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 “primitve,” “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, macrohiste 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 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.

Abnormalities involving the X chromosome are frequent in BLC

Whole genome alleleye 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 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.

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.
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 tumors.
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 nonheterochromatized 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 BRCA1/2 (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 BRCA1/2−/− 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 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). 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
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 expression 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...
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:

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.
(Xp22.32), VBP1 (Xq28), and ATRX (Xq13.1), along with another tumor (T137) that bore a heterozygous SNP in the ATRX gene. STS has been reported to escape XCI, while ATRX is normally silenced on Xi (Carrel and Willard, 2005). VBP1 was reported to be silenced in eight of nine human Xi in rodent/human somatic hybrids, suggesting that it is subject to XCI in most individuals (Carrel and Willard, 2005). cDNA, generated by gene-specific RT-PCR of RNA prepared from microdissected tumor in these cases, was analyzed in search of evidence of mono- versus biallelic expression. As expected, STS, the known escape gene, was biallelically expressed in the one informative tumor, T123 (Figures 6A and 6Bb). ATRX, which is normally silenced on Xi,

Figure 5. Analysis of X chromosome gene expression in BLC
A: Analysis of mean gene expression from individual chromosomes in normal female breast and in BLC with X isodisomy. The x axes indicate chromosome identity. The y axes depict log2-transformed normalized RNA expression levels. At: Results averaged for seven normal female breast bulk tissue samples. Ab: Average for eight BLC (bulk tumor) with X isodisomy.
B: Differential X chromosome gene expression in BLC. X chromosome genes with at least 1.2-fold overexpression in BLC and BRCA1-associated tumors relative to non-BLC and normal breast samples are shown. The X chromosome cytogenetic band pattern is on the left. In the middle panel, differentially overexpressed genes are indicated by black horizontal lines and plotted by location on the X chromosome with plus strand genes (oriented from p-terminus to q-terminus) on the left and minus strand genes (oriented q-terminus to p-terminus) on the right. Gray lines similarly indicate the positions of all other known X genes. Blue boxes mark chromosomal regions of enrichment of overexpressed genes relative to gene density. The right panel illustrates the relative gene expression with each column representing a tissue sample and each row demonstrating the results of a different gene. Mean levels of expression are depicted in white, overexpression is depicted in red, and underexpression is depicted in blue. Gene names are to the right. The reported X chromosome inactivation (XCI) status of each gene is indicated as follows: black solid circles, genes subject to XCI; open circles, genes that escape XCI in at least some individuals; dashes, genes that have not been analyzed for XCI (Carrel and Willard, 2005).
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 heterochromatinization 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 XXY) 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 Kliewerfelter’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 obtained from Dr. Kornelia Polyak. These array data were normalized with our expression array data of tumor and normal bulk tissue 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 methyl-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

DNA preparation was performed as described (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%; 90th percentile, 3.3%. 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://lpgws.nci.nih.gov/ pertl/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 a unique 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.cancercell.org/cgi/content/full/9/2/121/DC1/.

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References


Expression of Brca1 is associated with terminal differentiation of ectodermal and mesodermally derived tissues in mice. Genes Dev. 9, 2712–2722.


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 BRC (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.