

X chromosomal abnormalities in basal-like human breast cancer

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Summary

Sporadic basal-like cancers (BLC) are a distinct class of human breast cancers that are phenotypically similar to *BRCA1*-associated cancers. Like *BRCA1*-deficient tumors, most BLC lack markers of a normal inactive X chromosome (Xi). Duplication of the active X chromosome and loss of Xi characterized almost half of BLC cases tested. Others contained biparental but nonheterochromatinized X chromosomes or gains of X chromosomal DNA. These abnormalities did not lead to a global increase in X chromosome transcription but were associated with overexpression of a small subset of X chromosomal genes. Other, equally aneuploid, but non-BLC rarely displayed these X chromosome abnormalities. These results suggest that X chromosome abnormalities contribute to the pathogenesis of BLC, both inherited and sporadic.

Introduction

Gene expression analysis has defined sporadic basal-like cancer (BLC) as a distinct subtype of human breast carcinoma that accounts for ~15% of breast cancer cases (Perou et al., 2000; Sorlie et al., 2003). These tumors are high-grade, aneuploid, invasive ductal carcinomas that do not express estrogen receptor (ER), progesterone receptor (PR), or HER2; display a high incidence of p53 mutations; and express specific cytokeratins characteristic of the basal layer of breast epithelium (Abd El-Rehim et al., 2004; Palacios et al., 2005). Basal-like carcinomas are reported to have a worse prognosis than other breast tumor subtypes (Sorlie et al., 2001). Furthermore, without ER or HER2, options for targeted drug therapy in these patients are limited.

BLC have been shown to overexpress both cyclin E (Foulkes et al., 2004) and Skp2 (Signoretti et al., 2002). However, whether these S phase-regulated genes are important to the pathogenesis of BLC or are merely associated with a high proliferation rate is unclear.

Most *BRCA1*^{-/-} breast carcinomas fail to express ER, PR, and HER2 and often carry p53 mutations (Lakhani et al.,

2002). Moreover, they cluster with sporadic BLC in analysis of expression array profiles (Sorlie et al., 2003). These findings suggest that *BRCA1*^{-/-} and sporadic BLC are biologically related entities. Whether the less common cases of non-BLC (ER- or HER2-positive) that occur in *BRCA1* mutation carriers are truly *BRCA1*^{-/-} is unknown. Conceivably, some are sporadic tumors that are unrelated to an abnormal *BRCA1* genotype.

BRCA1 operates in the maintenance of genomic integrity, chromatin remodeling, transcription regulation, and cell cycle checkpoint control (Scully and Livingston, 2000; Venkitaraman, 2002). Another suspected role for *BRCA1* is as a breast epithelial cell differentiation regulator, permitting transition from a "primitive," undifferentiated, "basal" phenotype to a "mature," luminal phenotype (Foulkes, 2004; Lane et al., 1995; Xu et al., 1999a). It also participates in the maintenance of a normal, inactive X chromosome (Xi) (Ganesan et al., 2002). Despite containing two X chromosomes, *BRCA1* mutant cell lines, as well as those *BRCA1*-deficient human and murine breast tumors that have been reported, lack an X chromosome decorated by XIST RNA, macrohistone H2A 1.2, and histone H3 methylated at lysine 27 (H3mK27).

SIGNIFICANCE

Basal-like cancers are a recently recognized subtype that accounts for 10%–15% of sporadic human breast cancer. This subtype tends to be highly aggressive with a poor prognosis. BLC are notable for the absence of ER and HER2 receptor expression, and targeted therapies are not currently available for these patients. The molecular mechanisms leading to this tumor subtype are poorly understood. We report that BLC, both sporadic and *BRCA1*-associated, consistently display X chromosome abnormalities and increased expression of a small set of X chromosome genes. Similar abnormalities were rare in non-BLC. These results provide new insight into possible pathogenic mechanisms underlying both sporadic and *BRCA1*-associated basal-like breast cancer.

Earlier studies suggested that a subset of sporadic human breast cancers bear Xi abnormalities. Recent results have pointed to loss of an intact Xi and extensive X chromosomal loss of heterozygosity (LOH) in some *BRCA1* wild-type (wt) breast cancer cell lines and primary cancers (Sirchia et al., 2005; Wang et al., 1990). In the few primary tumors tested, evidence of partial, interstitial, X chromosome LOH was found, but the global status of Xi in these cases is unclear, and the subtype of these tumors is unknown. Furthermore, with the karyotypic instability of cancer cell lines, one cannot be certain whether the Xi abnormalities reported in the above-noted cell lines were present in the original tumors or whether they developed in culture. However, it has long been known that some human breast cancers lack identifiable Barr bodies—morphologic markers of an intact Xi (Moore and Barr, 1957). It was also observed that “Barr body negative” breast cancers were likely to fail hormonal treatment (Perry, 1971; Rosen et al., 1977; Scholl et al., 1968) and, thus, were likely ER negative, a feature shared with BLC.

Given these findings, we asked whether sporadic breast cancers, BLC in particular, display X chromosome abnormalities. The relevant experiments were aimed at learning whether an Xi-associated abnormality constitutes part of the BLC disease mechanism.

Results

Most BLC lack markers of a normal, inactive X chromosome

Frozen sections from 18 sporadic, primary BLC specimens and 20 sporadic, high-grade, non-BLC specimens were analyzed by RNA fluorescence in situ hybridization (FISH) for XIST and by immunofluorescence (IF) for H3mK27 (Figure 1A; Table S1 and Figure S1 in the Supplemental Data available with this article online). We chose to study high-grade non-BLC to control for proliferation rate, aneuploidy, and a poorly differentiated state found in BLC. This non-BLC comparison group included both HER2-positive and/or ER-positive ductal carcinomas. Of 18 BLC specimens evaluated, 15 lacked normal, monofocal XIST staining in tumor cells but contained these structures in adjacent normal cells. The tumor cells in these cases also lacked distinct, focal nuclear H3mK27 staining that would normally mark Xi (Plath et al., 2003). By contrast, only 2 of 20 high-grade non-BLC revealed loss of focal XIST or H3mK27 staining (Table S1). The XIST/Xi and H3mK27 differences between these two tumor groups were significant ($p < 0.0001$). We further analyzed an additional five cases of low- or intermediate-grade breast cancers, including specialized subtypes of lobular and mucinous carcinoma. All five displayed normal XIST (Table S1) and H3mK27 localization (Figure S2). Of four breast carcinomas from *BRCA1* mutation carriers that were analyzed, three resembled BLC in that they did not express ER, PR, or HER2. The fourth (T151) was ER and HER2 positive, i.e., an uncharacteristic *BRCA1*-associated tumor with a non-basal-like phenotype. All four *BRCA1*-associated cases were negative for XIST/Xi and monofocal H3mK27 staining (Table S1; Figure S3).

Sporadic BLC are wt for *BRCA1* and reveal normal nuclear localization of *BRCA1* p220

To determine whether sporadic BLC have aberrant expression or localization of *BRCA1*, IF analysis was performed on frozen sections of the 18 sporadic BLC and 11 high-grade, non-BLC

controls using a p220 *BRCA1* monoclonal antibody. *BRCA1* punctate, nuclear immunoreactivity was detected in tumor cells in 16 of 18 BLC samples and in all 11 high-grade, non-BLC controls analyzed, but it was absent in the *BRCA1*-associated tumors (Table S1; Figure 2A). To rule out the possibility that sporadic BLC harbor rare mutations in *BRCA1* that would preserve *BRCA1* localization, genotype was determined in five sporadic BLC, four *BRCA1*-associated cases, and nine non-BLC controls. At least one wt *BRCA1* allele was retained in the sporadic BLC and non-BLC cases (Table S1), but no wt allele was present in the four *BRCA1*-associated tumors, including in T151 with the non-basal-like phenotype. Thus, most sporadic BLC differed from *BRCA1*-deficient tumors in that, where tested, they were genetically wt for *BRCA1*, synthesize p220 *BRCA1* protein, and localize it in the nucleus normally.

Abnormalities involving the X chromosome are frequent in BLC

Whole genome allelotype and DNA copy number analysis were performed on all 18 BLC and *BRCA1*^{-/-} tumors and on 20 high-grade non-BLC using Affymetrix 10K single nucleotide polymorphism (SNP) arrays (Figure 1B). The analysis revealed that 44% of sporadic BLC (8 of 18 tumors) displayed LOH involving the entire X chromosome, while retaining two copies of X chromosome DNA. Results of interphase DNA FISH on a subset of these tumors confirmed the presence of two X chromosomes in tumor cells (Figures 2Ba and 2Bb and Table S2). Since these tumors lack focal, nuclear accumulation of XIST RNA and H3mK27, which normally mark Xi, these data are consistent with the presence of X chromosome isodisomy arising from duplication of the active X chromosome (Xa) and loss of Xi in these cancers (Figure 3Aa).

