Skewed X Chromosome Inactivation and Breast and Ovarian Cancer Status: Evidence for X-Linked Modifiers of BRCA1


Background
X chromosome inactivation, which silences gene expression from one of the two X chromosomes in females, is usually random. Skewed X inactivation has been implicated in both the expression and the suppression of X-linked disease phenotypes and has been reported to occur more frequently in breast and ovarian cancer patients, including BRCA1 or BRCA2 mutation carriers, than in control subjects.

Methods
We assessed the pattern of X chromosome inactivation using methylation-specific polymerase chain reaction amplification of the exon 1 microsatellite region of the X-linked androgen receptor (AR) gene in DNA from blood samples obtained from control subjects without a personal history of breast or ovarian cancer (n = 735), ovarian cancer patients (n = 313), familial breast cancer patients who did not carry mutations in BRCA1 or BRCA2 (n = 235), and affected and unaffected carriers of mutations in BRCA1 (n = 260) or BRCA2 (n = 63). We defined the pattern of X chromosome inactivation as skewed when the same X chromosome was active in at least 90% of cells. The association between skewed X inactivation and disease and/or BRCA mutation status was assessed by logistic regression analysis. The association between skewed X inactivation and age at cancer diagnosis was assessed by Cox proportional hazards regression analysis. All statistical tests were two-sided.

Results
The age-adjusted frequency of skewed X inactivation was not statistically significantly higher in ovarian cancer or familial breast cancer case subjects compared with control subjects. Skewed X inactivation was higher in BRCA1 mutation carriers than in control subjects (odds ratio [OR] = 2.7, 95% confidence interval [CI] = 1.1 to 6.2; P = .02), particularly among unaffected women (OR = 6.1, 95% CI = 1.5 to 31.8; P = .005). Among BRCA1 mutation carriers, those with skewed X inactivation were older at diagnosis of breast or ovarian cancer than those without skewed X inactivation (hazard ratio [HR] of breast or ovarian cancer = 0.37, 95% CI = 0.14 to 0.95; P = .04). Among BRCA2 mutation carriers, skewed X inactivation also occurred more frequently in unaffected carriers than in those diagnosed with breast or ovarian cancer (OR = 5.2, 95% CI = 0.5 to 28.9; P = .08) and was associated with delayed age at onset (HR = 0.59, 95% CI = 0.37 to 0.94; P = .03).

Conclusions
Skewed X inactivation occurs at an increased frequency in BRCA1 (and possibly BRCA2) mutation carriers compared with control subjects and is associated with a statistically significant increase in age at diagnosis of breast and ovarian cancer.

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**CONTEXT AND CAVEATS**

**Prior knowledge**
X chromosome inactivation silences gene expression from one of the two X chromosomes in females and is usually random. Non-random (ie, skewed) X inactivation has been implicated in both the expression and the suppression of X-linked disease phenotypes and has been reported to occur more frequently in breast and ovarian cancer patients, including BRCA1 mutation carriers, than in control subjects.

**Study design**
A molecular genetic analysis of X chromosome inactivation patterns using methylation-specific polymerase chain reaction amplification of the exon 1 microsatellite region of the X-linked androgen receptor gene in peripheral blood lymphocyte DNA from cancer-free female control subjects, ovarian cancer patients, familial breast cancer patients who did not carry mutations in BRCA1 or BRCA2, and unaffected and breast or ovarian cancer–affected carriers of mutations in BRCA1 or BRCA2.

**Contribution**
Skewed X inactivation occurs at increased frequency in unaffected BRCA1 (and possibly BRCA2) mutation carriers compared with control subjects and is associated with an older age at breast or ovarian cancer diagnosis in female BRCA1 mutation carriers.

**Implications**
Skewed X inactivation may be a mechanism that favors expression of an X-linked allele that protects against cancer and that may be increased in BRCA1 mutation carriers due to BRCA1 mutation–related overexpression of X-linked genes.

**Limitations**
Data interpretations were based on small numbers of individuals who displayed skewed X inactivation.

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Form the sex chromatin or Barr body. The active and inactive X chromosomes are then inherited by daughter cells through subsequent mitotic divisions (1). Because the initial process of X inactivation occurs at random in each cell of the early embryo, adult female tissues typically consist of an equal mixture of cells that contain an active paternal X chromosome and cells that contain an active maternal X chromosome (2). However, the phenomenon of nonrandom or skewed X inactivation, in which the same X chromosome is silenced in most or all cells of a tissue, has been observed in healthy control subjects as well as in individuals who are affected with a number of different diseases (2). Skewed X inactivation at the cut point of ≥90% (ie, 90% or more of cells in the tissue tested display the same active X chromosome) is generally accepted as biologically meaningful, although some studies [eg, (3)] have used lower cut points (ie, ≥80% or ≥75%).

Three hypotheses have been proposed to explain the mechanism of skewed X inactivation; all three hypotheses have evidence to support them. The first hypothesis—that skewed X inactivation is due to an X-linked allele that confers a proliferative advantage to a subset of peripheral lymphocytes (4,5). Support for the second hypothesis—that skewed X inactivation is due to a genetic predisposition—comes from heritability studies (6,7) that have estimated that about one-third of the variance in the X inactivation phenotype is due to genetic factors, from a study (8) that showed that skewed X inactivation in identical twins typically favors the same X chromosome, and from several studies (9–12) that reported linkage of skewed X inactivation to specific loci on the X chromosome. For the third hypothesis—that acquisition of skewed X inactivation is a protective mechanism to reduce expression of detrimental X-linked alleles—there is considerable evidence that skewed X inactivation can protect females from disease when the preferentially inactivated X chromosome carries a detrimental allele (13–15). However, in some instances, skewed X inactivation may be associated with a disease phenotype when the active X chromosome harbors a mutated X-linked gene (16,17); in such cases, the skewed X inactivation may have arisen to protect against a different X-linked mutation in trans.

