

ORIGINAL ARTICLE

Using next-generation sequencing to redefine *BRCAness* in triple-negative breast cancer

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Abstract

BRCAness is considered a predictive biomarker to platinum and poly(ADP-ribose) polymerase (PARP) inhibitors. However, recent trials showed that its predictive value was limited in triple-negative breast cancer (TNBC) treated with platinum. Moreover, tumors with mutations of DNA damage response (DDR) genes, such as homologous recombination (HR) genes, could be sensitive to platinum and PARP inhibitors. Thus, we aim to explore the relationship between mutation status of DDR genes and *BRCAness* in TNBC. We sequenced 56 DDR genes in 120 TNBC and identified *BRCAness* by array comparative genomic hybridization. The sequencing results showed that 13, 14, and 14 patients had *BRCA*, non-*BRCA* HR, and non-HR DDR gene mutations, respectively. Array comparative genomic hybridization revealed that *BRCA*-mutated and HR gene-mutated TNBC shared similar *BRCAness* features, both having higher numbers and longer length of large-scale structural aberration (LSA, >10 Mb) and similar altered chromosomal regions of LSA. These suggested non-*BRCA* HR gene-mutated TNBC shared similar characteristics with *BRCA*-mutated TNBC, indicating non-*BRCA* HR gene-mutated TNBC sensitive to platinum and PARP inhibitors. Among tumors with mutation of non-HR DDR genes, 3 *PTEN* and 1 *MSH6* mutation also contained significant LSAs (*BRCAness*); however, they had different regions of genomic alteration to *BRCA* and HR gene-mutated tumors, might explain prior findings that *PTEN*- and *MSH6*-mutated cancer cells not sensitive to PARP inhibitors. Therefore, we hypothesize that the heterogeneous genomic background of *BRCAness* indicates different responsiveness to platinum and PARP inhibitors. Direct sequencing DDR genes in TNBC should be applied to predict their sensitivity toward platinum and PARP inhibitors.

KEYWORDS

BRCAness, DNA damage response, PARP inhibitor, platinum, triple-negative breast cancer

Abbreviations: aCGH, array comparative genomic hybridization; ACMG, American College of Medical Genomics and Genetics; CI, confidence interval; CNV, copy number variation; DDR, DNA damage response; DSB, double-strand break; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; HR, homologous recombination; HRD, homologous recombination deficiency; LOH, loss of heterozygosity; LSA, large-scale genomic structural aberration; NGS, next-generation sequencing; PARP, poly(ADP-ribose) polymerase; PR, progesterone receptor; RFS, relapse-free survival; TNBC, triple-negative breast cancer.

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1 | INTRODUCTION

Triple-negative breast cancer, which lacks expression of ER, PR, and HER2, is an aggressive subtype associated with poor prognosis.¹ An important genetic feature of TNBC is the deleterious mutation of *BRCA1* and *BRCA2*. Mutations of *BRCA* genes cause HRD, which leads to an increased genomic instability.² Cancer cells with *BRCA1/2* mutations have displayed sensitivity to platinum-based chemotherapy and PARP inhibitors.³ Trials of PARP inhibitors indicated that the survival rate was significantly improved in patients with *BRCA*-mutated ovarian and breast cancer.⁴⁻⁶ However, only approximately 10% of TNBC patients carry *BRCA1/2* mutations. Therefore, many studies have attempted to explore whether a subset of non-*BRCA*-mutated tumors responded to platinum and PARP inhibitors.

Prior studies have reported that a subset of non-*BRCA*-mutated cancers show high-grade genomic instability, resembling tumors originated from germline *BRCA*-mutated carriers. These tumors show HRD as well, and these traits are collectively referred to as *BRCAness*.^{7,8} Array comparative genomic hybridization was used to classify *BRCAness* and non-*BRCAness* by assessing chromosome aberrations and measuring the severity of LOH, telomeric allelic imbalance, and large-scale state transition as an HRD score.^{2,9} The *BRCAness* feature was considered as a marker to predict responsiveness of platinum- and PARP inhibitors.^{2,10} However, the TNT phase III study showed that *BRCAness* did not correlate with the therapeutic response of carboplatin in TNBC.¹¹ Another recent clinical trial showed that *BRCAness* could only modestly predict the treatment outcome of PARP inhibitor-based therapy.¹² Therefore, it is uncertain whether *BRCAness* is an adequate marker for selecting breast cancer patients for treatment with platinum and PARP inhibitors.

Recently, preclinical experiments have indicated that platinum and PARP inhibitors can kill cancer cells with mutations in several DDR genes, such as *ATM*, *RAD51C*, and other genes involved in the HR repair pathway for DSB.¹³⁻¹⁵ In a phase II trial of prostate cancer, patients carrying deleterious mutation of HR genes had a higher response rate and a better survival rate when treated with PARP inhibitors.¹⁶ Furthermore, mutations of non-HR DDR genes, such as *MLH1*, *PTEN*, and *TP53*, causes an increase in genomic instability that resembled *BRCAness* features in pancreatic, ovarian, prostate, and other cancer types.¹⁷⁻²⁰ Yet, survival was not improved in *PTEN*-mutated ovarian cancer treated with platinum-based chemotherapy.²¹ These results suggested that mutations in HR genes could be used as biomarkers to predict sensitivity to PARP inhibitors. Whereas other DDR mutations might show *BRCAness*, their tumors do not necessarily respond to platinum and PARP inhibitors. In TNBC, the mutation incidence of HR genes and DDR genes is not known. Also, the relationship between *BRCAness* features and DDR gene mutations is uncertain. To explore these uncertainties, we sequenced DDR genes (including HR, non-homologous end-joining, base-excision repair,

nucleotide-excision repair, mismatch repair, and polymerases involved in DNA repair pathways) in TNBC, then we assessed their *BRCAness* by undertaking aCGH. Finally, we investigated the relationship between *BRCAness* and the mutation status of DDR genes.

