin. No secondary genetic alterations in *BRCA2* were identified in either of these tumors.

Taken together, our data demonstrate that secondary mutations that correct the frameshift caused by mutated *BRCA2* alleles are a mechanism of acquired resistance to cisplatin (Fig. S8). Testing for secondary mutations in platinum-treated *BRCA2*-mutated cancers may be clinically important, because tumors with secondary mutations are likely to be resistant to both cisplatin and PARP inhibitors. Theoretically, it might be possible to re-sensitize these tumors to cisplatin and to PARP inhibitors by treatment with drugs such as proteasome inhibitors that inhibit RAD51 recruitment to sites of DNA repair [25]. In contrast, platinum-resistant *BRCA2*-mutated tumors without secondary *BRCA2* mutations may remain sensitive to PARP inhibitors.

Treatment with cisplatin could facilitate secondary mutations by increasing the mutation rate via DNA damaging effects. Alternatively, since BRCA2 is involved in error-free DNA repair, HR, it is possible that BRCA2 deficiency itself promotes secondary mutations through compensatory utilization of more error-prone DNA repair

processes [26].

Secondary mutations are relevant as a mechanism of resistance only in those tumors that contain frameshift mutations in *BRCA2*, which occurs in only 5% of ovarian carcinomas. However, *BRCA2* mRNA expression is undetectable in 13% of ovarian carcinoma [27]. We speculate that other mechanisms yet to be identified could restore BRCA2 in acquired cisplatin resistance in these sporadic cases.

Our findings may be applicable to cancers other than ovary. Secondary mutations in *BRCA2* occurred in a mitomycin C-resistant human acute myeloid leukemia cell line with biallelic *BRCA2* mutations derived from an FA (D1 subtype) patient [28] and *BRCA2*-mutated Chinese hamster fibroblast lines [29], suggesting that secondary mutations in *BRCA2* may have a general role in resistance to DNA-crosslinking agents in cells derived from various organs.

Secondary mutation of mutated *BRCA2* is reminiscent of mechanisms of acquired resistance of Philadelphia-chromosome-positive leukemia to imatinib [30], such as BCR-ABL point mutations that prevent imatinib binding. In both cases, the disease-causing mutations increase tumor sensitivity to specific agents, but development of further mutations in the disease-causing genes during targeted drug treatment leads to drug resistance. It will be important to explore mechanisms for restoring drug sensitivity.

**METHODS SUMMARY**

HCC1428, Capan-1, and a lymphoblast cell line (HCC1428BL) were purchased from the American Type Culture Collections. Cisplatin-resistant Capan-1 clones were generated by long-term culture in cisplatin (2 μM)-containing medium. PCR-amplified *BRCA2* gene fragments were analyzed for sequence alterations with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). All nucleotide numbers refer to the wild-type cDNA human sequence of *BRCA2* (accession no. U43746; version U43746.1 GI: 1161383) and *BRCA1* (U14680.1).

For western blot analysis, anti-BRCA2 Ab-1 (1:100; OP95, EMD Biosciences), anti-BRCA2 Ab-2 (1:100; PC146, EMD Biosciences), and anti-FANCA (1:1000, (#7488) (a gift from Dr. M Hoatlin)) were used. For immunofluorescence microscopy, cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min, followed by permeabilization with 0.25% Triton-X-100 in PBS (10 min). A primary antibody (anti-RAD51 (Ab-1) (1:1000, PC130, EMD Biosciences)) and a secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG (H+L) antibody (A-11034, Invitrogen)) were used.

Cisplatin sensitivity and PARP inhibitor sensitivity of cells were determined either by crystal violet or XTT (3′-1-phenylaminocarbonyl-3,4-tetrazolium-bis(4-methoxy-6-nitrobenzenesulfonic acid) ) assays. A PARP inhibitor, AG14631 is a gift of Pfizer. Expression of targeted genes was knocked down by transient transfection of siRNA directed against *FANCA* (5'-AAGGGTCAAGAGGGAAAAATA-3'), *BRCA2* (5’-AACAACAATTACGAACCAAAC-3’), and negative control (5’-AATTCTCCGAACGTGTCACGT-3’). HR assay was done as previously described

[22]. A BAC clone, RP11-37E23, was used as a probe for FISH on metaphase chromosome preparations of Capan-1 cells. Three DNA samples from *BRCA2*-mutated ovarian cancer patients were obtained from Cedars-Sinai Medical Center (Los Angeles, CA) through the Pacific Ovarian Cancer Research Consortium, and two were from the tissue bank of University of Washington (Seattle, WA). The study was approved by Institutional Review Boards of Fred Hutchinson Cancer Research Center and University of Washington.

## METHODS

**Cell lines.** Capan-1, HCC1428BL and breast cancer cell lines (BT-474, BT-483, HCC38, HCC1500, HCC1428, HCC1395, MCF7, MDA-MB-231, MDA-MB-436, MDA-MB-468) were purchased from American Type Culture Collections (Manassas, VA). 21NT was a gift from Dr. K Polyak (Dana Farber Cancer Institute). HCC1937, 2008 and 2008+FANCF were described [31, 32]. HCC1428BL were grown in RPMI with 10% fetal calf serum (FCS) and other cell lines were grown in DMEM with 10% FCS. Gamma irradiation was delivered using a linear accelerator. 1.2 $x10^7$ Capan-1 cells ($10^6$ cells/15 cm tissue culture plate x 12 plates) were selected in cisplatin (2 μM)-containing medium. Resistant colonies, appearing after 5–6 weeks, were picked, and expanded in normal medium.

**RNA extraction.** Total RNAs were extracted using Trizol (Invitrogen; Carlsbad, CA), and 2 μg of total RNA was placed in 20 μL reaction and converted to first-strand cDNA using Superscript III RNase H(-) Reverse Transcriptase and oligo(dT)15 (Invitrogen).

**Genomic DNA extraction, PCR and Sequencing of *BRCA2* and *BRCA1*.** Genomic DNA was extracted from cell lines using QIAamp DNA Blood Mini Kit (Qiagen; Valencia, CA). PCR was performed using Expand High Fidelity PCR System (Roche). Sequences of PCR products were analyzed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems; Foster City, CA). Primers are listed in Tables S4 and S5. All nucleotide numbers refer to the wild-type cDNA human sequence of *BRCA2* (accession no. U43746; version U43746.1 GI: 1161383) and *BRCA1* (U14680.1) (GenBank database).

**Western blotting**. Cells were lysed with 1x sample buffer (50 mM Tris-HCl pH6.8, 86

mM 2-mercaptoethanol, 2% SDS), boiled for 5 min, and subjected to electrophoresis using NuPAGE 3-8% Tris-Acetate Gel (Invitrogen). Proteins were transferred to nitrocellulose membranes. After blocking with 5% non-fat dried milk in TBS-T (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20), the membrane was incubated with the primary antibody (BRCA2 Ab-1 (a middle part (a.a. 1651-1821) of BRCA2) (1:100; OP95, EMD Biosciences; San Diego, CA), BRCA2 Ab-2 (C-terminus (a.a.3245–3418) of BRCA2) (1:100; PC146, EMD Biosciences), FANCA (1:1000, #7488, gift from Dr. M Hoatlin, Oregon Health and Science University) diluted in TBS-T, and incubated with the appropriate horseradish peroxidase-linked secondary antibody (Amersham). Chemiluminescence was used for detection.

**Immunofluorescence microscopy.** Cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.25% Triton-X-100 in PBS. After blocking in blocking buffer (3% bovine serum albumin/0.05% Triton X-100/0.04% sodium azide/PBS), a primary antibody (RAD51 (1:1000, PC130, EMD Biosciences)) diluted in blocking buffer was added and incubated for 1-2 hours. A secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG (H+L) antibody (A-11034, Invitrogen)) (1:1000) was added and incubated for 1 hour. The nuclei were counterstained with 4', 6-diamidino-2-phenylindole dihydrochloride. Images were captured on a microscope (TE2000, Nikon, Tokyo) through a CCD camera and processed using MetaVue (Universal Imaging, Downington, PA) and Photoshop (Adobe). Cells with five or more RAD51 foci were scored as positive. At least 100 cells per experimental point were scored.

**Cytotoxicity assays.** Drug sensitivity was determined by crystal violet or XTT

(3′-1-phenylaminocarbonyl-3,4-tetrazolium-bis(4-methoxy-6-nitrobenzenesulfonic acid) ) assays as previously described [33-35]. Cells were seeded onto 12-well plates at 6 x $10^3$ cells/well in DMEM-10% FCS (1.5 ml). After cells attached, the medium was replaced with DMEM-10% FCS containing cisplatin or a PARP inhibitor, AG14631 (a gift of Pfizer, La Jolla, CA) [36], at various concentrations. After incubation for 10 days, monolayers were fixed for 5 to 10 min in 10% methanol and 10% acetic acid. Adherent cells were stained with 1% crystal violet in methanol (0.5 ml/well). The adsorbed dye was resolubilized with methanol containing 0.1% SDS (0.3 ml/well). Dye solution (200 µl) was transferred to 96-well plates and diluted (1:2) in methanol. Crystal violet concentrations were measured photometrically (595 nm) in a microplate reader. For XTT assays, cells were seeded in 96-well plates at 2 x $10^3$ cells/well (for HCC1428 and Capan-1 in Figure 1g) or 8 x $10^2$ cells/well (for Capan-1 and its clones in Figure 3c), and cisplatin or AG14631 was added at varying concentrations. After 5 or 6 days, the number of viable cells was measured by the XTT assay as previously described [37]. For HCC1428 cells, we only used XTT assays, because HCC1428 do not grow in the condition for the crystal violet assays.

**siRNA transfection.** HCC1428 cells (3x$10^5$ cells) were plated in 6-well plates at the time of transfection in 2 ml of complete media. siRNA oligos (*FANCA* (5'-AAGGGTCAAGAGGGAAAAATA-3') [38], *BRCA2* (5’-AACAACAATTACGAACCAAAC-3’) [38], and negative control (5’-AATTCTCCGAACGTGTCACGT-3’)) were diluted in 400 µL DMEM and were mixed with 12 µL of HiPerFect reagent (Qiagen). Complex formation was allowed to proceed for 10 min at room temperature prior to addition to cells. Final concentration of

siRNA was 50 nM. Two days after transfection, cells were seeded in 96-well plates for cisplatin sensitivity assay.

**Homologous recombination (HR) assay.** Nucleotide changes were incorporated into *BRCA2* cDNA **pcDNA3.1** subclones using the QuikChange kit (Stratagene; La Jolla, CA). Mutant partial cDNA fragments were subcloned into a FLAG-tagged full-length *BRCA2* cDNA **pcDNA3.1** plasmid. VC8-DR-GFP cells [22] were transfected with either **pcDNA3.1** vector, **pcBAsce1** vector containing the I-SceI restriction endonuclease gene, or **pcBAsce1** plus various FLAG tagged *BRCA2*-**pcDNA3.1** constructs. The percentage of GFP positive cells was quantitated by flow cytometric analysis 72 hours after transfection as described [22]. In parallel, the percentage of VC8-DR-GFP cells expressing the various constructs were determined by immunofluorescence with anti-FLAG antibody and were used to normalize the HR data for VC8 transfection efficiency.