Skewed X inactivation occurs in a very small percentage of the general population (ie, 2.7%–4.5%) (18–20). However, several studies (3,19,21) have reported an increased frequency of skewed X inactivation in patients with ovarian and breast cancer. For example, one study (3) found that skewed X inactivation (defined at the ≥75% cut point) was statistically significantly more common in patients with invasive ovarian cancer than in patients with borderline ovarian cancer (ie, patients with low malignant potential tumors) or healthy control subjects; however, the blood samples used for the analysis were not age matched. In that study, a subset analysis of the case patients who were screened for BRCA1 mutations (and were informative for the X inactivation assay due to heterozygosity at the X-linked androgen receptor [AR] locus) revealed that nine (82%) of 11 BRCA1 mutation carriers had skewed X inactivation (P < .008) and that loss of heterozygosity at BRCA1 was associated with skewed X inactivation (P = .04). The latter finding is particularly intriguing considering the evidence indicating that BRCA1 is involved in regulating the expression of a large number of X-linked genes (22,23) and may play a role in the maintenance of the inactive X chromosome (24,25), although there is some conjecture over the latter (26–29).

Two studies have described an association between skewed X inactivation and breast cancer. An association between skewed X inactivation (defined at the ≥90% cut point) and early-onset breast cancer (defined as onset at age 45 years or younger) was detected in Norwegian women (21). However, neither family history status nor the BRCA1 or BRCA2 mutation status of the breast cancer subjects was reported. A study of Swedish women suggested that skewed X inactivation is more common in younger breast cancer patients (ie, those younger than 55 years) with a first- or second-degree family history of breast cancer than in control subjects younger than 55 years of age (P = .001) (19). Although the latter study reported no statistically significant association between skewed X inactivation and BRCA1 or BRCA2 mutation status among the limited number of case subjects whose mutation status was tested, skewed X inactivation was approximately twice as common in the small pooled subset of BRCA1 and BRCA2 mutation carriers than among control subjects with no personal or family history of breast cancer. In contrast to these two breast cancer
studies (19,21), a North American study found no association with skewed X inactivation in breast cancer case subjects (n = 235) (the majority of whom had no family history of breast cancer), compared with age-matched control subjects (n = 253), and the results were similar when the analysis was restricted to women younger than 50 years of age (30). The aforementioned studies (3,19,21,30) assayed skewed X inactivation in DNA from peripheral blood cells. Another small study specifically examined the association between skewed X inactivation and BRCA1 mutation–positive breast cancer and also assessed the suitability of DNA extracted from Epstein–Barr virus–immortalized lymphoblastoid cell lines for the analysis of skewed X inactivation (31). The authors concluded that DNA from lymphoblastoid cell lines was suitable for the analysis of skewed X inactivation and that BRCA1 mutation status was not associated with skewed X inactivation.

The increased frequency of skewed X inactivation in subsets of breast and ovarian cancer case subjects suggests the existence of X-linked cancer susceptibility genes that cause breast cancer and, possibly, ovarian cancer de novo and/or that modify the onset of breast or ovarian cancer in BRCA1 mutation carriers. We examined the prevalence of skewed X inactivation using a methylation-specific polymerase chain reaction (PCR) technique (32) on bisulfite-treated DNA extracted from peripheral blood lymphocytes of female BRCA1 and BRCA2 mutation carriers (affected or unaffected by breast or ovarian cancer), familial breast cancer case subjects who were negative for mutations in BRCA1 or BRCA2, a population-based sample of women diagnosed with ovarian cancer, and cancer-free female control subjects.

Subjects and Methods

Subjects

**Cancer-free Control Subjects.** Two independent sets of control subjects were used in this study. The first set included women unsolicited for family history of cancer and with no reported personal history of any cancer who were recruited during 2005 through the Australian Red Cross Blood Service as described previously (33) (hereafter referred to as the blood donor control subjects) (n = 163). Each of these women completed a short questionnaire that assessed risk factor information. The second set of control subjects included women with no reported personal history of ovarian cancer or a bilateral oophorectomy (n = 572) who were frequency matched (by state of residence and age in 5-year groups) to women diagnosed with ovarian cancer who were recruited through the Australian Ovarian Cancer Study, a national population-based case–control study of ovarian cancer (see “Ovarian Cancer Case Subjects” below). These control subjects were randomly selected from January 2002 to December 2005 from the Australian Electoral Roll (enrollment to vote is compulsory in Australia).

**Ovarian Cancer Case Subjects.** Ovarian cancer case subjects were from the Australian Ovarian Cancer Study and have been described previously (34). Briefly, women aged 18–79 years who were newly diagnosed with epithelial ovarian cancer (including borderline tumors) were recruited from January 2002 through December 2005 primarily from specialized gynecologic oncology units and also through cancer registries. Participants completed a detailed health and lifestyle questionnaire that included information on their family history of cancer. The subset of individuals selected for this study (n = 313) were prioritized for inclusion if they reported a family history.

