

## ORIGINAL ARTICLE

**BRCA1/2 reversion mutations in a pan-cancer cohort**

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**Abstract**

Tumor sensitivity to platinum (Pt)-based chemotherapy and poly(adenosine diphosphate ribose) polymerase (PARP) inhibitors is increased by homologous recombination deficiency-causing mutations; in particular, reversion mutations cause drug resistance by restoring protein function. Treatment response is predicted by breast cancer susceptibility gene 1/2 (*BRCA1/2*) mutations; however, *BRCA1/2* reversion mutations have not been comprehensively studied in pan-cancer cohorts. We aimed to characterize *BRCA1/2* reversion mutations in a large pan-cancer cohort of Japanese patients by retrospectively analyzing sequencing data for *BRCA1/2* pathogenic/likely pathogenic mutations in 3738 patients with 32 cancer types. We identified somatic mutations

**Abbreviations:** BRCA, breast cancer susceptibility gene; cfDNA, cell-free DNA; delin, deletion–insertion; HRD, homologous recombination deficiency; HRR, homologous recombination repair; MMEJ, microhomology-mediated end-joining; NHEJ, nonhomologous end-joining; PALB2, partner and localizer of BRCA2; PARPi, poly(adenosine diphosphate ribose) polymerase (PARP) inhibitor; POLθ, DNA polymerase θ; Pt, platinum; SNV, single-nucleotide variant.

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in tumors or circulating cell-free DNA that could restore the ORF of adverse alleles, including reversion mutations. We identified 12 (0.32%) patients with somatic *BRCA1* ( $n=3$ ) and *BRCA2* ( $n=9$ ) reversion mutations in breast ( $n=4$ ), ovarian/fallopian tube/peritoneal ( $n=4$ ), pancreatic ( $n=2$ ), prostate ( $n=1$ ), and gallbladder ( $n=1$ ) cancers. We identified 21 reversion events—*BRCA1* ( $n=3$ ), *BRCA2* ( $n=18$ )—including eight pure deletions, one single-nucleotide variant, six multinucleotide variants, and six deletion–insertions. Seven (33.3%) reversion deletions showed a microhomology length greater than 1 bp, suggesting microhomology-mediated end-join repair. Disease course data were obtained for all patients with reversion events: four patients acquired mutations after PARP-inhibitor treatment failure, two showed somatic reversion mutations after disease progression, following Pt-based treatment, five showed mutations after both treatments, one patient with pancreatic cancer and *BRCA1* reversion mutations had no history of either treatment. Although reversion mutations commonly occur in *BRCA*-associated cancers, our findings suggest that reversion mutations due to Pt-chemotherapy might be correlated with *BRCA1/2*-mediated tumorigenesis even in non-*BRCA*-associated histologies.

**KEYWORDS**

*BRCA1/2*, homologous recombination deficiency, PARP inhibitor, platinum-based chemotherapy, reversion mutation

## 1 | INTRODUCTION

Platinum-based chemotherapy and PARPi treatment cause DNA damage-mediated cell death. Mutations in *BRCA1/2* hinder the repair of this damage and predict the response to these agents.<sup>1–5</sup> Tumor lineage affects Pt- and PARPi-responsive phenotypes in *BRCA*-mutant tumors. *BRCA* mutations act as the drivers of classic *BRCA*-associated tumors—including breast, ovarian, prostate, and pancreatic cancers—and exhibit zygosity dependence and selection for biallelic inactivation. These characteristics, in turn, suggest that PARP inhibition has a number of potential benefits.<sup>6</sup> However, *BRCA* mutations do not act as the drivers of oncogenesis in other noncanonical *BRCA* cancers, suggesting that PARP inhibition would be less beneficial in these cases.<sup>6</sup>

It remains unclear whether selected *BRCA*-mutant tumors with noncanonical histological features exhibit HRD, which is influenced by *BRCA* loss of function, despite the fact that overall analysis of noncanonical *BRCA*-mutant tumors reveal no signs of such phenotypes. A *BRCA*-dependent phenotype can be confirmed by detecting *BRCA* reversion mutations, subsequent to the selective pressure exerted by therapies that act through the DNA damage pathway.

In tumors exhibiting a *BRCA*-mediated HRD phenotype, somatic change in *BRCA1/2* could lead to resistance against Pt-based chemotherapy or PARP inhibition. Restoration of the ORF of *BRCA1/2* due to secondary reversion point mutations, insertions, or deletions not only enables *BRCA* genes to repair DNA damage induced by PARPi and Pt-based therapies but also causes resistance to PARPi<sup>5,7–10</sup> and

Pt-based therapy.<sup>8,11–15</sup> Thus, Pt-based chemotherapy and/or PARPi treatment applies a selective pressure on the original tumor with loss of *BRCA* function, causing secondary reversion mutations.

In this study, we used data from a cohort of 3738 patients with various tumor types, after germline genetic testing and matched tumor next-generation sequencing, to characterize *BRCA*-mediated HRD phenotypes in different types of cancers. We identified patients who showed reversion mutations indicating a *BRCA*-mediated HRD phenotype, following Pt-based chemotherapy and/or PARPi treatment.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and study design

We retrospectively recruited 3738 patients with solid tumors, listed in the Keio PleSSision Group Database (Keio University Hospital), based on the following criterion: patients who underwent a 324-gene somatic genomic profiling test (FoundationOne CDx; Foundation Medicine, Inc.) ( $n=3094$ ) or a 324-gene cfDNA-based comprehensive genomic profiling assay (FoundationOne Liquid CDx; Foundation Medicine, Inc.) ( $n=644$ ) between August 2020 and June 2023. In total, 1320 patients (35.3%) had canonical *BRCA*-associated tumors (breast, ovarian, peritoneal, pancreatic, and prostate cancers), and 2418 patients (64.7%) had noncanonical *BRCA*-associated tumors (colorectal and endometrial cancers). Table 1 summarizes the patient characteristics. This study was approved by the Ethics Committee

**TABLE 1** Characteristics of Japanese patients with solid tumors who underwent 324-gene somatic genomic profiling test (FoundationOne CDx) or 324-gene cell-free DNA-based comprehensive genomic profiling assay (FoundationOne Liquid CDx)