2 | MATERIALS AND METHODS

2.1 | Patients

This study enrolled patients who were diagnosed as stage I-III TNBC and had received surgical intervention in our hospital between 2003 and 2010. The clinical and pathologic characteristics were retrospectively recorded. Estrogen receptor, PR, and HER2 were determined by immunohistochemical staining. Estrogen receptor or PR was considered negative when less than 5% of tumor cells showed positive staining. For HER2 staining, a score of 0 or 1+ was considered negative; specimens with a score of 2+ were confirmed by FISH analysis. In addition, the tumor histological grade was defined using the Nottingham combined histological grading system. Finally, the study was approved by the Institutional Review Board of National Taiwan University Hospital (Taipei, Taiwan).

2.2 | DNA extraction and library preparation

After a patient had signed an agreement, their tumor sample was stored in either a liquid nitrogen tank or a -80°C refrigerator. As reported previously, DNA was extracted from resected tumors and used for library construction.²² Briefly, the purity and concentration of tumor DNA was checked by agarose gel electrophoresis, OD ratio, and a Qubit 2.0 Fluorometer (Life Technologies), and this was followed by a Covaris fragmentation. Size distribution of the fragmented DNA was confirmed using a Bioanalyzer 2100 (Agilent Technologies). Then DNA libraries were generated with the Truseq DNA Library Prep kits (Illumina), according to the manufacturer's manual. Finally, target gene libraries, containing a customized gene panel of 56 genes selected from DDR genes (Table S1), including HR, nonhomologous end-joining, base-excision repair, nucleotide-excision repair, mismatch repair, and polymerases involved in DNA repair pathways, were generated using a SeqCap EZ Target Enrichment System (Roche NimbleGen).^{22,23} Finally, the libraries were sequenced on an Illumina NextSeq 500 that generated paired-end reads of approximately 300 nt in lengths.

2.3 | Sequence data analysis

Previously, we have described the post-NGS bioinformatics.^{24,25} The raw sequencing data were aligned to the reference human

genome (Feb. 2009, GRCh37/hg19) by using BWA software (version 0.5.9) and SAMtools (version 0.1.18). Picard (version 1.54) was used to undertake the necessary data conversion, sorting, and indexing. Genome Analysis Toolkit (version 3.5) was used for variant calling through the parameters of UnifiedGenotyper, HaplotypeCaller, and VariantFiltration. Pindel (version 0.2.4) software was utilized to find structural variants, such as deletions, insertions, and duplications. Finally, ANNOVAR was used to annotate the genetic variants. Variants interpretation were based on the guideline of the ACMG.²⁶ Briefly, the frameshift insertion or deletion (indel), nonsense, uncorrected splice-site variants, large-scale deletions, and missense mutations with impaired protein function by functional assays were considered as pathogenic or like pathogenic variants. We used “deleterious mutation” to represent the “pathogenic” and “likely pathogenic” variants by the guidelines of the ACMG.²⁶ Only the deleterious mutations were used in the further analysis; variants of uncertain significance were not.

Large-scale deletions were detected by CNV analysis.²⁷ For the 56 genes, 15 samples without CNV were selected to construct the CNV reference.²² Briefly, the mean depth of each sample was normalized to the amount of DNA library loaded on the sequencing flow cells, target enrichment rate, and the total output of each sequencing run on Nextseq 500. If the reading depth lied outside the 95% confidence interval of the same region in the reference (Appendices S1 and S2, Figure S1), an exon region was considered CNV.

2.4 | Checking mutation origin

Germline DNA was extracted from PBMCs of patients. For the deleterious mutations (small insertion, deletion, or

nonsynonymous variants) harbored within tumors, we used Sanger sequencing to check whether the mutations existed in the germline DNA in the same patients. If the blood cells carried the same mutations, the mutation was categorized to be originated from the germline.

2.5 | Assessment of genomic instability

Genomic patterns of breast carcinomas were determined by aCGH; the array contained approximately 60 000 probes covering the whole genome with an average spacing of 40 kb. Briefly, fragmented DNA was labeled using the Agilent SureTaq Genomic DNA Labeling Kit (Agilent Technologies) with Cy5-dUTP (for the experimental sample) and Cy3-dUTP (for the reference) (PerkinElmer), according to the manufacturer's instructions. After cleaning and denaturing, the labeled samples were hybridized to a SurePrint G3 Custom CGH Microarray, 8 × 60K (G4126A; Agilent Technologies). After drying, the hybridized arrays were scanned on an Agilent DNA microarray scanner at 535 nm for Cy3 and 625 nm for Cy5 at a resolution of 2 μm. To quantify signal intensities and normalize the data for each feature, the scanned images were analyzed on the Agilent Genomic Workbench (Agilent Technologies) using the Feature extraction 10.5.1.1 software (Agilent Technologies).

