Clinical BRCA1/2 Reversion Analysis Identifies Hotspot Mutations and Predicted Neoantigens Associated with Therapy Resistance


ABSTRACT

Reversion mutations in BRCA1 or BRCA2 are associated with resistance to PARP inhibitors and platinum. To better understand the nature of these mutations, we collated, codified, and analyzed more than 300 reversions. This identified reversion "hotspots" and "deserts" in regions encoding the N and C terminus, respectively, of BRCA2, suggesting that pathogenic mutations in these regions may be at higher or lower risk of reversion. Missense and splice-site pathogenic mutations in BRCA1/2 also appeared less likely to revert than truncating mutations. Most reversions were <100 bp deletions. Although many deletions exhibited microhomology, this was not universal, suggesting that multiple DNA-repair processes cause reversion. Finally, we found that many reversions were predicted to encode immunogenic neoantigens, suggesting a route to the treatment of reverted disease. As well as providing a freely available database for the collation of future reversion cases, these observations have implications for how drug resistance might be managed in BRCA-mutant cancers.

SIGNIFICANCE: Reversion mutations in BRCA genes are a major cause of clinical platinum and PARP inhibitor resistance. This analysis of all reported clinical reversions suggests that the position of BRCA2 mutations affects the risk of reversion. Many reversions are also predicted to encode tumor neoantigens, providing a potential route to targeting resistance.

INTRODUCTION

Defects in genes that control homologous recombination (HR) DNA repair, such as BRCA1, BRCA2, RAD51C, RAD51D, and PALB2, are common in cancer and are enriched in high-grade serous ovarian cancers (HGSOc; ref. 1), triple-negative breast cancer (TNBC; refs. 2, 3), castration-resistant metastatic prostate cancer (4), and pancreatic cancer (5–7). Following the preclinical identification of synthetic lethality between BRCA1/2 mutation and PARP inhibitors (PARPi; refs. 8, 9), a number of clinical trials demonstrated that PARPi, as well as platinum, are effective in patients with either germline or somatic HR gene mutations, leading to the approval of four different PARPi for the treatment of HR-defective breast or ovarian cancers, and the increased use of platinum in a similar clinical context (8, 10–12).

Despite the clinical effectiveness of PARPi and platinum, drug resistance is a growing clinical problem, especially in those with advanced disease (8). The causes of drug resistance in HR-defective cancers are not fully understood, but...
the observation that platinum resistance in HGSOC is predictive of a poor response to PARPi (13) suggests that clinical platinum resistance can often result in cross-resistance to PARPi. One potential explanation for PARPi/platinum cross-resistance is that tumor cells have restored HR. In BRCA1-, BRCA2-, PALB2-, RAD51C-, or RAD51D-mutant cancers, this occurs via reversion mutations that restore the native reading frame of each gene (refs. 14, 15; Fig. 1A). When first seen in HR genes, true reversions (i.e., to wild-type sequence) as well as second-site reversions were identified (refs. 14, 15; Fig. 1A).

In many cases the second-site reversion was intragenic deletions, all of which were flanked by short regions (1–6 bp) of DNA sequence microhomology or accompanied by an insertion (14, 15). This microhomology-associated DNA sequence “scar” suggested that DNA-repair processes that utilize regions of microhomology to repair double strand breaks (DSB), such as microhomology end joining (MMEJ) or single-strand annealing (SSA; refs. 16, 17), could be the predominant cause of reversion.

Although reversion mutations have been associated with clinical PARPi and/or platinum resistance, their description has been limited to individual case reports or studies of relatively small clinical cohorts where low numbers make it difficult to detect any recurring patterns with confidence (Supplementary Table S1). Therefore, to better understand clinical reversions and to stimulate and enable the research community to report and analyze such events, we collated, codified, and analyzed more than 300 HR gene reversion events described in the literature and show how, by analyzing the sequence context of each of these reversions, some insight can be gained as to their origin and nature.

**RESULTS**

**Collation, Review, and Codification of Cases of HR-Gene Reversion Mutation**

To collate all of the available data on HR-gene reversions associated with PARPi or platinum resistance (Fig. 1A), we searched the literature (see Methods) up until March 13, 2020, identifying 29 publications that described 308 reversion mutation events in a total of 91 patients (Supplementary Table S2). The majority of patient-derived reversion mutations were in BRCA1 (n = 90, 29%) or BRCA2 (n = 211, 68%). We also included relevant studies identifying reversion mutations in tumor cell lines and patient-derived xenografts (PDX). The number of cases of PARPi or platinum resistance that are not explained by reversion mutations is difficult to determine, as there will be many unreported cases where a reversion is not detected, not investigated, or cannot be ruled out. Across all the studies that we collated, we identified a total of 96 cases (either cell line clones or patients with recurrent or platinum/PARPi-resistant cancer) where the presence of reversion mutations was assessed, but not detected (Supplementary Table S3).

Differences in nomenclature and annotation exist between publications. This often arises from the use of historical mutation nomenclature for BRCA1/2, and/or the varied use of either transcript-based or coding sequence (CDS)–based numbering across different studies. In addition, the nucleotide-based annotation of microhomologies at reversion deletions lacks a standard definition. Given this, we reannotated and codified all published reversion mutations, both in terms of nucleotide change and microhomology use (see Methods and Fig. 1B). In addition, we reviewed the clinical information provided for all reported cases. We collated all of this information as a singular, freely accessible database (http://reversions.icr.ac.uk).

In terms of disease subtype, the largest number of revertant cases was from patients with ovarian cancer (56 patients with 125 reversion events; Fig. 1C and D). Rather than reflecting a greater propensity for ovarian cancers to exhibit reversion mutations, the number of ovarian cancers in the collated dataset might reflect the longer period over which PARPi and platinum treatments have been in routine use in this disease. Most of the patients in the study had pathogenic mutations in BRCA1 (39 patients with 29 mutations) or BRCA2 (51 patients with 44 mutations), with one each for PALB2, RAD51C, and RAD51D (Fig. 1C). For the majority (84%) of patients, the pathogenic HR gene mutation was a confirmed germline mutation. Two patients (Lin 2018 SubjectID_63 and Carneiro 2018 Patient 1 in the database) had two different pathogenic alleles with reversions in each.