**Fluorescence In Situ Hybridization (FISH).** A BAC clone, RP11-37E23, was used as a probe for FISH on metaphase chromosome preparations of Capan-1 cells using procedures detailed previously [39].

**Clinical specimens.** Three DNA samples from *BRCA2*-mutated ovarian cancer patients were obtained from Cedars-Sinai Medical Center through the Pacific Ovarian Cancer Research Consortium, and two DNA samples were from the tissue bank of University of Washington. DNA from UW3548 was obtained after laser capture microdissection. The study was approved by Institutional Review Boards of Fred Hutchinson Cancer Research Center and University of Washington.

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**Supplementary Information** is available online.

**Acknowledgments**

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**Author Contributions**:

W.S. performed most of the experiments. E.M.S., B.Y.K., and N.U. provided clinical samples and expertise on ovarian cancer. E.M.S. performed laser capture micro dissection and DNA extractions. M.K.A., D.J.F. and F.J.C. performed homologous recombination assays. J.H. sequenced *BRCA2* in HCC1428. C.F. performed FISH analysis. E.V. performed siRNA experiments shown in Figs 1f and 1g. C.J. performed PARP inhibitor sensitivity assays in Fig 3c. T.T., W.S., and E.M.S. wrote the manuscript.

**Author Information**:

The authors declare that they have no competing financial interests. Correspondence and requests for materials should be addressed to T.T. (ttaniguc@fhcrc.org).

**Figure Legends**

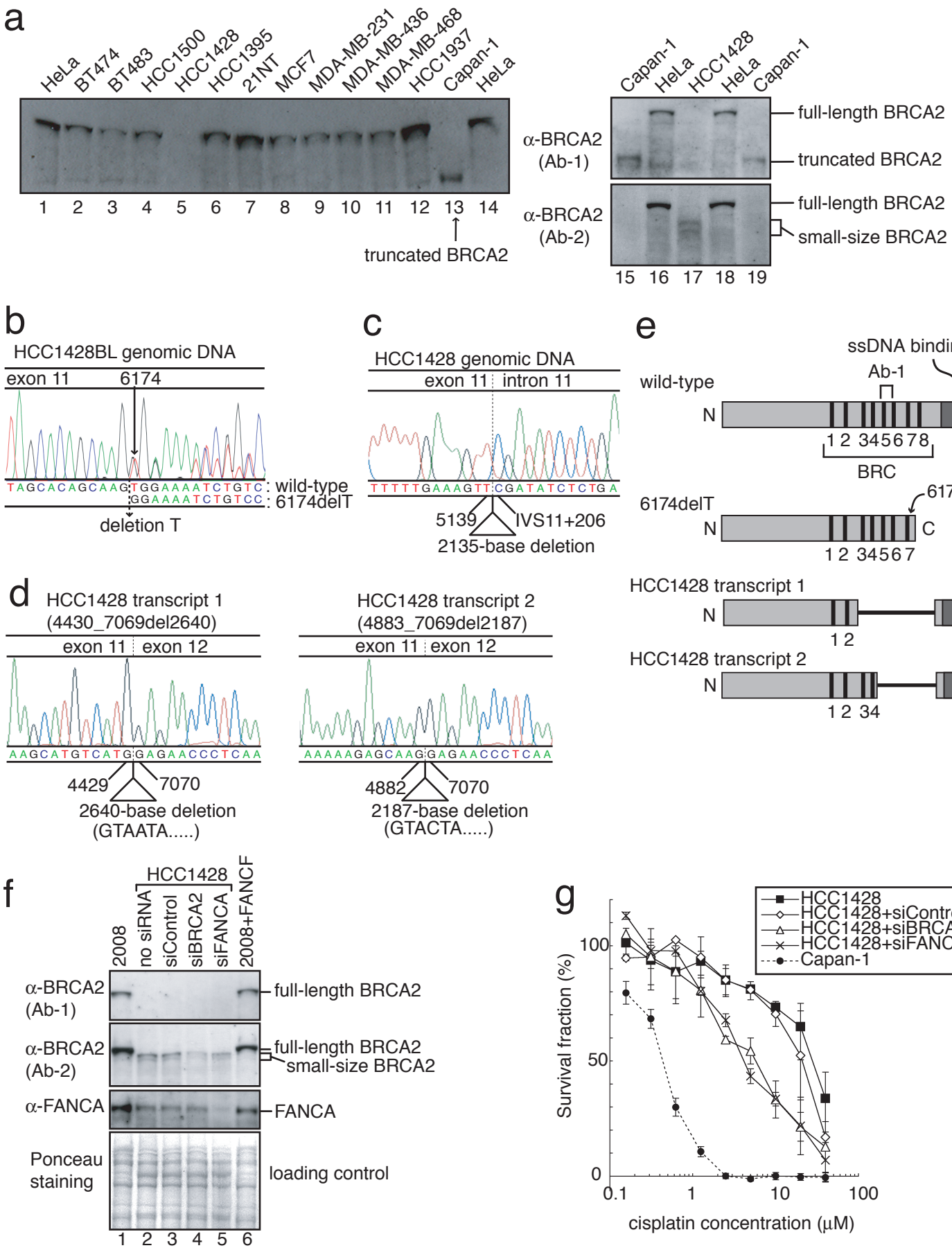
**Figure 1. HCC1428 is a cisplatin-resistant breast cancer cell line with a secondary *BRCA2* mutation.** (Full-length blots are presented in Supplemental Figure 9)
**a**, Cell lysates from HeLa, Capan-1 and indicated breast cancer cell lines were immunoblotted with BRCA2 antibodies. **b**, Genomic DNA sequence of *BRCA2* in HCC1428BL lymphoblast. A heterozygous mutation (6174delT) was identified. **c**, Genomic DNA sequence of *BRCA2* in HCC1428 breast cancer cell line. A homozygous 2135-base deletion from nt5140 (in exon 11) to IVS11+205 (in intron 11) was identified. **d**, Sequences of the two *BRCA2* transcripts in HCC1428 breast cancer cell line. The two RT-PCR products shown in Fig. S1b were purified from the gel, and sequenced separately. HCC1428 transcripts 1 and 2 had a 2640-base deletion (nt4430 to 7069) and a 2187-base deletion (nt4883 to 7069), respectively, suggesting activation of the cryptic splice donor sites in exon11. **e**, Schematic presentation of BRCA2 proteins. The regions that the BRCA2 antibodies (Ab-1 and Ab-2) recognize are also depicted. HCC1428 transcripts 1 and 2 encode BRCA2 lacking 881 amino acids and 730 amino acids, respectively, both of which have ssDNA binding domains, nuclear localization signals (NLS) and some of the BRC repeats. **f**, Cell lysates from HCC1428 cells treated with indicated siRNA were immunoblotted with BRCA2 and FANCA antibodies. 2008 and 2008+FANCF were used as controls. **g**, Cisplatin sensitivity assessed by XTT assay. HCC1428 was resistant to cisplatin, and depletion of BRCA2 or FANCA sensitized HCC1428 to cisplatin (mean ± SEM, n=3).

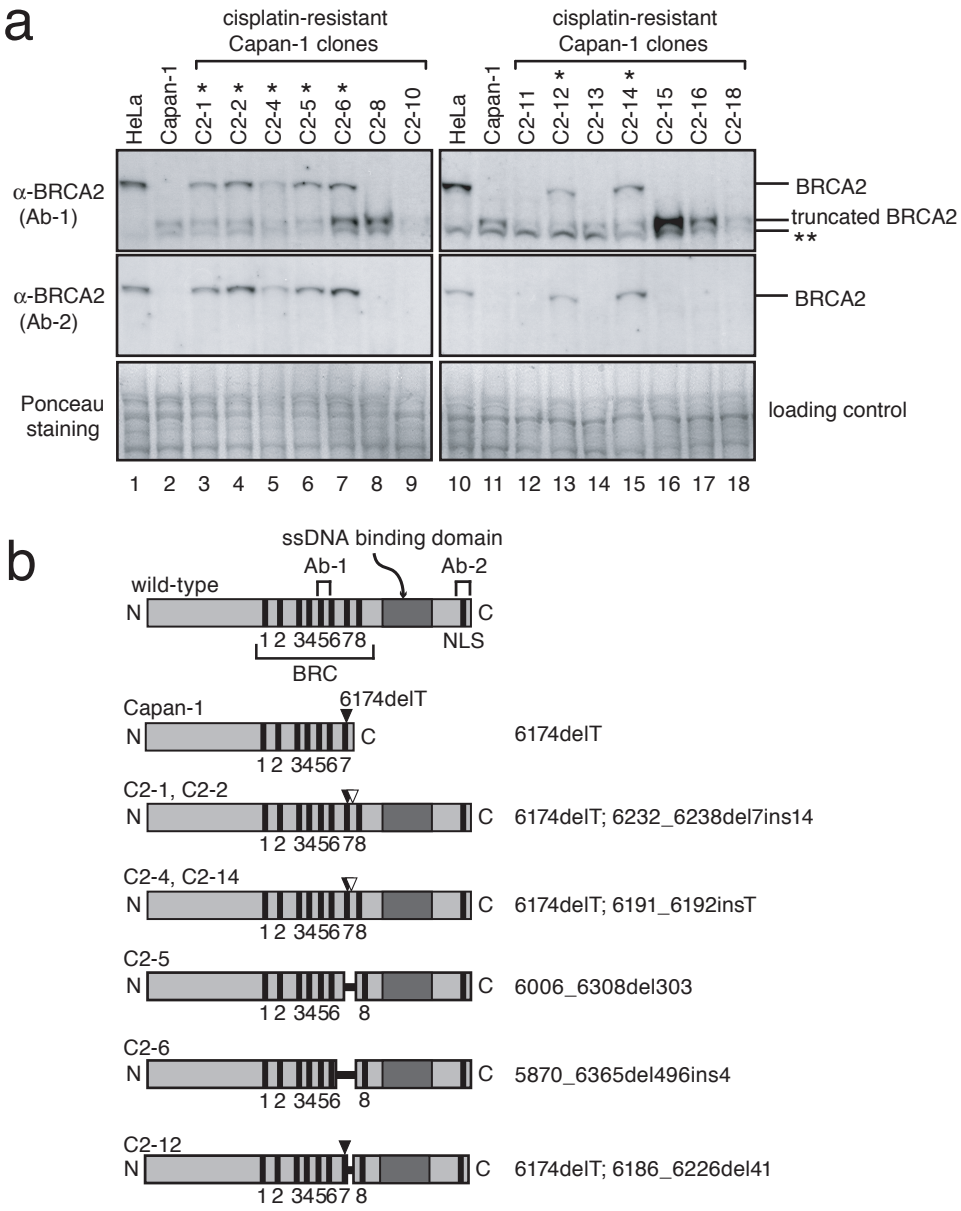
**Figure 2. Secondary genetic changes in mutated *BRCA2* in cisplatin-resistant clones of a pancreatic cancer cell line, Capan-1.** (Full-length blots are presented in Supplemental Figure 9) **a**, Seven Capan-1 clones indicated with a single asterisk (*) restored apparently full-length BRCA2 protein expression. Cell lysates from indicated cells were immunoblotted with BRCA2 antibodies (Ab-1 and Ab-2). A double asterisk (**) indicates a band presumed to be nonspecific. **b**, Schematic presentation of BRCA2 proteins encoded by transcripts in Capan-1 clones. In all of the BRCA2-restored Capan-1 clones, additional genetic changes in exon 11 of *BRCA2* were detected (Table S1, Figs. S4 and S5). All of these secondary genetic changes (shown as white arrow heads or black horizontal bars) cancel the frameshift caused by the original mutation (6174delT, black arrow heads), and the encoded BRCA2 proteins have intact C-terminal regions containing a single strand DNA (ssDNA) binding domain and nuclear localization signals (NLS).

