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Article

Somatic inactivation of breast cancer predisposition genes in tumors associated with pathogenic germline variants

Belle W. X. Lim, PhD,[1,2,‡] Na Li, PhD,[1,3,4] Sakshi Mahale, MSc,[1] Simone McInerny, BSc,[4] Magnus Zethoven, MSc,[1,5] Simone M. Rowley, BSc,[1] Joanne Huynh, BSc,[1] Theresa Wang, BSc,[1,4] Jue Er Amanda Lee, PhD,[1,3,6] Mia Friedman, BSc,[1,4] Lisa Devereux, MSc,[3,7] Rodney J. Scott, PhD,[8,9] Erica K. Sloan, PhD,[2,10] Paul A. James, PhD,[3,4,‡] Ian G. Campbell, PhD[1,3,7,*,‡]

[1]Cancer Genetics Laboratory, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia
[2]Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, Victoria, Australia
[3]Sir Peter MacCallum Department of Oncology, The University of Melbourne, Melbourne, Victoria, Australia
[4]Parkville Familial Cancer Centre, Peter MacCallum Cancer Centre and Royal Melbourne Hospital, Melbourne, Victoria, Australia
[5]Bioinformatics Core Facility, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia
[6]Molecular Genomics Core, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia
[7]Lifepool, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia
[8]Discipline of Medical Genetics and The Centre for Cancer Detection and Therapy, The University of Newcastle and Hunter Medical Research Institute, Newcastle, New South Wales, Australia
[9]Division of Molecular Medicine, New South Wales Health Pathology North, Newcastle, New South Wales, Australia
[10]Division of Cancer Surgery, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia

*Correspondence to: Ian Campbell, PhD, Cancer Genetics Laboratory, Research Division, Peter MacCallum Cancer Centre, 305 Grattan Street, Melbourne, VIC 3000, Australia (e-mail: ian.campbell@petermac.org).
‡Authors contributed equally to this work.

Abstract

Background: Breast cancers (BCs) that arise in individuals heterozygous for a germline pathogenic variant in a susceptibility gene, such as *BRCA1* and *BRCA2*, *PALB2*, and *RAD51C*, have been shown to exhibit biallelic loss in the respective genes and be associated with triple-negative breast cancer (TNBC) and distinctive somatic mutational signatures. Tumor sequencing thus presents an orthogonal approach to assess the role of candidate genes in BC development.

Methods: Exome sequencing was performed on paired normal-breast tumor DNA from 124 carriers of germline loss-of-function (LoF) or missense variant carriers in 15 known and candidate BC predisposition genes identified in the BEACCON case-control study. Biallelic inactivation and association with tumor genome features including mutational signatures and homologous recombination deficiency (HRD) score were investigated.

Results: *BARD1*-carrying TNBC (4 of 5) displayed biallelic loss and associated high HRD scores and mutational signature 3, as did a *RAD51D*-carrying TNBC and ovarian cancer. Biallelic loss was less frequent in *BRIP1* BCs (4 of 13) and had low HRD scores. In contrast to other established BC genes, BCs from carriers of *CHEK2* LoF (6 of 17) or missense (2 of 20) variant had low rates of biallelic loss. Exploratory analysis of BC from carriers of LoF variants in candidate genes such as *BLM*, *FANCM*, *PARP2*, and *RAD50* found little evidence of biallelic inactivation.

Conclusions: *BARD1* and *RAD51D* behave as classic BRCA-like predisposition genes with biallelic inactivation, but this was not observed for any of the candidate genes. However, as demonstrated for *CHEK2*, the absence of biallelic inactivation does not provide definitive evidence against the gene's involvement in BC predisposition.

Hereditary breast cancer (HBC) often clusters within families and can be attributed to germline variants in susceptibility genes directly or indirectly involved in DNA repair. The major contributors—*BRCA1*, *BRCA2*, and *PALB2* (1,2)—collectively explain less than half of the familial aggregation of BC (3). Exploratory case-control studies in the past have found that potentially pathogenic variants in individual candidate genes are rare (3-5), precluding any confident conclusion about their role in HBC based solely on this approach.

An orthogonal approach to assess if a candidate gene is driving tumorigenesis is through genomic analysis of the cancers from carriers of germline mutations. For example, approximately 90% of *BRCA1* and 50%-60% of *BRCA2* breast tumors from germline mutation carriers have a somatic "second-hit" (6-9), resulting in biallelic inactivation. Most commonly, this occurs through loss of heterozygosity (LOH) or, less frequently, through protein truncating somatic point mutations or promoter hypermethylation. Biallelic inactivation of genes such as *BRCA1* and *BRCA2* is almost invariably associated with specific somatic mutational signatures (10). The presence or absence of these tumor genomic features can provide strong evidence for or against a gene's cancer predisposition role, even if based on relatively few cancers as previously

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demonstrated for *PALB2*, *RAD51C*, and *ATM* (11-13). Recent large case-control studies involving more than 65 000 participants each confirmed the association of moderate risk genes *RAD51C*, *RAD51D*, and *BARD1* but not *BRIP1* with breast cancer (4,5).

In this study, we extend the tumor sequencing approach by performing exome sequencing on 124 BCs from individuals harboring germline variants in proposed and candidate HBC genes identified in the BEACCON case-control study (hereditary BrEAst Case CONtrol study) (3) to look for evidence of biallelic inactivation as a means of validating the role of these genes in BC predisposition.