**Reversion Mutations Are Frequently Unique Events**

Among the 91 patients from whom we collated data, most (68/91, 75%) had unique pathogenic mutations (Fig. 1E, annotated as “single-patient mutations,” and Supplementary Fig. S1). There were eight pathogenic mutations represented by multiple patients in the dataset, including common founder mutations such as BRCA2.c.6174delT (c.5946delT in our codified annotation, 5 patients in the dataset) and BRCA1.c.185delAG.

![Figure 1.](image-url)
BRCA Gene Reversions

A. Pathogenic mutation

- Original pathogenic mutation
- Premature STOP codon
- Out-of-frame sequence

B. Reversion mutations

- Second-site deletion/insertion restoring reading frame
- True reversion of mutation to wild-type
- In-frame deletion of original mutation

C. Gene

- BRCA1
- BRCA2
- PALB2
- RAD51C
- RAD51D

D. Site

- Breast
- Ovarian
- Pancreatic
- Prostate

E. Number of patients

- Single-patient mutations
- 60

F. Gene

- BRCA1
- BRCA2
- PALB2
- RAD51C
- RAD51D

- BRC repeats
- OB-folds
- TR2

- wt
- wt
- insCAA
- insTATC
- insA
- dupAAAA

- 10 bp

- Sakai 2008 UW3548
- Norquist 2011 UW304
- Edwards 2008 UK252
- Edwards 2008 UK199
- Carneiro 2018 Patient 1_9
- Carneiro 2018 Patient 1_8
- Carneiro 2018 Patient 1_7
- Carneiro 2018 Patient 1_6
- Carneiro 2018 Patient 1_5
- Carneiro 2018 Patient 1_4
- Carneiro 2018 Patient 1_3
- Carneiro 2018 Patient 1_2
- Carneiro 2018 Patient 1_1
Even where patients had the same founder pathogenic mutation, the DNA sequences of the reversion mutations that emerged in these patients were all unique, with the exception of true reversions to wild-type and two cases of reversion of the BRCA1:c.5266dupC founder mutation (Supplementary Fig. S2B), suggesting that there is not a strong propensity for any particular reversion mutation to arise from a particular pathogenic mutation (Fig. 1E; Supplementary Fig. S1). True wild-type reversions were recurrently observed for the BRCA1:c.68_69delAG (n = 3) and BRCA2:c.5946delT (n = 2) pathogenic mutations (Fig. 1F; Supplementary Fig. S2C).

For each of these common founder mutations, we noted that the reversions that emerged in these patients were generally localized to the 3′ flanking sequence of the original pathogenic mutation (transcriptionally downstream; Fig. 1F; Supplementary Fig. S2B and S2C). Several other sites in both BRCA1 and BRCA2 exhibited a predominant directionality in the deletion reversions that were associated with them (e.g., BRCA2:c.7355delA; Fig. 2A and B). However, other pathogenic mutations in BRCA1 or BRCA2 had reversion deletions that occurred on either side of the pathogenic mutation, suggesting that this was not a universal property but specific to certain pathogenic mutations (Fig. 2A; Supplementary Fig. S2D–S2G).

One possible explanation for the directionality of some reversion mutations is that there is critical amino acid sequence encoded by the DNA upstream of the pathogenic mutation that cannot be disrupted if a productive reversion allele is to be formed. However, we did not find any evidence for particular evolutionary conservation of the amino acid residues immediately upstream of the pathogenic mutation, as assessed by Conservation Score (see Methods; Fig. 2B).

Reversion Mutations in BRCA2 Exhibit Position Dependence

Although the reversion events that emerged in patients with the same founder pathogenic mutations tended to be unique, we assessed whether the propensity of a pathogenic mutant allele to acquire reversion mutations might depend on its position in either BRCA1 or BRCA2. To do this, we compared the CDS positions of pathogenic BRCA-gene mutations known to revert (i.e., those in our reversion dataset) to the CDS positions of pathogenic BRCA-gene mutations in a set of clinical sequencing studies (“Incidence” dataset, see Methods; Supplementary Tables S4 and S5) covering ovarian, breast, pancreatic, and prostate cancers—the predominant tumor types in our reversion dataset. In the case of BRCA1 mutations, the pathogenic mutations in the reversion dataset were distributed throughout the BRCA1 coding sequence, suggesting that reversion mutation is a possible resistance mechanism for pathogenic mutations at most positions (Fig. 2C) and their distribution was not significantly different from the distribution of BRCA1 mutations in the Incidence dataset (Fig. 2D, P = 0.23, two-sided Kolmogorov–Smirnov test).

In contrast to BRCA1, the position distribution of BRCA2 pathogenic mutations that reverted differed from the distribution in the Incidence data (Fig. 2C, P = 0.023; Kolmogorov–Smirnov test). Despite pathogenic truncating mutations in the region encoding the C terminus of BRCA2 being relatively common in large-scale tumor sequencing studies (22% of the pathogenic mutations in the Incidence dataset occurred in exon 16 onwards (CDS position 7617; Fig. 2D)), reversions of pathogenic mutations in this region were rare (Fig. 2C; four reversions from four patients, 7.8%, P < 0.015, permutation test). All but one of the reversions in this “desert” region were true reversions to wild-type (n = 2), or missense mutations (n = 1) rather than deletions (only one deletion observed, Supplementary Fig. S3). This might suggest that pathogenic mutations in the C-terminal coding sequence of BRCA2 are less able to be productively reverted by second-site mutations, particularly deletions, possibly because the surrounding sequence is important for HR function. This hypothesis is consistent with the known importance of the C terminus for HR function (18) and the high degree of amino acid sequence conservation in this region (Fig. 2B). This region of BRCA2 encodes the oligonucleotide/oligosaccharide binding (OB) folds, the nuclear localization signal (NLS), and the TR2 domain (Fig. 2E). Although loss of the TR2 domain causes only a moderate defect in HR deficiency (19–21), studies in BRCA2-mutant tumor cell lines with PARPi resistance indicated that reversion alleles that cause PARPi encode the TR2 domain even where they delete multiple C-terminal exons, suggesting that it is required for PARPi resistance (refs. 14, 15; Supplementary Fig. S4A and S4B).

