Secondary BRCA1 Mutations in BRCA1-Mutated Ovarian Carcinomas with Platinum Resistance

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Abstract

Although ovarian carcinomas with mutated BRCA1 or BRCA2 are sensitive to platinum compounds, such carcinomas eventually develop platinum resistance. Previously, we showed that acquired resistance to cisplatin in BRCA2-mutated tumors can be mediated by secondary intragenic mutations in BRCA2 that restore the wild-type BRCA2 reading frame. Here, we show that secondary mutations of BRCA1 also occur in BRCA1-mutated ovarian cancer with platinum resistance. We evaluated nine recurrent BRCA1-mutated ovarian cancers previously treated with platinum compounds, including five with acquired platinum resistance, one with primary platinum resistance, and three with platinum sensitivity. Four of the six recurrent platinum-resistant tumors had developed secondary genetic changes in BRCA1 that restored the reading frame of the BRCA1 protein, whereas none of the three platinum-sensitive recurrent tumors developed BRCA1 sequence alterations. We immunohistochemically confirmed restored expression of BRCA1 protein in two cases with secondary mutations. Intriguingly, the case with primary platinum resistance showed back mutation of BRCA1 in the primary tumor and showed another secondary mutation in the recurrent tumor. Our results suggest that secondary mutations in BRCA1 can mediate resistance to platinum in BRCA1-mutated ovarian tumors. [Cancer Res 2008;68(8):2581–6]

Introduction

DNA cross-linking agents such as cisplatin, carboplatin, melphalan, and cyclophosphamide are widely used anticancer drugs. Resistance to these agents is a major obstacle to effective cancer therapy. Patients with ovarian cancer usually respond well to initial chemotherapy with cisplatin and its derivative carboplatin, but over time the majority of patients become refractory to platinum compounds. Ultimately, progression of chemoresistant disease is the major cause of death in women with ovarian carcinoma (1).

BRCA1 and BRCA2 are tumor suppressor genes responsible for familial breast/ovarian cancer. BRCA1 or BRCA2 (BRCA1/2) mutation carriers have increased risks of developing breast and ovarian cancer (2). Ten percent to 15% of all epithelial ovarian cancers are associated with inherited mutations in BRCA1/2 (3, 4). The vast majority of tumors from women with germ-line BRCA1/2 mutations show loss of the wild-type BRCA1/2 allele and are considered to be BRCA1/2 deficient (5–7).

BRCA1/2-deficient cancer cells are hypersensitive to DNA cross-linking agents including cisplatin (8–10). Consistently, patients developing BRCA1/2-mutated ovarian cancer have a better prognosis compared with noncarriers, if they receive platinum-based therapy (8, 11). However, even BRCA1/2-mutated ovarian cancers frequently develop platinum resistance.

Recently, we reported that acquired resistance to cisplatin in BRCA2-mutated tumors can be mediated by secondary mutations in BRCA2 that restore the wild-type BRCA2 reading frame (12). BRCA2-mutated cancer cells selected in the presence of cisplatin acquired secondary genetic changes on the mutated BRCA2 allele, which canceled the frameshift caused by the inherited mutation and restored expression of functional nearly-full-length BRCA2 protein. Cells with secondary mutations were resistant to cisplatin (12). However, mechanisms of cisplatin resistance in BRCA1-mutated ovarian cancer remain unclear. Because both BRCA1 and BRCA2 are involved in homologous recombination DNA repair (13, 14) and are required for cellular resistance to cisplatin (9, 10), we hypothesized that similar secondary mutations of the mutated BRCA1 gene mediate acquired resistance to cisplatin in BRCA1-mutated ovarian cancer. Now we report that secondary mutations of BRCA1 also occur in BRCA1-mutated ovarian cancer with platinum resistance.