Methods

Case-control study and tumor sequencing

A total of 124 unique breast tumors were selected from cancers arising in individuals with a germline loss-of-function (LoF) or rare, likely pathogenic missense (MS) variant of interest in a known (*BARD1*, *BRIP1*, *CHEK2*, and *RAD51D)* or candidate (*BLM*, *CDK9*, *CTH*, *ERCC5*, *FANCM*, *MUTYH*, *PARP2*, *RAD50*, *RAD51B*, *WRN*, and *XRCC2*) BC predisposition gene detected in the BEACCON case-control study (3). LoF variants included stop-gained, frame-shift, or essential splice-site variants, and MS variants of interest were identified based on a combination of population frequency, in silico prediction, and location in key functional domains as detailed in Supplementary Tables 1-4 (available online). Two ovarian cancers from carriers of *BRIP1* and 1 of *RAD51D* LoF variants, respectively, were also included as these genes are known to be ovarian cancer predisposing genes. Since last reporting (5), this study has been expanded to include 6689 BRCA-negative female index familial BC patients and 14 381 cancer-free female participants (Supplementary Table 5, available online). Candidate genes were selected for this analysis based on an excess of rare coding variants in the case group. Microdissection, DNA extraction, and exome sequencing are described in the Supplementary Methods (available online). Tumor characteristics and personal and family history of the individuals selected for the current study are summarized in Supplementary Table 6 (available online).

Determination of variant allelic status and potential biallelic inactivation

For each tumor, the somatic sequencing data were assessed for the presence of somatic LoF or MS point mutations in the gene of interest as well as the allelic status of the germline variant as described previously (11,12). In summary, locus-specific LOH was determined by tumor variant allele frequency comparisons as adjusted according to estimated tumor purity. All cases had matched germline sequencing data for the gene of interest. Promoter hypermethylation, using targeted Twist Custom Panel methylation sequencing or Sanger sequencing on bisulfite converted DNA, was also assessed for cases where there was no somatic mutation or LOH across the gene of interest. Homologous recombination deficiency (HRD) scores were calculated for each tumor sample using copy number plots as a sum of the occurrence of telomeric allelic imbalances, large-scale state transitions, and homologous recombination deficiency–loss of heterozygosity from copy number plots as described previously (12), where a threshold of an HRD score of 42 or higher is defined as high-HRD (14,15). Mutational signatures were generated against COSMIC v2 catalogue (https://cancer.sanger.ac.uk/signatures/signatures_v2/) using the DeconstructSig package in R (16) on whole-exome sequenced samples.

Statistical analyses

Odds ratios and Fisher exact test (2-sided) were calculated in case-control analyses, with a 2-tailed *P* value of .05 or less defined as statistically significant. Confidence intervals (CIs) were calculated using a conditional maximum likelihood estimate. All calculations were carried out using R-in built function in R 3.3.2 (17).

Results

Tumor sequencing in individuals harboring a germline variant in known BC genes

Whole or targeted exome sequencing was performed on 41 tumors from individuals harboring germline LoF variants in genes commonly present in HBC panels: *BARD1* (n = 7), *BRIP1* (n = 13), *RAD51D* (n = 4), and *CHEK2* (n = 17) (Table 1). *BARD1*, which in the BEACCON case-control data (Figure 1) and other published data (4) is associated specifically with triple-negative breast cancer (TNBC), showed loss of the wild-type (WT) allele via LOH in 4 of 5 assessable triple negative (TN) tumors. A sixth TN tumor also had LOH, but it was not possible to determine which allele had been lost, and the only *BARD1* tumor to show loss of the mutant allele was estrogen receptor (ER) positive. The 6 *BARD1* TN tumors including 1 in heterozygous status showed high HRD scores and 4 with strong HRD-related mutational signature 3. Carriers of *RAD51D* LoF mutations, which are also associated with TNBC, were rare in the BEACCON study. One of the 2 TNBC showed LOH of the WT allele, whereas 2 ER-positive tumors remained heterozygous. An additional high-grade serous ovarian cancer (HGSOC) that was available for analysis (Supplementary Table 5, available online) showed biallelic inactivation through LOH. Nine carriers of rare *RAD51D* MS variants shortlisted based on likely pathogenicity assessment (Supplementary Table 1, available online) were also analyzed, but only 2 cases showed loss of the WT allele with only 1 of these being a TNBC. This case was a compound heterozygote that showed loss of the p.Ala313Val and retention of the p.Ala52Val allele; it had a high HRD score and a strong mutational signature 3.

The role of *BRIP1* in breast cancer predisposition is debated, and our analysis of *BRIP1* LoF variants identified an equal number of tumors showing loss of the WT or mutant alleles (4 cases each) with the remaining 5 remaining heterozygous. By comparison, analysis of 2 HGSOCs showed both had loss of the WT (Supplementary Table 5, available online), consistent with the established role of *BRIP1* in ovarian cancer predisposition. Both HGSOCs also showed high HRD scores compared with only 2 of 4 *BRIP1*-null BCs. Mutational signature 3 was observed only in 1 *BRIP1*-null BC and not in the HGSOCs.

LoF mutations in *CHEK2*, predominantly the c.1100delC variant, are well established to confer a two- to threefold increase in BC risk (20), with the association being strongest for ER-positive BC. The current tumor data do not provide evidence that *CHEK2* requires biallelic inactivation with the majority (9 of 17) of tumors remaining heterozygous, whereas only 6 showed loss of the WT allele, and 2 ER-positive tumors showed loss of the LoF allele. It appears that the 2 tumors with loss of pathogenic variants were not driven by *CHEK2* LoF, and given that *CHEK2* is only a moderate risk gene, several of the tumors without LOH could also not be driven by the *CHEK2* pathogenic variants. In addition, 20 tumors from individuals with rare germline *CHEK2* MS variants of interest (Table 1; Supplementary Table 2, available online) were analyzed. Most of these variants except for p.Ile157Thr and p.Arg117Gly are currently classified as variants