In contrast to the reversion “desert” at the region encoding the C terminus of BRCA2, we noted a large number of reversion

Figure 2. Directionality and hot and cold spots for reversion mutations. A, Scatter plots showing orientation (5′/upstream or 3′/downstream) of all reversions relative to original pathogenic mutation in BRCA1 (left) or BRCA2 (right). The start and end positions of each reversion mutation (i.e., the start and end of deleted regions) are joined by lines; insertions are not shown. All positions are shown in CDS coordinates. In a few cases deletions extend beyond the plot boundaries, denoted by lines without a terminating point. For the majority of pathogenic mutations, reversion mutations do not have a directional bias and are seen both upstream and downstream of the pathogenic mutation. However, for some pathogenic mutations, for example, BRCA2:c.5946delT and BRCA2:c.7355delA, second site reversions are biased to the DNA sequence downstream of the pathogenic mutation. There is some evidence of a hotspot for reversion mutations at BRCA2 position c.750–775 (highlighted in gray) and for a desert at the BRCA2 C terminus (highlighted in blue). Colors of points and lines denote different studies (colors are repeated). B, Conservation of amino acid sequence in BRCA1 (left) and BRCA2 (right) mapped onto CDS position for BRCA1 and BRCA2, defined by conservation scores (see Methods) determined by the alignment of 11 mammalian species. Notable peaks of conservation in BRCA2 are seen in the BRCA region and the C-terminal OB and TR2 domains. C, Histogram illustrating the frequency of pathogenic mutations in the reversion dataset annotated by CDS position in BRCA1 or BRCA2. Pathogenic mutations are shown in 40-bp bins. Two regions of BRCA2 are highlighted: the candidate reversion hotspot at c.750–775 (gray) and C-terminal region (blue). D, Histogram illustrating the frequency of pathogenic mutations in BRCA1 or BRCA2 clinical mutations covering breast, ovarian, pancreatic, and prostate cancers (“Incidence” data; see Methods), plotted as in C. The distribution of reversion mutations in BRCA1 (shown in C) was not significantly different from the distribution of BRCA1 mutations in the Incidence dataset (P = 0.21, two-sided Kolmogorov–Smirnov test). The frequency of reversions 3′ to CDS position 7617 of BRCA2 (exon 16 onward) was significantly lower than expected frequency based on The Cancer Genome Atlas mutation data (P < 0.015, permutation test). E, Domain structure of BRCA1 and BRCA2 proteins annotated by CDS position. F, Bar chart illustrating the frequency of different pathogenic mutation types among reversions (top) and compared with mutation types in incidence data (bottom).
**BRCA Gene Reversions**

**A**

**BRCA1-reversion mutations**

- c.68_69delAG

**BRCA2-reversion mutations**

- c.5946delT
- c.7355delA

**B**

Amino acid conservation score

**C**

Pathogenic mutations in revertants

**D**

Pathogenic mutations in incidence data

**E**

RING Ser-rich  
BRCT  
BRC repeats  
OB folds  
TR2

**F**

- Few reverted pathogenic missense mutations (n.s.)
- No reverted pathogenic splice mutations ($P = 0.001$)
mutations in the N-terminal c.750-775 region (61 reversions in total from 4 patients in four separate studies; Fig. 2A; Supplementary Fig. S5). These reversions were identified by circulating tumor DNA (ctDNA) sequencing, which might be more effective in identifying more reversion events per patient than, for example, the bulk sequencing of tumor cells from a solid-tumor biopsy (22). However, these mutations originated from 4 different patients, and this region of BRCA2 did not show a high frequency of pathogenic mutations in the Incidence dataset (Fig. 2D). This suggested that BRCA2 mutations in this region might show a greater propensity to acquire reversions and/or better tolerate the local disruption of the coding sequence in the reverted BRCA2 allele, although more data will be required to confirm this. Consistent with this hypothesis, the c.750-775 region is not a highly conserved region of BRCA2 compared with the C terminus of the protein (Fig. 2B).

Reversion of Pathogenic Missense Mutations Is Rare

Multiple types of known pathogenic BRCA1 and BRCA2 mutation exist, including frameshift or nonsense mutations, as well as well-characterized missense and splice-site mutations (23–26). We therefore investigated whether the propensity of a BRCA1-gene mutation to acquire reversion mutations might depend on the nature of the pathogenic mutation. Of the 74 BRCA1/2 pathogenic mutations in our reversion dataset, 49 were present in the BRCA Exchange database of reported mutations (23). All of these 49 mutations were classified as pathogenic by the ENIGMA (27) or ClinVar (26) criteria. All remaining mutations (n = 25) without an entry in the BRCA Exchange database were frameshift or nonsense mutations and therefore predicted to be pathogenic.

Interestingly, we noted very few missense pathogenic mutations in the set of reported reversions. For example, in the Incidence tumor sequencing datasets used previously, we found that 40 of 849 (4.7%) of these pathogenic BRCA1/2 mutations were missense variants; conversely, in the reversion dataset, only a single patient with a pathogenic missense mutation (BRCA1;p.C61S missense mutation, known to be pathogenic) was present (1/91, 1.1%; Fig. 2F). We also noted a patient with a BRCA1 p.M1I pathogenic mutation, which would result in loss of the translation start site. In each of these cases, the reversion seen was a true reversion to wild-type. Moreover, there were no splice-site pathogenic mutations among the reversion cases, despite such mutations constituting 7.3% of Incidence mutations. Splice-site mutations affect nucleotides critical for correct splicing; similarly, pathogenic missense mutations, by definition, affect amino acid residues that are critical for function. Thus, these classes of pathogenic mutations may be under similar constraints when it comes to reversion, and, in particular, are unlikely to be reverted productively by a deletion. The single missense mutation in the reversion dataset was not a statistically significant underrepresentation compared with the Incidence data (P = 0.08, Fisher exact test); however, the absence of reversions in splice-site mutations, or splice and missense mutations considered as a combined category, was significant (P = 0.001 and P = 0.0002, respectively, Fisher exact test; Fig. 2F).

