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Prevalence and predictors of loss of wild type *BRCA1* in estrogen receptor positive and negative *BRCA1*-associated breast cancers

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Abstract

Introduction: The majority of breast cancers that occur in *BRCA1* mutation carriers (*BRCA1* carriers) are estrogen receptor-negative (ER-). Therefore, it has been suggested that ER negativity is intrinsic to *BRCA1* cancers and reflects the cell of origin of these tumors. However, approximately 20% of breast cancers that develop in *BRCA1* carriers are ER-positive (ER+); these cancers are more likely to develop as *BRCA1* carriers age, suggesting that they may be incidental and unrelated to *BRCA1* deficiency. The purpose of this study was to compare the prevalence of loss of heterozygosity due to loss of wild type (wt) *BRCA1* in ER+ and ER- breast cancers that have occurred in *BRCA1* carriers and to determine whether age at diagnosis or any pathologic features or biomarkers predict for loss of wt *BRCA1* in these breast cancers.

Methods: Relative amounts of mutated and wt *BRCA1* DNA were measured by quantitative polymerase chain reaction performed on laser capture microdissected cancer cells from 42 ER+ and 35 ER- invasive breast cancers that developed in *BRCA1* carriers. *BRCA1* gene methylation was determined on all cancers in which sufficient DNA was available. Immunostains for cytokeratins (CK) 5/6, 14, 8 and 18, epidermal growth factor receptor and p53 were performed on paraffin sections from tissue microarrays containing these cancers.

Results: Loss of wt *BRCA1* was equally frequent in ER+ and ER- *BRCA1*-associated cancers (81.0% vs 88.6%, respectively; *P* = 0.53). One of nine cancers tested that retained wt *BRCA1* demonstrated *BRCA1* gene methylation. Age at diagnosis was not significantly different between first invasive ER+ *BRCA1* breast cancers with and without loss of wt *BRCA1* (mean age 45.2 years vs 50.1 years, respectively; *P* = 0.51). ER+ *BRCA1* cancers that retained wt *BRCA1* were significantly more likely than those that lost wt *BRCA1* to have a low mitotic rate (odds ratio (OR), 5.16; 95% CI, 1.91 to ∞). *BRCA1* cancers with loss of wt *BRCA1* were more likely to express basal cytokeratins CK 5/6 or 14 (OR 4.7; 95% CI, 1.85 to ∞).

Conclusions: We found no difference in the prevalence of loss of wt *BRCA1* between ER+ and ER- invasive *BRCA1*-associated breast cancers. Our findings suggest that many of the newer therapies for *BRCA1* breast cancers designed to exploit the *BRCA1* deficiency in these cancers may also be effective in ER+ cancers that develop in this population.

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Introduction

Sixty-four to 90% of breast cancers that occur in *BRCA1* mutation carriers (*BRCA1* carriers) are estrogen receptor-negative (ER-), progesterone receptor-negative (PR-) and lack HER2 protein overexpression and gene amplification, so called "triple negative" breast cancers [1-8]. These *BRCA1*-associated ER- tumors typically demonstrate characteristic pathologic features which include high grade ductal histology, a high mitotic rate, a prominent lymphocytic infiltrate, pushing or circumscribed margins, and geographic areas of necrosis or a central fibrotic focus [3,9,10]. In addition, these tumors often express "basal" biomarkers and cluster within the "basal-like" group in gene expression profiling studies [7,11-13].

Since *BRCA1* cancers are so often ER-, it has been suggested that ER negativity is intrinsic to *BRCA1* cancers and reflects the cell of origin of these tumors [14]. Preclinical models suggest that *BRCA1* can transcriptionally induce ER gene expression and that loss of *BRCA1* function is accompanied by loss of ER expression [15-17]. However, approximately 10 to 36% of breast cancers that occur in *BRCA1* carriers are estrogen receptor-positive (ER+) [4,6,8,18,19]. Further, as *BRCA1* carriers age, they are increasingly more likely to develop an ER+ breast cancer [14,20] following the trend seen in breast cancers that develop in the general population. It has, therefore, been suggested that ER+ *BRCA1*-associated breast cancers may actually be incidental or sporadic rather than caused by a complete loss of *BRCA1* function.

We have previously shown that the pathologic features of ER+ invasive breast cancers that arise in *BRCA1* carriers are significantly different than age-matched sporadic ER+ breast cancers in non-mutation carriers. When compared to sporadic ER+ cancers, ER+ *BRCA1*-associated cancers are more often of invasive ductal type and exhibit a high mitotic rate [20]. With the development of treatments such as poly(ADP-ribose) polymerase (PARP) inhibitors that are targeted to the specific defects in DNA repair pathways which exist in *BRCA1* deficient cancers [21], it is important to determine whether ER+ breast cancers that develop in *BRCA1* mutation carriers are incidental (that is, not directly related to the *BRCA1* mutation/*BRCA1* dysfunction) or if they are mutation-related in order to determine whether such *BRCA1*-targeted therapies might be effective in this population.

One way to address this issue is to analyze ER+ cancers that arise in *BRCA1* mutation carriers for loss of the wild type (wt) *BRCA1* allele. Several recent studies evaluating the prevalence of loss of heterozygosity (LOH) in *BRCA1*-associated breast cancers have noted that 50 to 90% of these cancers show LOH, with loss of wt *BRCA1* [22-24]. However, none of these studies was designed to specifically evaluate loss of wt *BRCA1* in relation to ER status in *BRCA1*-associated cancers. Therefore, we undertook a study to 1) determine the prevalence of loss of heterozygosity with loss of the wt *BRCA1* allele in ER+ cancers from *BRCA1* mutation carriers and compare it to that found in ER- *BRCA1*-associated cancers; and 2) determine whether any clinical factors (that is, age at diagnosis), pathologic features or biomarkers predict for loss of wt *BRCA1* in *BRCA1*-associated breast cancers.

Materials and methods

A series of 51 ER+ and 47 ER- invasive breast cancers was assembled from 88 women with deleterious *BRCA1* germ-line mutations who had undergone genetic testing at five high risk genetic programs.

Age at diagnosis of the breast cancer and determination of whether the cancer was a first or subsequent cancer for the patient was determined from medical record review. Specific *BRCA1* mutations were confirmed by review of genetic test reports.