	FoundationOne CDx	FoundationOne Liquid CDx	Total
Patients, <i>n</i>	3094	644	3738
Sex, <i>n</i> (%)			
Female	1611	272	1883
Male	1483	372	1855
Cancer type			
BRCA-associated cancer			
Breast	196	72	268
Ovary/fallopian tube/peritoneal	253	21	274
Pancreas	263	114	377
Prostate	254	147	401
Total	966	354	1320
Non-BRCA-associated cancer			
Colorectal	539	72	611
Biliary	229	55	284
Central nervous system	152	2	154
Sarcoma	138	12	150
Uterus	115	3	118
Uterine cervix	114	10	124
NSCLC	105	35	140
Head and neck	99	12	111
Stomach	85	15	100
Thyroid	78	4	82
Skin	75	1	76
Unknown primary	66	13	79
Esophagus	63	10	73
Urinary	63	8	71
Kidney	23	6	29
Thymus	14	2	16
Adrenal gland	11	1	12
Duodenum	11	2	13
SCLC	11	2	13
Neuroendocrine carcinoma	9	2	11
GIST	8	2	10
Neuroendocrine tumor	8	0	8
Mesothelioma	7	0	7
Liver	6	3	9
Soft tissue	6	3	9
Vagina	4	0	4
Testis	2	1	3
Others	87	14	101
Total	2128	290	2418

Abbreviations: GIST, gastrointestinal stromal tumor; NSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer.

of Keio University Hospital (approval number: 20211159). Due to the retrospective nature of this study, informed consent was not required and was waived. The study procedures involving human participants complied with the Declaration of Helsinki.

## 2.2 | Next-generation sequencing analysis

After obtaining written informed consent from patients, formalin-fixed, paraffin-embedded samples or plasma samples were

collected and subsequently assessed using FoundationOne CDx or FoundationOne Liquid CDx,<sup>16,17</sup>

### 2.3 | Analysis of *BRCA1/2* pathogenic mutations

The pathogenicity of the *BRCA1/2* variants was assessed according to the ClinVar classification (<http://www.ncbi.nlm.nih.gov/clinvar/>). Variants that had not been submitted to either database, and therefore were not defined as “likely to be pathogenic” or “pathogenic” using the Ambry five-tier variant classification protocol (<https://www.ambrygen.com/science/variant-classification>), were considered pathogenic based on American College of Medical Genetics and Genomics, Association for Molecular Pathology, and International Agency for Research on Cancer guidelines.<sup>18–20</sup>

### 2.4 | Confirmation of germline variants

For patients with potential reversion mutations in *BRCA1/2*, we performed genetic counseling and checked whether the primary *BRCA1/2* mutations were somatic or germline events. Germline *BRCA1/2* testing was carried out on the blood samples using single-site germline variant tests based on the somatic event or potentially comprehensive analysis of *BRCA1/2* using BRACAnalysis CDx (Myriad Genetics).

### 2.5 | Identification of *BRCA1/2* reversion mutations in tumor and cfDNA

We defined *BRCA* reversion mutations as follows: (i) a base substitution of a nonsense mutation that converted it to a missense mutation, or (ii) an insertion/deletion that restored the ORF. Confirmation that the insertion/deletion restored the ORF, and therefore could be considered a reversion mutation, required that the primary deleterious mutation in combination with the secondary mutation produced a nucleotide change divisible by 3. For example, if the primary mutation was a deletion of 2 bp and the secondary mutation was a deletion of 4 bp, either upstream or downstream, the two mutations would together constitute a net deletion of 6 bp, which is divisible by 3. Open reading frame restoration could also be defined as the loss of the primary deleterious mutation, due to a large intragenic deletion.

### 2.6 | Identification of the MMEJ signature

For determining the types of reversion mutations, we examined microhomologies in deletions according to the method outlined by Taheri-Ghahfarokhi et al.<sup>21</sup> Briefly, when checking for pure deletion mutations, we adjusted the position of the deleted sequence in the full sequence to determine whether the nucleotides before the deletion matched the last nucleotides of the deleted sequence. Next, we determined the

number of the contiguous nucleotides at the beginning of the deleted sequence that matched after the deletion. If the number of contiguously matched nucleotides matches the length of the microhomology, the maximum length was considered equal to the length of the deletion. Microhomologies of at least 2 bp were considered candidates for MMEJ repair (Figure 1) and were used to define the MMEJ signature.

### 2.7 | Statistical analysis

Fisher's exact test was used to compare the rates of *BRCA1/2* mutations in different tumor types.  $p < 0.05$  was considered statistically significant.

## 3 | RESULTS

### 3.1 | Study cohort and *BRCA1/2* pathogenic variants

Table 1 shows the patient characteristics and the number of patients with each cancer type. In the FoundationOne CDx cohort, the analysis involved 3094 patients, including 1611 men (52.1%) and 1483 women (47.9%). Of these, 31.2% ( $n = 966$ ) patients had *BRCA*-associated cancers (breast,  $n = 196$ ; ovarian/peritoneal,  $n = 253$ ; pancreatic,  $n = 263$ ; and prostate,  $n = 254$ ), and 68.8% ( $n = 2128$ ) patients had non-*BRCA*-associated cancers. In the FoundationOne Liquid CDx cohort, the analysis involved 644 patients, including 272 women (42.2%) and 372 men (57.8%). Of these, 55.0% ( $n = 354$ ) patients had *BRCA*-associated cancers (breast,  $n = 72$ ; ovarian/peritoneal,  $n = 21$ ; pancreatic,  $n = 114$ ; and prostate,  $n = 147$ ), and 45.0% ( $n = 290$ ) patients had non-*BRCA*-associated cancers. The most frequent tumors in non-*BRCA*-associated cancers were colorectal and biliary cancers in each cohort.