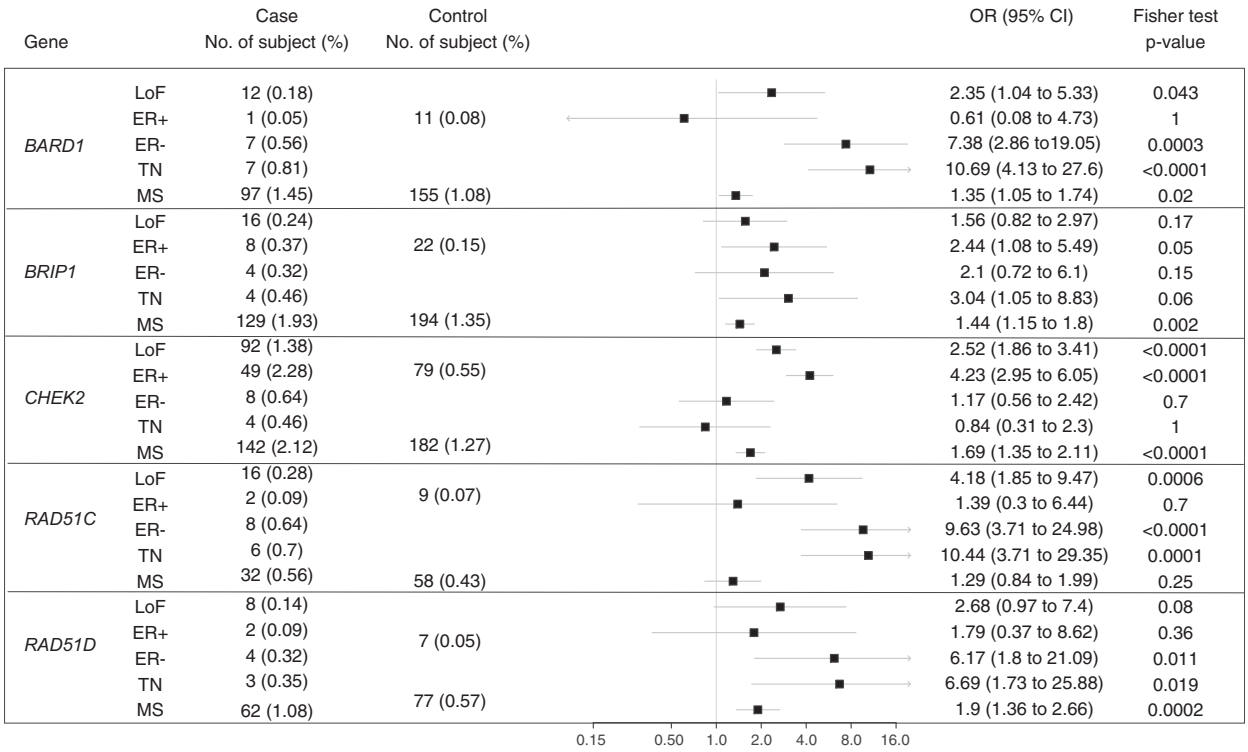

Figure 1. Case-control analysis of rare LoF variants (minor allele frequency [MAF] ≤ 0.005) and MS variants (MAF ≤ 0.001) in known or strongly proposed breast cancer genes, including subcategories of estrogen receptor–positive (ER+), ER-negative (ER-), and triple-negative (TN) breast tumor where diagnosis was available. ER+ and ER- groups were mutually exclusive, and the ER- groups include the TN samples. Participants without sufficient pathological information were only included in the overall LoF group and excluded from the subcategory analysis. *CHEK2*, *BARD1*, and *BRIP1* were screened in 6689 cases and 14 381 controls; *RAD51C* and *RAD51D* were screened in 5726 cases and 13 428 controls. The sample sizes of ER+, ER-, and TN were 2146, 1246, and 862, respectively. CI = confidence interval; LoF = loss of function; MS = missense; OR = odds ratio (3,4,11,12,18,19).

of unknown significance, and tumor sequencing showed that most retained heterozygosity with only 2 showing loss of the WT allele and 2 showing loss of the variant allele. In particular, all 3 carriers of the known pathogenic, but reduced penetrance, variant *CHEK2* p.Ile157Thr retained heterozygosity, and in the 2 tumors that were tested, neither were found to have promoter methylation.

Tumor sequencing in individuals with germline variants in candidate HBC genes

Tumor sequencing was performed on 57 BCs (Table 2) carrying LoF (n = 45) or rare MS (n = 16) variants in 11 genes that have been the subject of debate in the literature (*FANCM*, *RAD50*, *RAD51B*, and *XRCC2*) or were shortlisted from the BEACCON study (*BLM*, *CDK9*, *CTH*, *ERCC5*, *MUTYH*, *PARP2*, and *WRN*) (Supplementary Figure 1, available online). The 4 BCs from *RAD50* LoF mutation carriers remained heterozygous, consistent with the accumulating literature that it does not predispose to BC (21). Literature support for a role of *XRCC2* in BC predisposition is weak but with a potential association with ER-negative cancer (4,22,23). Of the 2 BCs from *XRCC2* LoF variant carriers, only 1 (a TNBC) showed biallelic inactivation with both a high HRD score and mutational signature 3. For *RAD51B*, only tumors from carriers of rare MS variants were available with 2 remaining heterozygous and 2 showing loss of the variant allele.

LoF mutations in *FANCM* have previously been reported to be associated with a small increase in BC risk (24-26), but 4 of 6 tumors from individuals with *FANCM* LoF variants remained heterozygous with only 1 having lost the WT allele and did not show a high HRD score. Similarly, there was no consistent loss of the WT allele in tumors associated with germline *FANCM* MS variants (Supplementary Table 3, available online). *BLM* has previously been implicated in BC predisposition (27-29) and in the BEACCON data (Supplementary Figure 1, available online), but 5 of the BCs with LoF variants remained heterozygous with no evidence of promoter hypermethylation, whereas 2 BCs lost the LoF allele.