Three additional BLC revealed whole X chromosome LOH with two copies of Xp and one copy of Xq, consistent with isodisomy of Xp and monosomy Xq (Figure 3Ab). Thus, 61% (i.e., 11) of 18 BLC had undergone LOH of the entire X chromosome associated with either complete X chromosome isodisomy or Xp isodisomy. Whole X chromosome isodisomy was found in only 2 of 20 high-grade non-BLC (Figure 1B). In an earlier study, X chromosome LOH was not observed in a similar-sized group of low- and intermediate-grade non-BLC (0 of 17) (Wang et al., 2004). Thus, X isodisomy appeared to be rare (2 of 37, 6%) in non-basal-like breast cancers analyzed, regardless of pathological grade.

Three BLC were free of significant X chromosome LOH and contained diploid X copy number by SNP array analysis (Figure 3Ac). This is consistent with retention of intact, biparental X chromosomes in these tumors. However, they lacked XIST/Xi and H3mK27/Xi staining, consistent with a significant alteration of the normal structure of Xi in these tumor cells. Another BLC contained one copy of Xp from each parent and isodisomy of Xq. This tumor was also negative for XIST/Xi and H3mK27 staining, consistent with loss of the transcriptionally active, XIST-encoding X inactivation center (located at Xq13) in this case (Figure 3Ad).

The other three BLC displayed proper, monofocal nuclear XIST and H3mK27 localization, consistent with the presence of an intact Xi. However, copy number analysis revealed that each had gained additional X chromosomal DNA (Figure 1B). One contained three X chromosomes (trisomy X; Figures 3Ae and 2Bc; Table S2), among which there was only a single Xi

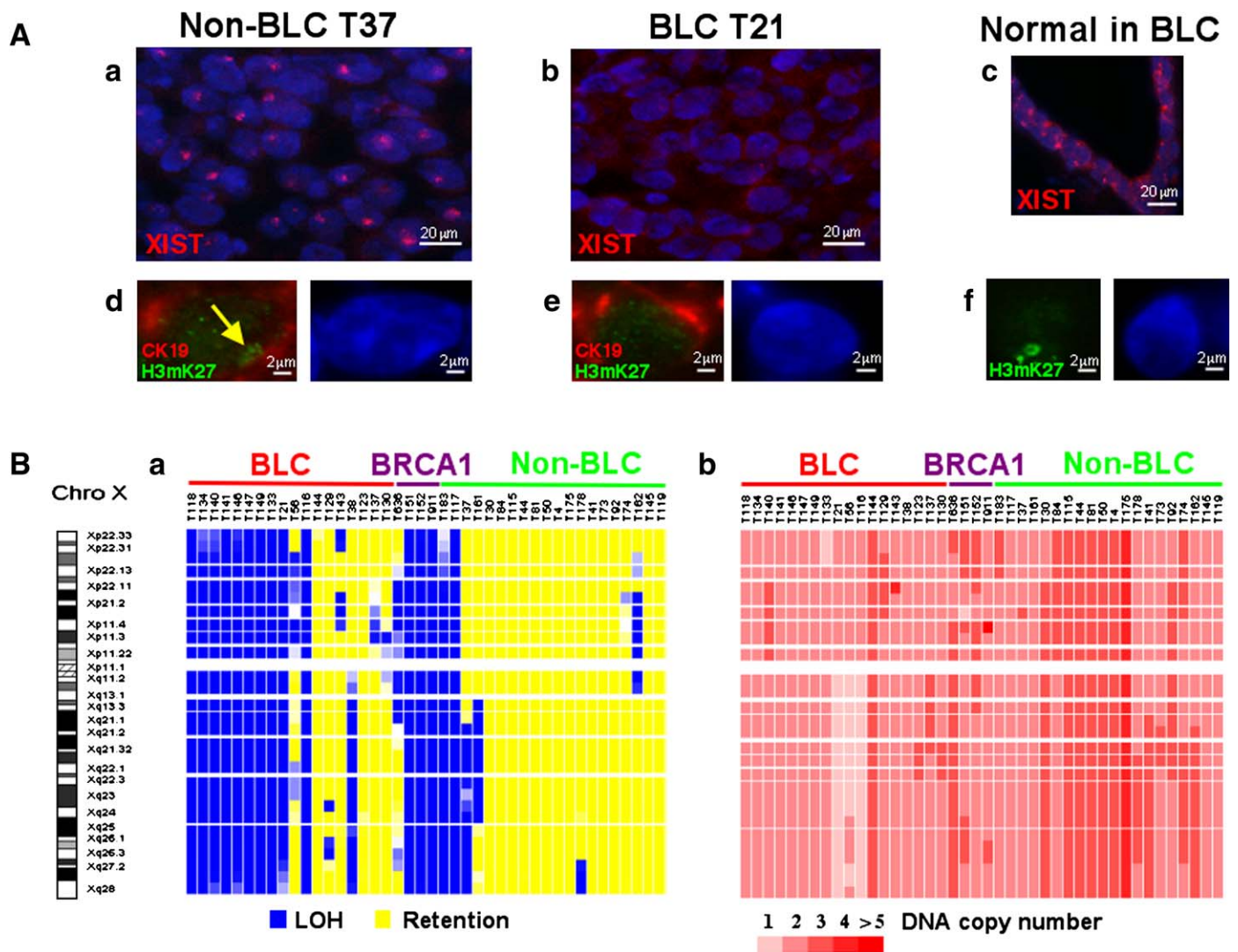


Figure 1. X chromosome analysis in BLC and non-BLC

A: BLC lack normal XIST and H3mK27 staining. FISH for XIST RNA (red) is shown for representative sections of non-BLC (**Aa**), BLC (**Ab**), and a normal duct in the BLC section (**Ac**); DAPI nuclear staining is blue. Tumor cells from a non-BLC (**Ad**), BLC (**Ae**), and a nontumor cell in the BLC section (**Af**) are shown stained for H3mK27 (green) and cytokeratin 19 (red). The yellow arrow points to the presumed Xi marked by focally enhanced H3mK27 staining. Cytokeratin 19, a keratin present in both BLC and non-BLC, is used to differentiate epithelial from stromal cells. DAPI nuclear staining is on the right in each case.

B: Analysis of 10K SNP array data obtained on microdissected BLC, BRCA1-deficient tumors, and non-BLC specimens. The left panel (**Ba**) shows LOH data for the X chromosome of the indicated tumors, with regions of LOH in blue, retention of heterozygosity in yellow, and noninformative regions in white. The panel on the right (**Bb**) shows the DNA copy number data for the same specimens, with increasing copy number indicated by increasing intensity of red.

identified by XIST and H3mK27 staining (Figure 3Ae; Figure S4, top panels). The others contained two X chromosomes that were biparental, along with a gain of additional Xp22 sequences (Figure 3Af). Whether these extra sequences are located on the active or inactive X, or elsewhere, is unknown. Of note, X trisomy was also detected in 5 of the 20 high-grade, non-BLC breast tumors that were analyzed. However, all five contained two inactive X chromosomes, as indicated by XIST and H3mK27 staining (Figure 3Bb and Figure S4, bottom panels), and therefore, only one active X chromosome.

A majority (three of four) of the BRCA1-deficient tumors that were studied displayed whole X isodisomy, including T151, which displayed the non-BLC phenotype. The fourth BRCA1-associated cancer, T636, displayed isodisomy over approximately half of the X chromosome (from Xp22 to Xq21) including

LOH of the XIST locus, but retained regions of heterozygosity at and near the Xp and Xq termini (Figure 1Ba). All four of these cases were negative for XIST and H3mK27 staining (Table S1 and Figure S3).

Thus, the tumor cells of all sporadic and BRCA1-associated BLC analyzed displayed an increase in the normally expected complement of nonheterochromatinized X chromosomal DNA. This arose by duplication of the active X chromosome and loss of Xi (61%; Figures 3Aa and 3Ab); by a major perturbation of the heterochromatic structure of one otherwise intact X chromosome—presumably the former Xi (22%; Figures 3Ac and 3Ad); or by gain of additional, presumably noninactivated X chromosomal territory (16%; Figures 3Ae and 3Af). These increases in nonheterochromatinized, X chromosomal DNA were rarely detected in the 20 cases of high-grade non-BLC

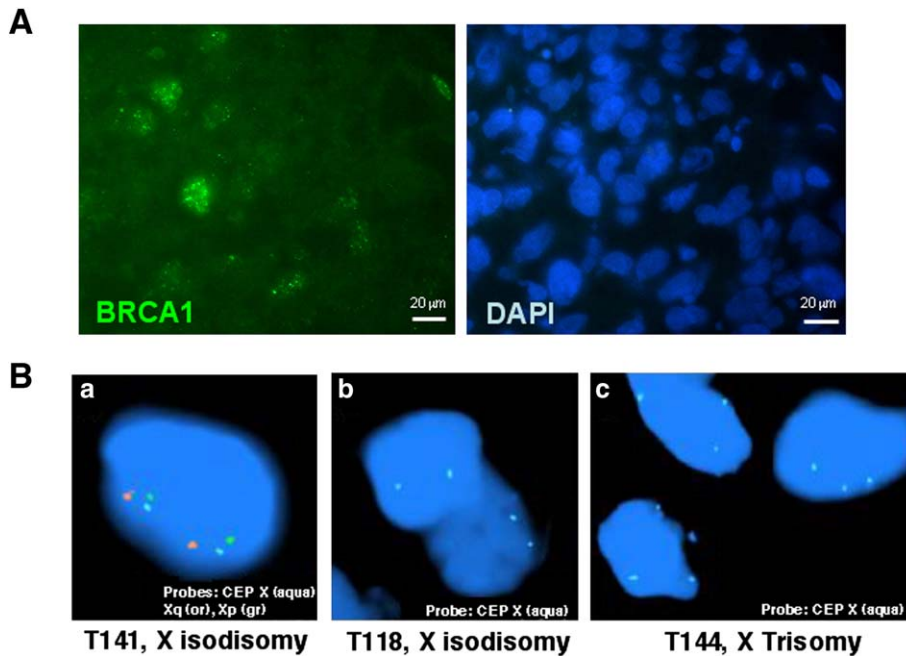


Figure 2. BRCA1 expression and X copy number in sporadic BLC

A: BRCA1 immunofluorescence using a monoclonal antibody against human BRCA1 is shown on the left for a representative frozen section of a BLC (T21). DAPI staining of nuclei is shown on the right.