The allelic frequency of gain or loss for each chromosomal region was calculated by the ratio thresholds of 0.25 and -0.25, respectively. We defined the regions of gains and losses that were >10 Mb as the hallmark of LSA.² The presence of LSA was considered as evidence of genomic instability. The genomic aberrations of regions <10 Mb were not included for further analysis and are not shown in Figure 1 or Figure S2. The number, length, and specific chromosomal regions of LSA were analyzed.

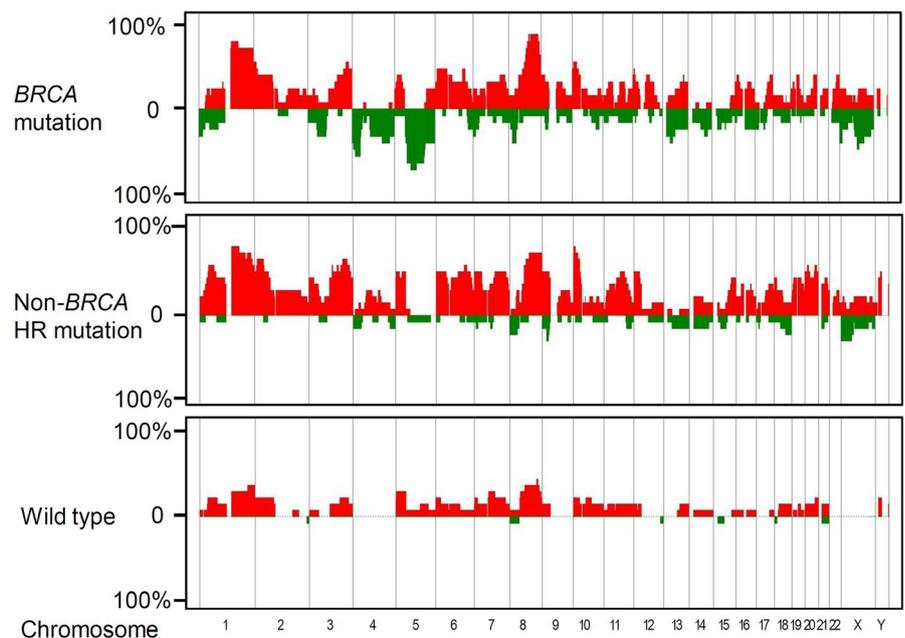


FIGURE 1 Three panels that show the frequencies of gain (red) or loss (green) of large-scale structural aberration in BRCA-mutated, non-BRCA homologous recombination (HR) gene-mutated, and control breast cancers

TABLE 1 Clinical and pathologic characteristics of patients with triple-negative breast cancer stratified by gene mutation

Characteristic	Without HR mutation (n = 79)	BRCA mutation (n = 13)	Non-BRCA HR mutation (n = 14)	Other mutation (n = 14)	P value
Age (mean ± SD)	53.2 ± 11.9	51.7 ± 8.9	52.0 ± 9.9	56.9 ± 10.3	.589
T					
T1	38	6	3	5	.433
T2	36	7	10	7	
T3-4	5	0	1	2	
N					
N0	41	10	6	12	.324
N1	20	2	4	1	
N2	6	0	2	1	
N3	12	1	2	0	
Grade					
1	3	2	0	2	.746
2	21	3	5	3	
3	49	7	8	8	
Undetermined	6	1	1	1	
TP53 mutation					
Wild type	29	0	2	7	.011
Mutation	50	13	12	7	
Family history					
All cancer	33	9	3	5	.085
Breast/ovarian	15	7	0	2	.004
Adjuvant chemotherapy					
No	11	0	1	3	.335
Yes	68	13	13	11	
Anthracycline	22	8	7	5	
Taxane	2	0	1	0	
Combination	43	5	5	3	
Others	1	0	0	3	

Abbreviations: Adjuvant chemotherapy: anthracycline alone; taxane, taxane-containing chemotherapy (taxane + anthracycline allowed). HR, homologous recombination.

2.6 | Statistical analysis

The χ^2 test and Fishers exact test were used to calculate the significance of variances between each group. Survival was estimated by Kaplan-Meier analysis. All *P* values are 2-sided, and *P* values <.05 are considered statistically significant.

3 | RESULTS

3.1 | Patients and mutation analysis

A total of 120 TNBC patients were analyzed. Based on the mutation status, the 120 TNBC patients were classified into 4 groups: control, *BRCA* mutations, non-*BRCA* HR gene mutations, and other DDR gene mutations. The demographic information is shown in Table 1.

Their clinical characteristics were similar in age, stage and grade distribution, and the use of adjuvant therapy. Patients with *BRCA* mutation had a higher prevalence of family history of cancer, but patients with non-*BRCA* HR gene mutations did not, compared with that of controls. Deleterious mutation of *TP53* was identified in 92 patients. The proportion of patients carrying *TP53* mutation was significantly higher among patients with mutations in either *BRCA* or other HR genes than those without HR mutation (*P* = .011).