Materials and Methods

Clinical specimens. Three DNA samples from recurrent BRCA1-mutated ovarian cancer patients were obtained from Cedars-Sinai Medical Center (Los Angeles, CA) through the Pacific Ovarian Cancer Research Consortium. Six DNA samples from BRCA1-mutated ovarian cancer patients with recurrent disease were obtained from the tissue bank of University of Washington (Table 1). DNA from UW80, UW40, UW27, and the recurrent tumor from UW91 were obtained after laser capture microdissection. The study was approved by Institutional Review Boards of Fred Hutchinson Cancer Research Center and University of Washington. Clinical data of UW40 patient and a cancer cell line derived from the same patient’s recurrent tumor (UWB1.289) have previously been reported (15). Platinum sensitivity was defined as a complete response to treatment maintained without progression for at least 6 mo after platinum therapy. Tumors that were resistant or refractory had progressive disease on platinum therapy, less than a complete response to platinum therapy or progression within 6 mo of completing platinum therapy.

Sequencing of BRCA1. PCR products of genomic DNA were analyzed for sequence alterations with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using ABI 3100 Genetic Analyzer (Applied Biosystems). All detected mutations were confirmed by sequencing of an...
Independent amplification of template. Information of all sequencing and PCR primers for BRCA1 is available in Supplementary Table S1. All nucleotide numbers refer to the wild-type cDNA human sequence of BRCA1 (U14680.1) as reported in the GenBank database.

**Immunohistochemical studies.** BRCA1 protein was detected in formalin-fixed paraffin-embedded sections using the mouse monoclonal antibody MS110 (previously called Ab-1; Oncogene Research Products) as previously described (16). The MS110 antibody recognizes an NH2-terminal epitope at BRCA1 (amino acid residues 89–222; ref. 16). Briefly, paraffin sections were deparaffinized, washed in PBS, and treated with steam heat epitope at BRCA1 (amino acid residues 89–222; ref. 16). The MS110 antibody recognizes an NH2-terminal epitope in BRCA1, it cannot immunohistochemical detection of BRCA1 (16). Because the MS110 antibody recognizes an NH2-terminal epitope in BRCA1, it cannot distinguish between wild-type BRCA1 protein and truncated nonfunctional protein resulting from frameshift mutations occurring distal to the antibody epitope. Thus, MS110 is not suitable for distinguishing functional versus nonfunctional BRCA1 protein in UW40, UW208, and UW317. Therefore, we evaluated immunohistochemistry only in those tumors with 185delAG mutations (proximal to the antibody epitope) for which we had paired primary and recurrent paraffin-embedded tumor samples.

**Results and Discussion.**

We analyzed nine clinical samples of recurrent BRCA1-mutated ovarian cancer previously treated with platinum (Table 1). Six of them were clinically platinum resistant and three were platinum sensitive. One case (UW40) showed primary resistance to platinum, whereas five cases were initially sensitive to platinum and acquired resistance during the disease course. Three of the five tumors with acquired platinum resistance (UW80, UW91, and UW27) and the primarily resistant tumor (UW40) had secondary genetic changes in BRCA1, whereas none of the three cisplatin-sensitive recurrent tumors showed secondary genetic changes in BRCA1.