B: X chromosome FISH using a probe for X chromosome centromere (aqua) on paraffin tissue sections of representative cases of BLC with X isodisomy (**Ba** and **Bb**) and a BLC with X trisomy (**Bc**). In panel **Ba**, two additional FISH probes directed against X chromosome q-arm (orange) and X chromosome p-arm (green) were used to detect subtelomeric segments of the X chromosome.

that were analyzed (Table S1). Furthermore, normal XIST and H3mK27 localization were seen in five of five low- or intermediate-grade non-BLC, including specialized breast carcinoma subtypes (Table S1 and Figure S2), and there was no evidence of an X chromosome abnormality in a prior analysis of lower-grade, non-basal-like breast cancer (Wang et al., 2004). Therefore, the gain of nonheterochromatinized X chromosomal DNA appears to be relatively specific to BLC.

BLC that lack normal nuclear XIST and H3mK27 staining also lack CpG island methylation of X-linked genes

CpG island methylation analysis of multiple X-linked genes normally subject to X chromosome inactivation (XCI) was performed on microdissected tumor cells in all eight cases of BLC with X isodisomy (cf. Figure 3Aa) and in four sporadic and one *BRCA*^{-/-} (T636) BLC with biparental but XIST- and H3mK27-negative X chromosomes (cf. Figures 3Ac and 3Ad). As expected, none of the BLC with X isodisomy revealed the presence of a methylated allele of the tested genes, unlike normal breast tissue (Figure 4). Surprisingly, BLC with retention of biparental X chromosomes but loss of XIST and H3mK27 staining lacked promoter-associated CpG island methylation at each of these genes (Figure 4). T636, the *BRCA*^{-/-} tumor with retained biparental X chromosome regions, also lacked CpG island methylation in X chromosome genes, including genes from the heterozygous regions (Figure S5). These data suggest a loss of methylation at these CpG islands on what was formerly Xi in the founder cell of these tumors.

Not surprisingly, BLC with intact XIST/Xi and H3mK27 staining and partial X chromosomal sequence gains revealed normal CpG island methylation in the tested genes, having each retained one Xi per cell (cf. Figures 3Ae and 3Af).

BLC also reveal frequent isodisomy of chromosomes 14 and 17

SNP array analysis of the BLC cohort also demonstrated whole chromosome 14 isodisomy in 10 of 18 tumors (56%) and whole

chromosome 17 isodisomy in 12 of 18 tumors (67%). Of the eight BLC with X isodisomy, seven revealed coexisting isodisomy of either or both chromosome 14 and 17. By contrast, only 1 of 20 (5%) non-BLC, high-grade tumors revealed chromosome 14 isodisomy, and only 4 of 20 (20%) revealed chromosome 17 isodisomy. In a previously reported study (Wang et al., 2004), whole chromosome 14 LOH was not observed in cases of low- or intermediate-grade non-BLC (0 of 17), although small, interstitial chromosome 14 LOH events were present in rare, intermediate-grade cases. Whole chromosome LOH of chromosome 17 was infrequent in low- and intermediate-grade non-BLC (2 of 17, 12%).

There was a low-level incidence of isodisomy of other autosomes in the BLC set, but the frequency was not significantly increased compared with that observed in high-grade non-BLC. Thus, whole chromosome LOH involving chromosomes X, 14, and/or 17 appeared to be relatively constant and specific characteristics of BLC.

BLC with X isodisomy do not reveal a global increase in X gene-encoded RNA

Nearly half of the 18 sporadic BLC analyzed contained two identical, noninactivated X chromosomes. One hypothetical outcome of such an occurrence is globally increased transcription of all expressed X chromosomal genes. To test for this possibility, RNA abundance was semiquantitatively analyzed, using Affymetrix U133 gene expression array data for the relevant tumors in the cohort and for normal breast tissue samples. A calculated global average of X chromosome-encoded RNA abundance was compared to a similar measure for each autosome within a given tissue set (normal or BLC). In normal breast tissue, the average and range of RNA levels were remarkably similar across all autosomes and the X chromosome (Figure 5Aa, green box). This suggests that the expression of normal X chromosome-encoded RNA, which, for most loci, originates from one gene copy on the active X chromosome, is up-regulated to reach average levels achieved by each autosome in

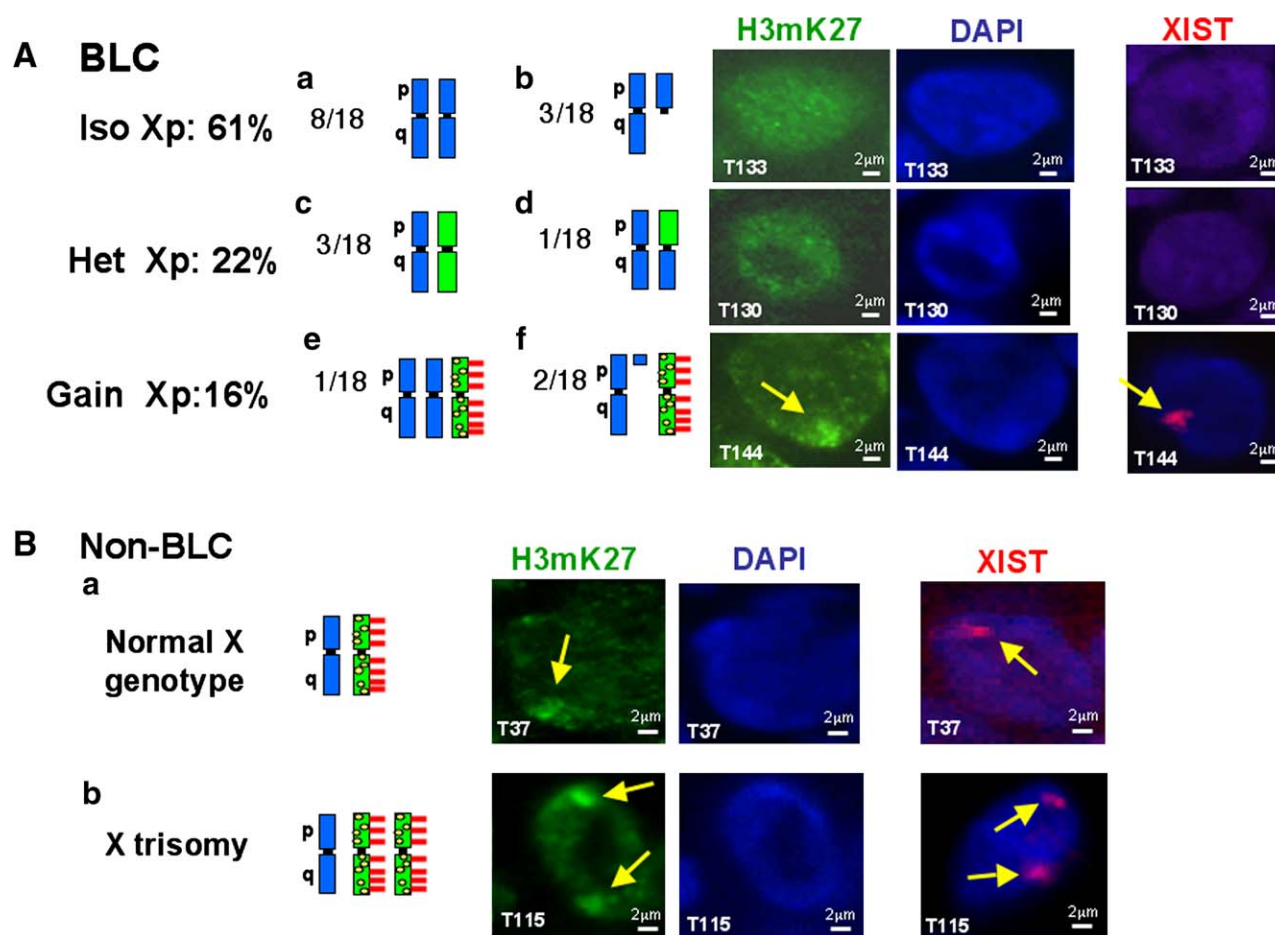


Figure 3. X chromosomal changes in BLC and non-BLC

A: Summary of X chromosomal karyotype in BLC. Cartoons of the different categories of X chromosomal alterations in BLC are shown on the left: **Aa**, whole X chromosome isodisomy; **Ab**, Xp isodisomy Xq monosomy; **Ac**, heterozygous for whole X chromosome; **Ad**, heterozygous for Xp, isodisomy of Xq; **Ae**, X trisomy with two Xi and one Xa; and **Af**, gain of Xp22 regions. Regions in blue are chromosomal territories of Xa; regions in green are territories of original Xi. Yellow circles and red lines indicate the presence of H3mK27 and XIST RNA, respectively. To the right are H3mK27 IF, DAPI staining, and XIST RNA FISH staining of tumor cells from representative cases of each category.