Histologic sections of *BRCA1*-associated ER- and ER+ breast cancers were reviewed by the study pathologists blinded to the ER status of the tumor prior to the determination of LOH status. Each cancer was scored for the following pathologic features: histologic type; Nottingham combined histologic grade, with each of the three components of grade (that is, tubule formation, nuclear grade and mitotic rate) recorded separately; presence of geographic necrosis or fibrotic focus; extent of lymphocytic infiltrate; and tumor margin characteristics (invasive or pushing).

Information regarding ER, PR and HER2 status, assessed as part of the routine clinical evaluation, was abstracted from institutional pathology reports. When information regarding the ER, PR or HER2 status for *BRCA1* tumors was missing from the pathology report, paraffin blocks were re-cut and sections were immunostained for ER (SP1 antibody, Neomarkers, Fremont, CA, USA), PR (PgR 636 antibody, Dako, Carpinteria, CA, USA) and HER2 (A085 antibody, Dako). For ER- tumors, ER expression was re-evaluated if the pathology report did not state that an appropriately staining internal positive control was present. Similarly, ER+ tumors with reports stating "faint" or "low" ER staining were re-evaluated for ER expression. If greater than 1% of breast cancer cells stained for ER, the tumor was considered ER+. If fluorescence *in situ* hybridization (FISH) for HER2 was available for a cancer, this result was used rather than the IHC result.

Tissue microarrays were constructed by obtaining three 0.6 mm cores from the formalin-fixed paraffin

embedded blocks of each *BRCA1* cancer and placing them in recipient paraffin blocks. Slides cut from the TMAs were immunostained for cytokeratin (CK) 5/6 (D5/16B4 antibody, Dako), CK14 (LL02 antibody, Neomarkers), CK7/8 (Cam5.2 antibody, BD Biosciences, San Jose, CA, USA), CK18 (DC10 antibody, Dako) and CK19 (RCK108 antibody, Dako) as well as for EGFR (EGFR pharmDX kit, Dako) and p53 (DO-7 antibody, Dako).

This study was approved by the institutional review boards of Dana Farber/Harvard Cancer Center and North Shore Medical Center.

Assessment of *BRCA1* LOH

Representative slides from each cancer were reviewed by a study pathologist and areas of normal tissue as well as invasive cancer were identified. Cells were isolated from hematoxylin and eosin stained tissue sections by laser capture microdissection. Approximately 2,000 pulses were used for each microdissection. As higher levels of normal cell contamination will reduce the proportion of mutant allele that is measured in a tumor with true LOH, for each tumor we attempted at least two microdissections performed at the same general region. For each case, an area of normal non-tumor tissue present in the same block as the cancer was microdissected to obtain germline DNA. The microdissected cells were lysed to release DNA by overnight digestion with proteinase K, followed by a second round of proteinase K digestion, and then heat inactivation was performed.

Screening for LOH was carried out by polymerase chain reaction (PCR) followed by Sanger dideoxy sequencing [25] or missense and nonsense alterations, while denaturing capillary analysis was performed for insertions and deletions. Exon specific oligonucleotide primers flanking known *BRCA1* mutations were designed using Primer3 software [26] and limited to a maximum product size of 200 base pairs in order to successfully amplify sheared DNA extracted from paraffin embedded tissue (sequences listed in Additional file 1). PCR primers were ordered [27] with universal sequence tags at the 5' end in order to allow for secondary amplification, sequencing with a universal primer and elimination of primer dimers.

For cancers with missense and nonsense mutations, bidirectional DNA sequencing in triplicate was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and universal primers. Purified sequencing products were analyzed in 3730xl DNA Analyzers (Applied Biosystems). Sequence data from triplicate forward and reverse reactions from each micro-dissected tumor and normal surrounding tissue were analyzed using the Mutation Surveyor program version 3.30 (SoftGenetics, State College, PA, USA). The NM_Score is a value assigned to each nucleotide in a sequencing chromatogram. This score is used to determine mutant allele contribution based on shape and height at each position in the sequence. Normal (that is, non-cancerous) tissue DNA from *BRCA1* carriers is generally heterozygous for *BRCA1* mutation, and therefore sequence NM_scores are less than 1.5 indicating a significant difference from homozygous wild-type. In tumors with LOH and loss of the wild type allele, the difference from homozygous wild-type becomes even more significant as the NM_score decreases further towards 0.

Analysis of LOH in tumors with insertions or deletions in *BRCA1* was performed by taking advantage of the difference in size of the wild type and mutant alleles. Primary amplification was performed using the same conditions described for sequencing analysis above. Secondary PCR amplification was used to add a 6-FAM fluorescent label onto the 5'-end of the primary PCR products. Reaction products were then analyzed on ABI3730XL (Applied Biosystems) instruments using POP7 denaturing polymer (Applied Biosystems). The relative height of the wild type and mutant peaks was measured using Dax software (Van Mierlo Software Consultancy, Eindhoven, NL) and was used to indicate LOH. An example of the data for 187delAG analysis is shown in Additional file 2. Further details of the methodology used for LOH determination are included in Additional file 3[28-30].

Analysis of *BRCA1* promoter methylation

Tumor DNA was subjected to bisulfite modification using EZ DNA methylation-gold kit (Zymo Research, Orange, CA, USA). Lymphocyte DNA treated with Sss bacterial methylase (New England BioLabs, Ipswich, MA, USA) was used as a positive control. Lymphocyte DNA treated with bisulfite served as a negative control. Bisulfite treated tumor DNAs were analyzed by methylation specific PCR for *BRCA1* promoter as in Matros *et al.* [31]. The methylation assay evaluates CpG sites within the *BRCA1* promoter at positions -37, -29, -21, -19 from the start site. PCR products were resolved by 3% agarose gel electrophoresis.