In all tumor types, 5.0% ( $n = 156$ ) of the patients in the FoundationOne CDx cohort had pathogenic variants for *BRCA1/2*

*BRCA2* c.2635\_2655del21 (p.S879\_D885del)

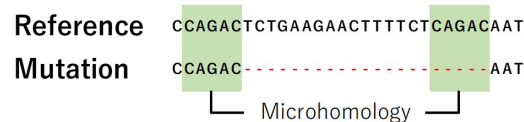


FIGURE 1 Identification of the microhomology-mediated end-joining (MMEJ) signature of the *BRCA2* gene. The reported mutation (deletion) was aligned with the reference sequence and, if necessary, the position of the deletion was recalibrated. For instance, if labeling the deletion as either “c.2635\_2655del” or “c.2630\_2650del” yielded an identical deleted sequence, it would cause an error; therefore, we opted to redefine the precise location of the deletion event. This adjustment allowed us to identify and report a microhomology of length 3, as opposed to being constrained to reporting a microhomology length of 2 in the absence of this procedural refinement.

(Table 2). The overall prevalence for patients with pathogenic mutations of *BRCA1*, *BRCA2*, and their comutants was 1.8% ( $n=55$ ), 3.1% ( $n=97$ ), and 0.13% ( $n=4$ ), respectively. The prevalence rates of *BRCA1/2* were significantly higher in *BRCA*-associated cancers (overall, 9.9% [ $n=96$ ]; breast, 8.7% [ $n=17$ ]; ovarian/peritoneal, 13.0%

[ $n=33$ ]; pancreatic, 4.9% [ $n=13$ ]; and prostate, 13.0% [ $n=33$ ]) than in non-*BRCA*-associated cancers (2.8% [ $n=60$ ]) ( $p<0.001$ ).

In all tumor types, 8.1% ( $n=52$ ) of patients in the FoundationOne Liquid CDx cohort had pathogenic variants in *BRCA1/2* (Table 3). The overall prevalence for pathogenic variants of *BRCA1*, *BRCA2*, and their

TABLE 2 *BRCA1/2* pathogenic variants in Japanese patients with solid tumors who underwent 324-gene somatic genomic profiling test (FoundationOne CDx)

		Total (n)	Patients with <i>BRCA1</i> pathogenic mutations (n)	Patients with <i>BRCA2</i> pathogenic mutations (n)	Patients with <i>BRCA1/BRCA2</i> pathogenic comutations (n)	Patients with <i>BRCA1/BRCA2</i> pathogenic mutations (n, %)
Patients, n		3094	55	97	4	156 (5.0)
Cancer type						
<i>BRCA</i> -associated cancer	Breast	196	2	15	0	17 (8.7)
	Ovary/fallopian tube/peritoneal	253	26	7	0	33 (13.0)
	Pancreas	263	1	12	0	13 (4.9)
	Prostate	254	3	30	0	33 (13.0)
	Total	966	32	64	0	96 (9.9)
Non- <i>BRCA</i> -associated cancer	Colorectal	539	4	6	1	11 (2.0)
	Biliary	229	3	4	2	9 (3.9)
	Central nervous system	152	1	4	0	5 (3.3)
	Sarcoma	138	1	3	0	4 (2.9)
	Uterus	115	3	2	1	6 (5.2)
	Uterine cervix	114	1	2	0	3 (2.6)
	NSCLC	105	0	3	0	3 (2.9)
	Head and neck	99	0	1	0	1 (1.0)
	Stomach	85	2	2	0	4 (4.7)
	Thyroid	78	0	0	0	0.0
	Skin	75	1	0	0	1 (1.3)
	Unknown primary	66	5	2	0	7 (10.6)
	Esophagus	63	0	3	0	3 (4.8)
	Urinary	63	1	1	0	2 (3.2)
	Kidney	23	0	0	0	0.0
	Thymus	14	0	0	0	0.0
	Adrenal gland	11	0	0	0	0.0
	Duodenum	11	1	0	0	1 (9.1)
	SCLC	11	0	0	0	0.0
	Neuroendocrine carcinoma	9	0	0	0	0.0
	GIST	8	0	0	0	0.0
	Neuroendocrine tumor	8	0	0	0	0.0
	Mesothelioma	7	0	0	0	0.0
	Liver	6	0	0	0	0.0
	Soft tissue	6	0	0	0	0.0
	Vagina	4	0	0	0	0.0
Testis	2	0	0	0	0.0	
Others	87	0	0	0	0.0	
Total		2128	23	33	4	60 (2.8)

Abbreviations: GIST, gastrointestinal stromal tumor; NSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer.

**TABLE 3** *BRCA1/2* pathogenic variants in Japanese patients with solid tumors who underwent 324-gene cell-free DNA-based comprehensive genomic profiling assay (FoundationOne Liquid CDx)

		Total (n)	Patients with <i>BRCA1</i> pathogenic variants (n)	Patients with <i>BRCA2</i> pathogenic variants (n)	Patients with <i>BRCA1/BRCA2</i> pathogenic covariants (n)	Patients with <i>BRCA1/BRCA2</i> pathogenic variants (n, %)
Patients, n		644	14	34	4	52 (8.1)
Cancer type						
<i>BRCA</i> -associated cancer	Breast	72	4	6	1	11 (15.3)
	Ovary/fallopian tube/peritoneal	21	4	2	0	6 (28.6)
	Pancreas	114	1	6	0	7 (6.1)
	Prostate	147	1	9	1	11 (7.5)
	Total	354	10	23	2	35 (9.9)
Non- <i>BRCA</i> -associated cancer	Colorectal	72	1	2	1	4 (5.6)
	Biliary	55	1	2	0	3 (5.5)
	Central nervous system	2	0	0	0	0 (0.0)
	Sarcoma	12	0	0	0	0 (0.0)
	Uterus	3	0	0	0	0 (0.0)
	Uterine cervix	10	0	2	0	2 (20.0)
	NSCLC	35	0	2	0	2 (5.7)
	Head and neck	12	0	0	0	0.0 (0.0)
	Stomach	15	0	1	0	1 (6.7)
	Thyroid	4	0	0	0	0 (0.0)
	Skin	1	0	0	0	0 (0.0)
	Unknown primary	13	1	1	1	3 (23.1)
	Esophagus	10	0	1	0	1 (10.0)
	Urinary	8	0	0	0	0 (0.0)
	Kidney	6	0	0	0	0 (0.0)
	Thymus	2	0	0	0	0 (0.0)
	Adrenal gland	1	0	0	0	0 (0.0)
	Duodenum	2	0	0	0	0 (0.0)
	SCLC	2	0	0	0	0 (0.0)
	Neuroendocrine carcinoma	2	0	0	0	0 (0.0)
	GIST	2	0	0	0	0 (0.0)
	Liver	3	0	0	0	0 (0.0)
	Soft tissue	3	0	0	0	0 (0.0)
Testis	1	0	0	0	0 (0.0)	
Others	14	1	0	0	1 (7.1)	
Total		290	4	11	2	17 (5.9)