A similar observation has been previously made in an analysis of the ARIEL2 clinical trial assessing the efficacy of the PARPi rucaparib in relapsed, platinum-sensitive high-grade ovarian carcinomas; out of a cohort of 112 patients, 4 had BRCA1-gene missense mutations and 10 possessed splice-site mutations. No reversions were found in any of these 14 patients, 5 of whom were platinum resistant or refractory at the start of the study (13).

Microhomology Use in Reversions Is Frequent, But Not Universal

When BRCA2 reversion mutations were originally identified in cultured tumor cell lines, each of the deletion-mediated second-site reversion events was characterized by the presence of DNA sequence microhomology at the ends of deleted regions (14, 15, 28). This suggested that DNA-repair processes that exploit regions of microhomology to repair DSBs could be responsible for the reversion events. From a mechanistic perspective, the loss of homologous recombination is known to cause increased use of MMEJ (29), suggesting that the microhomology-characterized reversions could even be a downstream effect of the loss of HR (14). Inhibition of DNA polymerase theta, which is involved in MMEJ, has been proposed as a strategy to target HR-defective cancers via their increased reliance on MMEJ (30–32). In subsequent reports of HR-gene reversion in patients, microhomology was also a frequent feature of reversions mediated by deletion, an observation that extended beyond BRCA1 or BRCA2 reversion to reversion events in PALB2, RAD51C, and RAD51D (14, 15, 22, 33–38). Therefore, to better understand the etiology of reversion mutations, we assessed the use of microhomology for the reversion events in our dataset. Such events can be recognized via their ambiguous alignments to the reference sequence, as the bases immediately adjacent to the deletion can be aligned equally well at either side of the deletion (Fig. 3A, alignment 1 and 2). Surprisingly, when we systematically assessed all of the reported reversion events, the use of microhomology-mediated deletions was clearly not universal. Only 56% (159 of 283 with sequence information) of the reversion cases across the whole dataset were deletions that had evidence of microhomology. In cases of BRCA1 reversion, only 47% of all reversions (including those not mediated by deletions) were deletions with evidence of microhomology use; for BRCA2 reversions, 60% showed microhomology use (Fig. 3B).

Overall, 71% of the BRCA1 reversions were mediated by deletions compared with 88% for BRCA2 (categories “deletion” and “microhomology deletion” in Fig. 3B). Therefore, BRCA1-mutant cells may use a wider range of pathways of DNA repair that lead to substitution or true wild-type reversions compared with BRCA2, where most events are deletion-mediated (Fig. 3B). When considering only reversions mediated by deletion, the fraction for which microhomology was present was similar between BRCA1 (67%) and BRCA2 (68%), but still approximately one third of deletions in each case did not exhibit microhomology (Fig. 3C). Taken at face value, this suggested that DNA repair or mutagenic processes that do not utilize regions of DNA microhomology could also play a major role in the formation of reversion deletion mutations in patients. There was no clear position effect on the type of reversions (Supplementary Fig. S6A and S6B), and deletions could revert by insertion and vice versa (Supplementary Fig. S6C).
Figure 3. Microhomology usage in reversion mutations. A, Example of a reversion mutation in BRCA2 associated with microhomology (patient 201 from Cruz and colleagues; ref. 52). The pathogenic G>T substitution leading to premature STOP codon (TAA) introduces a premature stop codon (TAA) as shown. The reversion mutation (c.145G>T) introduces a premature stop codon (TAA) as shown. The reversion mutation (c.145_168del24) is an in-frame deletion removing the mutated codon (shown in two different alignments). The existence of microhomology at this deletion is illustrated by the ambiguous alignment of the two nucleotides (TA) flanking it—these could be aligned equally well at either end as illustrated. B, Bar chart of reversion events classified by type. Reversions occurring via deletion are more frequent in BRCA2 (88%) than in BRCA1 (71%). C, Within deletion mutations, the use of microhomology occurs at a similar frequency in BRCA1 and BRCA2. Deletion mutations are plotted as in B for deleions only. D, Deletion sizes are generally larger in BRCA2 reversions (P = 0.0105, Wilcoxon rank sum test) with evidence of microhomology use. Total length of deleted sequence is shown for each reversion event, broken down by gene and presence of microhomology. E, BRCA2 reversions use longer lengths of microhomology compared with BRCA1. Frequency distribution of length of microhomology used in BRCA1 (red, left—mode 1 bp) compared with BRCA2 (blue, right—mode 2 bp) plotted for all secondary deletions.
Characteristics of Reversion Mutations Indicate Strong Selective Pressure for Close to Full-Length Proteins

BRCA2 reversion mutations identified in cell line models were often large intragenic deletions (>50 kb in some cases) that removed large segments of the coding sequence despite restoring the open reading frame of the gene and leading to expression of the C-terminal NLS and OB/TR2 domains (14). This might suggest that much of the BRCA2 coding sequence is dispensable for tolerance of PARPi or platinum, at least in cultured cells. In aggregate, deletions have been observed from CDS position 4203 to 9682, but reverted proteins retain the N-terminal PALB2 binding region, some of the BRC repeats, and the C-terminal TR2 domain (Supplementary Fig. S4B). For BRCA1, cell line–based studies suggest that much of the protein coded for by exon 11 (1,142 amino acids, 60% of the coding sequence) is dispensable for therapy resistance (39)—this is supported by the observation of potential reversion mutations in the splice donor of exon 11 in two cases (38, 40) that may cause skipping of exon 11 and the pathogenic mutation. However, and in contrast to the observations in preclinical models (14), the intragenic deletions seen in clinical reversion cases ranged from 1 to 2,541 base pairs (in cDNA coordinates), with most deletions being less than 50 bp and contained within a single exon (Fig. 3D; Supplementary Fig. S4). Therefore, although cells in culture appeared able to tolerate, for example, the loss of thousands of bases and multiple exons of the BRCA2 coding sequence, this does not appear to be recapitulated clinically. This may reflect a greater requirement or fitness advantage for tumor cells with near-full-length BRCA1 or BRCA2 proteins. It should be noted here that some next-generation sequencing technologies or variant calling pipelines may not be optimized to detect large intragenic deletions or fusion events.