UW80 patient is a heterozygous carrier of a frameshift BRCA1 mutation, 185delAG, which is common in the Ashkenazi Jewish population (Fig. 1A; ref. 17). In the primary tumor specimen, only BRCA1 sequence with 185delAG was detected, indicating that the tumor had lost the wild-type allele. Loss of heterozygosity (LOH) of an intragenic BRCA1 single nucleotide polymorphism (SNP) was also confirmed. In the microdissected recurrent tumor from the same patient after development of resistance to platinum, LOH of the BRCA1 SNP was confirmed, indicating that the recurrent tumor contained only the same single allele of BRCA1 as present in the primary tumor and that contamination of nontumor cells was negligible. In this recurrent sample, wild-type BRCA1 sequence represented >80% of the sequences at the mutation site, suggesting that the recurrent tumor had acquired wild-type BRCA1 by genetic reversion (back mutation to wild-type).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Inherited mutation</th>
<th>Specimen</th>
<th>Clinical platinum sensitivity</th>
<th>Secondary genetic change</th>
</tr>
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<tbody>
<tr>
<td>Paired specimens</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS1</td>
<td>185delAG</td>
<td>Pre-platinum</td>
<td>Sensitive</td>
<td>No</td>
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<tr>
<td></td>
<td></td>
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<td>Sensitive</td>
<td></td>
</tr>
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<td>CS4</td>
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<td>Sensitive</td>
<td>No</td>
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<tr>
<td></td>
<td></td>
<td>Post-platinum</td>
<td>Sensitive</td>
<td></td>
</tr>
<tr>
<td>UW91</td>
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<td></td>
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<td>Resistant</td>
<td></td>
</tr>
<tr>
<td>CS14</td>
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<tr>
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<td>Sensitive</td>
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<td>Post-platinum</td>
<td>Resistant</td>
<td></td>
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<tr>
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<td>Pre-platinum</td>
<td>Sensitive</td>
<td>Yes (back mutation)</td>
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<td>Post-platinum</td>
<td>Resistant</td>
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<td>UW208</td>
<td>3867G&gt;T (E1250X)</td>
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<td>UW317</td>
<td>3171insTGAGA</td>
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</table>
Similarly, in two more BRCA1.185delAG cases (UW91 and UWF27), the primary tumor showed only mutant sequence with loss of the wild-type allele. In the recurrent tumors, we mainly detected wild-type BRCA1 sequence, suggesting that these two platinum-resistant tumors acquired wild-type BRCA1 by genetic reversion (back mutation to wild-type; Fig. 1B). Both cases had confirmation of LOH at intragenic SNPs in the primary and recurrent tumors (Supplementary Fig. S1), indicating the presence

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Genetic reversion of BRCA1 mutation in three recurrent BRCA1-mutated ovarian cancer cases. A, DNA sequences of BRCA1 in peripheral blood lymphocytes and the primary and recurrent tumors from a patient (UW80) with BRCA1-mutated ovarian cancer. In the lymphocytes, a heterozygous SNP of the BRCA1 locus (4956A/G) was detected, in addition to a homozygous mutation (185delAG). In the primary tumor, a hemizygous mutation (185delAG) was detected. In the microdissected recurrent tumor, wild-type sequence was detected, suggesting that the recurrent tumor had acquired wild-type BRCA1 by genetic reversion (back mutation to wild-type). Residual 185delAG sequence could represent heterogeneity in the tumor. A speculative model of BRCA1 alleles in samples from this patient is also depicted. B, DNA sequences of BRCA1 in peripheral blood lymphocytes; the primary and recurrent tumors from two patients (UW91 and UWF27) with BRCA1-mutated ovarian cancer. In the lymphocytes, a heterozygous mutation (185delAG) was detected. In the primary tumors, a hemizygous mutation (185delAG) was detected. In the microdissected recurrent tumor specimens, wild-type sequence was detected, suggesting that the recurrent tumors had acquired wild-type BRCA1 by back mutation. Analyses of intragenic SNPs of these cases are shown in Supplementary Fig. S1.
Carcinoma was clinically resistant to platinum. After six cycles of cyclophosphamide, methotrexate, and fluorouracil that the patient previously received for breast cancer. Cyclophosphamide is a DNA cross-linking agent and could exert similar selection pressure for the correction of DNA repair defects as we have observed with exposure to platinum agents.

An alternative explanation for the existence of genetic reversion and other secondary mutations on a mutant allele in this primary tumor is that these events are more common than we appreciate but usually occur in only a small number of primary tumor cells before exposure to chemotherapy. Then, chemotherapy provides the selection pressure for expansion of these subclones in recurrent tumors. Perhaps some reports of retention of the wild-type BRCA1/2 allele in breast cancers (19) actually represent genetic reversion.