B: X chromosomal changes in non-BLC. Panels show H3mK27 IF, DAPI, and XIST RNA FISH staining of tumor cells: (**Ba**) non-BLC with two X chromosomes and (**Bb**) non-BLC with X chromosome trisomy and two apparent Xi. Arrows refer to focal staining areas of interest.

which two copies of most genes are expressed. Surprisingly, when this analysis was performed on the eight BLC with whole X chromosome isodisomy (Figure 5Ab), or in case T144 with X chromosome trisomy (Figure S6A), there was no measurable increase in mean X chromosome RNA relative to autosomes. Analysis of cases with either chromosome 14 or 17 isodisomy also showed a uniform level of RNA abundance across all chromosomes (Figures S6B and S6C). A similar analysis of mean chromosomal gene expression performed in cells and tissues from patients with trisomy 21 has been reported to detect only a minimal increase of chromosome 21 RNA (Mao et al., 2003).

BLC display overexpression of a small subset of X chromosomal genes

Although there was no increase in the overall abundance of X-encoded RNA in sporadic BLC, array analysis of X chromosome gene expression did reveal overexpression of a small subset (~3%) of the ~1200 X chromosome genes interrogated in BLC compared to high-grade non-BLC and normal breast tissue (Figure 5B). Other published data sets revealed overexpression

of some of the same X chromosomal genes in *BRCA1* mutation-associated tumors (van 't Veer et al., 2002) (<http://www.oncomine.org/>). Moreover, while up to 25% of all Xi genes are reported to escape silencing in some or all human Xi tested (Carrel and Willard, 2005), ~50% of the overexpressed X chromosome genes in BLC fell into this category; the others are uniformly silenced when on Xi. Two-thirds of the BLC overexpressed genes are localized in Xp22 and Xq26-28, and the remainder are scattered between these segments. Xp22 is particularly rich in escape genes (Carrel and Willard, 2005). Of the three BLC cases that displayed gains of X chromosomal DNA sequence (cf. Figures 3Ae and 3Af), the gains in two were limited to Xp22, consistent with the hypothesis that selection for expressional dysregulation of certain genes in this Xp22 region is an important feature of BLC.

Biallelic expression of a gene that is normally silenced on Xi

The four BLC bearing two biparental X chromosomes per cell revealed a major defect in X heterochromatinization and CpG

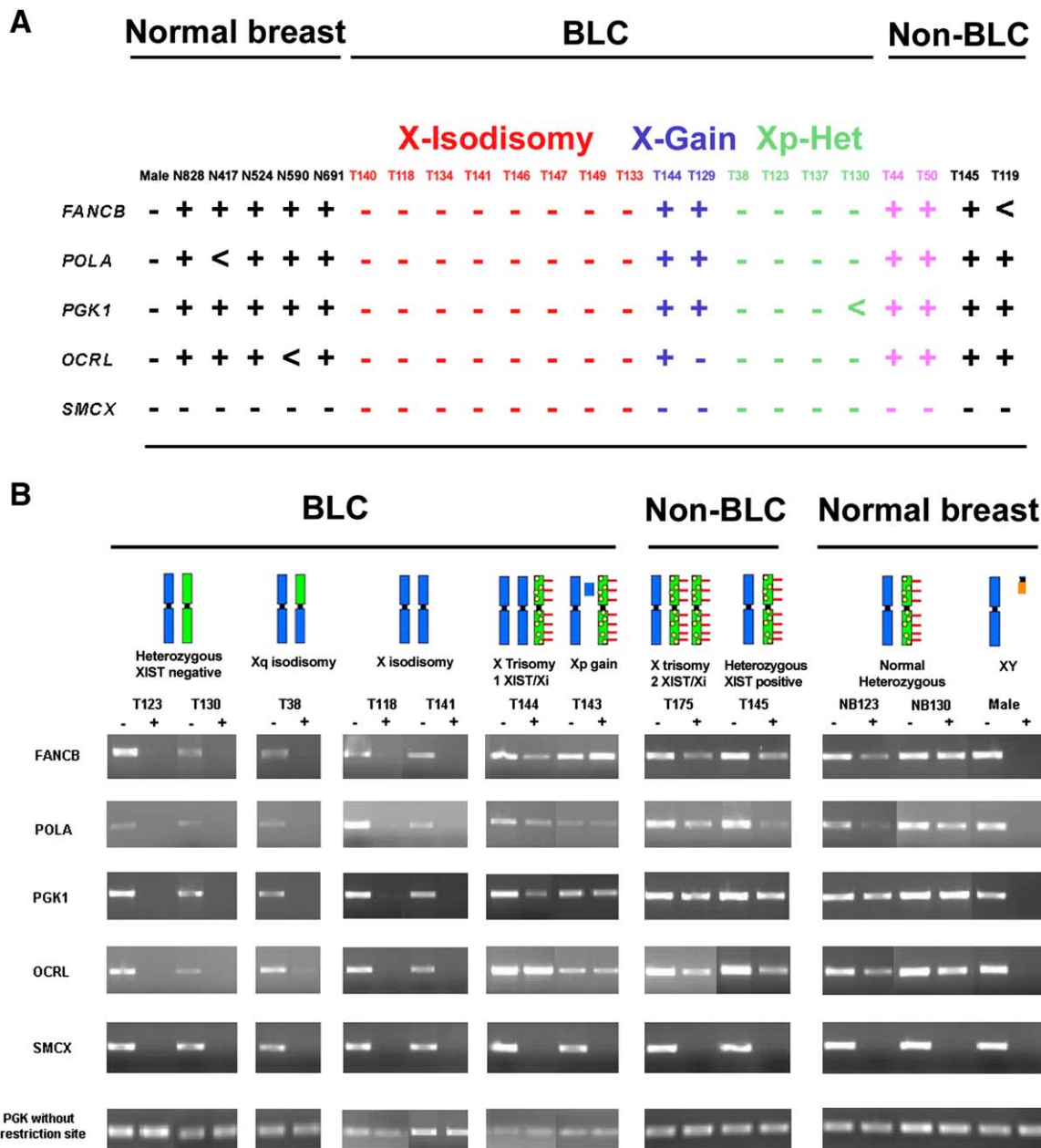


Figure 4. Methylation analysis of five representative X chromosomal genes in normal breast, BLC, and non-BLC cases

A: Results of gene/promoter methylation analysis are displayed graphically, with each row representing a different X chromosomal gene and each column a different tissue sample. Samples are color coded as follows: black, normal breast; red, BLC with X isodisomy; blue, BLC with chromosome X gains; green, BLC with biparental X chromosomes but loss of XIST staining; magenta, non-BLC with X trisomy; black, non-BLC with no chromosome X alterations. Cartoons of the X chromosome changes are shown above each group and are coded as in Figure 3. Samples were scored as follows: +, methylation; -, no methylation; <, hypomethylation.

B: Promoter methylation analysis of X chromosome genes in high-grade breast cancers representative of various X chromosome genotypes. Digestion of DNA from breast tumors with HpaII or HhaI, and without restriction enzyme, is indicated on the top as + and -, respectively. Promoter regions of X chromosome genes *FANCB* (Xp22), *POLA* (Xp21), *PGK1* (Xq13), and *OCRL* (Xq26) were amplified by PCR; the PCR products were analyzed by agarose gel electrophoresis, and the gels were stained with ethidium bromide. *SMCX* (Xp11), which escapes XCI, and part of the *PGK1* promoter region lacking HpaII and HhaI restriction sites were amplified as controls. Cartoons of the X chromosome changes are shown above each tumor category.

island methylation, raising the question of whether normal silencing of genes on what was Xi was disrupted in these tumors. To evaluate this possibility, databases were analyzed in search of known exonic SNPs in genes found to be expressed in BLC. Few exonic polymorphisms have been identified on the X chromosome, and the heterozygosity level on the X chromosome

has been observed to be below that of autosomes, a difference that has been argued to be a product of population genetic factors (Ross et al., 2005). Thus, despite an average of eight genes and 15 SNPs studied for each BLC sample, few were found to be informative in the four relevant BLC. However, one case (T123) was informative at transcribed SNPs in three genes: *STS*