Abbreviations: GIST, gastrointestinal stromal tumor; NSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer.

comutants was 2.2% ( $n=14$ ), 5.3% ( $n=34$ ), and 0.6% ( $n=4$ ), respectively. The prevalence rates of *BRCA1/2* were significantly higher in *BRCA*-associated cancers (overall, 9.9% [ $n=35$ ]; breast, 15.3% [ $n=11$ ]; ovarian/peritoneal, 28.6% [ $n=6$ ]; pancreatic, 6.1% [ $n=7$ ]; and prostate, 7.5% [ $n=11$ ]) than in non-*BRCA*-associated cancers (5.9% [ $n=17$ ]) ( $p<0.001$ ).

### 3.2 | Patients with reversion mutations

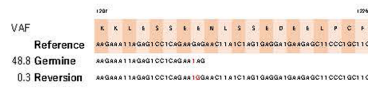
Among 208 patients with *BRCA1/2* pathogenic mutations, 21 reversion mutations were observed in 12 patients, including three patients with primary *BRCA1* mutations and nine patients with primary *BRCA2* mutations (Figure 2). Among the 12 patients with reversion

**FIGURE 2** List of reversion mutations in *BRCA1/2* carriers. The nucleotide and protein coding sequences for primary and reversion mutations are shown with respect to the reference genome, for patients with *BRCA1* ( $n=3$ ) and *BRCA2* ( $n=19$ ) reversion mutations in different types of cancers—ovarian, fallopian tubal, breast, pancreatic, gall bladder, and prostate cancers. VAF, variant allele frequency.

Case 1: Ovarian cancer

BRCA1 exon11

c.3640G>T (p.E1214\*)  
c.3640\_3641GA>TG (p.E1214W)



Case 2: fallopian tubal cancer

BRCA1 exon11

c.3958\_3982del25 (p.S1320fs\*8)  
c.3916\_3980>AAACACCCAGGATCCCTTTCTGATTG (p.L1306\_Q1327>KHGQSFLDW)



Case 3: Breast cancer

BRCA1 exon11

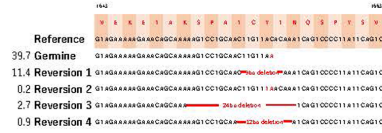
c.3640G>T (p.E1214\*)  
c.3548\_3767>GAGG (p.K1183\_T1256>RQ)



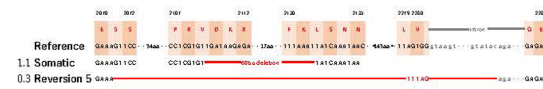
Case 4: Breast cancer

BRCA2 exon11

c.4965C>A (p.Y1655\*)  
c.4959\_4967del (p.C1654\_T1656del)  
c.4964\_4965AC>TA (p.Y1655L)  
c.4947\_4970del24 (p.K1649\_T1656del)  
c.4958\_4969delICTTTGATACACAA (p.T1653\_T1656del)



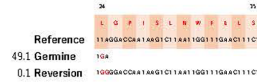
c.6328\_6395del68 (p.D2110fs\*3)  
c.6212\_6841+200>TTTAG (c.6212\_6841+200>TTTTAG)



Case 5: Breast cancer

BRCA2 exon3

c.71T>G (p.L24\*)  
c.71\_72TA>GG (p.L24W)



Case 6: Pancreatic cancer

BRCA2 exon10

c.1813del (p.I605fs\*9)  
c.1813\_1825ATACCGAAAGACC>TACCQAAAGACCA (p.I605\_Q609>YRKTKQ)



Case 7: Gallbladder cancer

BRCA2 exon11

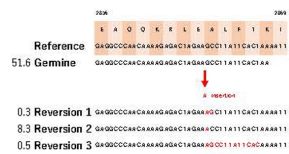
c.2641G>T (p.E881\*)  
c.2641\_2643GAA>TAT (p.E881Y)  
c.2635\_2655del21 (p.S879\_D885del)  
c.2633\_3364del732 (p.D878\_S1121del)  
c.1959\_4451del2493 (p.E653\_T1483del)



Case 8: Prostate cancer

BRCA2 exon20

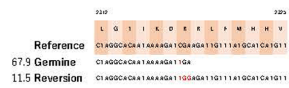
c.8589\_8590insA (p.A2864fs\*5)  
c.8590\_8591GC>AG (p.A2864S)  
c.8590G>A (p.A2864T)  
c.8590\_8601GCCTTATTCACT>AGCCCTATTCAAC (p.A2864\_T2867>SLIH)



Case 9: Ovarian cancer

BRCA2 exon13

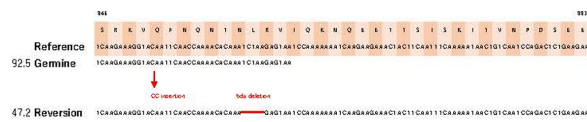
c.6952C>T (p.R2318\*)  
c.6952\_6954CGA>TGG (p.R2318W)



Case 10: Fallopian tubal cancer

BRCA2 exon11

c.2548\_2549insCC (p.Q850fs\*9)  
c.2568\_2572del (p.N856fs\*23)



Case 11: Breast cancer

BRCA2 exon10

c.1813del (p.I605fs\*9)  
c.1793\_1806CATCTATAAAGGA>AAAAACCGAAAGG (p.T598\_G602>KNTER)



Case 12: Pancreatic cancer

BRCA2 exon11

c.5709del (p.L1904fs\*5)  
c.5722\_5723del (p.L1908fs\*2)