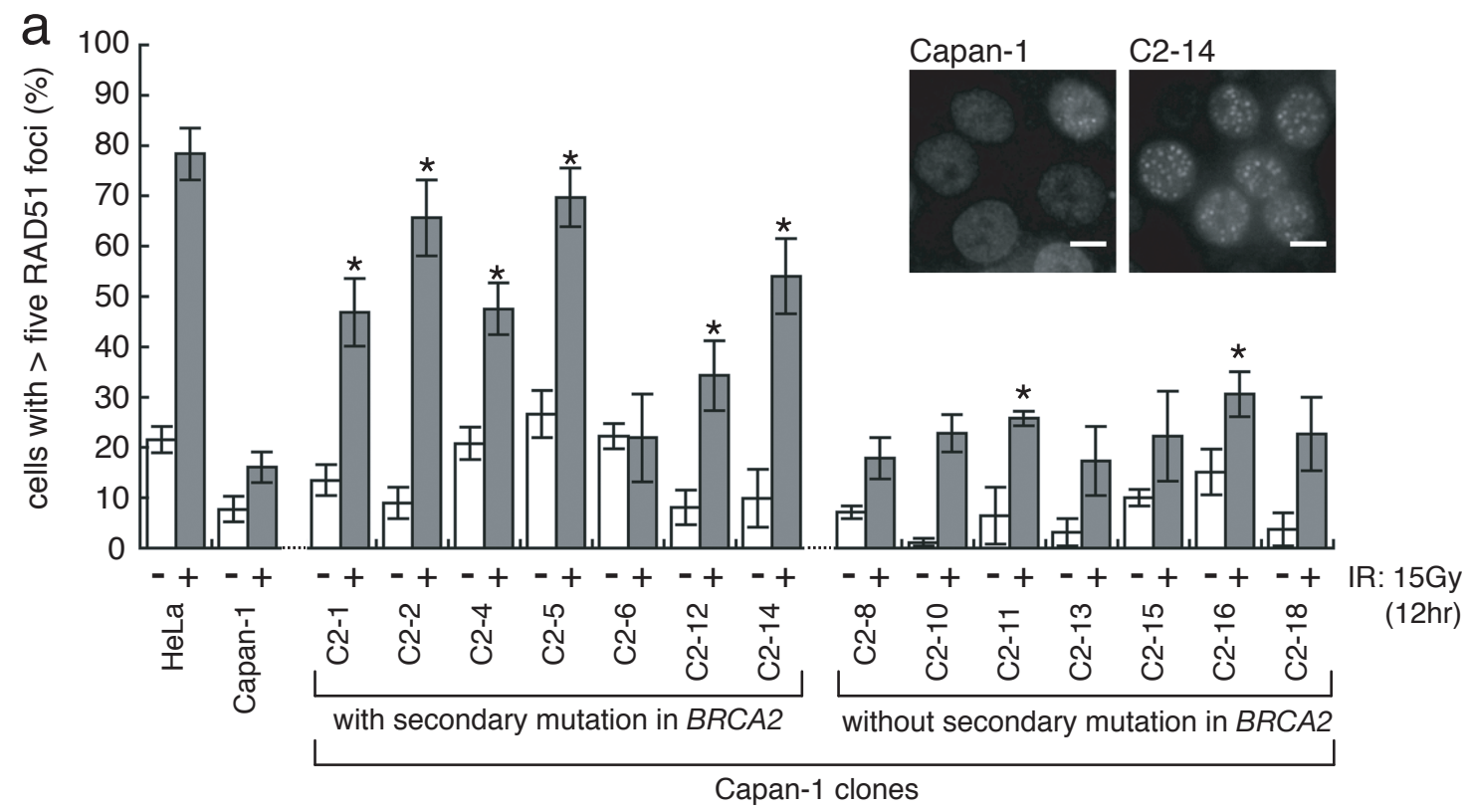
**Figure 3. Functional analyses of the restored BRCA2 proteins. a**, Ionizing radiation (IR)-induced RAD51 foci formation is restored in most of BRCA2-restored Capan-1 clones. Indicated cells were irradiated (15 Gy) and fixed 12 hours after IR. Cells were immunostained with RAD51 antibody. Representative pictures of immunostained cells after IR are shown, together with quantification of the cells with at least five RAD51 foci before (-, white bars) and 12 hours after IR (+, grey bars) (mean values of at least three independent experiments ± SEM). Asterisks (*) indicate significant difference with irradiated parental Capan-1 cells ($p<0.05$, unpaired t test). Scale bar = 40 μm. **b**, Quantitation of HR induced by I-SceI in VC8-DR-GFP cells transiently transfected with wild-type and mutant forms of FLAG-tagged human *BRCA2* cDNA. The proportion of

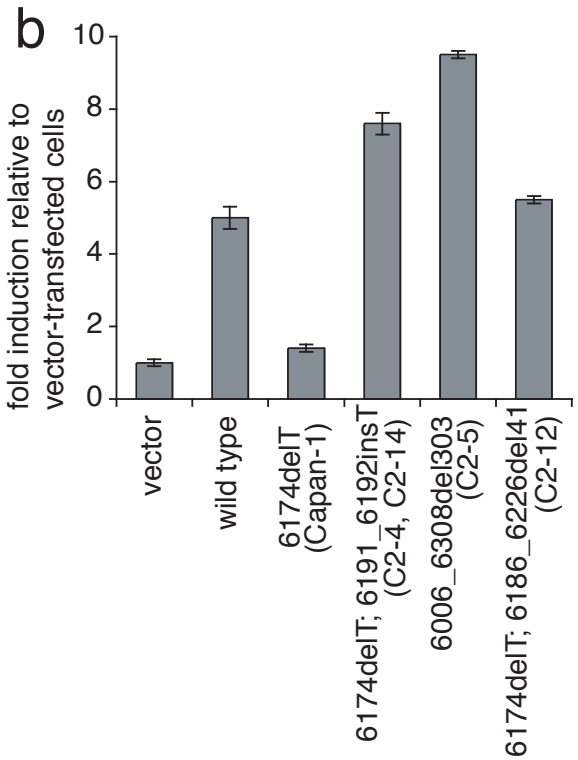
GFP-positive cells for each construct relative to vector control is shown (mean ± SEM, n=3). **c**, BRCA2-restored Capan-1 clones are resistant to a PARP inhibitor. Capan-1, its clones with secondary *BRCA2* mutation (C2-6, C2-12 and C2-14) and clones without secondary *BRCA2* mutation (C2-10, C2-11 and C2-13) were treated with a PARP inhibitor (AG14361) at the indicated concentrations for 6 days, and survival fraction was measured by XTT assay (mean ± SEM, n=6).

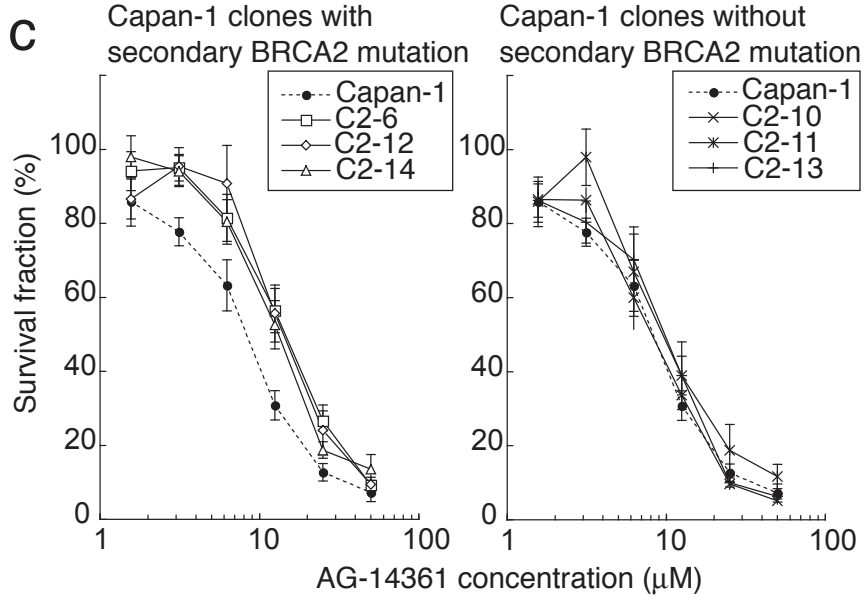
**Figure 4. Genetic reversion of *BRCA2* in platinum-resistant recurrent *BRCA2*-mutated ovarian cancer.** DNA sequences of *BRCA2* in peripheral blood lymphocytes and a microdissected specimen of the recurrent tumor from a patient (UW3548) with *BRCA2*-mutated ovarian cancer previously treated with cisplatin. In the lymphocytes, heterozygous single nucleotide polymorphisms (SNPs) of the *BRCA2* locus (IVS21-66C/T and IVS25-299C/G) were detected, in addition to a heterozygous mutation (6174delT). In the microdissected recurrent tumor specimen, loss of heterozygosity (LOH) of the SNPs was confirmed, but mixed sequences of 6174delT and wild-type *BRCA2* were detected.