Statistics

Determination of cutoff values for LOH

Tumors with insertion/deletion mutations The percentage of mutant *BRCA1* DNA (mutant *BRCA1* DNA/ mutant plus wt *BRCA1* DNA; m%) was determined by comparing peak heights specific for normal and mutant alleles determined from DNA extracted from each microdissected sample of normal tissue and tumor tissue. PCR reactions were run in triplicate for each microdissected normal and tumor tissue sample and those samples with three successful amplifications were

retained for further consideration. Coefficients of variation (CV) of the triplicate m% and 100-m% values were calculated for each microdissected sample and those with a CV greater than 20% were not considered further. For each microdissected sample, the average m% from the triplicate PCR reactions was determined. If multiple microdissected samples from a single tumor all showed acceptably low m% CV's then the results from the separate microdissections were averaged to give a single average m% for that tumor or normal specimen. Determination of LOH for *BRCA1* was guided by the assumption that the majority of normal tissue specimens should not have LOH, though there are data that some lobules from normal appearing breast tissue obtained from *BRCA1* carriers undergoing prophylactic mastectomies can have LOH with loss of wt *BRCA1* [32]. Two different strategies were adopted to determine the optimal m% cut-off for a determination of LOH. First, logistic regression was used to optimally discriminate between tumor and normal tissue yielding cut-off values for LOH. For this computation all specimens were assumed to be independent. In the second approach, the range of average m% values was determined for all the adjacent normal tissues dissected from tumors with insertion or deletion mutations. The 95% CI for average m% for normal tissue was determined within two standards of deviations. Tumors with an average m% above the upper limits of this 95% CI were determined to have LOH with loss of wt *BRCA1* and tumors with average m% below the limits of the 95% CI were determined to have LOH with loss of the mutant *BRCA1* allele (LOH^{mut}). Both methods yielded virtually identical results. An average m% > 60 was considered to represent LOH with loss of wt *BRCA1* and m% < 40 was considered to represent LOH^{mut} in the 59 cancers with small insertions or deletions.

The 1294del40 mutation results in a 40 bp deletion. The significant difference in size between the PCR products from the normal allele vs. the deletion mutant allele results in preferential amplification of the smaller mutant allele and a skewed ratio of mutant to normal PCR products. Therefore, the determination of LOH cut-off was performed separately for the 1294del40 mutations using the same process described above considering both the distribution of m% in normal 1294del40 heterozygous tissues as well as insight from logistic regression. An average m% of > 85 was established as an appropriate cut-off for determining loss of wt *BRCA1* for the 1294del40 cases.

Tumors with missense and nonsense mutations For cases with missense and nonsense mutations, the NM_Score (generated by the Mutation Surveyor program) was used to determine LOH. NM_scores from the forward sequencing triplicate data and reverse sequencing triplicate data were averaged separately for each case due to base and strand specific variation in the score. Samples with forward and reverse triplicate NM_score measurements showing a CV < 20% were included. Multiple microdissected samples from a single tumor with acceptably low NM_score CV's were averaged to give a single average NM_score for that case. The same process described above using logistic regression as well as 95% CI for normal tissue NM_score was used to determine the LOH cutoff for the 14 cancers with missense or nonsense mutations. An NM_score of < 0.6 was deemed to represent LOH with loss of wt *BRCA1* and NM_score > 1.4 was interpreted as LOH^{mut}.

Predictors of loss of wt *BRCA1*

The Wilcoxon Rank Sum test was used to determine *P*-value differences in mean age between *BRCA1* first invasive breast cancers with and without loss of wt *BRCA1*. The two-sided Fisher exact test was used to determine differences in the frequency of variables between cancers with and without loss of wt *BRCA1* on univariate analysis. The Bonferroni calculation identified *P* < 0.003 as significant after correction for multiple comparisons. In the multivariate analyses, exact logistic regression was used to evaluate the association between loss of wt *BRCA1* and the variables found significant in the univariate analyses. For these analyses, the cases with LOH and loss of wt *BRCA1* were compared to the combined group of cases showing either no LOH or LOH^{mut}.

Results

Prevalence of loss of wt *BRCA1*

Ninety-eight *BRCA1*-associated ER+ and ER- invasive breast cancers were used for this analysis. Of the 98 ER+ and ER- breast cancers from women with *BRCA1* mutations, the status of the wt *BRCA1* allele could not be obtained for 21 cancers (12 ER- and 9 ER+): in 4 cancers (1 ER- and 3 ER+), microdissection for isolation of a pure population of tumor cells was not feasible due to the intimate admixture of single tumor cells and normal cells; in 10 cancers (8 ER- and 2 ER+) PCR failed; in 7 cancers (3 ER- and 4 ER+) the results from triplicate PCR were too variable (that is, CV > 20%) for inclusion in analysis.

Thus, reliable determination of the status of the wt *BRCA1* allele was possible in 77 *BRCA1*-associated breast cancers (42 ER+, 35 ER-). Of these cancers 34/42 (81.0%) ER+ and 31/35 (88.6%) ER- had LOH with loss of the wt *BRCA1* allele; the difference in frequency of loss of wt *BRCA1* between ER+ and ER- cancers was not significant (*P* = 0.53).

Six cancers (three ER+ and three ER-) with insertion/deletion mutations had m% < 40% and one cancer (ER+) with a point mutation had an NM score > 1.4

consistent with LOH with loss of the mutant *BRCA1* allele. In sporadic breast cancers, particularly in ER- cancers, loss of heterozygosity involving large regions of chromosome 17, including the *BRCA1* locus, is seen in 49 to 57% of cases [33-35]. In the *BRCA1*-associated cancers in this study, LOH with loss of either the wt or mutant *BRCA1* allele was observed in 97% of ER- and 90% of ER+ cases. If LOH involving this region was unrelated to the *BRCA1* mutation, loss of the wt and mutant alleles would be expected to occur with equal frequencies. As shown in Additional file 4, the observed frequencies of loss of the wt and mutant alleles, determined by chi-square test, are significantly skewed toward loss of wt *BRCA1* ($P < 0.001$). There was no relationship between ER status and distribution of which allele was lost by LOH ($P = 0.81$). This result demonstrates the non-random relationship between LOH and selection for loss of wt *BRCA1* in both ER+ and ER- *BRCA1*-associated tumors.