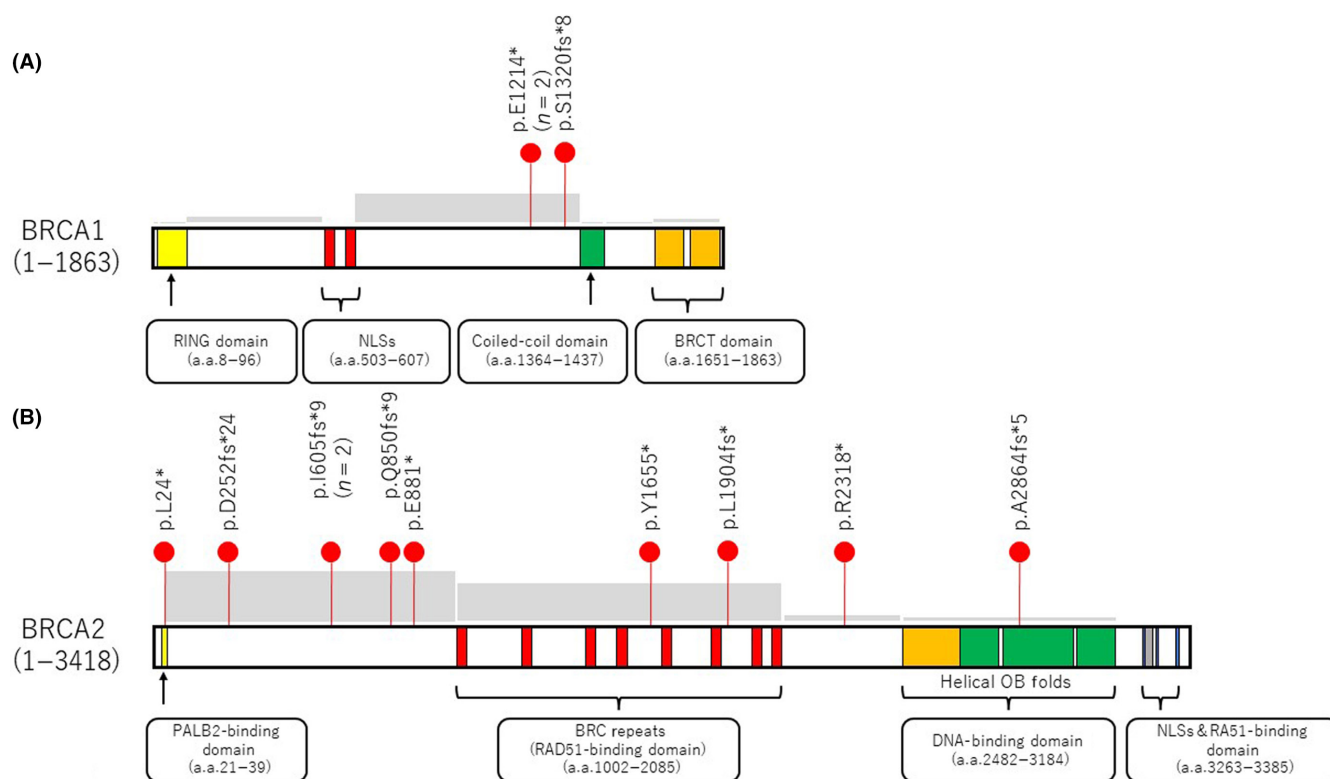


mutations, five different cancer types were observed (breast,  $n=4$ ; ovarian/fallopian tube/peritoneal,  $n=4$ ; pancreatic,  $n=2$ ; prostate,  $n=1$ ; and gallbladder,  $n=1$ ); case 4 even had two *BRCA2* primary mutations. Notably, one patient (case 6) did not undergo Pt-based or PARPi therapy treatments, while disease progression was observed in 11 patients after treatment with Pt-based or PARPi therapy. Among these 21 reversion mutations, three mutations were detected in *BRCA1* carriers, and 18 mutations were detected in *BRCA2* carriers. These corrected the original mutation in the tumor in some cases or re-established the mutation-induced disruption of the ORF of *BRCA*. Therefore, these secondary mutations can restore the expression of *BRCA* protein and are termed reversion mutations. Among the different tumor types, 5.8% ( $n=12$ ) of patients with a *BRCA* pathogenic mutation carried reversion mutations in all cohorts. Compared to patients with detectable reversions in *BRCA2*, fewer patients had detectable reversions in *BRCA1*—4.3% (*BRCA1*) versus 7.6% (*BRCA2*)—although the difference was not statistically significant.

Almost all primary *BRCA1/2* mutations in these patients were of germline origin (3/3 cases for *BRCA1* and 9/10 for *BRCA2*) and were predominantly detected in hotspot mutated regions within exon 11 of the gene (Figure 3). Additionally, for *BRCA2*, they were commonly located in regions that encode the DNA-binding domain, BRC repeats, or the N-terminal part of the protein, all of which occur between the

*PALB2*-interacting domain and DNA-binding domains (Figure 3).<sup>22</sup> Additionally, to facilitate comparison with the findings of previous studies on *BRCA* reversion mutations, specifically those reported by Pettitt et al.,<sup>12</sup> we have annotated the positions of the reversion mutations outlined in Pettitt et al.'s study within the domains of Figure 3. An analysis of these annotations revealed that the 13 reversion mutations identified in our study generally follow the same trends as those previously reported. Intriguingly, although reversion mutations within the *PALB2*-binding domain in *BRCA2* have not been previously documented, we observed such a mutation in case 5 with breast cancer, specifically a reversion at *BRCA2* p.L24\* within the *PALB2*-binding domain. These reversions were all found in tumors wherein the primary mutation produced a frameshift consisting of a secondary deletion, multinucleotide variant, or delin event, which thereby restored the ORF. In cases of reversion in which the primary mutation was a nonsense SNV, these occurred not only through secondary mutation, which involved another SNV, but also through deletions or delins.

Notably, three cases had multiple *BRCA* reversion mutations. Each reversion mutation showed frequency changes in different alleles, suggesting the potential expansion of certain clonal fractions and indicating the ability of *BRCA* reversion mutations to capture multiclonal heterogeneity. Reversion mutations were not dependent on treatment type and tended to accumulate in specific regions of either the *BRCA1* or *BRCA2* protein.