a
HeLa
BT474
BT483
HCC1500
HCC1428
HCC1395
21NT
MCF7
MDA-MB-231
MDA-MB-436
MDA-MB-468
HCC1937
Capan-1
HeLa
1 2 3 4 5 6 7 8 9 10 11 12 13 14
truncated BRCA2
Capan-1
HeLa
HCC1428
HeLa
Capan-1
α-BRCA2 (Ab-1)
full-length BRCA2
truncated BRCA2
α-BRCA2 (Ab-2)
full-length BRCA2
small-size BRCA2
15 16 17 18 19
b
HCC1428BL genomic DNA
exon 11
6174
TAGCACAGCAAGTGGAAAATCTGTC : wild-type
GGAAAATCTGTCC : 6174delT
deletion T
c
HCC1428 genomic DNA
exon 11
intron 11
TTTTTGAAAGTTCGATATCTCTGA
5139
IVS11+206
2135-base deletion
e
ssDNA binding domain
wild-type
Ab-1
Ab-2
N
C
1 2 345678
NLS
BRC
6174delT
6174delT
N
C
1 2 34567
HCC1428 transcript 1
N
C
1 2
HCC1428 transcript 2
N
C
1 2 34
d
HCC1428 transcript 1
(4430_7069del2640)
exon 11
exon 12
AAGCATGTCATGGAGAACCCTCAA
4429
7070
2640-base deletion
(GTAATA.....)
HCC1428 transcript 2
(4883_7069del2187)
exon 11
exon 12
AAAAAGAGCAAGGAGAACCCTCAA
4882
7070
2187-base deletion
(GTACTA.....)
f
HCC1428
2008
no siRNA
siControl
siBRCA2
siFANCA
2008+FANCF
α-BRCA2 (Ab-1)
full-length BRCA2
α-BRCA2 (Ab-2)
full-length BRCA2
small-size BRCA2
α-FANCA
FANCA
Ponceau staining
loading control
1 2 3 4 5 6
g
HCC1428
HCC1428+siControl
HCC1428+siBRCA2
HCC1428+siFANCA
Capan-1
Survival fraction (%)
100
50
0
0.1
1
10
100
cisplatin concentration (μM)

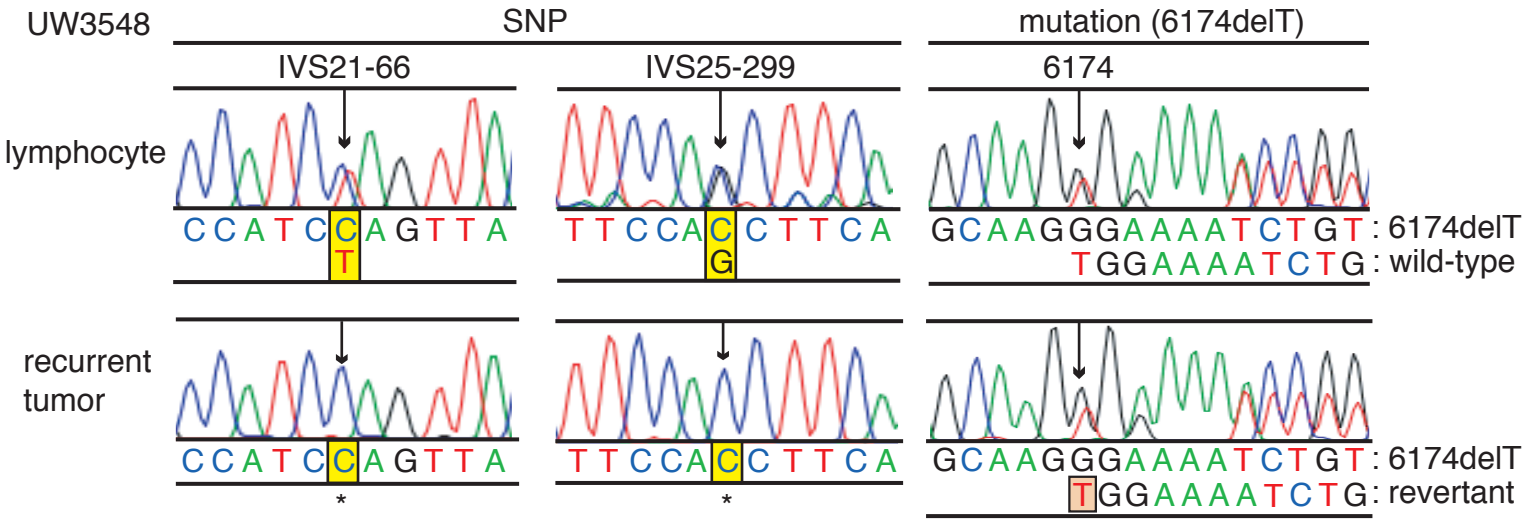

a
cisplatin-resistant Capan-1 clones
cisplatin-resistant Capan-1 clones
HeLa
Capan-1
C2-1 *
C2-2 *
C2-4 *
C2-5 *
C2-6 *
C2-8
C2-10
HeLa
Capan-1
C2-11
C2-12 *
C2-13
C2-14 *
C2-15
C2-16
C2-18
α-BRCA2 (Ab-1)
BRCA2
truncated BRCA2
**
α-BRCA2 (Ab-2)
BRCA2
Ponceau staining
loading control
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
b
ssDNA binding domain
Ab-1
Ab-2
wild-type
N
C
1 2 345678
NLS
BRC
Capan-1
6174delT
6174delT
C2-1, C2-2
6174delT; 6232_6238del7ins14
C2-4, C2-14
6174delT; 6191_6192insT
C2-5
6006_6308del303
C2-6
5870_6365del496ins4
C2-12
6174delT; 6186_6226del41


a
cells with > five RAD51 foci (%)
Capan-1
C2-14
IR: 15Gy (12hr)
HeLa
Capan-1
C2-1
C2-2
C2-4
C2-5
C2-6
C2-12
C2-14
C2-8
C2-10
C2-11
C2-13
C2-15
C2-16
C2-18
with secondary mutation in BRCA2
without secondary mutation in BRCA2
Capan-1 clones


b
fold induction relative to vector-transfected cells
vector
wild type
6174delT (Capan-1)
6174delT; 6191_6192insT (C2-4, C2-14)
6006_6308del303 (C2-5)
6174delT; 6186_6226del41 (C2-12)


c
Capan-1 clones with secondary BRCA2 mutation
Capan-1 clones without secondary BRCA2 mutation
Capan-1
C2-6
C2-12
C2-14
C2-10
C2-11
C2-13
Survival fraction (%)
AG-14361 concentration (μM)

Sakai, et al. Figure. 4



# Supplementary Information.

## Secondary mutations as a mechanism of cisplatin resistance in *BRCA2*-mutated cancers

Wataru Sakai, Elizabeth M. Swisher, Beth Y. Karlan, Mukesh K. Agarwal, Jake Higgins, Cynthia Friedman, Emily Villegas, Céline Jacquemont, Daniel J. Farrugia, Fergus J. Couch, Nicole Urban, Toshiyasu Taniguchi

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**1. Supplemental Figures.**

Sakai et al., Supplemental Figure1

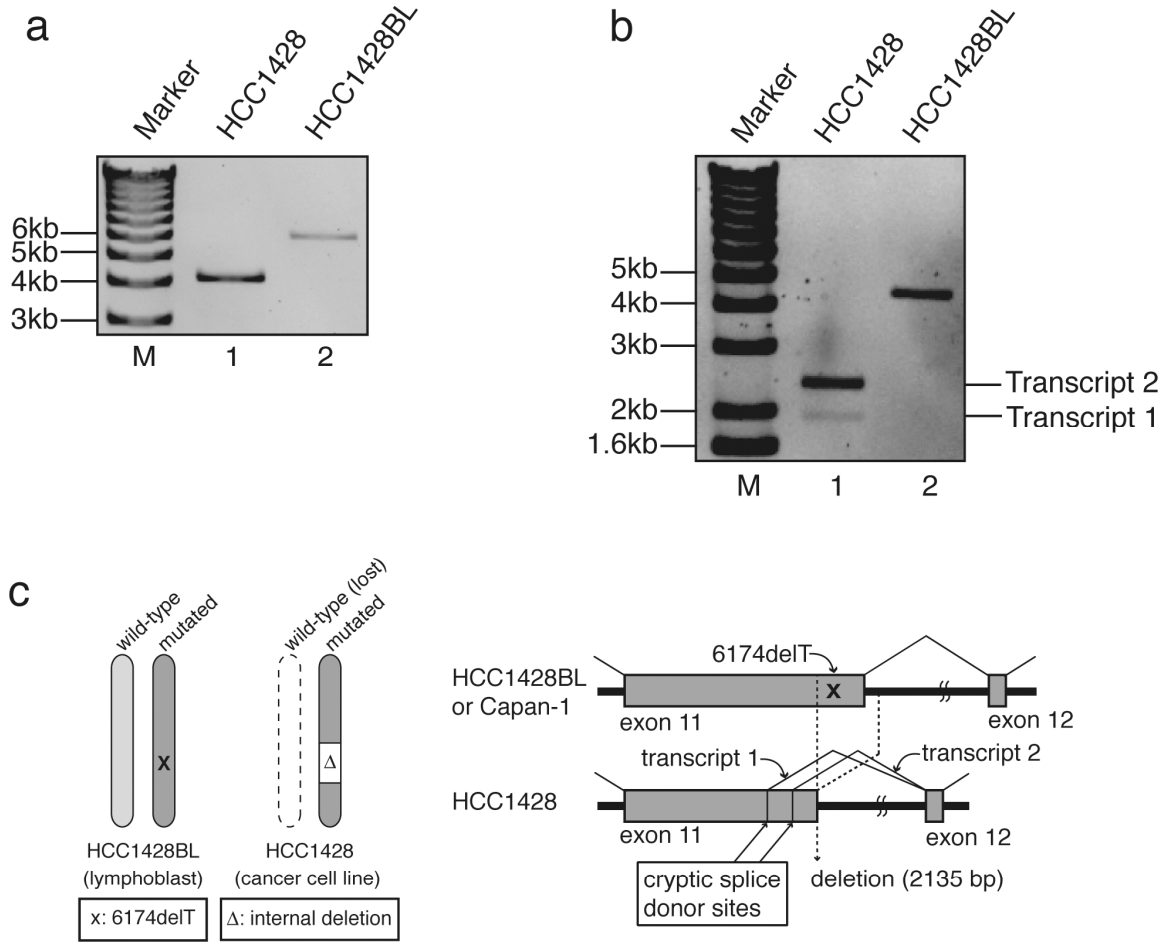


**Figure S1. *BRCA2* of HCC1428BL lymphoblast line and HCC1428 breast cancer cell line.** **a**, Ethidium bromide-stained agarose gel electrophoresis of the genomic PCR products from HCC1428 and HCC1428BL. The primers, B2F3785 and B2R6932, were used for the amplification. The molecular weight size standard is a 1000-bp ladder. A smaller PCR product was detected in HCC1428. **b**, Ethidium bromide-stained agarose gel electrophoresis of the RT-PCR products from HCC1428 and HCC1428BL. The primers, B2F3785 and B2R8452, were used for the amplification. The molecular weight size standard is a 1000-bp ladder. Two smaller RT-PCR products (transcripts 1 and 2) were detected in HCC1428. **c**, Schematic presentation of *BRCA2* alleles. HCC1428BL had wild-type and mutant (6174delT) alleles. HCC1428 lost the wild-type allele and retained the mutant allele with an additional internal deletion. The HCC1428's mutant allele had a 2135-bp deletion, which activates two cryptic splice donor sites in exon 11, resulting in the expression of transcripts 1 and 2.