**Breast Cancer Case Subjects.** All breast cancer case subjects and their family members were from high-risk breast cancer families that were ascertained through the Kathleen Cunningham Foundation Consortium for Research into Familial Breast Cancer (kConFab) (35). The cancer status of these subjects was based on self-report. The ascertainment criteria for recruitment into kConFab for families with mutations in BRCA1 or BRCA2 were that two or more individuals were known or likely (based on family mutation status and relationship to known carriers) carriers of pathogenic or consensus splice site mutations. Mutations were classified as pathogenic according to the criteria established by kConFab at the time of this analysis; namely, all truncating mutations were considered pathogenic unless there was clear evidence that the mutation was a single-nucleotide polymorphism (eg, a carboxy-terminal BRCA2 variant); a nontruncating variant that had been well characterized in family studies of multiple generations and was not found in control subjects and that resulted in a nonconservative amino acid substitution; or a nontruncating mutation that occurred in a functional domain of the protein and in an amino acid residue that is conserved across species (see Table 1 for sample

<table>
<thead>
<tr>
<th>Group</th>
<th>Total no. assessed for skewed X inactivation</th>
<th>Breast cancer</th>
<th>Ovarian cancer</th>
<th>No cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood donor control subjects</td>
<td>163</td>
<td>—</td>
<td>—</td>
<td>163</td>
</tr>
<tr>
<td>Population-based control subjects</td>
<td>572</td>
<td>—</td>
<td>—</td>
<td>572</td>
</tr>
<tr>
<td>Population-based ovarian cancer case subjects</td>
<td>313</td>
<td>—</td>
<td>313</td>
<td>—</td>
</tr>
<tr>
<td>BRCA1 (non-BRCA1/2) familial breast cancer case subjects</td>
<td>235</td>
<td>235</td>
<td>45 (26–78)</td>
<td>235</td>
</tr>
<tr>
<td>BRCA1 mutation carriers</td>
<td>260</td>
<td>123</td>
<td>41 (23–85)</td>
<td>17</td>
</tr>
<tr>
<td>BRCA2 mutation carriers</td>
<td>63</td>
<td>36</td>
<td>45 (23–73)</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>1606</td>
<td>394</td>
<td>333</td>
<td>879</td>
</tr>
</tbody>
</table>

* —, not applicable
sizes and characteristics). The ascertainment criteria for breast cancer families that were negative for mutations in BRCA1 and BRCA2 (hereafter referred to as BRCAX) were four or more cases of breast or ovarian cancer or two or more cases of breast or ovarian cancer if one case had “high-risk” features, such as a breast cancer diagnosis at age 40 years or younger, bilateral breast cancer, or ovarian and breast cancer diagnosed in the same woman. In both situations, two or more of the affected women had to be alive and for each family, four or more female unaffected first- or second-degree relatives older than 18 years had to be alive. The index case subjects, defined as the youngest living female family member with breast cancer, were tested for mutations in BRCA1 and BRCA2 by full sequence analysis or denaturing high-performance liquid chromatography of DNA from peripheral blood lymphocytes performed by diagnostic laboratories. For secondary analyses relating to treatment regimen, we accessed all available information on chemotherapy and radiation treatment for patients from questionnaire information and treatment records.

Ethical approvals were obtained from the Human Research Ethics Committees of the Queensland Institute of Medical Research, the Queensland University of Technology, the Peter MacCallum Cancer Institute, and all participating hospitals and cancer registries. Written informed consent was obtained from each participant.

**X Inactivation Analysis**

The most common method for analysis of X chromosome inactivation ratios (ratio of active to inactive allele in females) involves the X-linked AR gene, which contains a highly polymorphic (CAG) microsatellite repeat in exon 1, for two reasons. First, approximately 90% of women are heterozygous at this locus (36), which enables the two X chromosomes to be distinguished from each other after PCR. Second, the methylation status of this region correlates 100% with the activity state of the X chromosome (37). Bisulfite modification of DNA followed by methylation-specific PCR of this locus thus allows the calculation of the X chromosome inactivation ratio. The protocol used for this study was adapted from a protocol first described by Kubota et al. (32). Germline DNA extracted from peripheral blood lymphocytes was used for all subject groups, unless otherwise specified.

**PCR of DNA Samples for AR (CAG), Repeat Genotyping.** Primers encompassing the AR CAG trinucleotide repeat (CAG), were designed using the Primer3 program (38) based on the GenBank sequence (accession number M35844). The primers were the following: AR-UNTRF (forward): 5’T-GCGAGCGCACACTCCGGC-3′ and AR-UNTRR (reverse): 5’T-CAGGTAGGC CGCGGGGCTCTA-3′. PCR assays were carried out in a total volume of 20 µL that contained 15 ng genomic DNA from peripheral blood lymphocytes, 10 pmol of each primer (Genveworks, Adelaide, South Australia, Australia), 200 µM of each dNTP (Promega Corporation, Madison, WI), 1.5 mM MgCl2, 1 M betaine (Sigma Chemicals, Perth, Western Australia, Australia), and 1x PCR buffer and 1 U AmpliTaq Gold polymerase (both from PE Applied Biosystems, Foster City, CA). Touchdown PCR amplification conditions were as follows: denaturation at 94°C for 10 minutes, followed by two cycles of 94°C for 45 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; two cycles of 94°C for 45 seconds, 58°C for 30 seconds, and 72°C for 30 seconds; two cycles of 94°C for 45 seconds, 56°C for 30 seconds, and 72°C for 30 seconds; 35 cycles of 94°C for 45 seconds, 54°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 7 minutes. PCR products and no-template control reactions were visualized on a 1.5% agarose gel to confirm that PCR was successful and that there was no contamination from external DNA sources. Genotype data were generated from 2304 individuals in total. Analysis of skewed X inactivation was carried out, as described below, on the subset (88%) that were heterozygous at the AR locus.