Interestingly, deletion size was generally larger in reversion mutations that displayed evidence of microhomology use, an observation that appeared to be limited to reversion mutations occurring in BRCA2-mutant tumors (BRCA1, P = 0.97; BRCA2, P = 0.0105; Wilcoxon rank sum test; Fig. 3D) perhaps reflecting a greater extent of end resection and microhomology search in BRCA2-mutant tumors than in BRCA1-mutant tumors. One reason for the increased deletion size in BRCA2 reversion mutations with microhomology could be that longer regions of microhomology are required for DNA end joining in this context. Longer regions of microhomology would be expected to occur less frequently, resulting in increased DNA resection length during microhomology searching. Consistent with this hypothesis, BRCA2 reversion mutations did indeed exhibit longer regions of microhomology on average, peaking at 2 to 3 nt, when compared with BRCA1 reversion events (which predominantly utilized 1 bp of microhomology on each side of the reversion deletion; Fig. 3E). A general consensus of opinion is that although canonical non-homologous end joining utilizes either no DNA sequence microhomology or very short regions (1–3 bp) to repair DNA, MMEJ and SSA exploit somewhat longer regions (2–20 bp and >15 bp, respectively; refs. 16, 17). Taken at face value, this might suggest that differences in DNA-repair pathway usage could explain the differences in microhomology length associated with BRCA1 versus BRCA2 reversion deletions.

Proximity of Reversion Mutations to Original Truncating Mutation Suggests That Many Revertant Proteins Will Constitute Neoantigens

Compensatory frameshift reversions that do not restore the same codon as the original mutation (i.e., second-site reversions) will introduce out-of-frame stretches of novel amino acid sequence in the revertant protein that are not encoded by the wild-type allele and may not be stably expressed from the pathogenic allele. Overall, 50% of reversions restoring the reading frame occurred at a distance of at least 7 bp from the pathogenic mutation, ranging up to 105 bp (Supplementary Fig. S7A and S7B). This is consistent with the range of distances to out-of-frame stop codons, beyond which a reversion would not restore the reading frame (Supplementary Fig. S7C). Thus, most revertant proteins will contain some out-of-frame sequence of 2–30 amino acids, or at least a novel breakpoint amino acid junction. These amino acid sequences may not have previously been visible to the host immune system and could constitute neoantigens; this in turn could provide an opportunity to therapeutically target tumor cells presenting these candidate neoantigens, using approaches such as chimeric antigen receptor T-cell therapies, immune checkpoint inhibitors, or anticancer vaccines.

To assess this possibility, we first estimated, using the NetMHCpan-4.0 algorithm (41), how frequently in the general population neoantigens derived from the out-of-frame sequence following pathogenic mutations were predicted to be presented by HLA class I complexes. We found that for many pathogenic mutations, including common founder mutations such as BRCA2:c.5946delT, BRCA1:c.68_69delAG, and BRCA1:c.5266dupC, the associated neoantigens were likely to be presented in a sizable fraction of the population (Fig. 4A; Supplementary Table S6). Out-of-frame neoantigens can be shared to some extent by revertant sequences arising from the same pathogenic mutation and different downstream...
BRCA Gene Reversions

**A**

- **Wild-type protein**
- **Predicted truncated protein**
- **Out-of-frame sequence**

MHC binding prediction for out-of-frame sequence (right)

**B**

- **S1982fs**
- **c.5946delT**
- **c.5998_6008delTTTTCTGAAATinsCAA**
- **c.5994_5999delAGTGTTinsTATC**
- **c.5992_6005delCAAGTGTTTTCTGA**
- **c.5964_5998del35**
- **c.5959_5966delCAGGTATC**
- **c.5954_6090del137**
- **c.5949_5952dupAAAA**
- **c.5946_5990del45**
- **c.5944_5952delAGTGGAAAA**

**Out-of-frame amino acid sequence**

**C**

- **Neopeptide sequence**
- **Reverted protein**

Peptide (8-11 aa) surrounding potential neopeptide sequence

MHC binding prediction (NetMHCpan 4.0)

**D**

**Sequences in reversion mutant alleles**

- Median: 52%

**Gene**
- **BRCA1**
- **BRCA2**
- **PALB2**
- **RAD51C**
- **RAD51D**

**BRCA2:c.5946delT (99%)**

BRCA1:c.5266dupC (87%)

BRCA1:c.68,69delAG (66%)
reversions. For example, reversions observed downstream of the BRCA2:c.3946delT pathogenic mutations retain 3 to 15 amino acids of the original out-of-frame pathogenic sequence before the reading frame is restored (Fig. 4B). Neopeptides associated with the first 7 amino acids of the pathogenic out-of-frame sequence and shared by 3 of 10 revertant alleles were predicted to be presented by the MHC in at least 84% of individuals (based on a set of 1,261 individuals whose HLA alleles are known; see Methods) making them potential tumor antigens (Fig. 4C). This increased to 96% of individuals when considering a longer out-of-frame sequence (RENSRKYQMLHYKTQ) also shared by the same 3 revertant cases (Supplementary Fig. S8A).

In general, we observed that revertant sequences were associated with sets of neopeptides that, as a whole, were predicted likely to be presented by a considerable fraction of the general population [median 52%, interquartile range (IQR) 23–76; Fig. 4D; Supplementary Table S7], and this was also true when considering only neopeptides that were not potentially produced by the pathogenic allele (median 44%, Supplementary Fig. S8B). This raises the possibility that tumors with some revertant alleles may be targetable with either immunotherapies that relieve immune suppression or those that exploit the introduction of T-cell clones that recognize specific neopeptides. For some pathogenic mutations, it may be possible to vaccinate against the peptides predicted to be presented in revertant alleles, or exploit these as antigens for other immunotherapies, as a route to delay or prevent the emergence of therapy-resistant disease.