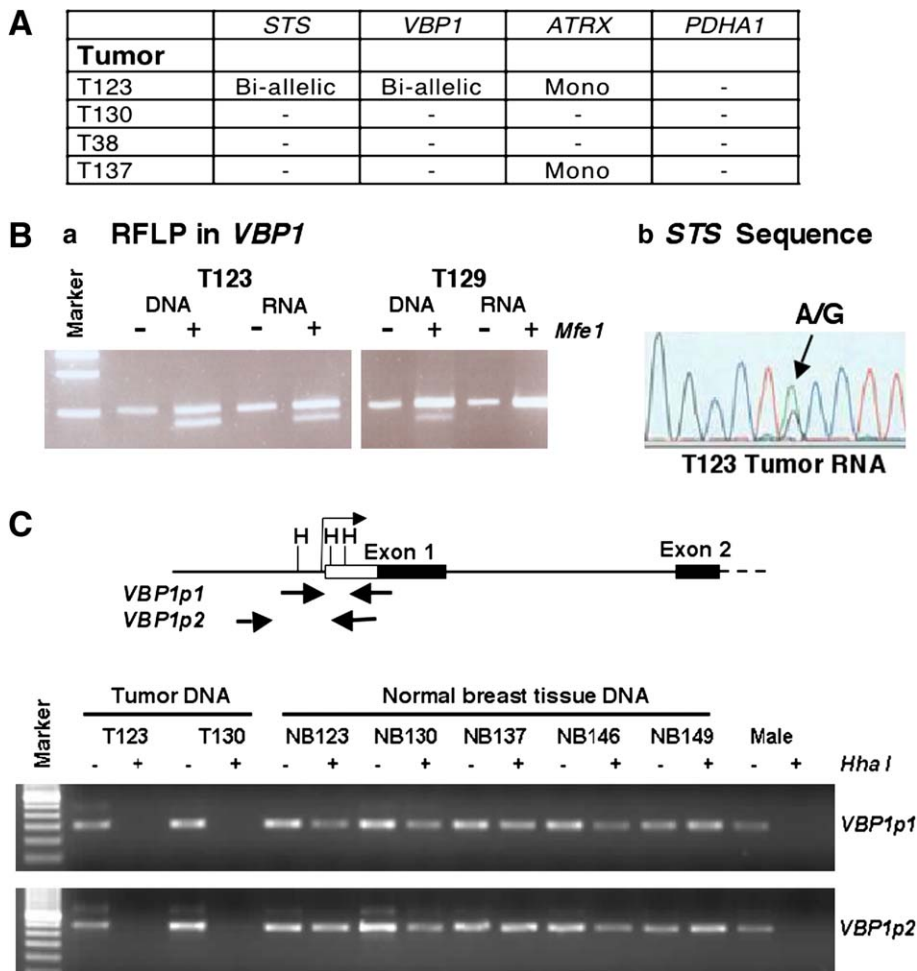


Figure 6. PCR analysis of RNA from microdissected tumors for biallelic X gene expression

Four BLC cases that contained biparental X chromosomes by SNP array analysis but lacked normal, focal XIST and H3mK27 staining were analyzed for evidence of biallelic expression of X chromosome genes. **A:** Table summarizing the results of experiments searching for evidence of mono- or biallelic expression from transcribed X chromosome SNPs in these tumors. *STS* is well known to be an escape gene. *VBP1* can sometimes escape XCI. *ATRX* and *PDHA1* have been shown to undergo XCI in all tested samples (Carrel and Willard, 2005). The – symbol means that the tumor of interest was noninformative at the indicated locus. **Ba:** Analysis of transcribed SNP-containing sequences in *VBP1*. A transcribed region of *VBP1* (Xq28) was independently amplified by PCR from both DNA and reverse-transcribed RNA derived from microdissected tumor. After amplification, the PCR products were digested with *Mfe1* (undigested and digested PCR/RT-PCR fragments are indicated on the top as – and +, respectively) and analyzed by agarose gel electrophoresis; gels were stained with ethidium bromide. The presence of two bands in the digested lanes indicates the presence of two alleles, one of which has a polymorphic *Mfe1* restriction site. T129, a BLC case with intact Xi/Xa and an extra copy of Xp22, contains two *VBP1* alleles in tumor DNA, but only one allele transcribed from tumor RNA. (Note: In heterozygous samples, the formation of heteroduplexes that are resistant to restriction digestion often results in the upper [uncut] band being more intense than the lower band; see Kutsche and Brown, 2000.) **Bb:** Representative sequencing data for *STS*. RNA isolated from tumor T123 (shown to be informative, at the DNA level, for a given polymorphic site) was isolated, reverse transcribed, and amplified by RT-PCR. Sequencing of the amplified fragment showed, as ex-

pected for an escape gene, heterozygous expression of two alleles (A and G), indicated by the presence of two peaks (highlighted with the arrow). The smaller peak likely represents the allele on the former Xi, since *STS*, like other escape genes, is likely expressed at a lower level from Xi than from Xa (Migeon et al., 1982). **C:** *VBP1* promoter methylation analysis. Promoter regions of *VBP1* were amplified by PCR using two different primer sets, as indicated in the top diagram. Restriction enzyme digestion was performed on DNA from BLC (T123 and T130) and from normal breast tissue (samples designated NB) that was part of the same surgical specimen as the tumor in each case. Digestion with *Hha1* and mock treatment were indicated as + and –, respectively.

was monoallelically expressed in the two informative tumors, T123 and T137 (Figure 6A). *ATRX* is expressed in BLC but was not differentially overexpressed relative to normal breast or non-BLC. *VBP1*, a gene that was often overexpressed in BLC (Figure 5B), was biallelically expressed in the tumor RNA of the one informative case, T123 (Figures 6A and 6Ba).

Since *VBP1* can infrequently escape XCI (Carrel and Willard, 2005), whether or not *VBP1* was subject to X inactivation in the normal breast tissue of multiple cases was examined. Methylation analysis demonstrated the presence of a methylated *VBP1* allele in the normal breast DNA of five patients, including in NB123, derived from the same patient from which tumor T123 was excised. Tumor DNA from two BLC, including T123, demonstrated lack of a methylated *VBP1* allele (Figure 6C). These data indicate that *VBP1* is methylated and silenced on Xi in normal breast tissue from patient 123 but is biallelically expressed in that patient's tumor cells. Thus, it is possible that loss of normal histone methylation and DNA methylation on Xi is associated with reexpression of some normally silenced genes on Xi in BLC. The extent of such an effect is not known, although the existing data imply that it is not complete.

Discussion

Sporadic BLC resemble *BRCA1*-deficient breast cancers in ways beyond their similar receptor profile and gene expression array signature. Indeed, in all BLC studied, whether sporadic or *BRCA1*^{-/-}, there was a major gain in nonheterochromatinized X chromosomal DNA sequences, a characteristic that was largely absent from non-BLC. Unexpectedly, most sporadic BLC are wt for *BRCA1* and display normal expression and localization of *BRCA1*. One explanation is that, although *BRCA1* is intact, BLC have developed defects in other genes involved in specific cellular pathways in which *BRCA1* also plays a key role, thus leading to a phenocopy of *BRCA1*-deficient tumor cells.

Data presented here show that BLC, whether inherited, rare, and *BRCA1* deficient, or sporadic, common, and wt for *BRCA1*, harbor defects in the maintenance of a proper number of noninactivated X chromosomes. Such X chromosome abnormalities are uncommon in non-BLC, regardless of tumor grade or subtype. Given the common and specific occurrence of this abnormality in BLC, it can now be argued that “misbehavior” manifest at the X chromosome constitutes a significant part of

the mechanism leading to the emergence of basal-like breast cancer—both sporadic and *BRCA1* mutant. Such a conclusion also suggests that the contribution to the Xi heterochromatin maintenance function of *BRCA1* p220 (Ganesan et al., 2002) may represent part of its tumor suppression activity. Whether this activity is yet another manifestation of or is linked to its known, intrinsic genome integrity/DNA repair/checkpoint activation function remains to be seen.

What is the nature of the alleged “misbehavior” at the X chromosome in BLC? First, X isodisomy was present in a majority of BLC. As expected, in X isodisomic tumors, there was no sign of CpG island methylation. Whether both copies of X genes in these isodisomic tumors are transcribed is unknown. In other BLC, there was a significant defect in the Xi heterochromatin superstructure in tumors bearing one X from each parent, accompanied by a gross defect in CpG island methylation. Similar changes have been reported in SuVAR 39H-deficient mouse cells, where a breakdown in pericentromeric heterochromatinization correlates with a promoter-associated DNA methylation defect and tumor development (Peters et al., 2001). In at least one of the BLC with retained biparental, but nonheterochromatinized X chromosomes, we found evidence consistent with reactivation of expression of a normally silenced gene on the former Xi.

Despite these widespread alterations, there was no detectable generalized increase in X chromosome-encoded RNA in the BLC studied. Thus, some form of transcription and/or RNA processing-associated downregulation may be directed at chromosome X in the BLC harboring two nonheterochromatinized X chromosomes. Alternatively, in BLC there might be a failure of a system long suspected of upregulating the expression of genes transcribed from the single active X chromosome so that they are equivalent in expression to the level observed for autosomal genes, which are normally transcribed from two chromosomes (Adler et al., 1997; Bhadra et al., 2005; Charlesworth, 1978; Jegalian and Page, 1998). Moreover, increasing evidence exists for such a mechanism in mammals (Adler et al., 1997; Nguyen and Disteche, 2006). Our results demonstrating that average expression levels from the single active X are equivalent to average expression levels from diploid autosomes in normal female breast cells further supports the existence of such a compensation mechanism on the Xa in humans. Conceivably, failure of such a compensatory mechanism during BLC development might lead to a selection for a second active X, through either the development of isodisomy or a failure of Xi heterochromatinization.