The distribution of m% values for normal and tumor samples (both ER+ and ER-) from insertion/deletion analysis are shown in Figure 1a. The mean and standard deviation of percent mutant allele from analysis of normal tissues was 50% ± 8.3%, indicating the expected proportion of mutant and wt alleles in heterozygous samples. The distribution of retained NM_scores from normal and ER+/ER- tumor samples with missense or nonsense mutation are shown in Figure 1b. The distributions for m% and NM_score for ER+ and ER- *BRCA1*-associated cancers were roughly similar. Several cases (seven ER- and two ER+) were noted to have an m% between 60 and 80%. Assuming a pure tumor sample, this intermediate level of mutant allele may indicate intratumoral heterogeneity for LOH, with an admixture of tumor cells with and without LOH. Alternatively, intermediate m% values may represent samples with slightly more normal cell contamination as an absolutely pure tumor sample is not always attainable, even with microdissection. Without performing an *in situ* LOH assay, it is not possible to distinguish these alternative explanations for any differences in distribution observed between ER+ and ER- cancers.

While the spectrum of *BRCA1* mutations was varied in this group of 77 cancers, 57% of the mutations were Ashkenazi mutations (Additional file 5) reflecting the significant Jewish population in the communities served by the participating hospitals. Fifty-nine cancers occurred in women with a small *BRCA1* insertion or deletion mutation, four additional women had a large 40 base-pair deletion (1294del40), seven had nonsense point mutations and three had a splice site mutation resulting in an in-frame deletion of an exon. Only four women had missense point mutations. Of the 12 cancers without loss of wt *BRCA1*, 10 (4 ER-, 6 ER+) had

protein truncating lesions and 2 (both ER+) had a splice site mutation resulting in an in-frame deletion (Additional file 5). Specific mutations for each cancer as well as the percentage of mutated *BRCA1* DNA (m%), NM_score, and ER status for each cancer is shown in Additional file 5.

Clinical, pathologic and immunohistochemical findings
All BRCA1-associated breast cancers (ER+ and ER- combined)

When pathologic features and biomarkers expression of all *BRCA1*-associated cancers (ER+ and ER- combined) were analyzed according to status of loss of wt *BRCA1* allele, in univariate analysis, breast cancers with loss of wt *BRCA1* were more likely to be of pure invasive ductal type ($P = 0.043$), to be histologic grade 3 ($P = 0.001$), and to have a higher mitotic rate ($P < 0.001$) (Table 1). Cancers with loss of wt *BRCA1* were also more likely to exhibit expression for CK5/6 ($P < 0.001$), CK14 ($P = 0.023$) or either CK5/6 or CK14 ($P < 0.001$) (Table 1). Cancers with loss of wt *BRCA1* were more likely to express either CK5/6 or CK14 even after Bonferroni adjustment for multiple comparisons and in multinomial logistic regression (odds ratio (OR), 4.7; 95% CI, 1.85 to ∞).

HER2 protein overexpression and/or gene amplification was found in four of the 77 *BRCA1*-associated breast cancers (one by IHC; three by FISH). Two of the three HER2 FISH amplified tumors did not show loss of wt *BRCA1*. The HER2 overexpressing tumor identified by IHC did demonstrate LOH for wt *BRCA1*; however, material was not available to analyze this case for HER2 gene amplification by FISH.

We also compared the frequency of pathologic features and biomarker expression of the 12 ER- and 9 ER + *BRCA1*-associated cancers for which wt *BRCA1* allele status could not be determined to ensure that this group was similar to those cancers which were successfully subjected to LOH analysis. No significant differences in any of the pathologic features or biomarkers were identified between the groups with and without successful *BRCA1* LOH analysis.

ER+ BRCA1-associated cancers

The age at which first ER+ breast cancers developed was analyzed according to wt *BRCA1* allele status. The mean age at diagnosis was 45.2 years for the 24 ER+ first breast cancers with loss of wt *BRCA1*, compared to 50.1 years in those 8 ER+ first cancers that retained a wt *BRCA1* allele. This difference was not statistically significant ($P = 0.51$) (Table 2).