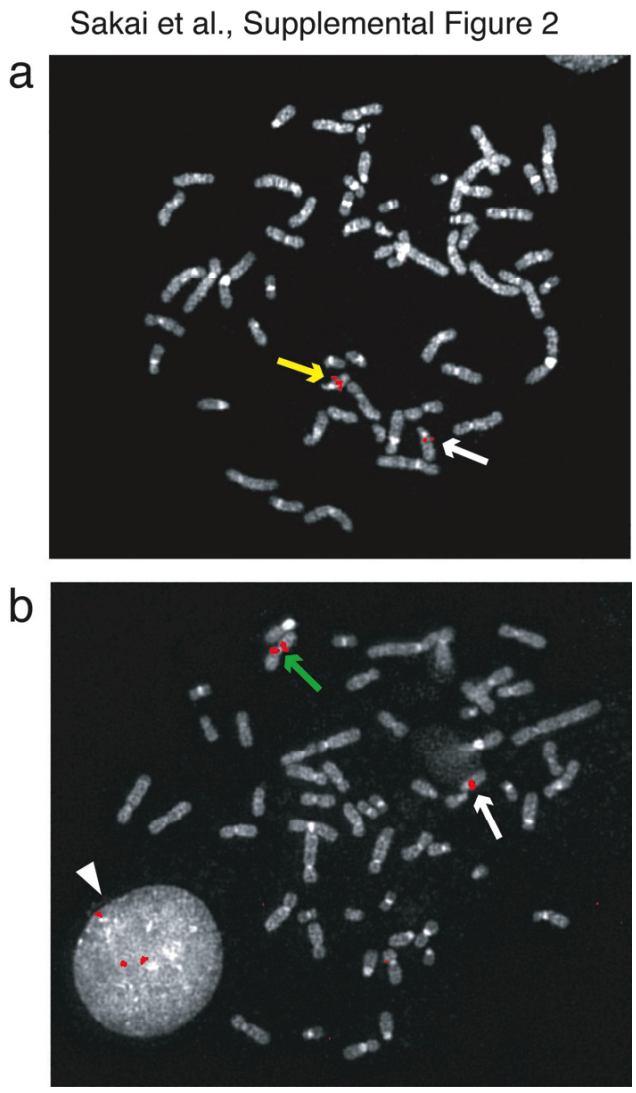


**Figure S2. Multiple copies of *BRCA2* gene in Capan-1.** Representative pictures of BRCA2 FISH on metaphase chromosomes of Capan-1 cells. **a**, This cell has a short chromosome 13 (yellow arrow) and a normal-looking chromosome 13 (white arrow), both of which have one BRCA2 signal (red). Therefore, the cell has at least 2 copies of *BRCA2* gene. **b**, This cell has a normal-looking chromosome 13 (white arrow) with one BRCA2 signal (red) and an isochromosome 13 (green arrow) with two *BRCA2* signals (red). Therefore, the cell has at least 3 copies of *BRCA2* gene. An interphase nucleus (white arrow head) also shows three BRCA2 signals (red).

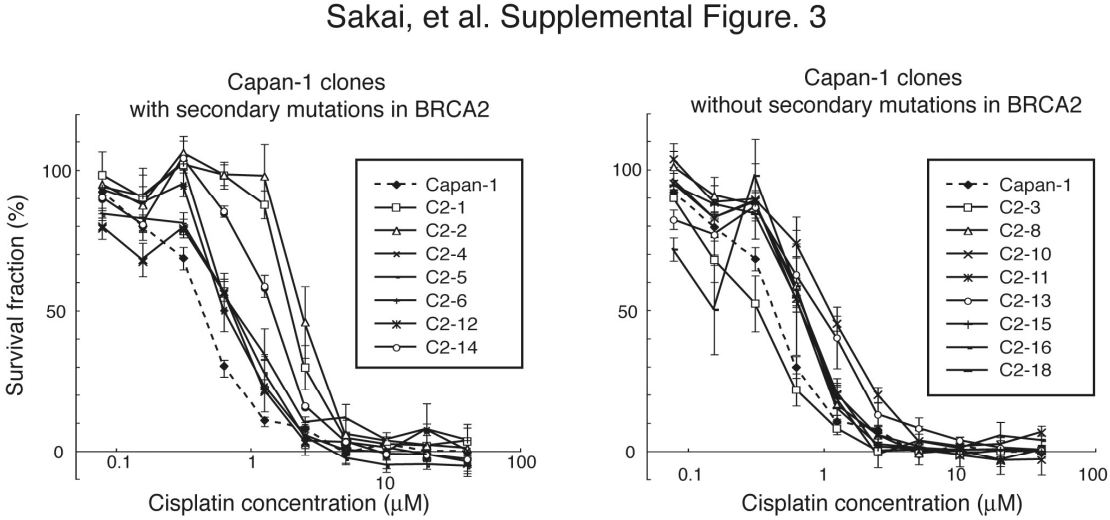


**Figure S3. Cisplatin-selected clones of a pancreatic cancer cell line, Capan-1.** Fifteen subclones of Capan-1 were generated by selecting the cells in the presence of cisplatin. Fourteen clones (except C2-3) were resistant to cisplatin compared to the parental Capan-1 cells. The cells were treated with cisplatin at the indicated concentrations for 10 days, and survival fraction was measured by crystal violet assay. Mean values of at least three independent experiments ± SEM are shown.

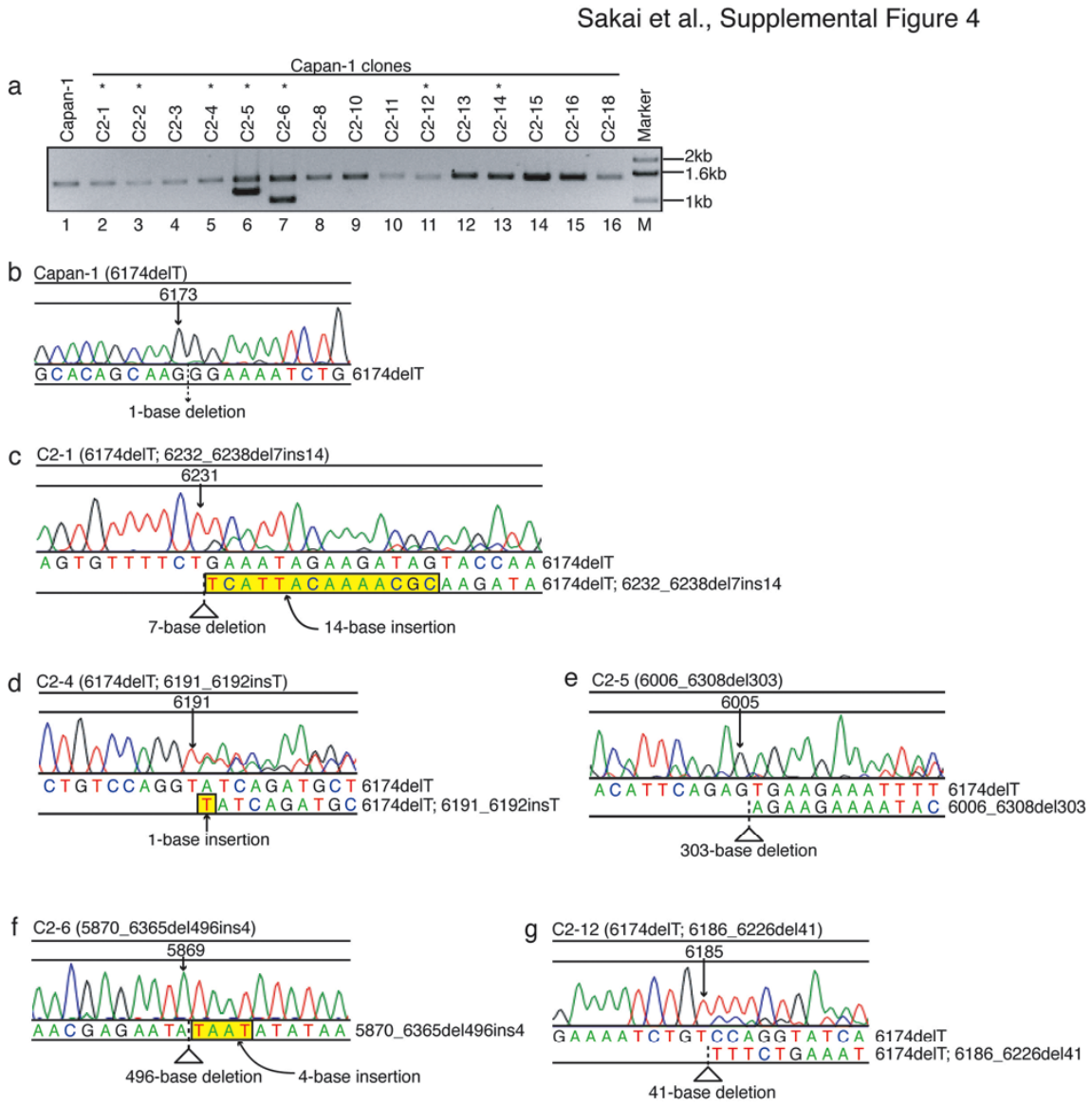


**Figure S4. *BRCA2* sequences of transcripts in Capan-1 clones.** **a**, Ethidium bromide-stained agarose gel electrophoresis of the RT-PCR products from Capan-1 and its clones. The primers, B2F5391 and B2R6932, were used for the amplification. The molecular weight size standard is a 1000-bp ladder. Asterisk (*) indicates clones with restored BRCA2 protein expression. In C2-5 and C2-6, smaller RT-PCR products, in addition to normal size products, were detected. **b**, Sequence of *BRCA2* transcript in parental Capan-1 cells. A *BRCA2* mutation (6174delT) was detected. **c**, *BRCA2* sequence in clone C2-1. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6174delT; 6232_6238del7ins14) were detected. C2-2 showed the same pattern (data not shown). **d**, C2-4. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6174delT; 6191_6192insT) were detected. C2-14 showed the same pattern (data not shown). **e**, C2-5. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6006_6308del303) were detected. The signals derived from the original sequence were much weaker than those from 6006_6308del303 sequence,

consistent with the ratio of intensities of 1.6kb (the original sequence, weak) and 1.3kb (6006_6308del303, strong) bands in lane 6 of Fig S4A. **f**, C2-6. The two RT-PCR products shown in Fig. S4A lane 7 were purified from the gel, and sequenced separately. In the smaller RT-PCR product (1.1kb), the sequence with an additional mutation (5870_6365del496ins4)) was detected. In the normal size product (1.6kb), the original sequence (6174delT) was detected (data not shown). **g**, C2-12. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6174delT; 6186_6226del41) were detected. All of these mutations were confirmed in genomic DNA as shown in Fig S5.