There were 25 deleterious mutations in HR genes, including 8 in *BRCA1*, 3 in *BRCA2*, 2 in *ATM*, 2 in *BARD1*, 1 in *CHEK2*, 1 in *FANCA*, 2 in *FANCB*, 1 in *FANCI*, 1 in *PALB2*, 2 in *RAD50*, and 2 in *RAD51D* (Table 2). Most *BRCA* and HR gene-mutated tumors originated from the germline. Among patients carrying insertion/deletion in non-*BRCA* HR genes, 2 patients carried mutations in other cancer-predisposing genes as well. Copy number variation analysis showed that 3 patients had large-scale somatic deletion in *BRCA1*, 4 deletions in *BRCA2*, and 11

TABLE 2 Pathogenic mutations in this cohort of patients with triple-negative breast cancer

Patient	Genetic variants			Large deletion
	Type	Gene	Location	Gene
<i>BRCA</i>				
#1	Frameshift	<i>BRCA1</i>	NM_007300:exon10:c.3770_3771del;p.E1257fs	
#2	Stopgain	<i>BRCA1</i>	NM_007300:exon10:c.T3257G;p.L1086X	
#3	Frameshift	<i>BRCA1</i>	NM_007300:exon10:c.3228_3229del;p.R1076fs	<i>FANCB</i>
#4	Frameshift	<i>BRCA1</i>	NM_007300:exon2:c.66dupA;p.E23fs	<i>FANCB, FANCG</i>
#5	Splicing	<i>BRCA1</i>	NM_007300:exon22:c.5395+1G>A	<i>FANCG</i>
#6	Stopgain	<i>BRCA1</i>	NM_007300:exon10:c.C928T;p.Q310X	
#7	Frameshift	<i>BRCA2</i>	NM_000059:exon22:c.8894delA;p.D2965fs	<i>BRCA2, FANCI</i>
#8	Frameshift	<i>BRCA2</i>	NM_000059:exon10:c.1806dupA;p.G602fs	
	Stopgain	<i>BRCA2</i>	NM_000059:exon11:c.C3109T;p.Q1037X	
#9	Frameshift	<i>BRCA1</i>	NM_007300:exon12:c.4356delA;p.K1452fs	
	Frameshift	<i>BRCA1</i>	NM_007300:exon10:c.865_866del;p.S289fs	
#10	Splicing	<i>FANCA</i>	NM_000135:exon21:c.1777-1G>C	<i>BRCA2</i>
#11				<i>BRCA1, FANCB, FANCC</i>
#12				<i>BRCA1, BRCA2, FANCM, RAD51C</i>
#13				<i>BRCA1, BRCA2, FANCI, ATM, CHEK2</i>
<i>Non-BRCA HR</i>				
#1 ^a	Frameshift	<i>BARD1</i>	NM_001282543:exon3:c.943_944del;p.K315fs	
#2	Splicing	<i>FANCB</i>	NM_152633:exon7:c.1327-2->T	
#3 ^b	Frameshift	<i>FANCB</i>	NM_152633:exon2:c.483delT;p.V161fs	
#4	Frameshift	<i>RAD51D</i>	NM_001142571:exon4:c.331_332insTA;p.K111fs	
#5	Frameshift	<i>RAD51D</i>	NM_001142571:exon4:c.331_332insTA;p.K111fs	
#6	Frameshift	<i>PALB2</i>	NM_024675:exon6:c.2555_2567del;p.P852fs	<i>FANCG, FANCM</i>
#7	Frameshift	<i>ATM</i>	NM_000051:exon35:c.5313delA;p.R1771fs	
#8	Frameshift	<i>BARD1</i>	NM_000465:exon4:c.628dupA;p.T210fs	
	Frameshift	<i>CHEK2</i>	NM_001005735:exon14:c.1580delC;p.P527fs	
#9	Frameshift	<i>FANCI</i>	NM_001113378:exon22:c.2252_2291del;p.E751fs	
#10	Frameshift	<i>RAD50</i>	NM_005732:exon13:c.2157delA;p.L719fs	
	Frameshift	<i>ATM</i>	NM_000051:exon56:c.8249_8268del;p.L2750fs	
#11	Frameshift	<i>RAD50</i>	RAD50:NM_005732:exon6:c.783_784del;p.N261fs	
#12				<i>FANCB</i>
#13				<i>FANCA</i>
#14				<i>FANCM</i>
<i>Other DDR genes</i>				
#1	Frameshift	<i>ERCC4</i>	NM_005236:exon4:c.607_608del;p.F203fs	
#2	Frameshift	<i>ERCC4</i>	NM_005236:exon5:c.891_892del;p.Y297fs	
#3	Stopgain	<i>MSH3</i>	NM_002439:exon4:c.C724T;p.Q242X	
#4	Splicing	<i>MSH3</i>	NM_002439:exon13:c.1764-2A>G	
#5	Stopgain	<i>MSH6</i>	NM_000179:exon4:c.C3013T;p.R1005X	
#6	Frameshift	<i>MSH6</i>	NM_000179:exon5:c.3254delC;p.T1085fs	
#7	Frameshift	<i>POLH</i>	NM_006502:exon8:c.978delA;p.P326fs	
#8	Frameshift	<i>POLK</i>	NM_016218:exon13:c.1631delA;p.E544fs	
#9	Frameshift	<i>PTEN</i>	NM_000314:exon5:c.489_492del;p.K163fs	
#10	Frameshift	<i>PTEN</i>	NM_000314:exon8:c.955dupA;p.L318fs	