**DISCUSSION**

Here, we show that by collating, codifying, and analyzing more than 300 HR-gene reversion mutations, a number of principles can be established. These include the unique nature of most reversions, positional “hotspots” and “deserts” in the N- and C-terminal coding regions of BRCA2, the paucity of missense and splice-site pathogenic mutations leading to reversions, and differences in microhomology use in BRCA1-related compared with BRCA2-related reversions. Finally, we found that many reverted alleles were predicted to encode highly immunogenic neopeptides, suggesting a route to treatment of reverted disease. We believe that by generating, analyzing, and expanding the reversion dataset, additional principles that govern how therapy resistance emerges in HR-defective cancers could be established.

One observation we noted was that the clinical reversion mutations seem to have a more restricted spectrum (<100 bp deletions, close to the pathogenic mutation; Fig. 2A; < Fig. 3D; Supplementary Fig. S7) compared with those previously seen in cell line and PDX studies, where large deletions predominate (14, 15, 42). Although some ascertainment bias in the detection of clinical reversions cannot be eliminated, it seems that the types of reversions seen in patients are more likely to preserve the majority of the coding sequence than those seen in preclinical models. Furthermore, in contrast to the ubiquitous microhomology at deletions in cell line studies, we found that microhomology usage in clinical reversions was not universal (67% of the deletion-mediated reversion mutations exhibiting microhomology; Fig. 3C). This suggests that multiple DNA-repair processes might drive reversion, implying that the design of therapeutic interventions that limit reversions might be more complex than originally thought. Tumor sequencing studies have assessed microhomology usage in somatic deletion mutations at a genome-wide level, finding, for example, that approximately 40% of deletions (IQR, 30–50) showed microhomology in BRCA1/2-mutant breast cancers, compared with approximately 20% in BRCA wild-type (43). Thus, the frequency of microhomology-associated BRCA-gene reversions is at the upper end of what might be expected at the genome-wide level in BRCA gene-mutant cancers, but still lower than that seen for reversions isolated from cell line models.

The observation of a possible hotspot for secondary mutations around position c.750–775 in BRCA2 has potential implications for patients with these mutations. This may indicate that patients with such mutations would be at higher risk of acquiring resistance via reversion mutations and should be monitored more closely. Conversely, patients with missense and splice-site mutations, or mutations in the BRCA2 C-terminal desert (exon 16 onward), may be at lower risk of developing resistance via reversion.

This study has several likely limitations and biases. There are several sources of bias in the data in terms of which tumor types have been studied, which treatments patients have received, and which methods were used to detect mutations. For example, the large number of reversions in the dataset that are derived from prostate cancers is somewhat out of proportion to the number of patients with prostate cancer who receive BRCA-targeted PARPi or platinum therapy, but reflects the number of prostate cancer studies where ctDNA sequencing has been used to detect reversions. Second, the identification of reversion mutations may be affected by the method used to detect them; although ctDNA sequencing is extremely sensitive and can often identify dozens of different reversion events in a single patient (thus reflecting clonal heterogeneity), singular biopsies from solid tumors often do not capture this heterogeneity and thus tend to lead to the identification of single reversions as opposed to many. Third, the method of reversion mutation detection might influence the size and type of reversion detected; large, multiple exon deletions may be more efficiently detected by RT-PCR, as in cell line studies (14, 15), compared with sequence capture approaches or Sanger sequencing around the site of pathogenic mutations. In addition, a major drawback of ctDNA sequencing is that true wild-type reversions are difficult to detect with confidence, due partly to the low prevalence of reversions relative to wild-type or nonreverted alleles in blood DNA, but also to the low likelihood that a linked SNP is available to link the wild-type reversion to the chromosome that originally bore the pathogenic mutation, either directly by being on the same sequencing read, or by inference using SNP allele frequencies (34, 36, 44). Thus, it is possible that the prevalence of wild-type reversions is underestimated.

The mechanism by which true wild-type reversions emerge is still unclear. Two possibilities are: (i) the sequence at these sites favors the specific wild-type reversion event; or (ii) the functional constraints on the sequence at the point of mutation are such that only a wild-type reversion can restore function (36). A third possibility is that the wild-type sequence is
BRCA Gene Reversions

directly copied from elsewhere in the genome by a process akin to gene conversion. However, BRCA-mutant tumors generally have loss of heterozygosity at the pathogenic mutation, meaning that the other allele is not available as a template for gene conversion even if it were to be used, and gene conversion would likely require some BRCA1/2-dependent RAD51 function, so this seems unlikely.

As more is understood about the prevalence and nature of reversion mutations, the question of how to treat cancers that acquire drug resistance via reversion can be addressed. There are several possibilities suggested by this analysis. First, as described above, inhibiting microhomology-mediated end joining, for example by inhibiting the MMEJ DNA polymerase POLQ (30–32), may be a way of preventing the emergence of some reversions, although this might not be a completely effective approach, given the frequency of non–microhomology-mediated events we observed. Targeting reverted proteins that differ from the wild-type BRCA protein might also serve some therapeutic purpose. For example, reverted BRCA proteins may, because of their altered amino acid sequence, have an increased dependence on chaperones such as HSPs to fold correctly, as suggested elsewhere (45). Where inserted or out-of-frame amino acid sequences are formed by reversion, these may be immunogenic. We show here that there is a high probability of presentation by the MHC across the general population for many of the revertant sequences, including at commonfounders such as BRCA2:c.5946delT (Fig. 4). Thus, immunotherapies (including cancer vaccines) may also be an option for direct targeting of the revertant protein. There are other possible approaches that are not related to the revertant protein per se, such as using WEE1 or ATR inhibitors, that have been empirically shown in preclinical models to target BRCA gene–mutant tumor cells even after the acquisition of reversion mutations (46), an effect likely mediated by the general replication stress that is likely to still exist in the tumor, despite reversion.