Whole X isodisomy was present in three of four *BRCA1*-deficient tumors analyzed, and loss of heterochromatinization of biparental X chromosome regions was found in the fourth *BRCA1*-associated case. *BRCA1* p220 promotes XIST and macro H2A decoration of Xi, and loss of p220 function might be expected to result in a failure of Xi heterochromatinization without a need for X isodisomy. However, *BRCA1* is implicated in multiple cellular functions that support genomic integrity, including mitosis (V. Joukov and D.M.L., unpublished data). Thus, loss of wt *BRCA1* might well promote the development of X chromosome isodisomy through a breakdown in chromosome segregation control, a process in which *BRCA1* also participates (Xu et al., 1999b), and possibly through selective pressure for the maintenance of two active X chromosomes per cell. From the small number of *BRCA1*-associated cases analyzed here, it is

unclear, as a general matter, whether X isodisomy or loss of heterochromatinization on biparental X chromosomes is the predominant mechanism underlying X misbehavior in these tumors.

The BLC cohort also revealed a high incidence of isodisomy of chromosomes 14 and 17, a phenomenon missing in high-grade, non-BLC tumors. Thus, these chromosomal abnormalities are also potentially specific contributors to the emergence of BLC. Isodisomy of autosomes might contribute to BLC development either by acting as a “second hit” for an acquired or germline mutation existing on one parental chromosome or by dysregulating certain genes subject to imprinting or allelic preference. Moreover, the frequent occurrence of isodisomy of three different chromosomes, while likely selected for during BLC development, is consistent with the presence of an underlying defect in chromosome segregation in the tumor progenitor cells of these cancers.

Nearly all BLC, by comparison with other types of aggressive breast cancer, displayed heightened expression of a small, distinct set of X chromosomal genes, some of which are concentrated in Xp22. Since the vast majority of BLC lack a normal Xi, and since in two sporadic BLC tumors there was selective gain of Xp22, one might hypothesize that increased expression of one or more Xp22 genes contributes to BLC development. Future studies will be needed to learn whether any one of these overexpressed genes contributes functionally to the neoplastic nature of BLC.

Of note, the presence of two potentially active X chromosomes has been reported in several other human cancers, including non-Hodgkin's lymphomas and germ cell tumors, supporting the possible role of a disorder at Xi in development of malignancy (Kawakami et al., 2003, 2004; Looijenga et al., 1997; McDonald et al., 2000; Spatz et al., 2004). Finally, genes that can sometimes escape XCI when located on Xi (Carrel and Willard, 2005) were overrepresented among the overexpressed, X-encoded genes in BLC. This suggests that X chromosomes in BLC cells have been, at least in part, subject to a breakdown in the system(s) that controls the level of biallelic expression of these escape loci. In this regard, the Xi-linked allele of genes that escape XCI is normally less efficiently expressed than that on the active X (Carrel and Willard, 2005). This observation suggests that the local chromatin environment of Xi affects the expression level of these escape loci (Carrel and Willard, 2005; Filippova et al., 2005). Thus, one wonders whether, in BLC, either loss of Xi and duplication of the active X chromosome, or an alteration of local heterochromatin structure on Xi, results in increased overall transcription of some of the genes that normally escape XCI.

Of note, cells of males with Klinefelter's syndrome (genotype XXiY) are predicted to experience biallelic expression of escape genes, unlike normal male somatic cells (XY), which cannot. Klinefelter's patients also experience gynecomastia and an increased incidence of breast cancer (Hultborn et al., 1997; Smyth and Bremner, 1998). Similarly, an excessive number of X chromosomes has been reported in some sporadic male breast cancers (Rudas et al., 2000; Teixeira et al., 1998). Although most sporadic male breast cancers are hormone sensitive and likely lack a basal phenotype, it would nonetheless be interesting to determine whether male breast cancers that harbor multiple active X chromosomes have a different pathological/clinical phenotype than those with a single X chromosome.

By contrast, in nonmosaic cases of Turner's syndrome (genotype XO) where there is monoallelic expression of all X chromosome genes, breast development is greatly suppressed, and this defect was not always fully corrected by estrogen therapy (Alves et al., 2003). These observations are consistent with the hypothesis that intact, biallelic expression of some X chromosomal genes may be required for normal breast development. Given our data from sporadic BLC, and the Klinefelter's and Turner's examples, one wonders whether dysregulation of X chromosome escape gene expression constitutes part of the operating disease mechanism in BLC.

An X chromosome abnormality and BRCA1 deficiency, while infrequently seen in tumors without a basal-like phenotype, are much more prevalent in basal-like tumors. The mechanism underlying the strong associations between these genotype abnormalities and the basal-like tumor phenotype is, as yet, unknown. However, one wonders whether they are the product of a specific ability of the basal-like precursor cell to survive after acquiring two active X chromosomes, following either loss of BRCA1 function or somatic acquisition of a BRCA1-like defect in an as yet undefined pathway(s). It is also possible that basal-like precursor cells are more susceptible than other precursors to the oncogenic effects arising from the development of more than one nonheterochromatinized X chromosome.

Experimental procedures

Cohort

Frozen tissue samples of 43 primary, sporadic, clinically and pathologically annotated breast tumors and four tumors from *BRCA1* mutation carriers were obtained as anonymous samples from the Harvard Breast SPORE blood and tissue repository. The Partners Hospital and Dana-Farber Cancer Institute Institutional Review Boards monitor this tumor repository, and patient consent is obtained for all identified specimens. The Partners IRB approved the use of deidentified samples for this study (protocol no. 2000-P-001448). Histologic sections of the actual frozen tissue blocks were reviewed for adequate tumor content (at least 70% by area served as a minimum). Seven samples of normal bulk breast tissue were processed identically to the tumor samples. Gene expression array data from 11 samples of normal breast organoid preparations (collagenase digested and enriched for epithelial elements) were obtained from Dr. Kornelia Polyak. These array data were normalized with our expression array data of tumor and normal bulk tissue prior to analysis. A spreadsheet of results of various assays on each sample is provided in Table S3.

RNA FISH

Frozen sections of tumor samples were fixed in 3% paraformaldehyde (PFA) and then processed for RNA FISH as previously described (Clemson et al., 1996). Tumors were scored as negative for XIST if <5% of tumor cells analyzed were positive. Adjacent normal cells in the same tissue sections served as an internal source of positive control cells. A Zeiss Axiophot fluorescence microscope and Zeiss CCD camera were used in the analyses.

Histone H3 methyl lysine 27 and BRCA1 p220 IF staining

Frozen tumor cryostat sections (5–10 μ m) were fixed by incubation with 3% PFA/PBS for 15 min at room temperature. A BRCA1 monoclonal antibody (SD118) was used at a 1:10 dilution of hybridoma supernatant; polyclonal antibodies to H3mK27 (Upstate), and cytokeratin 19 (Santa Cruz) were used at 1:500 dilution as described (Ganesan et al., 2002). All samples were mounted with Vectashield containing DAPI (Vector Labs) prior to viewing. All XIST FISH, as well as H3mK27, and BRCA1 IF analyses were scored by readers blinded to the subtype or *BRCA1* genotype of the tumor being analyzed.

X chromosome FISH

Paraffin tissue sections were hybridized with an X centromere and/or Xq and Xp subtelomere probes (Vysis, Downers Groves, IL). Probe signals per nucleus were counted and averaged from 100 cells of each tumor analyzed.

BRCA1 genotype

BRCA1 genotype was analyzed by heteroduplex-based mutation detection as described (Miron et al., 2000) or results obtained from deidentified and linked clinical diagnostic reports of gene sequencing.

DNA preparation

DNA preparation was performed as described (Wang et al., 2004).

SNP 10K array analysis

Genomic DNA was digested with XbaI, and fragments were amplified, fluorescence labeled, and hybridized to GeneChip 10K arrays (Affymetrix) using procedures recommended by the manufacturer. In the majority of cases, LOH was determined by comparing the SNP array genotype of the tumor specimen with that obtained for autologous normal cells, using dChip custom software (W.H. Wong and C. Li, <http://www.dChip.org/>). For the six cases in which matched normal samples were not available, LOH was determined using a dChip prediction function.

To analyze copy number, dChip software was also used, as described (Zhao et al., 2004). The locations of SNP loci were obtained from the University of California Santa Cruz Biotechnology genome assembly (<http://genome.ucsc.edu/>). The complete SNP array data set is available on the NCBI GEO database (accession no. GSE3743).

Methylation assay for Xi genes

CpG methylation status of the promoter regions of *FANCB*, *POLA*, *PGK1*, *OCRL*, and *SMCX* was examined by PCR amplification of the relevant DNA templates digested or undigested with HhaI (New England Biolabs, Beverly, MA) for *FANCB* (Meetei et al., 2004) and *VBP1*, and HpaII (New England Biolabs) for *PGK1* (van Kamp et al., 1992) and for *POLA*, *OCRL*, and *SMCX* as described (Allen et al., 1992). These two restriction enzymes are methylation sensitive, and informative restriction sites are located in the region between the relevant 5' and 3' PCR primers. After DNA digestion, only methylated DNA templates were amplified. *SMCX* is a gene that escapes X inactivation and was used as an unmethylated control to rule out incomplete enzyme restriction or nonspecific background amplification in the enzyme-restricted reactions. A primer pair that amplified a *PGK1* promoter region without a HpaII site was used as a positive control for the PCR reaction for each case. Primer pairs are listed in Table S4.