**FIGURE 3** Distribution of reversion mutations on the various domain structures of *BRCA1/2*. (A) Reversion mutations in the RING, nuclear localization signal (NLS), coiled-coil, and *BRCA1* C-terminal (BRCT) domains of *BRCA1*. (B) Reversion mutations in the partner and localizer of *BRCA2* (*PALB2*)-binding, *RAD51*-binding, DNA-binding, and NLS and *RA51*-binding domains of *BRCA2*. Red dots represent patients.  $n$  denotes the number of patients showing each reversion mutation. Gray boxes indicate the frequency of *BRCA* reversion mutations, as reported by Pettitt et al.<sup>12</sup>



Detailed clinical data were available for all 12 patients with reversion mutations. Seven of the 12 patients received first-line Pt therapy with an overall median time of 6.0 months (range, 4–30 months). The remaining four patients who did not receive first-line Pt therapy but received PARPi therapy instead and had an overall median time of 11.5 months (range, 8–13 months). Reversion mutation was also identified in one patient with pancreatic cancer who had not received prior treatment with a PARPi or Pt agent. The patient with gallbladder cancer (case 7) was on first-line Pt therapy for 10 months (Figure 4).

### 3.3 | Reversion mutations with large deletions and the MMEJ repair mechanism

Eight secondary “deletions” were identified in *BRCA2*. No secondary “deletions” were identified in *BRCA1*. Most deletion events were accounted for by the error-prone DNA repair mechanisms of end-joining, commonly by classical NHEJ, or alternative end-joining.

Of the multiple resistance mechanisms reported preclinically, MMEJ has been associated with *BRCA* reversion mutations.<sup>10</sup> Microhomology-mediated end-joining, involving POLθ, is an alternative DNA damage repair pathway for end-joining of small sequence microhomologies located closely around the breakpoint. Upon microhomology analysis on surrounding secondary deletions, there were eight “deletions” in four patients that had microhomologies of greater than 1 bp (cases 4, 7, 10, and 12), suggesting MMEJ repair.<sup>23</sup>

These deletions varied from small deletions of 2 bp up to large deletions of 2493 bp. Anywhere from 2 to 5 bp of microhomology were identified around the breakpoints, without any correlations between the deletion and microhomology lengths (Figure 5).

## 4 | DISCUSSION

In this genomic analysis of 3738 patients with various cancer histologies, we identified *BRCA1/2* reversion mutations across *BRCA*-associated tumor types. Furthermore, clinical data helped identify rare cases in which Pt-based therapy resulted in selective pressure, and consequent reversion mutations, in tumors showing noncanonical histologies—an initial phenotype associated with the loss of *BRCA* function. The *BRCA*-mediated HRD phenotype has been shown to be lineage-specific and is frequently identified in *BRCA*-associated tumor types.<sup>6</sup> Although the incidence of the *BRCA*-mediated HRD phenotype in noncanonical cancer has not been determined, a previous study on *BRCA* reversion mutations in a large pan-cancer cohort reported two patients with *BRCA* reversion mutations with tumors showing noncanonical histologies involving esophagogastric and lung cancer.<sup>24</sup>

Here, we analyzed a large database for *BRCA1/2* alterations and identified a *BRCA* reversion mutation in a patient with gallbladder cancer. Although gallbladder cancer is not considered a canonical *BRCA*-driven tumor, patients with gallbladder cancer have been shown to carry *BRCA* mutations.<sup>25</sup> A large registry-based study in

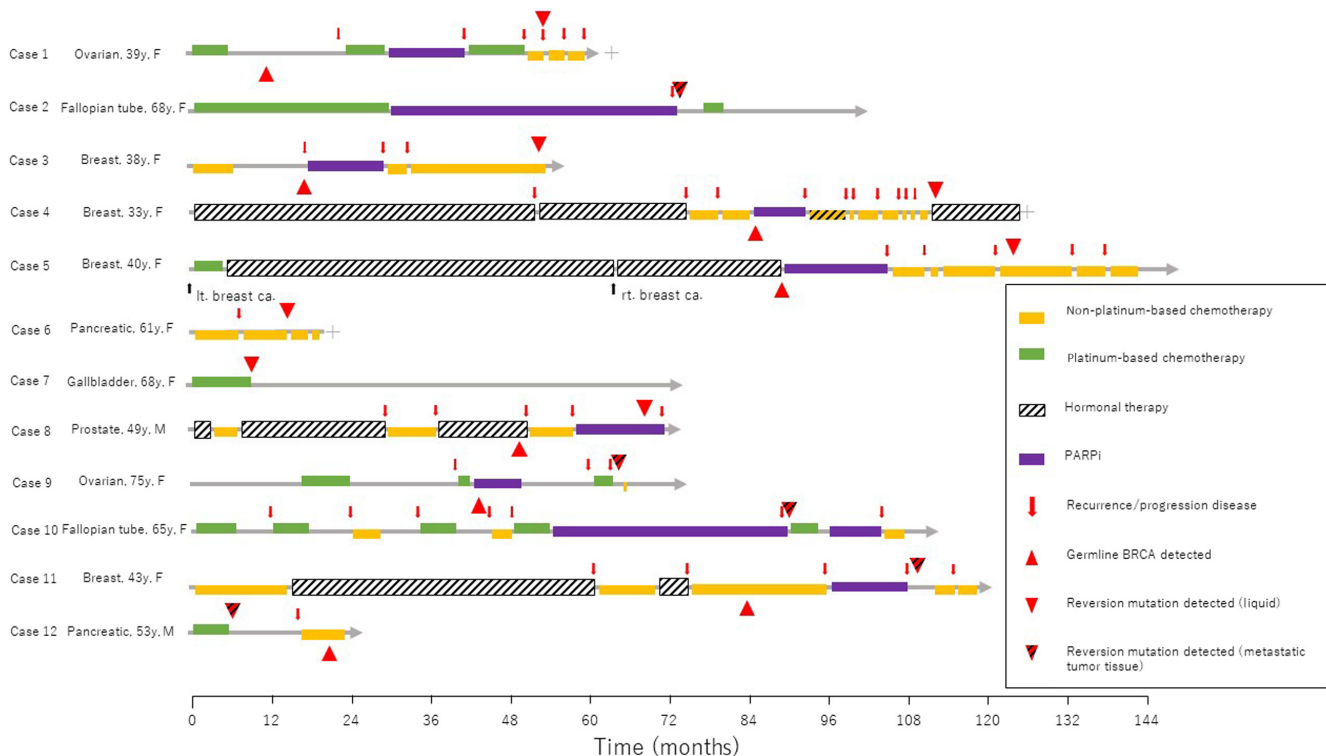
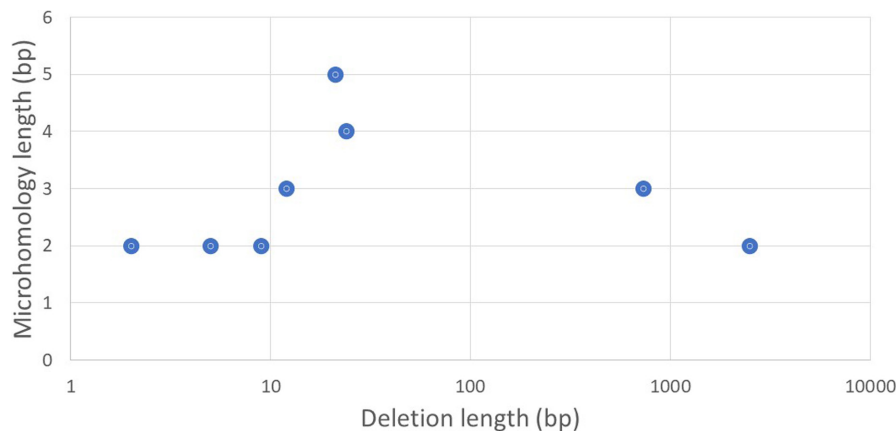