(Continues)

TABLE 2 (Continued)

Patient	Genetic variants			Large deletion
	Type	Gene	Location	Gene
#11	Stopgain	<i>PTEN</i>	NM_000314:exon5:c.G448T:p.E150X	
#12	Frameshift	<i>PTEN</i>	NM_000314:exon4:c.244dupA:p.F81fs	
#13	Frameshift	<i>STK11</i>	NM_000455:exon5:c.684delG:p.L228fs	
#14	Frameshift	<i>XPC</i>	NM_001145769:exon1:c.75dupC:p.K26fs	

Abbreviations: DDR, DNA damage response; HR, homologous recombination.

^aAlso had NM_000082(*ERCC8*):exon13:c.1123-2->T.

^bAlso had NM_000314(*PTEN*):exon6:c.602delA:p.E201fs.

deletions in non-*BRCA* HR genes. Overall, 13 patients had *BRCA* mutations and 14 patients had non-*BRCA* HR gene mutations. Beyond the *BRCA* and HR gene mutation, 14 patients had deleterious mutations in *ERCC4*, *ERCC8*, *MSH3*, *MSH6*, *POLH*, *POLK*, *PTEN*, *STK11*, and *XPC*.

We checked the origin of the deleterious mutations to determine whether they originated from the germline or if they were only acquired (somatic) in tumor cells (Figure S3). Peripheral blood DNA was available in 8 of the 9 *BRCA*-mutated patients, and Sanger sequencing of those DNA showed that 8 of the *BRCA1/2* mutations came from germline origin (all heterozygous). We then analyzed whether LOH occurred in tumor cells. The increased variant allele frequency was observed in patients #1 to #7 (Table S2), and LOH was highly suspected.²⁸ Patient #8 had 2 *BRCA2* mutations in tumor cells; 1 came from the germline, and the other was somatic. Double mutations might have suggested biallelic inactivation of *BRCA2* in tumors as well. Patient #9 also had double mutations in the *BRCA* gene. Therefore, we expected biallelic inactivation of *BRCA1* or *BRCA2* in these 9 tumors. For non-*BRCA* HR genes, peripheral blood DNA was available in 8 patients. The mutations in patients #2, #4, #5, and #6 originated from the germline, and LOH was also observed in their tumors (Table S2). This result is compatible with the previous finding that mutation of HR genes related to TNBC.^{29,30}

3.2 | Genomic patterns of non-*BRCA* HR gene-mutated tumors (vs *BRCA*-mutated)

We studied the genomic patterns by aCGH in the 13 *BRCA*-mutated, 14 non-*BRCA* HR gene-mutated, and 14 age- and clinical stage-matched control tumors without such mutations. Compared to the control tumors, large-scale (more than 10 Mb) gain or loss at specific chromosome locations were characterized in tumors with *BRCA* mutations and non-*BRCA* HR gene mutations; profiles are shown in Figure 1. We analyzed the numbers and lengths of LSA in each group, and we discovered that the LSA numbers and the lengths were significantly higher in the *BRCA*-mutated tumors than those in the control tumors (both $P < .001$, Figure 2A,B). Moreover, we observed that the LSA spectrum was similar between *BRCA*-mutated and non-*BRCA* HR gene-mutated tumors, either in LSA numbers or lengths (number, $P = .946$; length, $P = .918$; Figure 2A,B). These tumors contained

significantly more and longer LSAs compared with the control tumors (number, $P = .035$; length, $P = .022$; Figure 2A,B). Subsequently, we compared the LSA patterns in hereditary tumors with patterns in tumors with acquired somatic mutations. The LSA spectrums of the 4 cases with somatic *BRCA* mutations were like those of the 9 cases with germline *BRCA* mutations (number, $P = .460$; length, $P = .950$; Figure 2C,D). Also, the LSA spectrums showed no statistical differences between tumors carrying germline and tumors carrying somatic alterations of non-*BRCA* HR genes (number, $P = .678$; length, $P = .472$; Figure 2C,D). Both hereditary mutations and acquired HR gene mutations of tumors had significantly higher LSA numbers and lengths than those that do not have such mutation (all P value $< .05$) (Figure 2C,D). These results indicated that tumors with mutations in non-*BRCA* HR genes show a high grade of genomic instability, like those with *BRCA1/2* mutations.

The chromosomal regions of significant gains or losses between the 3 groups are highlighted in Table 3. Compared with control tumors, *BRCA*-mutated and non-*BRCA* HR gene-mutated tumors were associated with loss of Xp and gains of 1q, 2p21-25, 3q21-29, 8q, 10p, and 22q. Tumors with non-*BRCA* HR gene mutations contained more gains of 20p and 4q than the control tumors did, but *BRCA*-mutated tumors did not. While only *BRCA*-mutated tumors were significantly linked to gain of 12p, tumors with non-*BRCA* HR gene mutations did not show significant association with a gain of this region. These results suggest that, although some alterations were specific to TNBC tumors with either *BRCA* or non-*BRCA* HR gene mutations, numerous chromosomal alterations are shared.