**Bisulfite Modification of Genomic DNA.** The bisulfite modification protocol was adapted from Flanagan et al. (39). Genomic DNA extracted from peripheral blood cells (100 ng) was diluted to a volume of 20 µL with unautoclaved water for injections (AstraZeneca, London, UK; this water was used throughout the protocol), and 2 µL of freshly prepared 3 M NaOH (≥99% pure; Merck, Melbourne, Victoria, Australia) was added. The DNA samples were denatured by heating the mixture to 75°C for 20 minutes and then immediately placed on ice. Next, 14 µL of freshly prepared 10 mM hydroquinone (Sigma Chemicals) and 250 µL of freshly prepared 4.8 M sodium bisulfite (Sigma Chemicals) were added, the samples were mixed gently, and topped with two drops of paraffin oil. The samples were incubated at 55°C for 4–5 hours away from light and then placed on ice, and the DNA was purified using an AcroPrep 96-well filter plate with a molecular weight cutoff of 30 K (PALL Life Sciences, East Hills, NY) according to the following protocol. All centrifugations were performed at 1280 g for 10 minutes at room temperature. Plate wells were moistened with 100 µL of 1× TE (10 mM Tris–HCl [pH 8.0], 1 mM EDTA), and the plate was centrifuged. The samples were then added to the wells, and the plate was centrifuged. The wells were rinsed twice with 300 µL of 1× TE followed by centrifugation. Water (50 µL) was added to each well, and the plate was shaken gently for 2 minutes and then incubated at room temperature for 8 minutes. The liquid in each well was pipetted up and down six times and then aliquoted into a 1.5-mL tube that contained 5.5 µL of 3 M NaOH, and the tubes were incubated at room temperature for 5 minutes. The resulting bisulfite-modified DNA was precipitated by adding 2 µg of glycogen (Sigma Chemicals), 11 µL of 10 M ammonium acetate (98% pure; Merck), and 270 µL of 100% ethanol (Univar, Essex, UK) to each tube, and placing the tubes at −20°C overnight. The precipitated DNA was pelleted by centrifugation at 18000 g for 30 minutes at 4°C, washed with 75% ethanol, and recentrifuged. The samples were air-dried and resuspended in 20 µL of water.

**PCR of Bisulfite-Modified DNA Samples for Analysis of Skewed X Inactivation.** Initially, we performed two separate PCR assays on a randomly selected subset of 172 bisulfite-modified DNA samples: One assay used primers that were specific for the methylated allele of the AR (CAG), (and, therefore, for the inactive X chromosome); the other assay used primers that were specific for the unmethylated allele (and, therefore, for the active X chromosome). Primers for analysis of skewed X inactivation were described in Kubota et al. (32). Methylated DNA–specific PCR assays were
carried out in a total volume of 20 µL that contained 10 µL bisulfite-modified DNA, 10 pmol of each primer (AR-MF [forward primer, fluorescently labeled with 6FAM]: 5′-GCGAGCGTGAGTTTTTTCCGC-3′ and AR-MR [reverse primer]: 5′-AAACAAATACCTTTAAAACCTCTAGC-3′ (PE Applied Biosystems), 200 µM of each dNTP (Promega Corporation), 1.5 mM MgCl₂, 10 mM β-mercaptoethanol (Sigma Chemicals), and 1x PCR buffer and 1 U Hotstart Taq polymerase (both from Eppendorf, Hamburg, Germany). PCR amplification conditions were as follows: denaturation at 95°C for 5 minutes; followed by 12 cycles of 94°C for 15 seconds, 66°C for 15 seconds (decreased by 1°C/cycle), and 72°C for 55 seconds; 30 cycles of 94°C for 15 seconds, 52°C for 15 seconds, and 72°C for 15 seconds; and a final extension at 72°C for 7 minutes. Unmethylated DNA–specific PCR assays were carried out in a total volume of 20 µL that contained 5 µL bisulfite-modified DNA, 10 pmol of each primer: AR-UF (forward primer, fluorescently labeled with NED): 5′-GTTGAGTATGTATTGTTGGT-3′ and AR-UR (reverse primer): 5′-AAATAACCTTAAAACCTCTACA-3′ (PE Applied Biosystems), 200 µM of each dNTP (Promega Corporation), 1.5 mM MgCl₂, and 1x PCR buffer and 2 U AmpliTaq Gold polymerase (both from PE Applied Biosystems). PCR amplification conditions were the same as those for AR (CAG) genotyping. In an attempt to decrease costs associated with PCR analysis of skewed inactivation, we used linear regression analysis (see “Computational Analysis of AR (CAG), Genotype and Skewed X Inactivation” below) to derive a model that could be used to calculate skewed X inactivation from only one of these assays. On the basis of the results of this analysis, we used the unmethylated DNA–specific primers only to analyze skewed X inactivation by PCR on the remaining samples (n = 1432). A 5-µL aliquot of the PCR product was visualized on a 4% agarose gel to determine the dilution factor required for X inactivation analysis.

Analysis of AR (CAG), Genotype and Skewed X Inactivation. All PCR products were diluted with water, and 1 µL was mixed with 8.5 µL of formamide and 0.2 µL of ROX internal size standard (PE Applied Biosystems). The samples were denatured by heating at 95°C for 5 minutes, placed on ice for several minutes, and then subjected to microsatellite repeat analysis with the use of an ABI 377 capillary sequencer (PE Applied Biosystems) that was programmed using GeneScan software under the following parameters—dyeset: D, run module: Genescan 36_POP4, and analysis module: Genescan 500 analysis.gsp. All microsatellite data were analyzed using Genemarker software (version 1.3; Softgenetics, State College, PA). To calculate the X inactivation ratio, we applied the following formula [originally described by Kubota et al. (32)] to our methylated and unmethylated DNA–specific PCR data for 172 samples: \( \frac{D/B}{A} \), where \( A \) and \( B \) are the shorter and longer allele products of the methylated DNA–specific PCR, respectively, and \( C \) and \( D \) are the shorter and longer allele products of the unmethylated DNA–specific PCR, respectively. This method adjusts for the fact that shorter (CAG) repeats are preferentially amplified in PCR.