FIGURE 4 Treatment timeline of 12 patients whose solid tumors developed reversion mutations. ca., cancer; F, female; Lt., left; M, male; PARPi, poly(adenosine diphosphate ribose) polymerase (PARP) inhibitor; rt., right; y, years.



**FIGURE 5** Correlation between deletion and microhomology lengths in *BRCA1* and *BRCA2* reversion mutations.

Japan suggested that pathogenic variants of *BRCA1* and *BRCA2* were associated with the increased risk of cancers of the biliary tract, stomach, and esophagus, in addition to *BRCA*-associated cancers (breast, ovarian/fallopian tube/peritoneal, pancreatic, and prostate cancers).<sup>26</sup> The identification of mechanisms linking pathogenic variants and these cancer types for the potential efficacy of PARPi requires further investigation, given that HRR defects have been identified in cases of biliary and esophageal cancer.<sup>27</sup> Although it is unclear whether *BRCA* is, in fact, a pathogenic driver in these cancers, few studies have investigated *BRCA* reversion mutations in these cancers.<sup>24</sup> The patient with gallbladder cancer responded positively to Pt-based therapy before the detection of a reversion mutation, suggesting that the initial phenotype was *BRCA*-mediated HRD, with the reversion mutation restoring the DNA repair function of the tumor, thereby inducing resistance. This patient showed rapid clinical deterioration following testing with PARPi after developing the reversion mutation. This might be attributable to the effects of reversion in restoring WT *BRCA* function.

The functionally important roles of different protein domains in inducing resistance to treatment are well illustrated by mapping the consequences of reversion mutations in *BRCA* proteins. Reversion mutations in exon 11 of *BRCA2* produce very long deletions involving a considerable amino acid length. This region encodes BRC repeats and the binding domains for RAD51 recombinase that are essential in HRR for *BRCA2* function.<sup>28</sup> Two extreme cases resulted in the deletions of 732 bp (244 a.a.; deletion in eight BRC repeats) and 2493 bp (831 a.a.; deletion in eight BRC repeats) in patients with gallbladder cancer progression after Pt therapy. However, no reversion reported to date has deleted all *BRCA* repeats, which suggests that a minimum of two is required to ensure the preservation of *BRCA2* function and conferral of resistance to Pt or PARPi.<sup>29</sup> Consistent with this indication, mice with a homozygous deletion of *BRCA2* exon 11 have been found to be nonviable.<sup>30</sup> Accumulation of reversions involving the N-terminal or DNA-binding regions of *BRCA2* is limited to smaller deletions or mutations, suggesting greater constraints around the amino acid changes in these regions. This is despite the finding that some in vitro reversion events involve complete loss of the DNA-binding region of *BRCA2*.<sup>7</sup>

Notably, in our cohort, more deletions were observed among the *BRCA2* secondary mutations, especially for mutations greater than 1 bp. Although the limited number of cases prevented any focus on the mutational mechanisms driving reversion mutations through SNVs or delins, understanding the contribution of other DNA repair pathways, such as translesion synthesis, nucleotide excision repair, base excision repair, and mismatch repair, to such outcomes would be interesting.

Deletion is the primary mechanism underlying reversion mutations in *BRCA*. The prevalence of deletions suggests the existence of a DNA double-strand break intermediate, which is repaired by end-joining mechanisms. Regarding NHEJ, its mutational signature commonly involves small delins that are generated by limited DNA-end resection. This resection is itself imposed by the presence of Ku heterodimers bound to the ends of the DNA break. Therapeutic implications of this effect may follow, given the recent introduction of compounds that inhibit DNA-PK, a key protein kinase involved in NHEJ, in clinical settings.<sup>29</sup> In contrast, MMEJ repair signatures involve more extensive DNA-end resection and microhomologies (2–5 bp) flanking the break site. These can occur at distances of several hundred base pairs, resulting in large deletions and chromosomal translocations.<sup>31</sup> Notably, we identified significant microhomology in deletions affecting *BRCA* genes regardless of therapeutic regimen, which provides evidence of MMEJ repair mechanism, particularly in *BRCA2*. A critical component in MMEJ repair is POL $\theta$ , which is encoded by the *POLQ* gene.<sup>32</sup> POL $\theta$  is essential to cell survival in *BRCA*-deficient cell lines and competes for similar DNA repair substrates with HRR proteins. Preclinical efforts to generate POLQ inhibitors are now underway, and we look forward to learning whether the blockage of NHEJ and/or MMEJ repair in *BRCA*-mutant backgrounds potentially prevents the accumulation of reversion mutations and accordingly resistance to drug treatments.