3.3 | Cancer genes at LSA regions

We inspected the LSA regions listed in Table 3 for known cancer genes. A gain of chromosome 1q21 results in the amplification of S100 calcium-binding protein (*S100A*) family members *PI4KB*, *SHC1*, and *NCSTN*, which have been previously suggested as driver oncogenes of basal-type breast cancer.³¹ In addition, this amplification of 1q21 loci was linked to breast cancer recurrence (Figure 3A).^{31,32} Indeed, the *S-100* family was amplified at a higher proportion among the tumors with *BRCA* mutations ($N = 10$, 76.9%) and those with non-*BRCA* HR gene mutations ($N = 11$, 78.6%) ($P = .030$). The gain

FIGURE 2 Box and whisker diagrams comparing the numbers and lengths of large-scale structural aberration (LSA) among *BRCA*-mutated, non-*BRCA* homologous recombination (HR) gene-mutated, and control breast cancers. A, B, Tumors with *BRCA* mutations and non-*BRCA* HR gene mutations showed significantly higher numbers and lengths of LSA than those of the control tumors (*BRCA* vs WT, both $P < .001$; non-*BRCA* HR vs WT, number $P = .035$; length $P = .022$). C, D, Tumors with mutation were stratified by germline and acquired somatic mutation. Numbers and lengths of LSA were not different either between germline and somatic *BRCA* mutations, or between germline and somatic non-*BRCA* HR gene mutations

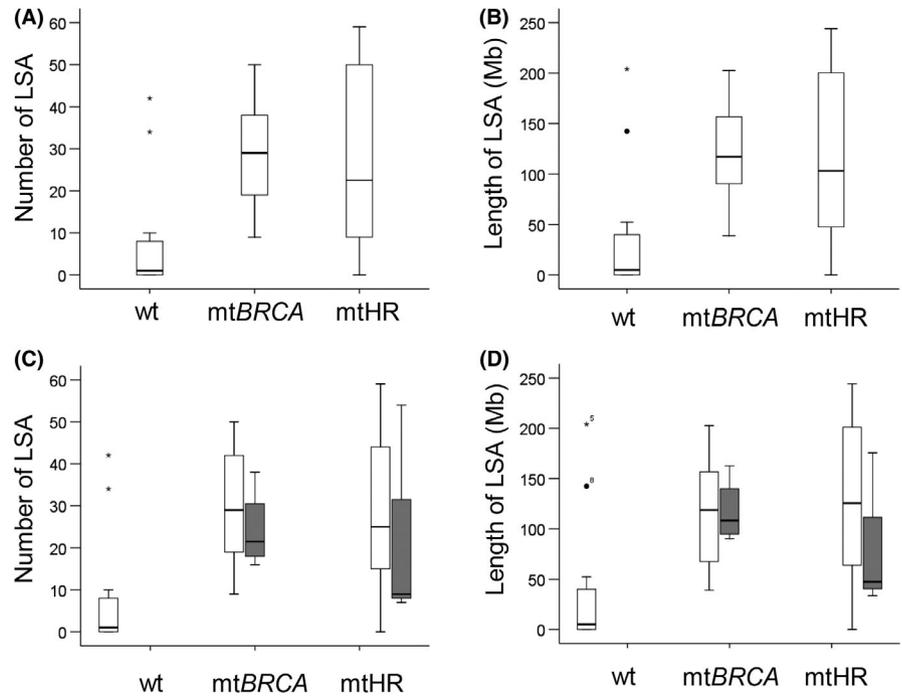


TABLE 3 Significant chromosomal regions between triple-negative breast tumors with *BRCA*-mutated, non-*BRCA* homologous recombination (HR) gene mutations, and WT

	<i>BRCA</i> (%)	Non- <i>BRCA</i> HR (%)	WT (%)	P value		
				<i>BRCA</i> vs HR	<i>BRCA</i> vs wt	HR vs wt
Loss						
Xp	38.46%	28.57%	0.00%	.586	.01	.031
Gain						
1q21-25	76.92%	78.57%	28.57%	.918	.012	.008
2p21-25	53.85%	64.29%	21.43%	.581	.081	.022
3q21-29	53.85%	71.43%	21.43%	.345	.081	.008
8q	84.62%	78.57%	35.71%	.686	.025	.053
10p	53.85%	71.43%	21.43%	.345	.081	.008
22q	38.46%	28.57%	0.00%	.586	.01	.042
20p	15.38%	57.14%	14.29%	.025	.936	.018
4q	0.00%	35.71%	0.00%	.017	1	.014
12q	38.46%	7.14%	0.00%	.05	.01	.309

of chromosomal 8q also caused an amplification of the *c-MYC* oncogene. This was discovered significantly in the tumors with *BRCA* mutations ($N = 11$, 84.6%) and tumors with non-*BRCA* HR gene mutations ($N = 11$, 78.6%) (Figure 3B), compared to the control tumors ($N = 5$, 35.7%; $P = .039$). *CRKL*, an oncogene located in 22q11.1 that mediates cellular proliferation and metastasis, was amplified in both *BRCA*-mutated ($N = 5$, 38.5%) and non-*BRCA* HR gene-mutated ($N = 4$, 28.6%) tumors ($P = .042$, Figure 3C). The gain of 3q and 10p and loss of Xp, which are significantly associated with *BRCA* and non-*BRCA* HR gene mutations, were related to carcinogenesis and reduced survival of patients.³³

3.4 | Genomic patterns and cancer genes of other DDR gene-mutated tumors

Genomic patterns were examined in 10 tumors with mutations of non-HR genes and tumor suppressor genes as well (Table 2 and Figure S2). Three of the 4 tumors carrying *PTEN* mutations showed high numbers of LSA, similar to those with *BRCA* (number $P = .106$; length, $P = .263$) or other HR gene mutations (number, $P = .294$; length, $P = .477$). *MSH6* mutation was observed in 1 tumor, and it contained large numbers of LSA. Nevertheless, tumors with mutations in *XPC*, *ERCC8*, or *MSH3* did not.