Linear regression analysis was then used to derive a model to predict skewed X inactivation from a single PCR assay (ie, using primers specific for methylated or unmethylated template DNA). A correlation of 97% to results from the Kubota formula was provided by a model that included the following fixed terms: the peak area ratio for the alleles as assayed using the unmethylated DNA assay (P2); the square of P2; the individual allele repeat lengths for the AR gene polymorphism; the square of the difference in allele repeat length for the AR CAG polymorphism; and peak areas for the two alleles. The prediction model estimated skewed X inactivation at the ≥90% cut point with no bias in control samples, as estimated from the subset of samples for which skewed X inactivation results had previously been experimentally determined using results from both the methylated DNA–specific and unmethylated DNA–specific PCR (n = 172). The prediction model was applied universally to all samples to estimate skewed X inactivation from the unmethylated DNA–specific PCR assay only. We used ≥90% as a cut point for skewed X inactivation for the primary analyses for two reasons: first, such extreme levels of skewed X inactivation are considered to be the most biologically relevant, and second, the SD of the X inactivation ratio is much smaller at these levels, resulting in more robust classifications when assays are repeated. Using estimates of X inactivation ratios based on both methylated and unmethylated DNA–specific PCR assays (the full Kubota method) and comparing data from repeat assays on 41 samples, we found that the SD for the X inactivation ratio was much smaller for samples with skewed X inactivation levels of 90% or greater than for samples with skewed X inactivation of less than 80% (0.76 vs 5.7; \( P = .095 \)) or less than 90% (0.76 vs 2.3; \( P = .007 \)). Therefore, only individuals with expression of one allele in at least 90% of their cells, as measured by lack of methylation for this allele, were considered to exhibit skewed X inactivation for the majority of subsequent analyses. Alternative cut points were used for secondary analyses (Supplementary Tables 1–4, available online).

Statistical Analysis
The association between age at blood draw and skewed X inactivation was assessed with the trend test, using cut points for age that were based on tertiles for all the samples analyzed (categorized into subgroups <50, 50–60, and >60 years). Unconditional logistic regression with adjustment for age at blood draw as a categorical variable was used to compare the frequency of skewed X inactivation in the pooled control sample with that in each of the other sample groups (ie, ovarian cancer case subjects, familial breast cancer case subjects who were negative for mutations in BRCA1 and BRCA2 [BRCAX], BRCA1 mutation carriers [unstratified or stratified by cancer status], and BRCA2 mutation carriers [unstratified or stratified by cancer status]). All P values were calculated using exact methods. The association between skewed X inactivation and disease risk in BRCA1 and BRCA2 mutation carriers was assessed using Cox proportional hazards regression analysis, and the assumption of proportionality of the data was verified by visual inspection of the Kaplan–Meier plots. Subjects with a first primary invasive breast cancer diagnosis were considered to be affected, whereas subjects with no reported breast or ovarian cancer were censored at their age at blood draw or at a prior prophylactic mastectomy. Subjects with a first primary ovarian cancer diagnosis were either excluded or censored as affected at their age at ovarian cancer diagnosis. The endpoint was time to diagnosis of first breast cancer or, for secondary analyses, to diagnosis of primary ovarian cancer.
cancer. Hazard ratios (HRs) were estimated separately for BRCA1 and BRCA2 mutation carriers, with adjustment for year of birth (categorized into three subgroups [1910–1949, 1950–1959, and 1960–1979] for BRCA1 mutation carriers and two subgroups [1910–1949 and 1950–1989] for BRCA2 mutation carriers). The specific categories for year of birth were defined by subsidiary analyses assessing confounding by year of birth by decade in this sample of carriers. The 95% confidence intervals (CIs) for the hazard ratios were calculated using a robust variance approach to account for the lack of independence among individuals in the same family (40). Kaplan–Meier plots were used for graphical presentations of age at diagnosis of breast and ovarian cancer for subjects with and without skewed X inactivation. SPSS (version 13.0; SPSS Inc., Chicago, IL) and R (version 1.9.0, specifically, the eglm package for logistic regression and the survival package for Cox proportional hazards regression analysis; R Foundation, Vienna, Austria) software were used for statistical analyses, and all statistical tests were two-sided.

Results

We first genotyped a total of 2304 DNA samples for the AR exon 1 (CAG), repeat to determine heterozygosity for skewed X inactivation analysis. The heterozygosity frequency in this study was 89.4%, in the range of 88%–90% reported previously in the literature (41,42). Overall, the number of CAG repeats ranged from 8 to 36; the mean number of CAG repeats for shorter alleles, longer alleles, and sum of shorter and longer alleles were 20.3, 23.6, and 21.9, respectively. The genotype distribution in the entire study population was approximately normal. These results are comparable to those reported previously for white women (41,42).

The frequencies of skewed X inactivation (defined according to the ≥90% cut point) in normal control subjects, the ovarian cancer case subjects, the familial breast cancer case subjects who were negative for mutations in BRCA1 and BRCA2, the BRCA1 mutation carriers, and the BRCA2 mutation carriers are shown in Table 2. The two control groups had similar frequencies of skewed X inactivation (3.1% for the blood donors vs 2.8% for the population-based control subjects; P = .8) and were therefore combined for the subsequent statistical analyses. Skewed X inactivation was somewhat more common among study subjects who were older at blood draw, with statistically significant trends observed for control subjects (P\text{trend} = .02) and the BRCA1 mutation carriers (P\text{trend} = .006).