For the candidate genes, loss of the WT allele was not observed for the majority. The BCs carrying LoF or MS variants in the candidate genes *CDK9* and *CTH* (Supplementary Table 4, available online) remained heterozygous although promoter methylation assessment was not successful for these genes. Of the 4 *PARP2* BCs with LoF mutations, 2 showed loss of the LoF allele with the other 2 remaining heterozygous. The 2 *ERCC5* BCs showed loss of the LoF allele. In contrast, the *WRN* gene, which was found to have a statistcally significant association with ER-positive BC in the BEACCON study (unadjusted $P = .003$), showed loss of the WT allele in 2 of 6 ER-positive cases. A *WRN*-heterozygous BC also carried an *ATM* germline variant that had experienced biallelic loss, suggesting that the *ATM* variant was instead responsible for the tumor. Overall, evidence of loss of the WT allele was rare among candidate genes despite evidence in case-control frequencies.

Discussion

The frequent observation of loss of the WT allele in BCs carrying germline pathogenic mutations in *BRCA1* and *BRCA2* supports the model of biallelic inactivation being required for BC predisposition, at least in some high penetrance genes. Recent studies

Table 1. Tumor sequencing data for 69 tumors from individuals heterozygous for a germline LoF (n = 41) or MS (n = 29) variant in a known breast cancer predisposition gene (*BARD1*, *BRIP1*, *CHEK2*, and *RAD51D*)

ID[a]	Gene	Germline variant	Variant type	Variant allelic status[b]	BC subtype	HRD	Somatic *TP53*	Somatic *PIK3CA*	Mutation signature 3[c]	Dominant mutation signature	Promoter hypermethylation
3530	*BARD1*	c.1135A>T, p.Lys379Ter	LoF	Mutant loss	ER+/HER2-	na	na	na	na	na	Failed
3977	*BARD1*	c.1212C>G, p.Tyr404Ter	LoF	WT loss	TN	50	na	na	Strong	3, 11	DNT
1531	*BARD1*	c.1652C>G, p.Ser551Ter	LoF	WT loss	TN	83	LoF	—	Weak	19, 30	DNT
3828	*BARD1*	c.1652C>G, p.Ser551Ter	LoF	WT loss	TN	92	MS	—	Strong	1, 3	DNT
425	*BARD1*	c.1652C>G, p.Ser551Ter	LoF	LOH	TN	80	na	na	Strong		na
3496	*BARD1*	c.1905G>A, p.Trp635Ter	LoF	Het	TN	82	MS	—	Strong	3	Failed
1272	*BARD1*	c.2078_2079insTAATA, p.Lys693AsnfsTer23	LoF	WT loss	TN	76	LoF	—	No	19	DNT
2439	*BRIP1*	c.93 + 1G>T	LoF	WT loss	TN	59	LoF	—	Strong	3, 19	DNT
4160	*BRIP1*	c.103G>T, p.Gly35Ter	LoF	Mutant loss	ER-/HER2+	30	MS	—	Strong	1, 3	na
3259	*BRIP1*	c.1426del, p.Thr476LeufsTer50	LoF	WT loss	ER+/HER2-	41	—	MS	No	12, 20	DNT
3597	*BRIP1*	c.1888dup, p.Thr630AsnfsTer9	LoF	Het	ER+/HER2-	12	—	MS	No	30	Negative
3093	*BRIP1*	c.2298_2301delTGAG, p.Ser766ArgfsTer14	LoF	Het	ER+/HER2-	5	—	MS	na	na	na
227	*BRIP1*	c.2392C>T, p.Arg798Ter	LoF	WT loss	TN	58	MS	—	No	20, 21	DNT
1325	*BRIP1*	c.2392C>T, p.Arg798Ter	LoF	Mutant loss	TN	57	LoF	—	na	na	na
786	*BRIP1*	c.2392C>T, p.Arg798Ter	LoF	Het	ER+/HER2-	1	LoF	—	na	na	Failed
1928	*BRIP1*	c.2392C>T, p.Arg798Ter	LoF	Het	ER+/HER2-	11	MS	—	na	na	Negative
3829	*BRIP1*	c.2400C>G, p.Tyr800Ter	LoF	Mutant loss	TN	50	—	—	Weak	6	na
3635	*BRIP1*	c.2400C>G, p.Tyr800Ter	LoF	Het	ER+/HER2-	32	LoF	—	Weak	1	na
3354	*BRIP1*	c.2492_2492 + 5delGGTAAG	LoF	WT loss	ER+/HER2-	31	MS	MS	Weak	1	DNT
3468	*BRIP1*	c.3715del, p.Ser1239ProfsTer15	LoF	Mutant loss	ER+/HER2-	37	LoF	—	Weak	1, 13	na
4152	*CHEK2*	c.629_732delCAGT, p.Ser210PhefsTer6	LoF	Mutant loss	ER+/HER2-	47	MS	—	No	3	Negative
2320	*CHEK2*	c.630delA, p.Val211PhefsTer6	LoF	Het	na	7	—	—	Weak	5, 30	na
290	*CHEK2*	c.902delT, p.Leu301TrpfsTer3	LoF	Mutant loss	ER+/HER2-	27	—	—	Weak	1,6	na
3587	*CHEK2*	c.1100delC, p.Thr367MetfsTer15	LoF	WT loss	ER+/HER2-	34	na	na	No	11	DNT
1825	*CHEK2*	c.1100delC, p.Thr367MetfsTer15	LoF	Het	ER+/HER2-	32	LoF	—	Strong	3	na
3174	*CHEK2*	c.1100delC, p.Thr367MetfsTer15	LoF	Het	ER+/HER2-	29	—	—	No	6, 30	na
2182	*CHEK2*	c.1100delC, p.Thr367MetfsTer15	LoF	Het	ER+/HER2-	45	—	—	No	11, 19	Failed
2410	*CHEK2*	c.1100delC, p.Thr367MetfsTer15	LoF	Het	ER+/HER2-	27	—	—	No	1, 30	Failed
2475	*CHEK2*	c.1100delC, p.Thr367MetfsTer15	LoF	WT loss	ER+/HER2-	80	—	—	No	6, 19	DNT
1300	*CHEK2*	c.1100delC, p.Thr367MetfsTer15	LoF	WT loss	ER+/HER2-	36	—	MS	No	10	Failed
2326	*CHEK2*	c.1100delC, p.Thr367MetfsTer15	LoF	WT loss	ER+/HER2-	21	—	—	No	19, 30	Failed
2711	*CHEK2*	c.1100delC, p.Thr367MetfsTer15	LoF	WT loss	ER+/HER2+	10	—	—	No	1, 11	Failed
3500	*CHEK2*	c.1100delC, p.Thr367MetfsTer15	LoF	Het	ER+/HER2unknown	37	—	—	Strong	3	na
2351	*CHEK2*	c.1100delC, p.Thr367MetfsTer15	LoF	Het	ER-/HER2+	4	na	na	Weak	19, 30	DNT
3076	*CHEK2*	c.1100delC, p.Thr367MetfsTer15	LoF	Het	ER-/HER2+	1	—	—	No	1	na
1732	*CHEK2*	c.1100delC, p.Thr367MetfsTer15	LoF	WT Loss	TN	17	MS	MS	No	6	DNT
1853	*CHEK2*	c.1696delC, p.Thr533GlnfsTer33	LoF	Het	ER+/HER2-	28	MS	—	na	na	na
2625	*CHEK2*	c.14C>T, p.Ser5Leu	MS	Het	ER+/HER2-	85	—	—	Weak	19	Failed
1993	*CHEK2*	c.190G>A, p.Glu64Lys	MS	Het	ER+/HER2-	91	LoF	—	Weak	1, 6	na
811	*CHEK2*	c.349A>G, p.Arg117Gly	MS	Het	ER+/HER2+	0	—	MS	Weak	5	Negative
1103	*CHEK2*	c.349A>G, p.Arg117Gly	MS	Mutant loss	ER+/HER2-	16	—	MS	Weak	3	Negative
616	*CHEK2*	c.349A>G, p.Arg117Gly	MS	Het	ER-/HER2+	17	—	MS	Strong	1, 3	na
787	*CHEK2*	c.442A>G, p.Arg148Gly	MS	Het	ER+/HER2-	na	na	na	na	na	Failed
2531	*CHEK2*	c.470T>C, p.Ile157Thr	MS	Het	ER+/HER2-	34	—	—	Weak	19	Negative