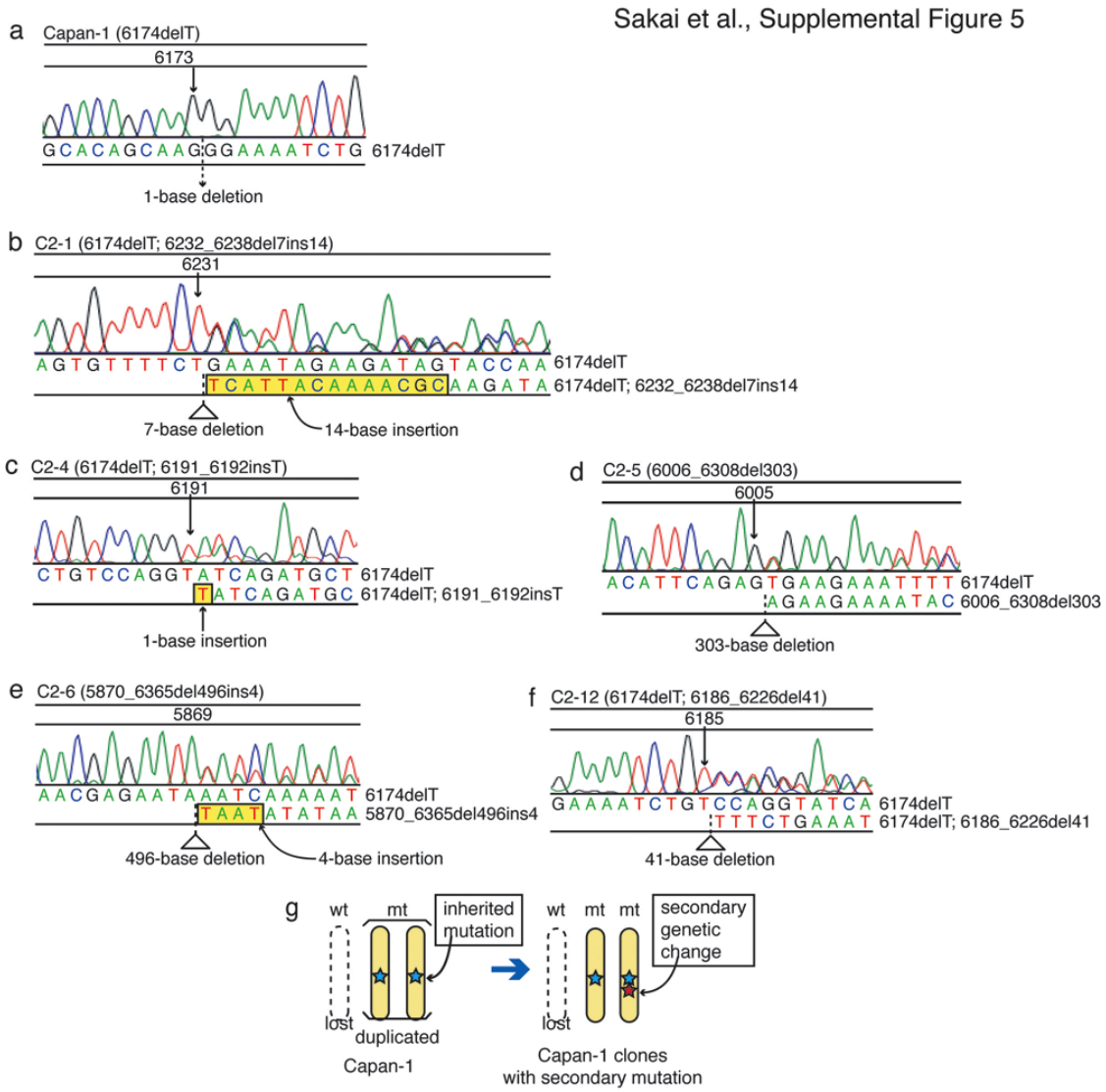


**Figure S5.** ***BRCA2*** **sequences of genomic DNAs from Capan-1 clones.**
**a**, Parental Capan-1 cells. A *BRCA2* mutation (6174delT) was detected. **b**, C2-1. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6174delT; 6232_6238del7ins14) were detected. C2-2 showed the same pattern (data not shown). **c**, C2-4. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6174delT; 6191_6192insT) were detected.

C2-14 showed the same pattern (data not shown). **d**, C2-5. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6006_6308del303) were detected. **e**, C2-6. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (5870_6365del496ins4) were detected. **f**, C2-12. Mixed sequences of the original sequence (6174delT) and the sequence with an additional mutation (6174delT; 6186_6226del41) were detected. **g**, Schematic model of the secondary mutations in Capan-1 clones. Capan-1 has lost a wild-type *BRCA2* allele, but has retained an allele with the inherited mutation (6174delT). This mutant allele has been duplicated (or triplicated), according to the FISH data shown in Fig. S2. In Capan-1 clones with secondary mutations, the secondary genetic change occurs only on one of the mutant *BRCA2* copies. This model explains why we see mixed sequences of *BRCA2* in all of the Capan-1 clones with secondary *BRCA2* mutations.

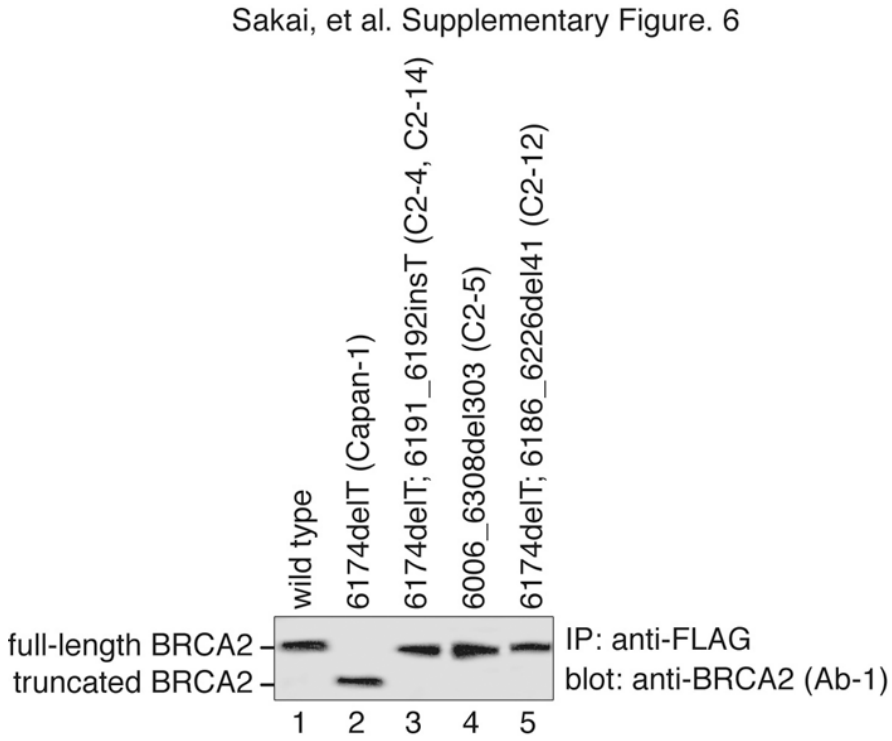


**Figure S6. Expression of FLAG-BRCA2 mutant proteins used for the DR-GFP homologous recombination assay.** FLAG-tagged BRCA2 variants used for the DR-GFP homologous recombination assay shown in Fig 3b were transiently expressed in 293T cells, immunoprecipitated with anti-FLAG (M2) (Sigma), and analyzed by Western blotting using a BRCA2 antibody (Ab-1, EMD Biosciences).

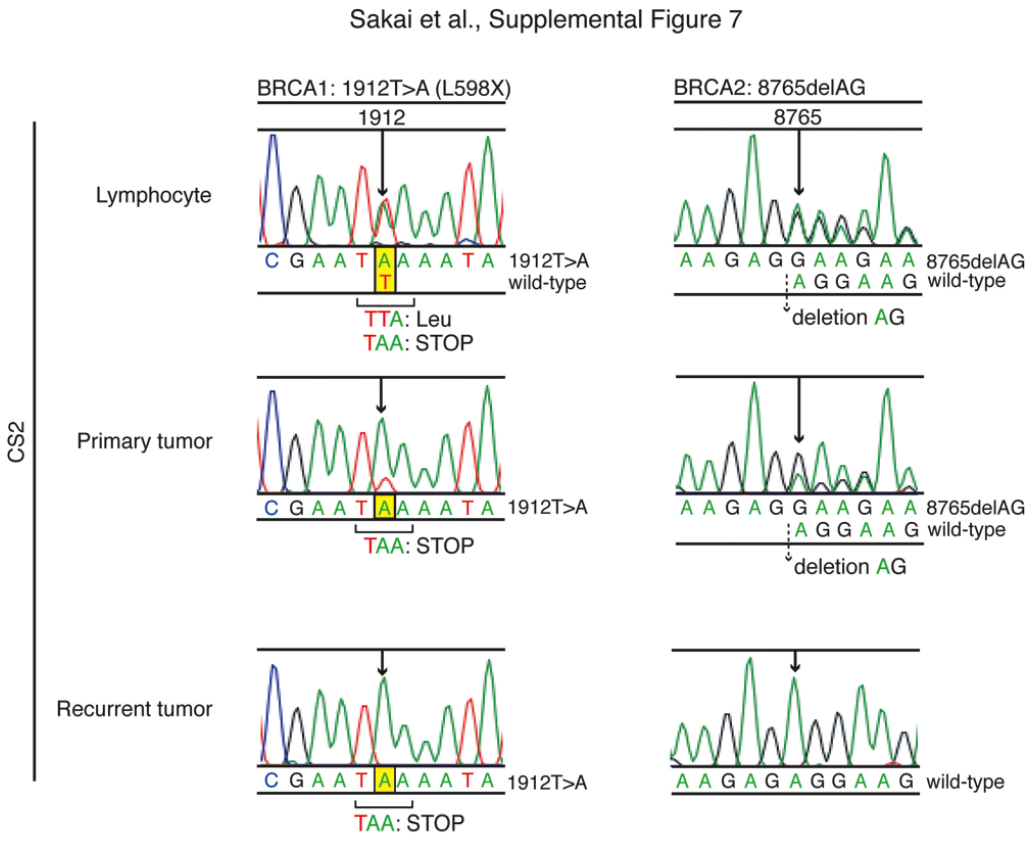


**Figure S7. DNA sequences of *BRCA1* and *BRCA2* in peripheral blood lymphocytes, and pre-treatment and recurrent tumors from a patient (CS2) with *BRCA1-BRCA2*-doubly mutated ovarian cancer.** The lymphocytes showed heterozygous mutations of both *BRCA1* (1912T>A) and *BRCA2* (8765_8766delAG). Before treatment, the primary tumor showed LOH of *BRCA1* (loss of wild-type *BRCA1*), and hetrozygosity of *BRCA2*. The post-treatment recurrent tumor showed LOH of *BRCA1* (loss of wild-type *BRCA1*) and LOH of *BRCA2* (loss of mutant *BRCA2*).