We next compared the frequency of skewed X inactivation in control subjects with that in the other subject groups after adjustment for age at blood draw (Table 3). There was no association between skewed X inactivation and disease for ovarian cancer case patients overall, or for the most common ovarian cancer subgroups defined by tumor behavior (invasive subgroup, n = 257; 85% of ovarian cancer case subjects) or tumor histological subtype (serous subtype, n = 184; 59% of ovarian cancer case subjects) (Table 3). Given a previous report (3), we also examined whether an association between skewed X inactivation and the risk of ovarian cancer was limited to case subjects with a strong family history of this disease. Of the 305 ovarian cancer case subjects for whom family history information was available, 109 had at least one first- or second-degree relative affected by breast or ovarian cancer and only one of these subjects, who reported having a single affected second-degree relative, displayed skewed X inactivation (P = .7).

We also observed no association between skewed X inactivation and disease for the familial breast cancer case subjects who were negative for mutations in BRCA1 and BRCA2. When we assessed associations stratified by age group to compare our results with associations reported previously (19,21), we observed no statistically significantly increased frequency of skewed X inactivation.

Table 2. Frequency of skewed X inactivation at the ≥90% cut point in subject groups, overall and by age at blood draw

<table>
<thead>
<tr>
<th>Group</th>
<th>Total no. analyzed</th>
<th>No. with skewed X inactivation (%)</th>
<th>Fraction with skewed X inactivation by age at blood draw (%)</th>
<th>P\text{trend}†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined control subjects</td>
<td>735</td>
<td>21 (2.9)</td>
<td>1/201 (0.5)</td>
<td>9/282 (3.2)</td>
</tr>
<tr>
<td>Population-based ovarian cancer case subjects</td>
<td>313</td>
<td>5 (1.6)</td>
<td>0/57 (0)</td>
<td>3/121 (2.5)</td>
</tr>
<tr>
<td>Invasive ovarian cancer case subjects</td>
<td>267</td>
<td>5 (1.9)</td>
<td>0/41 (0)</td>
<td>3/102 (2.9)</td>
</tr>
<tr>
<td>Serous subtype cancer case subjects</td>
<td>184</td>
<td>5 (2.7)</td>
<td>0/33 (0)</td>
<td>3/65 (4.6)</td>
</tr>
<tr>
<td>BRCA1 (non-BRCA1/2) familial breast cancer case subjects</td>
<td>235</td>
<td>4 (1.7)</td>
<td>1/78 (1.3)</td>
<td>1/96 (1.3)</td>
</tr>
<tr>
<td>BRCA1 mutation carriers</td>
<td>260</td>
<td>12 (4.6)</td>
<td>5/171 (2.9)</td>
<td>1/52 (1.9)</td>
</tr>
<tr>
<td>Breast or ovarian cancer affected†</td>
<td>140</td>
<td>6 (4.3)</td>
<td>3/76 (4.0)</td>
<td>0/56 (0)</td>
</tr>
<tr>
<td>Cancer unaffected</td>
<td>120</td>
<td>6 (5.0)</td>
<td>2/96 (2.1)</td>
<td>1/16 (6.3)</td>
</tr>
<tr>
<td>BRCA2 mutation carriers</td>
<td>63</td>
<td>3 (4.8)</td>
<td>2/24 (5.9)</td>
<td>0/15 (0)</td>
</tr>
<tr>
<td>Breast or ovarian cancer affected§</td>
<td>39</td>
<td>1 (2.6)</td>
<td>0/16 (0)</td>
<td>0/13 (0)</td>
</tr>
<tr>
<td>Cancer unaffected</td>
<td>24</td>
<td>2 (8.3)</td>
<td>2/18 (11.1)</td>
<td>0/2 (0)</td>
</tr>
</tbody>
</table>

* For BRCA1 mutation carriers with skewed X inactivation, unaffected individuals did not differ statistically significantly from affected individuals in age distribution (P = .5); Unaffected individuals ranged in age from 42 to 88 years at interview (mean = 61.3 y; SD = 19.0 y), with age at blood draw within 1 year of censor age; affected individuals ranged in age from 33 to 85 years at diagnosis of first cancer (mean = 52.7 y; SD = 19.6 y), and age at blood draw ranged from 1 to 22 years (average 11 y) after censor age defined by first diagnosis age. For BRCA2 mutation carriers with skewed X inactivation, age at blood draw was similar to censor age irrespective of cancer status.
† Two-sided.
‡ Includes 17 ovarian case subjects (seven aged <50 y, five aged 50–60 y, and five aged >60 y at blood draw), none of whom displayed skewed X inactivation.
§ Includes three ovarian case subjects (one aged <50 y, one aged 50–60 y, and one aged >60 y at blood draw), one of which displayed skewed X inactivation.
in case subjects younger than 45 years at blood draw relative to age-matched control subjects, with 0/54 case subjects and 0/154 control subjects in this group displaying skewed X inactivation. Similarly, case subjects younger than 55 years at blood draw displayed no statistically significant difference in the frequency of skewed X inactivation relative to age-matched controls (2/129 [1.6%] vs 3/329 [0.9%], respectively; \( P = .9 \)).

After adjustment for age at blood draw, \( BRCA1 \) mutation carriers unstratified by cancer status were statistically more likely than control subjects to have skewed X inactivation (odds ratio [OR] = 2.7, 95% CI = 1.1 to 6.2; \( P = .02 \)). Stratification of the \( BRCA1 \) mutation carriers by breast cancer status revealed that unaffected \( BRCA1 \) mutation carriers were statistically more likely to display skewed X inactivation than control subjects (OR = 6.1, 95% CI = 1.5 to 31.8; \( P = .005 \)), whereas \( BRCA1 \) mutation carriers with breast cancer showed a non–statistically significant association of a lower magnitude (OR = 2.3, 95% CI = 0.7 to 6.9; \( P = .1 \)). None of the seventeen \( BRCA1 \) mutation carriers with ovarian cancer demonstrated skewed X inactivation.