Interestingly, case 4 had two *BRCA2* primary mutations (one germline and one somatic), and both mutations were genetically reverted in the tumor sample. To the best of our knowledge, this is the first case showing individual reversion mutations against two *BRCA* primary mutations. Notably, one patient (case 6) did not undergo either Pt-based or PARPi treatments. It has been previously reported

that a patient with Pt-resistant primary ovarian cancer and a *BRCA1* reversion mutation had a history of breast cancer treated with an interstrand DNA cross-linking agent, cyclophosphamide, which could exert selection pressure for the correction of DNA repair defects.<sup>33</sup> However, in our cohort, this patient had no history of another cancer treated with such a drug.

Our study has a few limitations. First, although *BRCA* reversion mutations resulting from selective pressure from DNA-damaging agents suggest a *BRCA*-mediated phenotype, a possibility remains that reversion mutations in some tumors are random events. In this regard, one of our patients with pancreatic cancer and a reversion mutation in *BRCA1* had no history of either PARPi or Pt-based therapy, which are generally associated with reversion mutations due to their targeting of the DNA damage repair processes. This patient had been treated with gemcitabine (a DNA synthesis-inhibiting agent) and paclitaxel (an antimicrotubular agent) and with secondary chemotherapy with irinotecan (a DNA topoisomerase I inhibitor) and 5-fluorouracil (pyrimidine analog antineoplastic chemotherapy agent). However, whether the reversion mutation in this patient with pancreatic cancer emerged spontaneously or due to resistance to these chemotherapies remains unknown. This patient had a high mutational burden due to *MLH1* methylation, and it is possible that the *BRCA2* mutation was a passenger mutation. Second, given the small sample size, the data should be interpreted carefully. Third, we could not verify our results with any public databases because, to the best of our knowledge, there are no public databases exclusively on *BRCA* reversion mutations. Even if multiple mutations were reported in *BRCA*, we cannot determine whether these are reversion mutations as we do not have information on the treatment course or timing of sample collection for sequencing.

Another limitation of our analysis was that we did not investigate the "back mutations to wild type" *BRCA* reversion mutation. Many previous studies have reported *BRCA1/2* reversion mutations as back mutations to WT.<sup>33,34</sup> Therefore, to distinguish the occurrence of back mutations from retention of WT alleles or contamination of cells with WT alleles, examination of intragenic SNPs is essential. Furthermore, regarding liquid biopsy, if the cfDNA amount was small, in such cases during chemotherapy, the somatic mutation was not detected, which produced false negative results. Therefore, our analysis likely underestimates the prevalence of reversion mutations.

In conclusion, although most *BRCA*-driven tumors occur in breast, ovarian, pancreatic, or prostate cancers, we analyzed *BRCA* reversion mutations in a large pan-cancer cohort and identified rare cases of gallbladder cancer mediated through the loss of *BRCA* function. Reversion mutations due to Pt therapy could reflect a rare *BRCA*-mediated phenotype in tumors showing noncanonical histologies. Therefore, monitoring the HRR mutation status along the course of disease progression and treatment could be beneficial in identifying resistance mechanisms and guiding the choice of subsequent therapies.

#### AUTHOR CONTRIBUTIONS

**Kohei Nakamura:** Conceptualization; writing – original draft.  
**Hideyuki Hayashi:** Data curation. **Ryutaro Kawano:** Data

curation. **Marin Ishikawa:** Data curation. **Eriko Aimono:** Data curation. **Takaaki Mizuno:** Data curation. **Hajime Kuroda:** Formal analysis; investigation; resources. **Yasuyuki Kojima:** Formal analysis; investigation; resources. **Naoki NIIKURA:** Formal analysis; investigation; resources. **Aya Kawanishi:** Formal analysis; investigation; resources. **Kei Takeshita:** Formal analysis; investigation; resources. **Shinsuke Suzuki:** Formal analysis; investigation; resources. **Shinichi Ueno:** Formal analysis; investigation; resources. **Kosuke Okuwaki:** Formal analysis; investigation; resources. **Jiichiro Sasaki:** Formal analysis; investigation; resources. **Masatoshi Yamaguchi:** Formal analysis; investigation; resources. **Kenta Masuda:** Formal analysis; investigation; resources. **Tatsuyuki Chiyoda:** Formal analysis; investigation; resources. **Wataru Yamagami:** Formal analysis; investigation; resources. **Chihiro Okada:** Data curation. **Sachio Nohara:** Data curation. **Shigeki Tanishima:** Data curation. **Hiroshi Nishihara:** Conceptualization; writing – review and editing.

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#### CONFLICT OF INTEREST STATEMENT

Hiroshi Nishihara is a member of the *Cancer Science* Editorial Board. Sachio Nohara, Chihiro Okada, Shigeki Tanishima, and Hiroshi Nishihara are employees of Mitsubishi Electric Software Corporation. Sawa Nohara (wife of Sachio Nohara) is an employee of Kaina Home Care Station. Naomi Tanishima (wife of Shigeki Tanishima) is an employee of INOUE Co., Ltd. Hideaki Tanishima (child of Shigeki Tanishima) is an employee of MARUI & Co., Ltd. Mai Tanishima (child of Shigeki Tanishima) is an employee of Konan Medical Center. Takaaki Mizuno is employee of Rakuten Medical K.K. Takaaki Mizuno from CLINIAL Co., Ltd (corporate stocks). Tatsuyuki Chiyoda received a research grant from Takeda Pharmaceutical Company. The other authors declare no conflict of interest.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are not available as the study participants did not consent to public sharing of their data.

#### ETHICS STATEMENT

Approval of the research protocol by an institutional review board: This study was part of a research project approved by the Ethics Committee of Keio University Hospital (approval number: 20211159). The study procedures involving human participants complied with the principles of the Declaration of Helsinki.

Informed consent: All patients in this study provided opt-out consent.

Registry and the registration no. of the study/trial: N/A.

Animal studies: N/A.

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