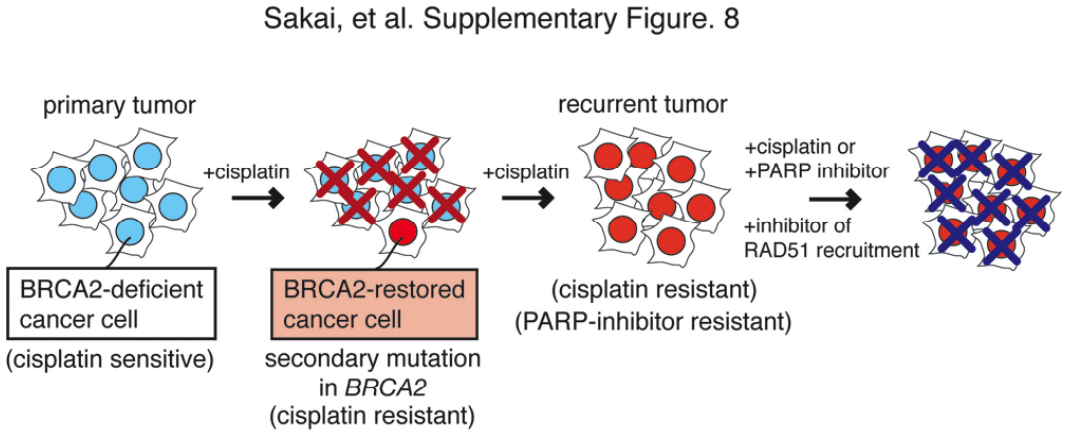


**Figure S8. Schematic model for involvement of a secondary *BRCA2* mutation in development of resistance to cisplatin in a *BRCA2*-mutated tumor.** See text for detail.

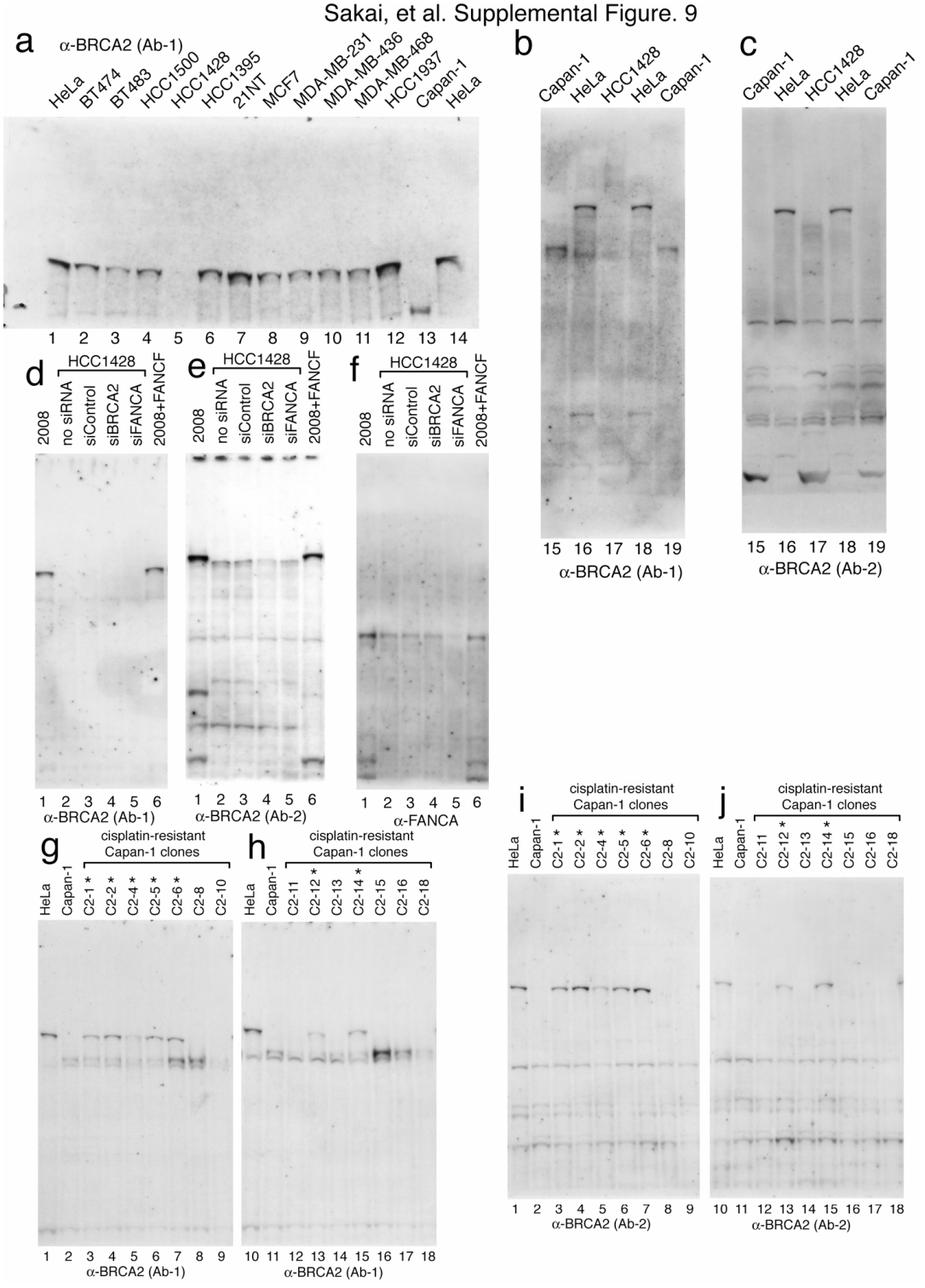


**Figure S9. Non-cropped pictures of blots.**
**(a, b, c)** Non-cropped pictures of blots presented in Figure 1a.
**(d, e, f)** Non-cropped pictures of blots presented in Figure 1f.
**(g, h, i, j)** Non-cropped pictures of blots presented in Figure 2a.

## 2. Supplemental Tables

**Table S1. Summary of characterization of cisplatin-selected Capan-1 clones.** *BRCA2* sequence, BRCA2 protein expression, and cisplatin sensitivity of Capan-1 and its clones are summarized.

Table S1. Characterization of cisplatin-selected Capan-1 clones.

| Name of clone | originated from | BRCA2 mutation inherited | BRCA2 mutation additional | Type of secondary mutation | BRCA2 protein expression | Cisplatin sensitivity | IC50 (mean±SEM) (uM) |
|---|---|---|---|---|---|---|---|
| C2-1 | plate #1 | 6174delT | 6232_6238del7ins14 | second site deletion and insertion | + | R | 2.09±0.15 |
| C2-2 | plate #1 | 6174delT | 6232_6238del7ins14 | second site deletion and insertion | + | R | 2.49±0.39 |
| C2-3 | plate #2 | 6174delT | not found | N/A | - | S | 0.34±0.08 |
| C2-4 | plate #3 | 6174delT | 6191_6192insT | second site insertion | + | R | 0.73±0.16 |
| C2-5 | plate #3 | 6174delT | 6006_6308del303 | in-frame deletion surrounding nt6174 | + | R | 0.72±0.08 |
| C2-6 | plate #3 | 6174delT | 5870_6365del496ins4 | in-frame deletion surrounding nt6174 | + | R | 0.86±0.15 |
| C2-8 | plate #4 | 6174delT | not found | N/A | - | R | 0.75±0.12 |
| C2-10 | plate #5 | 6174delT | not found | N/A | - | R | 1.16±0.06 |
| C2-11 | plate #6 | 6174delT | not found | N/A | - | R | 0.69±0.05 |
| C2-12 | plate #7 | 6174delT | 6186_6226del41 | second site deletion | + | R | 0.71±0.08 |
| C2-13 | plate #8 | 6174delT | not found | N/A | - | R | 1.07±0.28 |
| C2-14 | plate #9 | 6174delT | 6191_6192insT | second site insertion | + | R | 1.48±0.10 |
| C2-15 | plate #10 | 6174delT | not found | N/A | - | R | 0.74±0.07 |
| C2-16 | plate #11 | 6174delT | not found | N/A | - | R | 0.73±0.18 |
| C2-18 | plate #12 | 6174delT | not found | N/A | - | R | 0.71±0.07 |
| Capan-1 | N/A | 6174delT | N/A | N/A | - | S | 0.46±0.03 |

abbreviations: N/A; not applicable,

ND; not done, R; resistant, S;

sensitive

**Table S2. Evaluation of homology-directed repair of the FLAG-BRCA2 mutants.**

| | Vector | Wild-type | 6174delT | C2-4, C2-14 | C2-5 | C2-12 |
|---|---|---|---|---|---|---|
| Mean GFP positive cells ± SEM | 214 ± 8 | 1073 ± 69 | 316 ± 9 | 1114 ± 39 | 1771 ± 25 | 1540 ± 27 |
| Relative transfection efficiency (compared to wild-type BRCA2 construct) determined by immunofluorescence with anti-FLAG antibody | ~ | 1.0 | 1.1 | 0.7 | 0.9 | 1.3 |
| Normalized GFP positive cells | ~ | 1073 | 297 | 1620 | 2024 | 1173 |
| Fold induction relative to vector ± SEM | 1.0 ± 0.1 | 5.0 ± 0.3 | 1.4 ± 0.1 | 7.6 ± 0.3 | 9.5 ± 0.1 | 5.5 ± 0.1 |

Raw data of Figure 3b.