No statistically significant association with skewed X inactivation was detected for the \( BRCA2 \) mutation carriers unstratified by cancer status, although the risk estimate was of similar magnitude to that observed for \( BRCA1 \) mutation carriers. The small total sample size, and particularly the small number of individuals with skewed X inactivation in this group (\( n = 3 \)), precluded reliable estimates of the association among \( BRCA2 \) mutation carriers stratified by first cancer. One \( BRCA2 \) mutation carrier with skewed X inactivation had been diagnosed with ovarian cancer at age 72 years, and the remaining two were unaffected, resulting in a borderline statistically significant association between being an unaffected carrier and displaying skewed X inactivation (OR = 5.2, 95% CI = 0.5 to 28.9; \( P = .08 \)).

Given that the frequency of skewed X inactivation was considerably greater in unaffected \( BRCA1 \) mutation carriers than in breast cancer–affected \( BRCA1 \) mutation carriers, we also assessed the relationship between skewed X inactivation and age at breast cancer diagnosis in \( BRCA1 \) mutation carriers. A Cox proportional hazards regression analysis that excluded ovarian cancer–affected individuals revealed that \( BRCA1 \) mutation carriers who displayed skewed X inactivation were older at breast cancer diagnosis than those who did not (HR of breast cancer = 0.38, 95% CI = 0.15 to 0.97; \( P = .04 \)). Results were similar when the analysis included \( BRCA1 \) mutation carriers who were diagnosed with either breast or ovarian cancer (HR = 0.37, 95% CI = 0.14 to 0.95; \( P = .04 \)) (Figure 1, A). A similar relationship, albeit of lower magnitude, was observed in the small sample of \( BRCA2 \) mutation carriers (HR = 0.59, 95% CI = 0.37 to 0.94; \( P = .03 \)) (Figure 1, B). However, the findings observed for \( BRCA2 \) mutation carriers should be interpreted cautiously because evidence for increased skewed X inactivation in familial breast cancer case subjects or ovarian cancer case subjects at the ≥85% or ≥75% cut points. In fact, case subjects with invasive ovarian cancer were statistically significantly less likely than control subjects to have skewed X inactivation at the ≥85% cut point (OR = 0.5, 95% CI = 0.24 to 0.96; \( P = .03 \)) but not at the ≥75% cut point (OR = 1.2, 95% CI = 0.85 to 1.63; \( P = .3 \)). Using both the ≥85% and ≥75% cut points, \( BRCA1 \) mutation carriers were more likely than control subjects to display skewed X inactivation, particularly the unaffected \( BRCA1 \) mutation carriers ≥85% cut point: OR = 2.5, 95% CI = 1.2 to 5.7; \( P = .01 \); ≥75% cut point: OR = 1.6, 95% CI = 1.0 to 2.7; \( P = .06 \), and \( BRCA1 \) mutation carriers with skewed X inactivation were diagnosed at an older age than those without skewed X inactivation (≥85% cut point: HR = 0.7, 95% CI = 0.4 to 1.2; \( P = .2 \); ≥75% cut point: HR = 0.6, 95% CI = 0.4 to 0.8; \( P = .005 \)). Although similar relationships with skewing were observed for unaffected \( BRCA2 \) mutation carriers at both the ≥85% cut point (OR = 2.3, 95% CI = 0.4 to 8.7; \( P = .2 \)) and the ≥75% cut point (OR = 1.4, 95% CI = 0.4 to 3.8; \( P = .6 \)), the association between skewed X inactivation and older age at diagnosis in \( BRCA2 \) mutation carriers was not robust to adjustment for year of birth and to different estimates of variance that account for familial clustering (data not shown). This observation highlights the need for cautious interpretation of the results for the much smaller sample of \( BRCA2 \) mutation carriers.

Finally, we assessed whether the treatment regimen of affected individuals was associated with skewed X inactivation. There was no statistically significant difference in frequency of skewed X inactivation among affected \( BRCA1 \) mutation carriers after stratification by record or report of chemotherapy treatment or radiation at or before age at blood draw (\( P ≥ .3 \)). None of the three \( BRCA2 \) mutation carriers with skewed X inactivation (two unaffected individuals and one ovarian cancer–affected Table 3. Odds ratios for skewed X inactivation, adjusted for age at blood draw*