(continued)

Table 1. (continued)

ID[a]	Gene	Germline variant	Variant type	Variant allelic status[b]	BC subtype	HRD	Somatic *TP53*	Somatic *PIK3CA*	Mutation signature 3[c]	Dominant mutation signature	Promoter hypermethylation
1420	*CHEK2*	c.470T>C, p.Ile157Thr	MS	Het	ER+/HER2-	28	MS	MS	No	6	Negative
3240	*CHEK2*	c.470T>C, p.Ile157Thr	MS	Het	ER+/HER2-	16	—	—	Strong	3, 6	na
807	*CHEK2*	c.1036C>T, p.Arg346Cys; c.499G>A, p. Gly167Arg	MS MS	WT loss; mutant loss	ER+/HER2unknown	52	—	—	Weak	1	na
2091	*CHEK2*	c.1067C>T, p.Ser356Leu	MS	Het	ER+/HER2-	47	—	—	Weak	1, 19	Failed
2689	*CHEK2*	c.1211A>G, p.Tyr404Cys	MS	WT loss	ER+/HER2+	28	—	MS	Strong	3, 30	DNT
2221	*CHEK2*	c.1270T>C, p.Tyr424His	MS	WT loss	ER+/HER2-	13	—	MS	No	20	DNT
1830	*CHEK2*	c.1312G>T, p.Asp438Tyr	MS	Het	ER+/HER2-	17	—	—	na	na	na
2257	*CHEK2*	c.1312G>T, p.Asp438Tyr	MS	Het	ER+/HER2-	0	—	MS	Weak	6	Negative
200	*CHEK2*	c.1312G>T, p.Asp438Tyr	MS	Het	na	5	—	—	Strong	3	na
4164	*CHEK2*	c.1427C>T, p.Thr476Met	MS	Het	ER+/HER2-	13	—	—	No	11, 19	na
1410	*CHEK2*	c.1447C>T, p.His483Tyr	MS	Mutant loss	ER+/HER2-	30	MS	—	na	na	DNT
2345	*CHEK2*	c.1525C>T, p.Pro509Ser	MS	Mutant loss	ER+/HER2+	7	LoF	—	na	na	Negative
1198	*CHEK2*	c.1604G>A, p.Arg535His	MS	Het	TN	22	MS	—	No	6, 30	Failed
1897	*RAD51D*	c.616C>T, p.Arg206Ter	LoF	Het	ER+/HER2-	4	—	—	na	na	Negative
2734	*RAD51D*	c.754C>T, p.Arg252Ter	LoF	WT loss	TN	42	LoF	—	na	na	DNT
2866	*RAD51D*	c.808delC, p.His270ThrfsTer2	LoF	Het	TN	7	—	—	na	na	na
3500	*RAD51D*	c.863G>A, p.Trp288Ter	LoF	Het	ER+/HER2-	8	—	—	Weak	30	na
506	*RAD51D*	c.26G>C, p.Cys9Ser	MS	Het	ER+/HER2-	0	—	—	na	na	na
30	*RAD51D*	c.26G>C, p.Cys9Ser	MS	Het	ER+/HER2-	20	MS	—	na	na	na
2936	*RAD51D*	c.26G>C, p.Cys9Ser	MS	Het	ER+/HER2-	8	—	—	No	5	DNT
1980	*RAD51D*	c.26G>C, p.Cys9Ser	MS	Het	TN	3	LoF	—	No	1	Negative
2224	*RAD51D*	c.137C>G, p.Ser46Cys	MS	Het	ER+/HER2-	4	—	—	na	na	Negative
2219	*RAD51D*	c.155C>T, p.Ala52Val; c.938C>T, p. Ala313Val	MS compound homozygous	WT loss; mutant loss	TN	42	LoF	—	Strong	3	Negative
1686	*RAD51D*	c.308C>T, p.Ala103Val	MS	Het	TN	17	MS	—	na	na	Negative
3095	*RAD51D*	c.472A>C, p.Asn158His	MS	Mutant Loss	ER+/HER2+	20	—	—	na	na	na
2606	*RAD51D*	c.551T>C, p.Leu184Pro	MS	WT Loss	ER+/HER2-	11	MS	—	na	na	DNT