The pathologic features of the ER+ *BRCA1* cancers with ($n = 34$) and without ($n = 8$) loss of wt *BRCA1* are compared in Table 2. In univariate analysis, those ER+ *BRCA1* cancers retaining wt *BRCA1* more often had a low mitotic rate (100% vs 27%; $P < 0.001$), were less
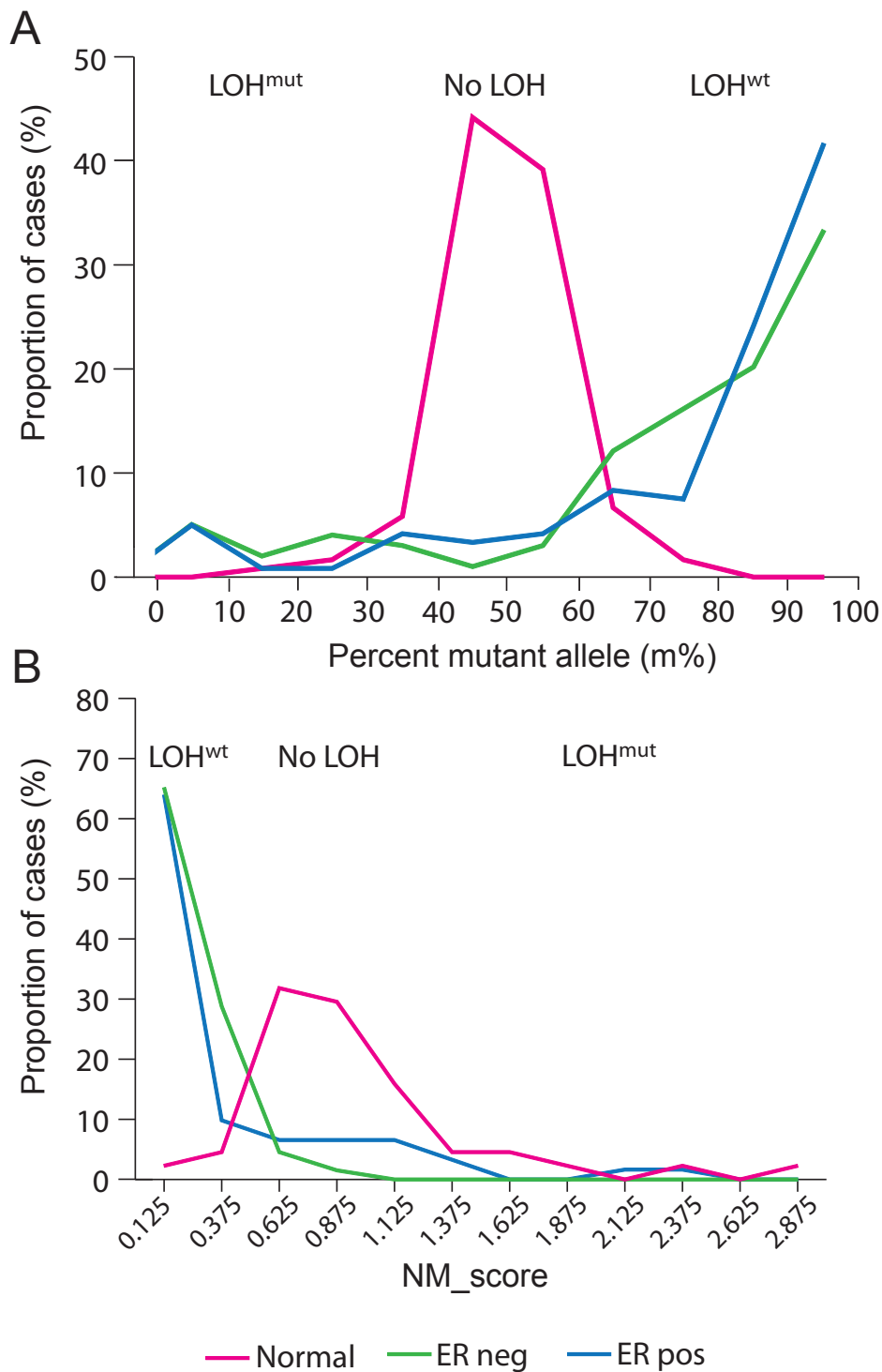
Figure 1 The distributions of the percent mutant allele are similar for ER+ and ER- *BRCA1*-associated breast cancers. The frequency distributions of percent mutant allele (m% values) for cases with insertion or deletion mutations in *BRCA1* but excluding 1294 del40 cases **(a)** and the frequency distributions of NM_scores for cases with missense or nonsense point mutations **(b)** are shown for adjacent normal tissue samples (pink), ER+ tumor samples (blue), and ER- tumor samples (green). Distributions are calculated using all triplicate measurements from those samples passing quality assessment (that is, CV < 20%). In panel A, higher m% values indicate LOH with loss of wt *BRCA1* allele (LOH[wt]) and lower values indicate LOH with loss of mutant *BRCA1* allele (LOH[mut]). For panel B, lower NM_scores indicate loss of wt *BRCA1* and higher NM_scores indicate loss of mutant *BRCA1*.

Table 1 Pathologic and immunohistochemical characteristics that were significantly different in *BRCA1*-associated breast cancers with and without loss of wt *BRCA1*

Variables	Loss of wt *BRCA1* N(%)	No loss of wt *BRCA1* N(%)	*P*-value
Sample Size	65	12	
Histology			
Ductal*	59 (90.8)	8 (66.7)	**0.043**
Ductal/Lobular	4 (6.1)	3 (25)	
Other	2 (3.1)	1 (8.3)	
Mitoses/10 high power field			
0 to 5*	10 (15.4)	8 (66.7)	**< 0.001***
6 to 10	14 (21.5)	0	
> 10	41 (63.1)	4 (33.3)	
Grade			
1 (3 to 5 points)	5 (7.7)	5 (41.7)	
2 (6 to 7 points)	7 (10.8)	3 (25)	
3 (8 to 9 points)*	53 (81.5)	4 (33.3)	**0.001***
CK5/6	58	11	
Positive	29 (50)	0 (0)	**< 0.001***
Negative	29 (50)	11 (100)	
CK14	58	9	**0.023**
Positive	23 (39.7)	0	
Negative	35 (60.3)	9 (100)	
CK5/6 or CK14	60	12	**< 0.001***
Positive	35 (58.3)	0 ((0)	
Negative	25 (41.7)	12 (100)	
CK5/6 and/or CK14 and/or EGFR	61	12	**0.023**
Positive	39 (63.9)	3 (25)	
Negative	22 (36.1)	9 (75)	

Abbreviations: ER, estrogen receptor; CK, cytokeratin; EGFR, epidermal growth factor receptor.
Loss of wt: cancers with LOH with loss of wt *BRCA1* allele.
No loss of wt: includes cancers with no LOH and LOH[mut].
P-value retained significance with Bonferroni adjustment for multiple comparisons.
Percentages calculated by column.

often of pure ductal histology (50% vs 85%; *P* = 0.05), and less often grade 3 (0% vs 68%; *P* < 0.001). The differences in mitotic rate and grade retained significance even after Bonferroni adjustment. In multinomial logistic regression, ER+ *BRCA1*-associated cancers retaining wt *BRCA1* were significantly more likely than cancers without wt *BRCA1* to have a low mitotic rate (OR, 5.16; 95% CI, 1.91 to ∞).

Results of biomarker studies were available for most of the *BRCA1*-associated cancers (Table 3). None of the eight ER+ cancers that retained wt *BRCA1* showed expression of basal cytokeratins CK5/6 or CK14. In contrast, 11 of 33 ER+ cancers with loss of wt *BRCA1* (33.3%) showed expression for one or the other of these basal cytokeratins. However, this difference was not statistically significant, possibly due to the small number of cases.

ER- *BRCA1*-associated cancers

In ER- cancers, the mean age at diagnosis of first invasive cancers with or without loss of wt *BRCA1* was 42 years and 33.5 years, respectively. This difference was not statistically significant (*P* = 0.15) (Table 2).

No significant histopathologic differences were noted between the 31 ER- cancers with loss of wt *BRCA1* and the 4 ER- cancers with retention of wt allele. Like the ER- cancers with loss of wt *BRCA1*, the four ER- cancers retaining wt *BRCA1* were high grade ductal cancers with a high mitotic rate. There were also no significant differences between tumors with or without loss of wt *BRCA1* with regard to those features considered to be characteristic of ER- *BRCA1*-associated cancers, including geographic necrosis, pushing margins, and moderate-marked lymphocytic infiltrate (Table 2).