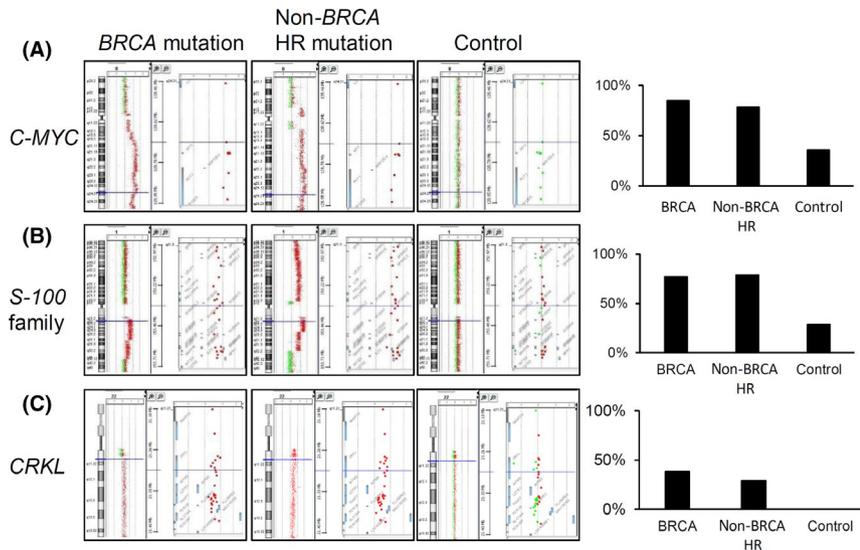


FIGURE 3 Representative array comparative genomic hybridization (aCGH) profiles of cancer-related genes in chromosome regions significantly different between *BRCA*-mutated, non-*BRCA* HR gene-mutated, and control breast cancers. (A) *MYC*, (B) *S100* family, and (C) *CRKL* were significantly amplified in *BRCA*-mutated and non-*BRCA* homologous recombination (HR) gene-mutated tumors, compared to those in control tumors. Green dot, loss; red dot, gain. Bar graphs represent the percentages of tumors containing amplifications of the target genes in each group

Among the frequently altered chromosomal regions of *BRCA* and HR-mutated tumors, *PTEN*-mutated tumors were not associated with loss of Xp or gains of 2p21-25, 3q21-29, and 22q (Figure S2). Two of the 4 *PTEN*-mutated tumors contained amplified chromosome 1q21 (*S100* family) and 8q (*c-MYC*); the other 2 did not. Among the 4 *PTEN*-mutated tumors, the *CRKL* gene was not amplified. In the *MSH6*-mutated tumor, frequent chromosomal loss was observed, such as 1p, 11p, and 12p. In contrast, they were not altered in *BRCA* and HR-mutated tumors. These results might suggest different biologic characteristics of *PTEN*-mutated and *MSH6*-mutated tumors, compared to *BRCA* and HR-mutated tumors. Nevertheless, all those types of tumors had high-grade genomic instability.

3.5 | Clinical relevance

The 5-year RFS was 78.7% (95% CI, 70.9%-86.5%) in the overall cohort. The RFS was 85.3% (95% CI, 73.3%-97.2%), 83.3% (95% CI, 72.1%-94.4%), and 57.1% (95% CI, 36.9%-77.2%) in patients with stage I, II, and III TNBC ($P = .006$), respectively. The 5-year RFS in the 27 patients with *BRCA* and non-*BRCA* HR gene mutations was 73.5 (95% CI, 56.6%-90.4%), which was nonsignificantly inferior to those without the mutations (RFS = 80.4%; 95% CI, 71.6%-89.2%) (Figure S4).

4 | DISCUSSION

Through direct sequencing, our study showed an overall 34.2% mutation rate in TNBC (10.8% of *BRCA*, 11.7% of non-*BRCA* HR gene, and 11.7% non-HR DDR gene). As tumors with mutations of 2 non-HR DDR genes, *PTEN* and *MSH6*, show *BRCAness* and are not sensitive to PARP inhibitors or platinum, we hypothesize that the heterogeneous genomic background of *BRCAness* indicates different responsiveness to platinum and PARP inhibitors.