Gene expression array analysis

RNA extraction, cRNA synthesis, and hybridization to Affymetrix Human Genome U133 Plus 2.0 Arrays were performed as described previously (Signoretto et al., 2002; Wang et al., 2004). Raw expression data obtained using Affymetrix GENECHIP software was normalized and analyzed using DNA-Chip Analyzer (dChip) custom software (W.H. Wong and C. Li, <http://www.dChip.org/>). Array probe data were normalized to the mean expression level of each probe across a sample set. Where indicated, tumors were classified as BLC or non-BLC on the basis of their expression array characteristics, using dChip hierarchical clustering analysis as previously described (Matros et al., 2005; Wang et al., 2004). Comparisons between results obtained on BLC or *BRCA1* tumors, non-BLC tumors, and normal breast samples were performed using the dChip "Compare Sample" function. A threshold of 1.2-fold overexpression in BLC and *BRCA1* tumors was applied with 90% confidence. Of 1271 gene probes that map to the X chromosome, 60 satisfied these overexpression criteria with a range of fold difference from 1.35 to 5.11. The false discovery rate (number) of 1000 permutations was as follows: median, 0% (0); 90th percentile, 3.3% (2). Of the 60 probes, 19 were redundant (two or more probes mapping to the same gene) and excluded, leaving 41 gene-specific probes for use in the expression plot of Figure 5B. The complete gene expression array data set is available on the NCBI GEO database (accession no. GSE3744).

Analysis of global gene expression by chromosome

For analysis of global gene expression associated with a given chromosome, raw probe intensities obtained using Affymetrix GENECHIP software were

normalized, and expression levels of all probe sets were calculated by the GCRMA algorithm from the BioConductor project (<http://www.bioconductor.org>). The chromosomal locations of the 54,000 probe sets on the microarray were extracted from the NetAffx annotation files provided by Affymetrix. There are 40,201 probe sets for which there is chromosomal location information existing in the gene information files. Probe sets that had not been mapped genomically were excluded from this analysis. Among the remaining probe sets, 2007 mapped to more than one location. Probe sets that mapped to both X and Y chromosomes, or to X and an autosome, were assigned to the X chromosome for this analysis. For probe sets that mapped to multiple, separate autosomal locations (<5% of the total), the chromosome location was assigned randomly to one of the mapped locations. The analysis was also performed after eliminating these multiply assigned probe sets, and the results were similar. The expression levels of all probe sets in normal breast samples and in BLC tumor samples were averaged and log₂ transformed. Box plots of expression levels of each chromosome were plotted by R language (Chambers et al., 1983).

Analysis of transcribed SNPs by PCR

To search for evidence of biallelic versus monoallelic expression in a subset of BLC with retention of biparental X chromosomes, public SNP databases (<http://www.ncbi.nlm.nih.gov/projects/SNP/> and <http://pgws.nci.nih.gov/perl/snpbr/>) were mined for relevant, validated exonic SNPs. Analysis of individual SNPs was performed either by sequencing or by restriction enzyme digestion of PCR/RT-PCR products containing an appropriate polymorphic site(s). Informative (heterozygous) cases were identified by testing normal DNA from each patient. Laser-capture microdissected tumor cells were isolated from frozen tumor sections and confirmed to have retained an informative polymorphism(s) in the tumor DNA. Where indicated, microdissected tumor RNA was subjected to RT-PCR and analyzed for the presence of relevant SNP(s). RNA samples were treated with DNase I after extraction using an RNA nanoprep kit (Stratagene). First strand cDNA synthesis was carried out using Superscript II reverse transcriptase (Invitrogen) with random primer oligonucleotides (Invitrogen). The reaction conditions followed the manufacturer's instructions. In each case, controls were performed in which cDNA synthesis was omitted to ensure the absence of any genomic DNA contamination. Primer pairs are listed in Table S4. Purified PCR/RT-PCR products were sequenced at the Dana-Farber Sequencing Core facility. For the *VBP1* gene, PCR/RT-PCR amplification and restriction enzyme digestion were carried out as described (Kutsche and Brown, 2000). Products of the digestion were resolved in 4% agarose gels and visualized by ethidium bromide staining.

Supplemental data

The Supplemental Data include six supplemental figures and four supplemental tables and can be found with this article online at <http://www.cancer.org/cgi/content/full/9/2/121/DC1/>.

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References

Abd El-Rehim, D.M., Pinder, S.E., Paish, C.E., Bell, J., Blamey, R.W., Robertson, J.F., Nicholson, R.I., and Ellis, I.O. (2004). Expression of luminal

and basal cytokeratins in human breast carcinoma. *J. Pathol.* 203, 661–671.

Adler, D.A., Rugarli, E.I., Lingenfelter, P.A., Tsuchiya, K., Poslinski, D., Liggitt, H.D., Chapman, V.M., Elliott, R.W., Ballabio, A., and Disteché, C.M. (1997). Evidence of evolutionary up-regulation of the single active X chromosome in mammals based on *Cic4* expression levels in *Mus spretus* and *Mus musculus*. *Proc. Natl. Acad. Sci. USA* 94, 9244–9248.

Allen, R.C., Zoghbi, H.Y., Moseley, A.B., Rosenblatt, H.M., and Belmont, J.W. (1992). Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. *Am. J. Hum. Genet.* 51, 1229–1239.

Alves, S.T., Gallicchio, C.T., Guimaraes, M.M., and Santos, M. (2003). Gonadotropin levels in Turner's syndrome: correlation with breast development and hormone replacement therapy. *Gynecol. Endocrinol.* 17, 295–301.

Bhadra, M.P., Bhadra, U., Kundu, J., and Birchler, J.A. (2005). Gene expression analysis of the function of the male-specific lethal complex in *Drosophila*. *Genetics* 169, 2061–2074.

Carrel, L., and Willard, H.F. (2005). X-inactivation profile reveals extensive variability in X-linked gene expression in females. *Nature* 434, 400–404.

Chambers, J.M., Cleveland, W.S., Kleiner, B., and Tukey, P.A. (1983). *Graphical Methods for Data Analysis* (Bemont, CA: Wadsworth & Brooks/Cole).

Charlesworth, B. (1978). Model for evolution of Y chromosomes and dosage compensation. *Proc. Natl. Acad. Sci. USA* 75, 5618–5622.

Clemson, C.M., McNeil, J.A., Willard, H.F., and Lawrence, J.B. (1996). XIST RNA paints the inactive X chromosome at interphase: evidence for a novel RNA involved in nuclear/chromosome structure. *J. Cell Biol.* 132, 259–275.

Filippova, G.N., Cheng, M.K., Moore, J.M., Truong, J.P., Hu, Y.J., Nguyen, D.K., Tsuchiya, K., and Disteché, C.M. (2005). Boundaries between chromosomal domains of X inactivation and escape bind CTCF and lack CpG methylation during early development. *Dev. Cell* 8, 31–42.

Foulkes, W.D. (2004). BRCA1 functions as a breast stem cell regulator. *J. Med. Genet.* 41, 1–5.

Foulkes, W.D., Brunet, J.S., Stefansson, I.M., Straume, O., Chappuis, P.O., Begin, L.R., Hamel, N., Goffin, J.R., Wong, N., Trudel, M., et al. (2004). The prognostic implication of the basal-like (cyclin E high/p27 low/p53+/glomeruloid-microvascular-proliferation+) phenotype of BRCA1-related breast cancer. *Cancer Res.* 64, 830–835.

Ganesan, S., Silver, D.P., Greenberg, R.A., Avni, D., Drapkin, R., Miron, A., Mok, S.C., Randrianarison, V., Brodie, S., Salstrom, J., et al. (2002). BRCA1 supports XIST RNA concentration on the inactive X chromosome. *Cell* 111, 393–405.

Hultborn, R., Hanson, C., Kopf, I., Verbiene, I., Warnhammar, E., and Weimarck, A. (1997). Prevalence of Klinefelter's syndrome in male breast cancer patients. *Anticancer Res.* 17, 4293–4297.

Jegalian, K., and Page, D.C. (1998). A proposed path by which genes common to mammalian X and Y chromosomes evolve to become X inactivated. *Nature* 394, 776–780.

Kawakami, T., Okamoto, K., Sugihara, H., Hattori, T., Reeve, A.E., Ogawa, O., and Okada, Y. (2003). The roles of supernumerical X chromosomes and XIST expression in testicular germ cell tumors. *J. Urol.* 169, 1546–1552.

Kawakami, T., Zhang, C., Taniguchi, T., Kim, C.J., Okada, Y., Sugihara, H., Hattori, T., Reeve, A.E., Ogawa, O., and Okamoto, K. (2004). Characterization of loss-of-inactive X in Klinefelter syndrome and female-derived cancer cells. *Oncogene* 23, 6163–6169.

Kutsche, R., and Brown, C.J. (2000). Determination of X-chromosome inactivation status using X-linked expressed polymorphisms identified by database searching. *Genomics* 65, 9–15.