There were significant differences in the frequency of expression of basal cytokeratins CK5/6 and CK14 in ER- cancers with and without loss of wt *BRCA1* in univariate analysis (Table 3). Expression of either CK5/6 or CK14 was significantly more frequent in the 27 ER- cases with loss of wt *BRCA1* compared to the 4 cases without (89%

Table 2 Pathologic characteristics and their association with loss of wt *BRCA1* in ER-positive and ER-negative *BRCA1*-related breast cancers

Variable	ER+			ER-		
	Loss of wt *BRCA1* N(%)	No loss of wt *BRCA1* N(%)	*P*-value	Loss of wt *BRCA1* N (%)	No loss of wt *BRCA1* N(%)	*P*-value
Sample Size	34	8		31	4	
First cancers	24 (70.6)	8 (100)		28 (90.3)	2 (50)	
Mean age (range)	45.2 (29 to 68)	50.1 (33 to 72)	0.51	42 (28 to 73)	33.5 (31 to 36)	0.14
Second cancers	10 (29.4)	0		3 (9.7)	2 (50)	
Mean age (range)	48.7 (34 to 63)			56 (53 to 61)	57 (56 to 58)	
Histology						
Ductal*	29 (85.3)	4 (50)	**0.05**	30 (96.8)	4 (100)	1.00
Ductal/Lobular	3 (8.8)	3 (37.5)		1 (3.2)	0	
Other	2 (5.9)	1 (12.5)		0	0	
Mitoses/10 high power field						
0 to 5*	9 (26.5)	8 (100)	**< 0.001***	1 (3.2)	0	1.00
6 to 10	12 (35.3)	0		2 (6.5)	0	
> 10	13 (38.2)	0		28 (90.3)	4 (100)	
Grade						
1 (3 to 5 points)	5 (14.7)	5 (62.5)		0	0	
2 (6 to 7 points)	6 (17.6)	3(37.5)		1 (3.2)	0	
3 (8 to 9 points)*	23 (67.6)	0	**< 0.001***	30 (96.8)	4 (100)	1.00
Geographic Necrosis/Fibrotic Focus						
Yes	9 (26.5)	0	1.00	22 (71.0)	2 (50)	0.574
No	25 (73.5)	8 (100)		9 (29.0)	2 (50)	
Margins						
Invasive	29 (85.3)	8 (100)	1.00	15 (48.4)	2 (50)	1.00
Pushing/Circumscribed	5 (14.7)	0		16 (51.6)	2 (50)	
Lymphocyte Infiltrate						
Moderate/Severe	4 (11.8)	0	1.00	13 (41.9)	2 (50)	1.00
None/Mild	30 (88.2)	8 (100)		18 (58.1)	2 (50)	
PR						
Positive*	29 (85.3)	8 (100)	1.00	1 (3.6)	0	1.00
Positive	26 (76.5)	7 (87.5)		0		
Low Positive[a]	3 (8.8)	1 (12.5)		1 (3.6)		
Negative	5 (14.7)	0		27 (96.4)	4 (100)	
Unknown	0	0		3 (9.8)	0	
Her2/Neu IHC						
Positive	1 (2.9)	0	1.00	0	0	1.00
Negative	24 (70.6)	7 (87.5)		25 (80.6)	3 (75)	
Her2/Neu FISH						
Positive	1 (2.9)	1 (12.5)		0	1 (25)	
Negative	6 (17.6)	0		5 (16.1)	0	
Equivocal[d]	2 (5.9)	0		1 (3.2)	0	

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization.

Loss of wt: cancers with LOH with loss of wt *BRCA1* allele.

No loss of wt: includes cancers with no LOH and LOH[mut] .

Percentages calculated by column.

[a] Low PR positive: 1 to 10% cells show nuclear staining for PR.

[b] HER2 positive: HER2:CEP17 ratio by FISH > 2.2 or IHC 3+ (no FISH performed).

[c] HER2 negative: HER2:CEP17 ratio by FISH < 1.8 or IHC < 3+ (no FISH performed).

[d] HER2 equivocal: HER2:CEP17 ratio by FISH 1.8 to 2.2.

* *P*-value retained significance with Bonferroni adjustment for multiple comparisons.

Table 3 Immunohistochemical characteristics and their association with loss of wt *BRCA1* in ER-positive and ER-negative *BRCA1*-related breast cancers

Variable	ER+			ER-		
	Loss of wt *BRCA1* N(%)	No loss of wt *BRCA1* N(%)	*P*-value	Loss of wt *BRCA1* N(%)	No loss of wt *BRCA1* N(%)	*P*-value
Cytokeratins						
CK5/6	(31)	(7)	0.164	(27)	(4)	**0.010**
Positive	9 (29.0)	0		20 (74.1)	0	
Negative	22 (71.0)	7 (100)		7 (25.9)	4 (100)	
CK7/8	(31)	(8)	—	(28)	(4)	1.0
Positive	31 (100)	8 (100)		25 (89.3)	4 (100)	
Negative	0	0		3 (10.7)	0	
CK14	(31)	(6)	0.566	(27)	(3)	0.054
Positive	5 (16.1)	0		18 (66.7)	0	
Negative	26 (83.9)	6 (100)		9 (33.3)	3(100)	
CK5/6 or CK14	(33)	(8)	0.083	(27)	(4)	**0.001***
Positive	11 (33.3)	0		24 (88.9)	0	
Negative	22 (66.7)	8 (100)		3 (11.1)	4 (100)	
CK 18	(32)	(7)	——	(27)	(4)	1.00
Positive	32 (100)	7 (100)		26 (96.3)	4 (100)	
Negative	0	0		1 (3.7)	0	
CK19	(33)	(6)	——	(28)	(4)	0.125
Positive	33 (100)	6 (100)		28 (100)	3 (75)	
Negative	0	0		0	1 (25)	
EGFR	(30)	(7)	0.560	(26)	(4)	0.360
Positive	5 (16.7)	0		24 (92.3)	3(75)	
Negative	25 (83.3)	7 (100)		2 (7.7)	1 (25)	
CK5/6 and/or CK14 and/or EGFR	(33)	(8)	0.083	(28)	(4)	0.125
Positive	11 (33.3)	0		28 (100)	3 (75)	
Negative	22 (66.7)	8 (100)		0	1 (25)	
P53	(30)	(7)	1.00	(26)	(4)	0.601
Positive	14 (46.7)	4 (57.1)		13 (50)	3 (75)	
Negative	16 (53.3)	3 (42.9)		13 (50)	1 (25)	