Prior studies considered “*BRCAness*”, a characteristic with high-grade genomic instability, as a marker to guide treatment of PARP inhibitors and platinum in ovarian cancer.³⁴ Both TNBC with mutations of *BRCA* or non-*BRCA* HR genes were found to show *BRCAness* features in our study, which could explain why some non-*BRCA*-mutated *BRCAness* tumors respond to platinum and PARP inhibitors. However, recent studies reported that *BRCAness* only possesses a modest value in positively predicting outcomes of breast cancer treated with PARP inhibitors.¹² In addition, carboplatin was an effective treatment in *BRCA*-mutated metastatic TNBC in the TNT phase III trial, but carboplatin was not an effective treatment for non-*BRCA*-mutated tumors with *BRCAness*.³⁵ Therefore, the clinical utility of *BRCAness*, in predicting the response rate to platinum and PARP inhibitors in breast cancer, still needs to be clarified. One possible explanation was that an epigenetic inactivation of the *BRCA1* gene, such as promoter methylation, caused a subset of *BRCAness* tumors.^{12,35,36} The status of methylation is changeable, so these tumors might not actually consistently respond to platinum. In this study, we found that *BRCAness* features not only resulted from HR/*BRCA* alteration, but also resulted from non-HR DDR gene mutations, like *MSH6* and *PTEN*-mutated tumors (Figure S2). Although *PTEN* mutation was previously reported as a marker to predict sensitivity to PARP inhibitors,³⁷ more recent evidence shows that *PTEN* mutation will cause an activation of the PI3K pathway. Hence, *PTEN*-mutated tumors would not respond to monotherapy of PARP inhibitors.^{38,39} Neither are *PTEN*-mutated tumors sensitive to platinum-based treatment in chemotherapy.²¹ These clinical features were significantly dissimilar to HR gene-mutated tumors, suggesting different biologic characteristics between *PTEN*- and HR-mutated tumors.²¹ Furthermore, we found different regions of chromosomal gain or loss between *PTEN*- and HR gene-mutated tumors. *MSH6* mutation was only found in 2 cases of our cohort, and their pathology was compatible with previous reports (ER-negative, high histologic grade, and hypermutated).⁴⁰ Mutation of Lynch syndrome-related genes (*MSH6*, *MLH1*, *MSH2*, and *PMS2*)

were rare in breast cancer.⁴⁰ As only 1 *MSH6*-mutated breast cancer was analyzed by aCGH (DNA from the other tumor was not adequate for aCGH), it is not conclusive that all *MSH6*-mutated breast tumors have high-grade genomic instability. Still, *MSH6* mutation might not be a marker for PARP inhibitor, because olaparib was not effective to treat colon cancer with Lynch syndrome.⁴¹ The case that the genomic background of *BRCAness* was heterogeneous could provide another rationale for why certain *BRCAness* TNBC did not respond well to platinum and PARP inhibitors.

In addition to genomic instability assessed by aCGH, various methods have been described to identify *BRCAness*, including gene expression, specific rearrangement signature, and RAD51 foci analysis by immunostaining.^{2,42-44} Currently, the concordance of mentioned methods is uncertain, and it is unclear which method most appropriately predicts the therapeutic response. A gene expression profile could identify those tumors with *BRCAness*, but the predictive value of the therapeutic response to PARP inhibitors would only be moderate.⁴⁵ "Signature 3", a specific rearrangement signature, is a marker associated with the mutations of *BRCA1/2*, *PALB2*, and *RAD51C* promoter methylation.⁴⁴ Although tumors that contain mutation of *ATM* or *CHEK2* could respond to PARP inhibitors, "Signature 3" is not associated with the mutations of *ATM* or *CHEK2*.⁴² As RAD51 foci deficiency indicates that the tumor cells have lost the ability to repair DSB, RAD51 could be a good marker to guide treatment with PARP inhibitors. Biallelic alteration of HR genes was significantly associated with RAD51 foci deficiency ($P < .001$),⁴³ indicating that HR gene-mutated breast tumors lost the ability to repair DSB. However, assay of RAD51 foci requires a primary cancer cell culture treated with radiation and other medications, which is difficult to undertake in clinical practice. Therefore, to identify patients who can benefit from PARP inhibitors and platinum-based chemotherapy, direct sequencing of all HR genes would be a more appropriate method. In the era of precision medicine and with the availability of NGS tools, sequencing is a highly rewarding strategy that can be applied to various cancer types.^{16,46}

A recent meta-analysis suggested that *BRCA* mutation is associated with worse overall survival.⁴⁷ Data from The Cancer Genome Atlas database showed that breast cancer patients with mutations in 12 DDR genes were associated with poor prognosis.⁴⁸ Our study found that patients with mutations of HR genes had a higher trend of recurrence. Even though the underlying molecular mechanism of the poor prognosis is still not well understood, our study revealed that both *BRCA*-mutated and non-*BRCA* HR-mutated tumors were associated with amplification of oncogenes. For example, *MYC*, *S-100* family genes, and *CRKL* were all related to poor prognosis. Hence, they might be some of the important reasons for worse disease outcome.^{31,32,49,50}

Our study indicated that the genomic background of *BRCAness* was heterogeneous. Certain DDR gene mutations led to *BRCAness* features. Yet, their clinical and biologic characteristics were different from *BRCA* and HR gene-mutated tumors. To identify TNBC patients who might benefit from treatment with platinum and PARP inhibitors, the direct sequencing of HR genes could lead to a more accurate prediction of the responsiveness to platinum and PARP

inhibitors (Figure S5). Thus, this strategy should be considered in precision medicine.

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DISCLOSURE

The authors have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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