[a] Subject 3093 carried 2 variants of interest in *BRIP1*; subject 2219 carried 2 variants in *RAD51C*; subject 3500 carried a variant of interest in both *CHEK2* and *RAD51D*. "—" signifies feature not present. BC = breast cancer; DNT = did not test; na = not available; ER+ = estrogen receptor—positive breast cancer; HER2- = HER2 negative; HER2+ = HER2 positive; HRD = homologous recombination deficiency score; LoF = loss of function; MS = missense; TN = triple-negative; WT = wild type.

[b] WT loss, somatic loss of the WT allele; mutant loss, somatic loss of the allele carrying the known germline variant; Het, heterozygous. Case 425 showed loss of heterozygosity across the gene regions but unable to determine which allele had been lost.

[c] Proportion of mutational signature 3 (COSMIC v2, assessed on whole-exome sequenced tumors only) above 25% is classified as "strong", under 25% as "weak".

Table 2. Sequencing results of 57 tumors from individuals heterozygous for a germline LoF (n = 43) or MS (n = 16) variant in a candidate breast cancer predisposition gene (*BLM*, *CDK9*, *CTH*, *ERCC5*, *FANCM*, *MUTYH*, *PARP2*, *RAD50*, *RAD51B*, *WRN*, and *PARP2*)

ID[a]	Germline gene	Variant	Variant type	Variant status[b]	Subtype	HRD	Somatic *TP53*	Somatic *PIK3CA*	Mutation signature 3[c]	Dominant signature	Hypermutated
2660	*BLM*	c.318_319insT, p.Leu107PhefsTer36	LoF	Het	ER+/HER2-	1	—	—	na	na	Negative
1471		c.768_769delCT, p.Leu258GlufsTer7	LoF	Mutant Loss	ER+/HER2unknown	5	—	MS	na	na	Negative
462		c.1624delG, p.Asp542ThrfsTer2	LoF	Mutant Loss	TN	44	LoF	—	na	na	Negative
3093		c.2695C>T, p.Arg899Ter	LoF	Het	ER+/HER2-	5	—	MS	na	na	na
2287		c.2875C>T, p.Arg959Ter	LoF	Het	ER+/HER2-	11	—	—	na	na	Negative
2083		c.3210 + 2delT	LoF	Het	ER+/HER2-	6	—	—	na	na	Failed
35		c.3558 + 1G>T	LoF	Het	ER+/HER2unknown	17	—	MS	na	na	Negative
1245	*CDK9*	c.130delA, p.Lys44ArgfsTer4	LoF	Het	ER+/HER2-	13	MS	—	na	na	Failed
2345		c.274delT, p.Tyr92IlefsTer23	LoF	Het	ER+/HER2+	4	LoF	—	na	na	Negative
3075		c.620_621insC, p.Ile210HisfsTer2	LoF	Het	TN	56	LoF	—	na	na	Failed
2723		c.689_690insG, p.Asn232GlnfsTer20	LoF	Het	ER+/HER2+	0	—	—	na	na	na
2045	*CTH*	c.465G>A, p.Trp155Ter	LoF	Het	ER+/HER2-	19	—	—	na	na	Failed
1322		c.465G>A, p.Trp155Ter	LoF	Het	ER+/HER2+	20	MS	—	na	na	Failed
3119		c.465G>A, p.Trp155Ter	LoF	Het	ER+/HER2+	0	—	—	na	na	na
1865		c.1064delC, p.Thr355IlefsTer19	LoF	Het	ER+/HER2-	36	MS	—	na	na	Failed
1092		c.230C>T, p.Ala77Val	MS	Het	ER+/HER2-	20	ESS	—	No	5, 12	Failed
307		c.323T>C, p.Ile108Thr	MS	Het	TN	24	LoF	—	na	na	Failed
2593		c.620T>C, p.Met207Thr	MS	Het	na	9	—	—	na	na	na
1065		c.718C>G, p.Gln240Glu	MS	Het	ER+/HER2-	0	—	—	na	na	na
4142		c.794G>A, p.Arg265Gln	MS	Het	ER+/HER2-	10	LoF	—	na	na	Failed
3197		c.1124G>A, p.Arg375Gln	MS	Het	ER+/HER2-	19	—	—	Weak	1, 12	na
2260	*ERCC5*	c.589delC, p.Pro198LeufsTer3	LoF	Mutant Loss	TN	41	—	—	Weak	15	Failed
901		c.1774_1775insAAGCA, p.Val592GlufsTer8	LoF	Mutant Loss	TN	83	—	MS	No	11	na
1367	*FANCM*	c.2267G>A, p.Arg756His	LoF	Het	ER+/HER2-	24	—	MS	No	6	Failed
1709		c.3589delG, p.Asp1197MetfsTer18	LoF	Mutant Loss	TN	26	MS	—	Weak	6	Failed
3147		c.5101C>T, p.Gln1701Ter	LoF	Het	ER+/HER2-	6	—	—	Weak	3, 6	Failed
691		c.5791C>T, p.Arg1931Ter	LoF	Het	ER+/HER2-	2	MS	MS	No	6	na
1172		c.5791C>T, p.Arg1931Ter	LoF	Het	ER+/HER2+	0	—	—	No	1	Failed
2771		c.5791C>T, p.Arg1931Ter	LoF	WT Loss	TN	18	LoF	—	No	1, 26	DNT
1127		c.163G>A, p.Asp55Asn	MS	Mutant Loss	TN	51	—	—	Weak	3	Failed
2094		c.2267G>A, p.Arg756His	MS	Het	TN	40	MS	—	Strong	1, 3	Failed
1879		c.2267G>A, p.Arg756His	MS cpd	Het	ER+/HER2-	72	LoF	—	Weak	1, 19	DNT
1222		c.3998A>C, p.Gln1333Pro	MS	WT Loss	TN	48	LoF	—	Weak	5	DNT
901		c.5108A>G, p.His1703Arg	MS	Het	TN	83	—	MS	No	5	na
2743	*MUTYH*	c.925-2A>G	LoF	Het	TN	10	MS	—	na	na	Failed
2727[‡]		c.925-2A>G	LoF	Het	TN	36	LoF	—	na	na	Failed
1253		c.384G>A, p.Trp128Ter	Biallelic LoF	Germline homozygous	TN	na	—	—	na	na	na
1474	*PARP2*	c.979_980insTT, p.Ser328CysfsTer8	LoF	Mutant Loss	ER+/HER2-	67	—	MS	na	na	DNT
2294		c.985_986insA, p.Ile331AsnfsTer11	LoF	Het	ER-/HER2+	81	—	—	na	na	Failed
333		c.1109_1110insT, p.Leu372ProfsTer2	LoF	Mutant Loss	ER+/HER2-	na	—	—	na	na	na
1185		c.1304delG, p.Val436TrpfsTer4	LoF	Het	ER-/HER2+	30	MS	MS	na	na	Failed
1327		c.965G>A, p.Arg322Gln	MS	WT Loss	ER+/HER2-	28	—	—	na	na	DNT
2883	*RAD50*	c.1291_1297delGAGATAA, p.Asp434LysfsTer7	LoF	Het	ER+/HER2-	47	—	—	No	16	Failed
2193		c.1958C>A, p.Ser653Ter	LoF	Het	ER+/HER2-	23	—	—	Strong	3	Failed
2251		c.2467C>T, p.Arg823Ter	LoF	Het	ER+/HER2-	24	—	MS	Strong	3, 5	na
1031		c.3207delA, p.Asn1070IlefsTer6	LoF	Het	ER+/HER2+	8	—	MS	No	25	Failed
2923	*RAD51B*	c.103C>T, p.Pro35Ser	MS	Het	TN	32	MS	—	na	na	na
1932		c.277G>A, p.Ala93Thr	MS	Het	TN	2	—	—	na	na	Failed