LOH of three intragenic BRCA1 SNPs (2201C/T, 2430T/C, and 2731C/T) that flank the mutation site was confirmed in both the primary and recurrent tumors (Fig. 3A and B, and data not shown), indicating that contamination by nontumor cells was negligible and that both the primary and recurrent tumors had lost one BRCA1 allele. Intriguingly, in the primary tumor, both wild-type BRCA1 sequence and BRCA1 sequence with 2594delC were detected (Fig. 3A and B). Careful laser microdissection of a separate second sample of this tumor revealed the same result. The presence of both wild-type BRCA1 sequence and mutant sequence on one allele in the primary tumor suggests that genetic reversion (back mutation to wild-type) occurred on one copy of the mutant allele. We speculate that the presence of the genetically reverted wild-type allele in the primary tumor contributed to the unusual initial platinum resistance of this tumor. The selective pressure for the genetically reverted tumor cells in the primary ovarian tumor is unknown, but may be related to the 11 months of cross-linking agent and could exert similar selection pressure as we have observed with exposure to platinum agents.

An alternative explanation for the existence of genetic reversion and other secondary mutations on a mutant allele in this primary tumor is that these events are more common than we appreciate but usually occur in only a small number of primary tumor cells before exposure to chemotherapy. Then, chemotherapy provides the selection pressure for expansion of these subclones in recurrent tumors. Perhaps some reports of retention of the wild-type BRCA1/2 allele in breast cancers (19) actually represent genetic reversion.

Figure 2. BRCA1 protein expression is restored in recurrent ovarian tumors with genetic reversion of BRCA1 mutation. A, schematic presentation of BRCA1 proteins encoded by 185delAG and wild-type BRCA1. Functional domains of BRCA1 protein [RING finger, nuclear localization signals (NLS), and BRCT domains] are depicted. The region that the BRCA1 antibody (MS110) for immunohistochemical staining recognizes (amino acids 89–222; ref. 16) is also depicted. B, immunohistochemical staining of BRCA1 in a primary tumor with BRCA1.185delAG mutation and a recurrent tumor with genetic reversion of the BRCA1 mutation (UW91 patient). The primary tumors are negative for BRCA1 immunostaining, whereas the recurrent tumors are positive for nuclear BRCA1 immunostaining. Bar, 10 μm.

The mechanism for genetic reversion of 185delAG mutations in all three tumors is not clear. At the genomic level, the reinsertion of two missing nucleotides would seem less likely to occur than a secondary downstream deletion that corrects the frameshift and restores the open reading frame. Several possible explanations for this phenomenon are not mutually exclusive. First, we speculate that because 185delAG is in a critical portion of the BRCA1 gene close to the RING finger (Fig. 2A), a change of one single amino acid here is likely to have deleterious effect on protein function. Similarly, missense mutations in the RING finger such as C61G can be deleterious (18). Therefore, restoration of BRCA1 function may require complete reversion of a mutant in this domain to wild-type. Another possibility is that the particular genomic sequence surrounding 185delAG somehow facilitates the genetic reversion. A third possibility is that other downstream mutations do occur in some tumors but are not detected with our relatively short PCR amplifications. We are unable to do long-range PCR that might identify larger genomic events because of the fragmented nature of our DNA. Thus, it is easier to identify genetic reversion in our samples than larger events that restore the open reading frame.

UW40 patient is a heterozygous carrier of a frameshift BRCA1 mutation, 2594delC (Fig. 3A and B). The mutant allele encodes an 844-amino-acid protein that lacks BRCT domains (Fig. 3C). The patient had a past history of breast cancer at the age of 42 years and developed ovarian cancer at age 54 years. The primary ovarian carcinoma was clinically resistant to platinum. After six cycles of platinum and Taxol, the patient was treated with Taxol for three additional cycles with development of progression. She was retreated with topotecan, then with gemcitabine and Adriamycin to obtain a partial remission. Recurrent tumor was then obtained at the time of surgery for a fistula.