The analysis presented here demonstrates the value of a codified set of secondary mutation sequences from clinical observations. We have provided this dataset online at http://reversions.icr.ac.uk along with the analysis presented in this article. This will be updated as more reversion events are reported in the literature to assess whether the conclusions and hypotheses here still apply as the numbers of reported cases increase. As PARPi and platinum are now in routine clinical use for several indications, it is possible that some reversions will no longer be considered novel enough to be reported, risking that these are lost from the literature. We provide a facility to directly report further cases for inclusion in the database at the web portal above, and would be happy to receive submissions from further clinical cases of resistance.

METHODS

Collation, Annotation, and Standardization of Reversion Mutations

Studies for this analysis were collated by searching the PubMed database for BRCA1, BRCA2, RAD51C, RAD51D, or PALB2 and “Secondary Mutation” or “Reversion.” These studies, or others referenced in these papers, describing mutations in cell lines, patients, or PDX models were included (13–15, 22, 33–38, 40, 47–64). Some studies only reported mutations in cell lines (including reversions generated by CRISPR mutagenesis) and PDX (28, 39, 42, 46, 65). These are included in the database but not the analysis described in this article. Where we identified patients whose reversion mutations were reported in multiple studies, these were included only once per reversion event. Reversions were detected by targeted sequencing of circulating free DNA. In one case a reversion was detected at the first cycle of the investigational regimen (olaparib combined with an AKT inhibitor, capivasertib); in the other 4 patients, the reversion was found at the end of treatment.

To aid with the overall analysis, a single transcript was used to annotate all the mutations for a gene. For BRCA1 and BRCA2 we used the same reference transcripts as the ARUP and BRCA Exchange databases; for other genes we chose the longest Consensus Coding Sequence (CCDS) annotated transcript. The transcripts used for codified annotations are: BRCA1, NM_007294.3; BRCA2, NM_000059.3; RAD51C, NM_058216.2; RAD51D, NM_002878.3; and PALB2, NM_024675.3. Where sequence information was available in the original publication this was used to annotate the mutation; otherwise, the reported annotations were checked for correspondence with the reference transcript chosen for each gene. The original annotation in the publication is provided for cross-referencing purposes, along with patient or case identifiers where used in the published paper. If no case/patient identifiers were used in the original publication, these were constructed for the purposes of our analysis based on the study and sequentially numbered reversion events. In the database we list both forms of annotation for the original mutation, the reversion mutations and the chromosomal location (where available). Where a chromosomal location was not annotated in the original report, we have back-calculated this from the CDS annotation using the Ensembl Variant Effect Predictor (VEP; ref. 66).

Once the original and reversion mutations were mapped for each case, we calculated the distance between the mutations as well as noting evidence of microhomology use. The distance between the original mutation and the reversion was measured as the shortest distance, specifically the bases between the last base of one mutation and the first base of the other. Where the reversions are deletions that span the original mutation, the distance is recorded as zero. We also annotated mutations with evidence of microhomology use (Fig. 3A), requiring at least one base pair homology. Microhomology is not reported for complex mutations such as insertion/deletions.

Genomic coordinates (hg38) were retrieved using the HGVs CDS annotation on the transcripts above via the Ensembl VEP (67). In annotations of the original pathogenic mutation, we aligned deletions in repetitive regions to the 3′ end of the deletion, and annotated small insertion as duplications where appropriate, to ensure compatibility with annotations in the BRCA Exchange database. Reversion mutation alleles were annotated relative to the reference sequence, including the original pathogenic mutation where this was retained. Deletions that encompassed or were immediately adjacent to the pathogenic mutation (or an alternative valid annotation of the pathogenic mutation) were annotated as a single deletion relative to the reference sequence.

The database records reversion mutations on a “per-event” basis, an event being a single observation of a reversion mutation in a patient with a pathogenic mutation in an HR gene. Where individual patients possessed multiple, distinct, reversions [as seen in 37 (40%) patients described in the database], each reversion was recorded as a different event. In addition, we also recorded clinical information, including, where available, information pertaining to cancer type, stage, and treatment history (Fig. 1B).

Mutation Data from Tumor Sequencing Studies

The reference set of BRCA1 and BRCA2 pathogenic mutations was assembled from several sources. Some studies were identified from published literature describing identification of BRCA4 mutations in relatively large cohorts of confirmed cases of breast, ovarian, pancreatic, or prostate cancer (10, 35, 68–71). These mutations were curated...
in the same way as the reversion mutations and annotations standardized where necessary. Both germline and somatic mutations were included. All patients studied by Lin and colleagues (13) were also included in this dataset (including the patients in whom reversions were identified). BRCA1/2 mutations were also downloaded from a series of studies available in cBioPortal (Supplementary Table S4) and filtered to retain only mutations that were classified as pathogenic or likely pathogenic by either the ENIGMA or ClinVar projects. The full set of mutations is given in Supplementary Table S5.

For comparisons with pathogenic mutations in the reversions dataset, pathogenic mutations consisting of deletion or rearrangement of entire or multiple exons were removed (there were no such mutations present in the reversion data). To assess underrepresentation of mutations in the BRCA2 C terminus, the Incidence data were randomly sampled (n = 51; the number of patients with at least one reversion mutation in BRCA2) and the number of mutations falling in the desert region (CDS position > 7617) calculated. This was repeated 1,000 times to calculate a p value for observing ≥ 4 mutations in this region. Fisher exact tests, Wilcoxon tests, and Kolmogorov–Smirnov tests were performed in R.

Conservation Analysis

Multiple sequence alignments of BRCA1 and BRCA2 orthologs across 11 mammalian species were downloaded from EGGNOG (72) and visualized using JalView. Sequences with large gaps relative to the human protein were removed and a consensus score was generated (73).