(continued)

Table 2. (continued)

ID[a]	Germline gene	Variant	Variant type	Variant status[b]	Subtype	HRD	Somatic TP53	Somatic PIK3CA	Mutation signature 3[c]	Dominant signature	Hypermutated
1795		c.436G>A, p.Ala146Thr	MS	Mutant Loss	TN	30	LoF	—	na	na	na
3024		c.553T>G, p.Cys185Gly	MS	Mutant Loss	TN	76	—	—	na	na	DNT
3054	*WRN*	c.171C>A, p.Tyr57Ter	LoF	Het	ER+/HER2-	24	—	MS	Weak	5	Failed
2963		c.944_948delTAAAC, p.Leu315PhefsTer5	LoF	Het	ER+/HER2-	24	—	—	Weak	5	Failed
1115		c.3961C>T, p.Arg1321Ter	LoF	Het	ER+/HER2-	40	MS	MS	No	5, 6	Failed
1847		c.4216C>T, p.Arg1406Ter	LoF	WT Loss	ER+/HER2-	53	—	MS	Strong	3	DNT
2562		c.4216C>T, p.Arg1406Ter	LoF	Het	ER+/HER2-	29	—	—	Strong	3, 11	Failed
3093		c.4216C>T, p.Arg1406Ter	LoF	WT Loss	ER+/HER2-	5	—	MS	na	na	na
1349		c.4216C>T, p.Arg1406Ter	LoF	Het	TN	43	—	—	Strong	3	Failed
863	*XRCC2*	c.39 + 1G>A	LoF	Het	ER-/HER2+	12	LoF	—	Strong	3	Failed
3062		c.794T>A, p.Leu265Ter	LoF	WT Loss	TN	44	LoF	—	Strong	3	DNT

[a] Subject 2727 also carried a germline ATM variant that had biallelic loss in tumor. Subject 3054 carried a germline *RAD51C* that had biallelic loss in tumor. Subject 3093 carried variants of interest in *BLM* and *WRN*. Subject 901 carried variants in both *ERCC5* and *FANCM*. "—" signifies feature not present. BC = breast cancer; DNT = did not test; na = not available; ER+ = estrogen receptor–positive breast cancer; HER2- = HER2 negative; HER2+ = HER2 positive; HRD = homologous recombination deficiency score; LoF = loss of function; MS = missense; TN = triple-negative; WT = wild type.

[b] WT loss, somatic loss of the wild-type allele; Mutant loss, somatic loss of the allele carrying the known germline variant. Het, heterozygous. Case 425 showed loss of heterozygosity across the gene regions but unable to determine which allele had been lost.

[c] Proportion of mutational signature 3 (COSMIC v2, assessed on whole-exome sequenced tumors only) above 25% is classified as "strong," below 25% as "weak."

indicate that biallelic inactivation is also common in BCs carrying pathogenic mutations in *PALB2* (12,30,31) and *ATM* (13) and has been used as a biomarker to support the role of *RAD51C* (11) as a TNBC predisposition gene. These examples suggest tumor sequencing can provide a useful orthogonal approach to validate new BC genes and rare MS variants in known genes.

In this study, tumor sequencing demonstrated that TN tumors from *BARD1* LoF mutation carriers frequently exhibit biallelic inactivation consistent with data from case-control studies (3-5) that indicate that *BARD1* pathogenic variants are associated with predisposition to TNBC. Recent case-control studies have also provided support for the role of *RAD51D* in TNBC predisposition (4). Although 1 of 2 LoF BCs studied here showed loss of the WT allele, the rarity of its variants precluded any confident conclusion to be drawn. *RAD51D* MS variants as a group showed an excess in the BEACCON case-control analysis, but most of the tumors from rare MS variant carriers showed no evidence of a second hit, and the 2 cases with loss of the WT allele had low HRD scores suggesting they are benign variants.