Lakhani, S.R., Van De Vijver, M.J., Jacquemier, J., Anderson, T.J., Osin, P.P., McGuffog, L., and Easton, D.F. (2002). The pathology of familial breast cancer: predictive value of immunohistochemical markers estrogen receptor, progesterone receptor, HER-2, and p53 in patients with mutations in BRCA1 and BRCA2. *J. Clin. Oncol.* 20, 2310–2318.

- Lane, T.F., Deng, C., Elson, A., Lyu, M.S., Kozak, C.A., and Leder, P. (1995). Expression of *Brca1* is associated with terminal differentiation of ectodermally and mesodermally derived tissues in mice. *Genes Dev.* 9, 2712–2722.
- Looijenga, L.H., Gillis, A.J., van Gurp, R.J., Verkerk, A.J., and Oosterhuis, J.W. (1997). X inactivation in human testicular tumors. XIST expression and androgen receptor methylation status. *Am. J. Pathol.* 151, 581–590.
- Mao, R., Zielke, C.L., Zielke, H.R., and Pevsner, J. (2003). Global up-regulation of chromosome 21 gene expression in the developing Down syndrome brain. *Genomics* 81, 457–467.
- Matros, E., Wang, Z.C., Lodeiro, G., Miron, A., Iglehart, J.D., and Richardson, A.L. (2005). BRCA1 promoter methylation in sporadic breast tumors: relationship to gene expression profiles. *Breast Cancer Res. Treat.* 91, 179–186.
- McDonald, H.L., Gascoyne, R.D., Horsman, D., and Brown, C.J. (2000). Involvement of the X chromosome in non-Hodgkin lymphoma. *Genes Chromosomes Cancer* 28, 246–257.
- Meetej, A.R., Levitus, M., Xue, Y., Medhurst, A.L., Zwaan, M., Ling, C., Roimans, M.A., Bier, P., Hoatlin, M., Pals, G., et al. (2004). X-linked inheritance of Fanconi anemia complementation group B. *Nat. Genet.* 36, 1219–1224.
- Migeon, B.R., Shapiro, L.J., Norum, R.A., Mohandas, T., Axelman, J., and Dabora, R.L. (1982). Differential expression of steroid sulphatase locus on active and inactive human X chromosome. *Nature* 299, 838–840.
- Miron, A., Schildkraut, J.M., Rimer, B.K., Winer, E.P., Sugg Skinner, C., Futreal, P.A., Culler, D., Calingaert, B., Clark, S., Kelly Marcom, P., and Iglehart, J.D. (2000). Testing for hereditary breast and ovarian cancer in the southeastern United States. *Ann. Surg.* 231, 624–634.
- Moore, R., and Barr, M. (1957). The sex chromatin in human malignant tissues. *Br. J. Cancer* 11, 384–390.
- Nguyen, D.K., and Distech, C.M. (2006). Dosage compensation of the active X chromosome in mammals. *Nat. Genet.* 38, 47–53.
- Palacios, J., Honrado, E., Osorio, A., Cazorla, A., Sarrío, D., Barroso, A., Rodríguez, S., Cigudosa, J.C., Diez, O., Alonso, C., et al. (2005). Phenotypic characterization of BRCA1 and BRCA2 tumors based in a tissue microarray study with 37 immunohistochemical markers. *Breast Cancer Res. Treat.* 90, 5–14.
- Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A., et al. (2000). Molecular portraits of human breast tumours. *Nature* 406, 747–752.
- Perry, P.M. (1971). Evaluation of breast tumour cellular sex chromatin (Barr body) as an index of survival and response to pituitary ablation. *Br. J. Surg.* 58, 294–295.
- Peters, A.H., O'Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schofer, C., Weipoltshammer, K., Pagani, M., Lachner, M., Kohlmaier, A., et al. (2001). Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* 107, 323–337.
- Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H., de la Cruz, C.C., Otte, A.P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. *Science* 300, 131–135.
- Rosen, P.P., Savino, A., Menendez-Botet, C., Urban, J.A., Mike, V., Schwartz, M.K., and Melamed, M.R. (1977). Barr body distribution and estrogen receptor protein in mammary carcinoma. *Ann. Clin. Lab. Sci.* 7, 491–499.
- Ross, M.T., Grafham, D.V., Coffey, A.J., Scherer, S., McLay, K., Muzny, D., Platzer, M., Howell, G.R., Burrows, C., Bird, C.P., et al. (2005). The DNA sequence of the human X chromosome. *Nature* 434, 325–337.
- Rudas, M., Schmidinger, M., Wenzel, C., Okamoto, I., Budinsky, A., Fazeny, B., and Marosi, C. (2000). Karyotypic findings in two cases of male breast cancer. *Cancer Genet. Cytogenet.* 121, 190–193.
- Scholl, A., Fischbach, H., Morl, F., Rieckert, H., and Bohle, A. (1968). *Verh. Dtsch. Ges. Pathol.* 52, 426–430.
- Scully, R., and Livingston, D.M. (2000). In search of the tumour-suppressor functions of BRCA1 and BRCA2. *Nature* 408, 429–432.
- Signoretto, S., Di Marcotullio, L., Richardson, A., Ramaswamy, S., Isaac, B., Rue, M., Monti, F., Loda, M., and Pagano, M. (2002). Oncogenic role of the ubiquitin ligase subunit Skp2 in human breast cancer. *J. Clin. Invest.* 110, 633–641.
- Sirchia, S.M., Ramoscelli, L., Grati, F.R., Barbera, F., Coradini, D., Rossella, F., Porta, G., Lesma, E., Ruggeri, A., Radice, P., et al. (2005). Loss of the inactive X chromosome and replication of the active X in BRCA1-defective and wild-type breast cancer cells. *Cancer Res.* 65, 2139–2146.
- Smyth, C.M., and Bremner, W.J. (1998). Klinefelter syndrome. *Arch. Intern. Med.* 158, 1309–1314.
- Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., et al. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc. Natl. Acad. Sci. USA* 98, 10869–10874.
- Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J.S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., et al. (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc. Natl. Acad. Sci. USA* 100, 8418–8423.
- Spatz, A., Borg, C., and Feunteun, J. (2004). X-chromosome genetics and human cancer. *Nat. Rev. Cancer* 4, 617–629.
- Teixeira, M.R., Pandis, N., Dietrich, C.U., Reed, W., Andersen, J., Qvist, H., and Heim, S. (1998). Chromosome banding analysis of gynecomastias and breast carcinomas in men. *Genes Chromosomes Cancer* 23, 16–20.
- van 't Veer, L.J., Dai, H., van de Vijver, M.J., He, Y.D., Hart, A.A., Mao, M., Peterse, H.L., van der Kooy, K., Marton, M.J., Witteveen, A.T., et al. (2002). Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 415, 484–485.
- van Kamp, H., Fibbe, W.E., Jansen, R.P., van der Keur, M., de Graaff, E., Willemze, R., and Landegent, J.E. (1992). Clonal involvement of granulocytes and monocytes, but not of T and B lymphocytes and natural killer cells in patients with myelodysplasia: analysis by X-linked restriction fragment length polymorphisms and polymerase chain reaction of the phosphoglycerate kinase gene. *Blood* 80, 1774–1780.
- Venkitaraman, A.R. (2002). Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* 108, 171–182.
- Wang, N., Cedrone, E., Skuse, G.R., Insel, R., and Dry, J. (1990). Two identical active X chromosomes in human mammary carcinoma cells. *Cancer Genet. Cytogenet.* 46, 271–280.
- Wang, Z.C., Lin, M., Wei, L.J., Li, C., Miron, A., Lodeiro, G., Harris, L., Ramaswamy, S., Tanenbaum, D.M., Meyerson, M., et al. (2004). Loss of heterozygosity and its correlation with expression profiles in subclasses of invasive breast cancers. *Cancer Res.* 64, 64–71.
- Xu, X., Wagner, K.U., Larson, D., Weaver, Z., Li, C., Ried, T., Hennighausen, L., Wynshaw-Boris, A., and Deng, C.X. (1999a). Conditional mutation of *Brca1* in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. *Nat. Genet.* 22, 37–43.
- Xu, X., Weaver, Z., Linke, S.P., Li, C., Gotay, J., Wang, X.W., Harris, C.C., Ried, T., and Deng, C.X. (1999b). Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells. *Mol. Cell* 3, 389–395.
- Zhao, X., Li, C., Paez, J.G., Chin, K., Janne, P.A., Chen, T.H., Girard, L., Minna, J., Christiani, D., Leo, C., et al. (2004). An integrated view of copy number and allelic alterations in the cancer genome using single nucleotide polymorphism arrays. *Cancer Res.* 64, 3060–3071.

Accession numbers

The complete SNP array data set and the complete gene expression array data set are available on the NCBI GEO database (accession numbers GSE3743 and GSE3744, respectively).

Note added in proof

One patient in which a seemingly sporadic BLC (T147) arose was subsequently found to have a germline BRCA1 mutation. This tumor was one of two sporadic BLC that were negative for BRCA1 expression by IF. This tumor has X chromosome isodisomy, but the tumor itself has not been genotyped for BRCA1. This information does not affect any of the conclusions in this study.