HLA Presentation Score Predictions

Given a gene and a mutational event (primary or reversion), we used an in-house python script (https://github.com/GeneFunctionTeam/neopeptides/) to generate all peptides of length 8-11 amino acids associated with the mutation(s). For primary events, we generated the set of all non–wild-type peptides associated with the primary mutation (Fig. 4A); for reversions, we generated the set of all non–wild-type peptides associated with the reversion (Fig. 4D) and the set C of peptides in B that are not in A (i.e., unique to the revertant sequence; Supplementary Fig. S8B). We then calculated the Best Rank (BR) HLA class I presentation score of the mutation with respect to each HLA allotype in a list of 195 HLA-A/B/C allotypes total found among 1,261 individuals from the 1000 Genomes study (74). We defined the BR by predicting the eluted ligand likelihood percentile rank for each peptide associated to the mutation using the program NetMHCpan-4.0 (41) and taking the minimum elution rank among all peptides (75), excluding those with a wild-type NetMHC predicted Icore (76). We defined an individual’s best rank (IBR) for a mutation m as the minimum BR of the mutation across all HLA class I allotypes of the individual. The percentage of individuals likely to present at least one peptide associated with m is then calculated as the percentage of individuals for which IBR < 0.5 when considering a set of 1,261 individuals from the 1000 Genomes project (74).

Data Availability

All data used in this study, along with updated analysis including any cases reported in future, are available to download from reversions.icr.ac.uk.

Disclosure of Potential Conflicts of Interest

S.J. Pettitt reports grants from Cancer Research UK, Breast Cancer Now, and Schottlander Trust during the conduct of the study. In addition, S.J. Pettitt has a patent for targeting neoantigens in BRCA1/2 reversions pending. M. Punta reports grants from Wellcome Trust (105104/Z/14/Z) and Schottlander Research Charitable Trust (Innovation Award: funding for high risk/high gain pilot projects at the ICR) during the conduct of the study. In addition, M. Punta has a patent for targeting neoantigens in BRCA reversions pending. S. Lise reports grants from Wellcome Trust (grant 105104/Z/14/Z) during the conduct of the study. In addition, S. Lise has a patent for targeting neoantigens in BRCA reversions pending. T.A. Yap reports grants, personal fees, and non-financial support from AstraZeneca during the conduct of the study and outside the submitted work; and research support (to institution: Artios, Bayer, Clovis, Constellation, Cyteir, Eli Lilly, EMD Serono, Forbius, F-Star, GlaxoSmithKline, Genentech, ImmuneSensor, Ipsi, Joun, Karyopharm, Kyowa, Merck, Novartis, Pfizer, Ribbon Therapeutics, Regeneron, Repare, Sanofi, Scholar Rock, Seattle Genetics, Tesaro, and Vertex Pharmaceuticals; consultancies: Almac, Aduro, Aтранн, Axiom, Bayer, Bristol-Myers Squibb, Calithera, Clovis, Cybexra, EMD Serono, F-Star, Guidedepoint, Ignyta, I-Mab, Janssen, Merck, Pfizer, Repare, Roche, Rubisch, Schrodinger, Seattle Genetics, Varian and Zai Labs). A.N.J. Tutt reports other support from AstraZeneca (financial support to my academic and hospital institutions for costs associated with global academic study chair and local site costs for Olympic trial); other support from AstraZeneca (local study site costs associated with VIOLETTE trial), Merck KGaA (local site trial support costs associated with clinical trial DNA inhibitors), Medivation (financial support for research at ICR), Myriad Genetics (financial support for research at ICR), and AstraZeneca (travel expenses covered by AstraZeneca for any trial-related meetings or trial commitments abroad); personal fees from Pfizer (advisory board related to targeted therapies in DNA repair–deficient cancers), Vertex (advisory board related to targeted therapies in DNA repair–deficient cancers), Artios (advisory board related to targeted therapies in DNA repair–deficient cancers), Prime Oncology (advisory board related to targeted therapies in DNA repair–deficient cancers), Inbiomotion (scientific advisory board function and stock options), CRUK (honorary associated with function as Deputy Chair and reviewer for CRUK clinical research committee), and MD Anderson (Moonshot Breast Cancer scientific advisory board honoraria) outside the submitted work; grants from Breast Cancer Now charity (grant funded to study homologous recombination–deficient breast and other cancers, Breast Cancer Now receive payments from AstraZeneca related to PARP inhibitor patents), CRUK (grant funded to study homologous recombination–deficient breast and other cancers, CRUK receive payments from AstraZeneca related to PARP inhibitor patents), and AstraZeneca (payments to institution through Breast International Group for trial conduct in Olympic trial and through CRO’s for commercial PARP inhibitor trial) during the conduct of the study; in addition, A.N.J. Tutt has a patent for AstraZeneca with royalties paid from Institute of Cancer Research (use of PARP inhibitors in DNA-deficient cancers, licensee – AstraZeneca; payments to Institute of Cancer Research and personally through ICR rewards to Inventors scheme). C.J. Lord reports grants and personal fees from AstraZeneca, Merck KGaA, Artios, and Vertex; personal fees from Syncona, Sun Pharma, Gerson Lehrman Group, Tongo (stock ownership), 3rd Rock, and Ono Pharma, and other support from Ovibio (stock ownership) outside the submitted work. In addition, C.J. Lord has a patent for PARP inhibitors and stands to gain as part of the ICR (Rewards to Inventors) scheme licensed to AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

S.J. Pettitt: Conceptualization, data curation, software, formal analysis, validation, investigation, methodology, writing-original draft, project administration, writing-review and editing. J.R. Frankum: Data curation, formal analysis, validation, investigation, writing-review and editing. M. Punta: Software, formal analysis, validation, investigation, writing-review and editing. S. Lise: Software, supervision. J. Alexander: Data curation, formal analysis, validation. Y. Chen: Software. T.A. Yap: Data curation. S. Haider: Software, supervision. A.N.J. Tutt: Supervision. C.J. Lord: conceptualization, supervision, investigation, writing-original draft, writing-review and editing.
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REFERENCES


Johnson N, Johnson SF and BRCA2 and resistance to PARP inhibitors and platinum. JCO Precis Oncol 2018;2:1–6. https://doi.org/10.1200/PO.17.00169


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