Abbreviations: ER, estrogen receptor; CK, cytokeratin; EGFR, epidermal growth factor receptor.
Loss of wt: cancers with LOH with loss of wt *BRCA1* allele.
No loss of wt: includes cancers with no LOH and LOH^{mut} .
*P-value retained significance with Bonferroni adjustment for multiple comparisons.
Percentages calculated by column.

vs. 0%, *P* = 0.001). This difference was significant even after Bonferroni correction. In multinomial logistic regression, ER- cancers with loss of wt *BRCA1* were significantly more likely to show expression of CK5/6 or CK14 (OR 5.48; 95% CI 1.79 to ∞). There were no significant differences in EGFR or p53 expression between ER-cancers with and without loss of wt *BRCA1* (Table 3).

BRCA1 methylation
BRCA1 promoter methylation is a potential alternative mechanism to LOH for providing the "second hit" to inactivate wt *BRCA1* in *BRCA1*-associated cancers. *BRCA1* promoter methylation analysis was performed by methylation-specific PCR on 28 cases for which remaining tumor DNA was available, including 6 of the 8 ER+ and 3 of the 4 ER- cancers which did not demonstrate loss of wt *BRCA1*. Methylation was identified in a single tumor sample, an ER+ low-grade (tubular) carcinoma without genomic loss of wt *BRCA1*.

Discussion
In this study, we found that 81.0% of ER+ *BRCA1*-associated breast cancers showed LOH with loss of the wt *BRCA1* allele. The prevalence of loss of wt *BRCA1* in these ER+ tumors was similar to that seen in the ER- *BRCA1*-associated cancers (88.6%). This is the first study, to our knowledge, that has specifically examined loss of wt *BRCA1* in a large cohort of *BRCA1*-associated breast cancers in relation to ER status. Our results are consistent with those reported in previous smaller studies [22-24]. Only two of these prior studies

included any ER+ *BRCA1* cancers and reported loss of wt *BRCA1* in 75% and 83% of such cancers [22,24].

Only one cancer of the 28 evaluated in our study demonstrated *BRCA1* promoter methylation, an ER+ cancer with retention of wt *BRCA1*. These results are consistent with the lower cumulative methylation observed in *BRCA1*-associated cancers compared to sporadic cancers [36]. Our results are also comparable to those of Dworkin *et al.* who found that none of seven of their *BRCA1* cancers without LOH showed methylation as a "second hit" [22]. The one ER+ cancer demonstrating *BRCA1* methylation in our series was not typical of the ER+ cancers with loss of wt *BRCA1*, being a low grade (tubular) carcinoma with a low mitotic rate. Studies of sporadic breast cancers have not found reproducible associations between *BRCA1* promoter methylation and tumor phenotype [31,37]. Furthermore, somatic methylation may be related to increasing age in some cases [36,38]. *BRCA1* promoter methylation has been found in germline DNA in five to seven percent of individuals regardless of health or *BRCA1/2* status and showed no association with development of breast cancer [39]. Whether the *BRCA1* promoter methylation found in the tumor DNA of the one patient in our series is indicative of loss of wt *BRCA1* function is uncertain and its clinical significance is unclear.

Previously, we have described that ER+ *BRCA1*-associated cancers are more often high grade ductal cancers compared to age matched ER+ sporadic breast cancers [20]. Our current findings that ER+ cancers with loss of wt *BRCA1* are significantly more often higher grade cancers is a consistent extension of our original results. Interestingly, a recent study found that ER+ cancers which develop in *BRCA2* carriers are of higher grade than age matched ER+ sporadic cancers [40]. Combined with our data, it appears that loss of *BRCA1* or *BRCA2* function results in a more proliferative luminal cancer when an ER+ cancer develops.

It has been suggested that basal-like cytokeratin expression in triple negative tumors is a good predictor of *BRCA1* mutation status [7]. Rakha *et al.* [41] examined seventeen *BRCA1*-associated ER-, HER2- breast cancers and found that only one of seventeen (5.9%) did not show expression of either CK5/6 or EGFR, also considered to be a basal marker. To distinguish between those ER- *BRCA1*-associated breast cancers that did or did not have loss of the wt *BRCA1* allele, however, we found the combination of CK5/6 and CK14 most useful. Adding EGFR staining increased the sensitivity of identifying ER- cancers with loss of wt *BRCA1* (28/28; 100%), however it lowered the specificity as three of four of the ER- cancers without loss of wt *BRCA1* (75%) stained for EGFR.

Immunostains were less helpful in distinguishing ER+ cancers with and without loss of the wt *BRCA1* allele. It is noteworthy that none of the ER+ cancers that retained wt *BRCA1* expressed CK5/6 or 14, while the only ER+ cancers expressing these basal cytokeratins had lost wt *BRCA1*. However, as the majority of the ER + cancers with loss of wt *BRCA1* did not express either basal cytokeratin, this difference was not significant. Larger studies are necessary to explore the possibility that basal epithelial markers may mark ER+ cancers that have lost wt *BRCA1*.

Our results regarding HER2 overexpression/gene amplification in *BRCA1*-associated cancers are consistent with prior studies that have found that HER2 overexpression and amplification are uncommon in these tumors [5,6,42]. In the 77 *BRCA1* cancers we studied, only one *BRCA1*-associated cancer with loss of wt *BRCA1* demonstrated HER2 gene amplification by FISH.

There may be mechanisms other than LOH or methylation by which the wt *BRCA1* allele is inactivated in *BRCA1*-associated cancers which were not examined in this study (for example, somatic mutation of the second allele elsewhere in the *BRCA1* gene or ID4 modulation of *BRCA1* expression [37,43]). However, significant phenotypic differences were observed between ER+ cancers with or without loss of wt *BRCA1*. Assuming phenotype is linked to gene function, these phenotypic differences suggest that ER+ cancers with a wt *BRCA1* allele are likely to also have retained *BRCA1* function and have not inactivated wt *BRCA1* by an alternative mechanism.