The role of *BRIP1* in BC predisposition is debated with most, but not all, published case-control studies failing to identify a statistically significant excess of LoF mutations in cases (4,5). Tumor sequencing did not find evidence to support a role for *BRIP1* in BC predisposition with most BCs remaining heterozygous and, importantly, an equivalent number of cases losing the WT and LoF alleles. Previous tumor sequencing studies on *BRIP1* BCs are limited, but our findings are consistent with a previous report on 3 *BRIP1* BC where only 1 was found to have biallelic inactivation (10). Overall, our results for *BARD1*, *RAD51D*, and *BRIP1* are consistent with the findings of 2 recent large case-control studies cited previously (4,5) where *BARD1* and *RAD51D* are associated with BC, specifically TNBC, whereas no causative link was identified for *BRIP1*.

Based on the data for *BARD1* and the other previously studied BC predisposing genes *BRCA1*, *BRCA2*, *PALB2*, and *ATM*, it might be extrapolated that biallelic inactivation is a typical feature for all BC predisposition genes. However, the data for *CHEK2*, which has highly robust case-control evidence supporting its role as a moderate penetrance BC gene, suggest that this is not true in all cases. Of the 17 BCs with germline *CHEK2* LoF mutations, only 6 showed loss of the WT allele, and the majority (53%) showed no evidence of biallelic loss. The established low penetrance *CHEK2* variant p.Ile157Thr was also not detected with WT allele loss. This is consistent with previous studies that found that LOH across *CHEK2* in BCs from LoF mutation carriers was infrequent (32 of 93, 34%) (32-37) and occurs at a similar rate in sporadic BCs (40%, n = 560) (38). A recent sequencing-based study reported that 13 of 16 (81%) BC from *CHEK2* LoF carriers had biallelic inactivation (32), however, only 5 of the 8 (63%) *CHEK2*-null tumors were of ER-positive and HER2-negative ductal histological subtype that are known to be associated with *CHEK2* predisposition. Our data based on 17 LoF and 20 MS *CHEK2* variant-carrying tumors suggested that although *CHEK2* displayed a selective predisposition to ER-positive ductal BC, there was no consistent biallelic inactivation, and the BCs had low mutational burden and were not consistently associated with a characteristic mutational signature or somatic driver mutations. These data suggest that the effect of pathogenic variants in *CHEK2* is possibly mediated by haploinsufficiency (39), which has implications for the reliability of using biallelic inactivation as an indicator of disease association.

Candidate genes analyzed in this study were identified in the BEACCON case-control and included *BLM*, *PARP2*, and *WRN*, which showed statistically significant association with BC with odds ratios of 2.5, 5.0, and 2.0, respectively, whereas other

candidate genes *CDK9*, *CTH*, and *XRCC2* also showed relatively high odds ratios despite the small number of cases. The BEACCON study included more than 11 500 subjects with enrichment for high-risk familial cases, however, LoF variants in candidate genes were still too rare to confidently assert a genuine association with BC predisposition. Inclusion of tumor sequencing from 57 cases did not provide definitive evidence for their roles in BC predisposition: 1 of 2 *XRCC2* and 2 of 7 *WRN* BCs were among the minority that showed loss of the WT allele. Despite multiple studies reporting an association with *BLM*, no instance of biallelic inactivation has been found in *BLM*-carrying tumors to date, including our study of 7 tumors and the previous evaluation of 22 cases across 3 studies (28,34,40). Despite early studies and inclusion of *RAD50* in many HBC gene panels (41,42), recent large studies have demonstrated that it is not a BC predisposition gene (4,5), consistent with the findings in the 4 *RAD50* tumors analyzed here, which all remained heterozygous.

Findings from this study have demonstrated that tumor sequencing is useful in validating BC predisposing genes that operate in carcinogenesis through a mechanism of biallelic inactivation, such as *BARD1*. However, based on the evidence from *CHEK2*-associated tumors, the absence of biallelic inactivation does not appear to preclude a role in BC predisposition. It is interesting to note that most of the established HBC genes that have been shown to undergo frequent biallelic inactivation in breast tumors, such as *BRCA1*, *BRCA2*, *PALB2*, and *RAD51C*, are highly penetrant and/or predispose selectively to TNBC, currently the sole exception being *ATM*, which is a moderate penetrance and ER-positive BC-associated gene. Interestingly, similar to *CHEK2*, the lower penetrance *BRCA2* variant p.Lys3326Ter does not appear to require biallelic activation. A recent study of 26 *BRCA2* p.Lys3326Ter-associated breast tumors found no instance of LOH (43).

Despite the ready availability of archival formalin-fixed, paraffin-embedded tumor blocks from BEACCON study participants, the main limitation to this study, was the small sample size for rare genes. In addition, the quality of formalin-fixed, paraffin-embedded tumor samples may cause potential errors in the determination of LOH. Tumor purity, especially in tumors that have high levels of infiltrating lymphocytes, may also introduce further complexity to the interpretation of allele frequency and copy number status, as addressed in the methods. Lastly, because of the greater requirements in terms of tumor DNA quality and quantity, methylation sequencing was not able to be carried out for all samples, therefore promoter hypermethylation cannot be ruled out for those samples, although in this study, no such instance was found.

In summary, this study demonstrates the utility of inclusion of tumor sequencing in HBC gene discovery and validation, but the absence of consistent biallelic inactivation in *CHEK2* suggests this approach might not be reliable for lower penetrance genes.

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Data availability

All sequencing data has been deposited to the European Genome-phenome Archive under accession numbers Study: EGAS00001006532 and Dataset: EGAD00001009299. Standard R codes were used. Code requests should be addressed to Prof. Ian Campbell.

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