**Table S3. Summary of genetic changes in clinical specimens of recurrent *BRCA2*-mutated ovarian cancer treated with platinum.**

Table S3. Summary of genetic changes in clinical specimens of recurrent *BRCA2*-mutated ovarian cancer treated with platinum

| Patient | specimen | Clinical platium response at the time of sampling | Mutated gene | Inherited mutation | LOH | Secondary genetic change |
|---|---|---|---|---|---|---|
| | *Paired specimens* | | | | | |
| CS9 | pre-platinum | sensitive | *BRCA2* | 6174delT | Yes (loss of WT) | No |
| | post-platinum | sensitive | *BRCA2* | 6174delT | Yes (loss of WT) | No |
| CS15 | pre-platinum | sensitive | *BRCA2* | 2699delT | Yes (loss of WT) | No |
| | post-platinum | sensitive | *BRCA2* | 2699delT | Yes (loss of WT) | No |
| CS2 | pre-platinum | sensitive | *BRCA1* | 1912T>A (L598X) | Yes(loss of WT) | No |
| | | | *BRCA2* | 8765_8766delAG | No | No |
| | post-platinum | refractory | *BRCA1* | 1912T>A (L598X) | Yes (loss of WT) | No |
| | | | *BRCA2* | 8765_8766delAG | Yes (loss of mt) | Yes (loss of mt) |
| | *Non-paired post-treatment specimens* | | | | | |
| UW174 | post-platinum | refractory | *BRCA2* | 5578_5579delAA | Yes (loss of WT) | No |
| UW3548 | post-platinum | refractory | *BRCA2* | 6174delT | Yes (LOH of SNPs) gain of wild-type sequence | Yes (back mutation) |

abbreviations: LOH; loss of heterozygosity, mt: mutant allele, SNP; single nucleotide polymorphism, WT; wild-type allele

**Table S4. Primers used for *BRCA2* sequencing and PCR.**

| # | Primer | Seq 5' to 3' |
|---|---|---|
| 1 | B2Pro1F | TCCGAATCCTAAGAATGCAAA |
| 2 | B2Pro1R | GCGAGGGCTTGGTAGTATCT |
| 3 | B2Pro2F | CACCCAGGCCTGACTTCC |
| 4 | B2Pro2R | CACGCTGGACTGGGACTG |
| 5 | B2Pro3F | GGTAGTGGGTTGGGACGAG |
| 6 | B2Pro3R | ATGGCGTCATCTGGGACA |
| 7 | B2Pro4F | CTAGCCACGCGTCACTGGT |
| 8 | B2Pro4R | TCTGCAAAACAGAAGACCAAAA |
| 9 | B2Pro5F | ACTGGCCCCTTGACTAGCAG |
| 10 | B2Pro5R | GCCAGGCTGGTCTCAAACT |
| 11 | B2F-125 | TCTGAAACTAGGCGGCAGAG |
| 12 | B2R32 | AATGTTGGCCTCTCTTTGGA |
| 13 | B2F420 | TGAAAGTCCTGTTGTTCTAC |
| 14 | B2R586 | TTGACCAAGACATATCAGGA |
| 15 | B2F806 | CATCAGGGAATTCATTTAAAGT |
| 16 | B2R848 | ATGTGGTCTTTGCAGCTATTTA |
| 17 | B2F1178 | GAATGGTCTCAACTAACCCT |
| 18 | B2F1561 | TCAGGTCATATGACTGATCC |
| 19 | B2F1934 | GAAGCTGTTCACAGAATGAT |
| 20 | B2R2208 | TGCAGCCAAGACCTCTTCTT |
| 21 | B2F2314 | ACTCCTACTTCCAAGGATGT |
| 22 | B2R2365 | GCAGGCATGACAGAGAATCA |
| 23 | B2F2689 | GAAAGGAATAATCTTGCTTTAG |
| 24 | B2F3016 | GGAGGTAGCTTCAGAACAGC |
| 25 | B2F3173 | AACTGAGCAAGCCTCAGTCAA |
| 26 | B2F3398 | CAAGCTACATATTGCAGAAG |
| 27 | B2R3548 | TCAAATTGCTTGCTGCTGTC |
| 28 | B2F3785 | CAAGTAAATGTCATGATTCTGTTG |
| 29 | B2F4190 | AAGCATGTCATGGTAATACTTC |
| 30 | B2R4382 | GGAAAAGTTATGCAATTCTTCTGG |
| 31 | B2R4528 | GTTGTCCCTGGAAGGTCACT |
| 32 | B2F4600 | ATTGCAAAGGAATCTTTGGA |
| 33 | B2R4840 | TAGGTGGCACCACAGTCTCA |
| 34 | B2F4985 | CAGTCATTGAAAATTCAGCCTTAG |
| 35 | B2R5202 | TTCGGAGAGATGATTTTTGTCAT |
| 36 | B2F5391 | AAATGCATACCCACAAACTG |
| 37 | B2R5534 | CTAAATGCAGGTGGCCCTAC |

| | | |
|---|---|---|
| 38 | B2F5741 | GCACGCATTCACATAAGGTTT |
| 39 | B2F5796 | TAACCAAAATATGTCTGGATTGGAG |
| 40 | B2R5859 | TTCCAAACTAACATCACAAGGTG |
| 41 | B2F5836 | TCACCTTGTGATGTTAGTTTGGA |
| 42 | B2F5925 | TGGGATTTTTAGCACAGCAA |
| 43 | B2R5928 | CCCACAAGTATTTGCAGATGAG |
| 44 | B2R5990 | CTTGCGTTTTGTAATGAAGCA |
| 45 | B2R6076 | TGAGCTGGTCTGAATGTTCG |
| 46 | B2F6193 | CAAGTTTCCATTTTAGAAAGTTCCT |
| 47 | B2R6202 | TGGAAACTTGCTTTCCACTTG |
| 48 | B2F6592 | GAAACTTTTTCTGATGTTCCTGTGA |
| 49 | B2R6496 | CCAACTGTTGTTTGTCTTGTTGA |
| 50 | B2R6692 | GCAATTTCTACTGCTTCTGTTTCA |
| 51 | B2R6932 | GGAGTGCTTTTTGAAGCCTTT |
| 52 | B2F7000 | CCCTTTCGCACAACTAAGGA |
| 53 | B2F7402 | GTAACTTTCACAAAGTGTGAAGA |
| 54 | B2F7810 | CTGTGTGACACTCCAGGTGT |
| 55 | B2R8452 | CACAACCAACATTTCCTCCA |
| 56 | B2F8565 | GGAGGCCCAACAAAAGAGAC |
| 57 | B2R8923 | CAATACGCAACTTCCACACG |
| 58 | B2F8960 | TGAGTATTTGGCGTCCATCA |
| 59 | B2F9362 | CTGCAAGCAACCTCCAGTG |
| 60 | B2F9905 | GGAGTTGTGGCACCAAATACGAAAC |
| 61 | B2F10259 | CATTTGCAAAGGCGACAATA |
| 62 | B2R10303 | CTGGAAAGGTTAAGCGTCAA |
| 63 | B2F10396 | GTGTATCGGGCAAAAATCGT |
| 64 | B2F10663 | TCTTTGGATTTGATCACTACAAGT |
| 65 | B2R10797 | GCTAAACTAAAAGGAATTATCTGCATC |
| 66 | B2F10933 | CAATTCTTCATCCTTAAGTCAGCA |
| 67 | B2F11129 | TTGCTTTCAAATTGGCACTG |
| 68 | B2R11158 | CAGTGCCAATTTGAAAGCAA |
| 69 | B2F.IVS10-237 | TACACCACCACACCCAGCTA |
| 70 | B2F.IVS12-221 | TGCTGATTTCTGTTGTATGCTTG |
| 71 | B2R.IVS13+278 | TGATTCGGAGCAATTTCCTT |
| 72 | B2F.IVS16-59 | TTGAATTCAGTATCATCCTATGTGG |
| 73 | B2R.IVS17+135 | GAGAACAGCAGTGTGGGATG |
| 74 | B2F.IVS18+1419 | CTCAGAGGCTTGTTGGGAAA |
| 75 | B2R.IVS18+1769 | AGGCCAGGTACAGTGGTTCA |
| 76 | B2F.IVS18+2497 | TGCCATGTTTTATAGATTTGTCTTT |

| | | |
|---|---|---|
| 77 | B2R.IVS18+2914 | TCACATCTGCACCTTTCTCTG |
| 78 | B2F.IVS19-174 | GGGGTTTCATCATGTTGGTC |
| 79 | B2R.IVS20+179 | TGTCCCTTGTTGCTATTCTTTG |
| 80 | B2F.IVS20-1695 | GGGAGGCCTCAGGAAACTTA |
| 81 | B2R.IVS20-1271 | TGATTCCAACCCTCATGGAT |
| 82 | B2R.IVS21-206 | GGAGGATCATTTTTGCCGTA |
| 83 | B2F.IVS24+127 | GCCTCTTTGAACCTCTGATTTT |
| 84 | B2R.IVS24+551 | CGGTGGCGGGTATATGTAGT |
| 85 | B2F.IVS25-492 | CCAGTTGTGATTTCTCAAGCA |
| 86 | B2R.IVS25-76 | AAAGCAGAAATGAAAAGTTTGGA |

**Table S5. Primers used for *BRCA1* sequencing and PCR.**

| # | Primer | Seq 5' to 3' |
|---|---|---|
| 1 | B1F3 | CTCGCTGAGACTTCCTGGAC |
| 2 | B1R418 | TCCAAACCTGTGTCAAGCTG |
| 3 | B1F878 | AGCTGAGAGGCATCCAGAAA |
| 4 | B1R972 | GCTGTAATGAGCTGGCATGA |
| 5 | B1F1311 | TCACATGATGGGGAGTCTGA |
| 6 | B1F1641 | CCTACATCAGGCCTTCATCC |
| 7 | B1R2114 | GTTTCTGCTGTGCCTGACTG |
| 8 | B1F2095 | CAGTCAGGCACAGCAGAAAC |
| 9 | B1F2280 | TTTGTCAATCCTAGCCTTCCA |
| 10 | B1F2494 | GGAAGGCAAAAACAGAACCA |
| 11 | B1R2504 | TTTTGCCTTCCCTAGAGTGC |
| 12 | B1F2717 | CCAGTCATTTGCTCCGTTTT |
| 13 | B1R2743 | GGATTTGAAAACGGAGCAAA |
| 14 | B1R2921 | CTGACCAACCACAGGAAAGC |
| 15 | B1F2997 | GGCAACGAAACTGGACTCAT |
| 16 | B1R3247 | TTGCTTGAGCTGGCTTCTTT |
| 17 | B1R3361 | TTCAATTTTGGCCCTCTGTT |
| 18 | B1F3524 | GCCTATGGGAAGTAGTCATGC |
| 19 | B1F3653 | CAAAAGCGTCCAGAAAGGAG |
| 20 | B1R3876 | ACAGACACTCGGTAGCAACG |
| 21 | B1F4100 | GTCTGAAAGCCAGGGAGTTG |
| 22 | B1F4305 | CAGAGGGATACCATGCAACA |
| 23 | B1F4530 | GGCCTTTCTGCTGACAAGTT |
| 24 | B1F5120 | GTTTGCCAGAAAACACCACA |
| 25 | B1R5668 | AGCTCCTGGCACTGGTAGAG |

| | | |
|---|---|---|
| 26 | B1F101-378 | ATTGGTGTTGGTTTGGTCGT |
| 27 | B1F101-159 | TCTTTAAAAATAAAGGACGTTGTCA |
| 28 | B1R199+143 | TTCAGTTAAGAAAATCAGCAATTACA |
| 29 | B1R199+451 | TCTCGATCTCCTGACCTCGT |
| 30 | B1F200-352 | GATGAGATGTGCACCCACAG |
| 31 | B1F200-206 | GCTCACTGAAGGTAAGGATCG |
| 32 | B1R253+190 | TTACCAGGAACTATGATTACAACCAA |
| 33 | B1R253+451 | AGGTGGATCACAAGGTCAGG |
| 34 | B1F.IVS10-294 | CTGATGGCCAATCTGCTTTT |
| 35 | B1R.IVS11+33 | ACTGGGGCAAACACAAAAAC |
| 36 | B1F.IVS15-47 | TTCAACATTCATCGTTGTGTAAA |
| 37 | B1R.IVS16+1 | CAAATTCTTCTGGGGTCAGG |