It has been previously reported that ER+ breast cancers are more likely to develop in *BRCA1* carriers as they age, suggesting that some of these may be incidental breast cancers occurring in *BRCA1* carriers. However, we did not see a significant difference in age at diagnosis between first ER+ breast cancers with and without loss of wt *BRCA1*. In addition all 10 ER+ second cancers that developed in *BRCA1* carriers demonstrated loss of wt *BRCA1*. It is possible that these findings are due to limited numbers, but it is also possible that some mechanism other than incidental development of breast cancer, with functioning wt *BRCA1*, is needed to explain why the development of ER+ breast cancers is more common as *BRCA1* mutation carriers age.

Further, it is apparent that the presence of wt *BRCA1* is not required for ER expression in cancer tissues, in contrast to what has been suggested by some preclinical studies [16]. Other studies have proposed wt *BRCA1* is essential for differentiation of mammary stem cells to ER+ luminal cells and that loss of wt *BRCA1* causes an expansion of ER-negative mammary stem cells, offering a mechanism for the common ER-negativity of *BRCA1* breast cancers [32]. However, this model does not address the origin of ER+ *BRCA1*-associated breast cancers. Another recent study has found expansion of a

committed luminal progenitor population, containing both ER+ and ER- cells, in preneoplastic tissues of *BRCA1* mutation carriers and proposed the luminal progenitor cells as the cell of origin of *BRCA1*-associated cancers [44]. In mouse models of tumorigenesis produced by deletion of *BRCA1*, the expression of ER in the resulting tumors appears to depend on whether *BRCA1* is deleted at an earlier or later stage of cell differentiation [32,45,46]. Our findings are consistent with these models and suggest that *BRCA1*-deficient ER+ tumors may derive from *BRCA1* loss in an ER-positive luminal progenitor cell.

This study cannot resolve whether ER+ breast cancers without loss of wt allele that develop in *BRCA1* carriers are equivalent to ER+ sporadic breast cancers that occur in non-carriers. It is possible that no breast cancer that develops in a *BRCA1* mutation carrier is really "incidental" or sporadic even if one functioning wt *BRCA1* allele is retained and expressed. Haploinsufficiency of *BRCA1* may predispose both to the development of breast cancer as well as to a specific histopathologic and or immunohistochemical profile. However, testing for differences between *BRCA1*-associated ER+ cancers with a retained wt *BRCA1* and ER+ sporadic breast cancers would require larger numbers of these ER+ *BRCA1* cancers as well as age matched ER+ sporadic controls.

Conclusions

In conclusion, in this study of 77 *BRCA1*-associated breast cancers, we found similar frequencies of LOH with loss of wt *BRCA1* in ER+ and ER- breast cancers. In addition, loss of wt *BRCA1* results in higher grade ductal cancers with higher proliferative rates, and a greater propensity to express basal cytokeratins. Many of the new therapies being evaluated in *BRCA1* breast cancers, such as poly(ADP-ribose) polymerase (PARP) inhibitors and cisplatin [47,48], are designed to take advantage of the defect in homologous recombination that *BRCA1* deficiency causes in these cancers. The results of our study suggest that allele-specific LOH analysis to evaluate for loss of wt *BRCA1* is more likely to predict response to such therapies than estrogen receptor expression. In addition to family history and age of onset, identification of women who may carry a previously undetected *BRCA1* mutation has recently focused on the triple negative subset as a population enriched for *BRCA1* mutation carriers. Our results suggest that the high grade ER+ luminal cancers also may be enriched for tumors with *BRCA1 or BRCA2* deficiency. A concerted effort should be made to identify these women so that they are not deprived of potentially effective new therapies.

Additional material

Additional file 1: Sequence Primers. Sequences of primers used to amplify the regions surrounding each mutation analyzed in the study are provided.

Additional file 2: Indel Analysis by denaturing capillary electrophoresis. An example of loss of the wt *BRCA1* allele (LOHwt) in a tumor from a patient with a 187delAG mutation is provided.

Additional file 3: Supplementary methods. Additional information describing the methodology used to determine LOH is supplied.

Additional file 4: Distribution of allele loss in tumors with LOH. This table presents the observed compared to the expected frequencies of loss of the wt and mutant *BRCA1* alleles in ER+ and ER- *BRCA1* breast cancers analyzed.

Additional file 5: LOH result for ER+ and ER- *BRCA1*-associated breast cancers analyzed. For each breast cancer analyzed, the following associated features are presented: specific mutation; age at diagnosis; ER status; m% or NM_score; LOH result; and *BRCA1* promoter methylation.

Abbreviations

CI: confidence interval; CK: cytokeratin; CV: coefficient of variation; ER: estrogen receptor; FISH: fluorescence *in situ* hybridization; HPF: high powered field; IHC: immunohistochemistry; LOH: loss of heterozygosity; m%: mutant *BRCA1* DNA/mutant plus wt *BRCA1* DNA; NM_score, normal/mutant allele; OR: odds ratio; PARP: poly(ADP-ribose) polymerase; PCR: polymerase chain reaction; PR: progesterone receptor; TMA: tissue microarray; wt: wild type.

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Authors' contributions

NT wrote the manuscript (with AM and ALR), shared in study design, oversaw the collection of data and pathology material and reviewed the data. AM shared in the design of the study, performed the LOH analyses and shared in writing the manuscript. SJS shared in study design, reviewed (with LC) all pathology material, reviewed data and helped in editing the manuscript. SG performed statistical analyses; KF served as the study coordinator and data manager. JK aided in collection and review of

pathology material as well as analyses of IHC stains. JK, YY, AB, JYK, AS, RT and ZCW performed laser capture microdissection, DNA extraction and methylation studies. LC (with SS) reviewed all study pathology material. KK, RDL, JB, PDR and DS identified cases and supplied pathologic material. DPS helped in study design and manuscript editing. JEG helped in study design, identification of study material, and manuscript editing. ALR co-wrote the manuscript, oversaw microdissections and methylation studies, and shared in study design and data review.

Competing interests

The authors declare that they have no competing interests.

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