Of a single BRCA1 allele that underwent a secondary genetic alteration.

BRCA1.185delAG encodes a severely truncated protein with 38 amino acids, and the back mutation should restore full-length BRCA1 protein (Fig. 2A). Consistently, immunohistochemical study revealed that BRCA1 protein expression was absent in the primary tumors of UW80 and UW91, but strongly positive in their recurrent tumors (Fig. 2B and data not shown).

The primary tumors are negative for BRCA1 immunostaining.
Only careful haplotyping distinguishes tumors with retention of the wild-type allele from those with genetic reversion.

The genetically reverted wild-type allele was then lost at some point during the disease course. In the recurrent tumor, we could no longer detect wild-type sequence, but we found a second site small deletion (2606_2628del23) in addition to the inherited mutation (2594delC). The second mutation corrects the frameshift caused by the inherited mutation and the allele BRCA1.2594-delC;2606_2628del23 encodes a BRCA1 protein with intact COOH-terminal region, which may be functional (Fig. 3C). The actual genomic DNA sequence of BRCA1 in the UW40 recurrent tumor is a mixture of 2594delC;2606_2628del23 and 2594delC without a...
secondary mutation (Fig. 3A). The mixture of sequences on one allele in both the primary and recurrent tumors may have occurred by secondary genetic alterations (reversion to wild-type in the primary tumor and 23-bp deletion in the recurrent tumor) occurring on just one of the duplicated mutant alleles (Fig. 3B), similar to the situation occurring in Capan-1 clones with secondary mutations of BRCA2 observed in the in vitro experiment in the previous report (12). Alternatively, the two sequences could represent tumor heterogeneity, with only a subset of tumor cells acquiring a secondary mutation on the mutant allele. We favor the explanation of tumor heterogeneity in this case, given that a cell line developed from this recurrent tumor contains only mutant sequence without secondary genetic changes (15).

Taken together, secondary mutations that restore the reading frame of mutated BRCA1 alleles were frequently observed in ovarian tumors with resistance to platinum. These results suggest that secondary mutations of BRCA1 can be a mechanism of acquired resistance to cisplatin in BRCA1-mutated ovarian cancer. In addition, the observation of the back mutation of BRCA1 in cisplatin-resistant primary tumor of UW40 patient suggests that secondary mutations of BRCA1 can be a mechanism of primary resistance to cisplatin.

Our present and previous (12) studies emphasize that BRCA1 and BRCA2 are not only cancer susceptibility genes but are also critical determinants of clinical sensitivity and resistance to chemotherapy. These studies support the general concept that defective DNA repair leads to chemosensitivity, whereas restoration of functional DNA repair contributes to acquired chemoresistance in tumor cells in vitro and in vivo.

Testing for secondary mutations in platinum-treated BRCA1-mutated cancers may be clinically important because tumors with secondary mutations are likely to be resistant to platinum and could show cross-resistance to other agents that exploit BRCA1 defects, such as poly(ADP-ribose) polymerase 1 inhibitors. A larger clinical study is warranted to further determine the prevalence and clinical significance of secondary mutations of BRCA1 in BRCA1-mutated cancer.

Our finding could have implications for sporadic ovarian carcinoma. Although somatic mutation of BRCA1/2 is rare in sporadic ovarian carcinoma, BRCA1 is reported to be down-regulated in a subset of sporadic ovarian carcinomas by promoter methylation and other unknown mechanisms (20). BRCA2 mRNA expression is reported to be undetectable by reverse transcription PCR in 13% of ovarian carcinoma (20). Therefore, it is tempting to hypothesize that down-regulation of BRCA1/2 causes initial sensitivity to cisplatin, and restoration of functional BRCA1/2 expression by demethylation of the BRCA1 promoter or other mechanisms leads to acquired resistance. Testing this hypothesis will be important to elucidate the more general roles of BRCA1/2 in platinum sensitivity and resistance.

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References

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