<table>
<thead>
<tr>
<th>Group</th>
<th>OR (95% CI)</th>
<th>( P \text{t} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined control subjects</td>
<td>1.0 (referent)</td>
<td></td>
</tr>
<tr>
<td>Ovarian cancer case subjects</td>
<td>0.5 (0.2 to 1.3)</td>
<td>.2</td>
</tr>
<tr>
<td>Invasive subgroup</td>
<td>0.6 (0.2 to 1.5)</td>
<td>.3</td>
</tr>
<tr>
<td>Serous subtype</td>
<td>0.8 (0.3 to 2.3)</td>
<td>.7</td>
</tr>
<tr>
<td>( BRCA1 ) (non-( BRCA1/2 )) case subjects</td>
<td>0.7 (0.2 to 1.9)</td>
<td>.4</td>
</tr>
<tr>
<td>( BRCA1 ) mutation carriers</td>
<td>2.7 (1.1 to 6.2)</td>
<td>.02</td>
</tr>
<tr>
<td>Unaffected</td>
<td>6.1 (1.5 to 31.8)</td>
<td>.005</td>
</tr>
<tr>
<td>Breast or ovarian cancer affected†</td>
<td>2.0 (0.6 to 5.6)</td>
<td>.2</td>
</tr>
<tr>
<td>Breast cancer affected only</td>
<td>2.3 (0.7 to 6.9)</td>
<td>.1</td>
</tr>
<tr>
<td>( BRCA2 ) mutation carriers</td>
<td>2.2 (0.4 to 8.6)</td>
<td>.2</td>
</tr>
<tr>
<td>Unaffected</td>
<td>5.2 (0.8 to 28.9)</td>
<td>.08</td>
</tr>
<tr>
<td>Breast or ovarian cancer affected$</td>
<td>1.1 (0.03 to 7.8)</td>
<td>1.0</td>
</tr>
<tr>
<td>Breast cancer–affected only$</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* OR, odds ratio; CI, confidence interval; \( BRCAX \), non-\( BRCA1/2 \) familial breast cancer cases; NA, not applicable.
† Two-sided \( P \) from logistic regression analysis.
‡ No ovarian cancer–affected \( BRCA1 \) mutation carriers displayed skewed X inactivation.
§ No breast cancer–affected \( BRCA2 \) mutation carriers displayed skewed X inactivation.
Discussion

Three studies have reported an increased frequency of skewed X inactivation in patients with early-onset breast (19,21) and/or ovarian (3) cancer. However, these findings have either not been replicated (30) or, for ovarian cancer, investigated further. We set out to investigate the occurrence of skewed X inactivation in well-characterized breast and ovarian cancer study populations, as well as in a large group of control subjects. We focused on the assessment of extremely skewed X inactivation (defined using a cut point of ≥90%) and used a sensitive allele-specific PCR-based method to quantify X chromosome inactivation. Importantly, our study included familial breast cancer case subjects and ovarian cancer case subjects who had information on their family history to specifically explore the association between skewed X inactivation and risk of familial cancer. The frequency of skewed X inactivation among the control subjects in our study was 2.9%, which is comparable to that reported in other studies (2.7%–4.5%) (18–20). In addition, our study confirmed previous reports of an increased incidence of skewed X inactivation with increasing age at blood draw among control subjects (4,5,20) and provided evidence for an increased incidence of skewed X inactivation with age in other case subject groups.

Buller et al. (3) are the only group, to our knowledge, to have studied skewed X inactivation in ovarian cancer to date; they reported an elevated frequency of skewed X inactivation (defined at the ≥75% cut point) in 213 invasive ovarian cancer subjects compared with 50 control subjects. They also reported a statistically significantly increased prevalence of skewed X inactivation in the small subset of BRCA1 mutation-positive ovarian cancer case subjects (9 of 11 were positive; P < .008) compared with control subjects, a finding of particular interest given a later study (22) that described a role for BRCA1 in X-linked gene regulation. Our assessment of larger sample sizes of invasive ovarian cancer case subjects (n = 267) and control subjects (n = 735), using what we consider to be a more technically robust and biologically meaningful cut point of ≥90%, revealed no association between skewed X inactivation and risk of ovarian cancer, either overall or within subgroups defined by tumor biology, tumor histological subtype, or reported family history of cancer. Furthermore, although our results provide support for increased incidence of skewed X inactivation in BRCA1 mutation carriers overall compared with control subjects, and so might be interpreted to be in accordance with results of Buller et al. (19), none of the 17 BRCA1 mutation–positive ovarian cancer case subjects in our sample displayed skewed X inactivation.

Three studies to date have investigated the association between skewed X inactivation and breast cancer. Kristiansen et al. (19,21) reported that subjects with early-onset breast cancer (ie, those 45 years or younger at diagnosis and with an unspecified family history [n = 40] and those younger than 55 years at diagnosis with a first- or second-degree family history [n = 107]) showed a statistically significantly higher frequency of skewed X inactivation than age-matched control subjects (n = 95 and 259, respectively). By contrast, we found no association between skewed X inactivation and early-onset breast cancer in either age group when we assessed breast cancer case subjects with a family history and no detected BRCA1 or BRCA2 mutations. It is possible that the positive associations reported by Kristiansen et al. (19,21) were due, in part, to the presence of undetected BRCA1 mutations, especially because early onset is a feature of BRCA1-related disease and mutation screening in those studies was incomplete. Alternatively, there may be an X-linked breast cancer predisposition allele that occurs at
much higher frequency in these Swedish and Norwegian founder populations than in North American and Australian populations. Yet another possibility is that skewed X inactivation may be increased in particular subgroups of breast cancers, particularly the basal-like breast tumors, which comprise only 10%–15% of sporadic breast tumors (43). It is interesting that basal-like breast tumors cluster with BRCA1–mutations–positive breast cancers in microarray analyses (44) and display many of the same X chromosome abnormalities as BRCA1–mutant tumors (23). Unfortunately, we were unable to examine whether the basal-like breast tumor subtype was more common in breast cancer case subjects displaying skewed X inactivation in our study because we had limited data available on tumor features for the familial breast cancers included in this study. A third study of almost 500 breast cancer case subjects and control subjects reported no association between skewed X inactivation and breast cancer (30).

Only one study has, to our knowledge, specifically examined the association of skewed X inactivation with BRCA1–mutation–related breast cancer; that study found no association with mutation status using DNA derived from Epstein–Barr virus–immortalized lymphoblastoid cell lines of only 38 female carriers and 41 noncarrier control subjects (31). However, we found no correlation in the ratios of X chromosome inactivation measured in DNA from two sources—lymphoblastoid cell lines and peripheral blood lymphocytes—for any of seven individuals, indicating that germline and age-acquired methylation cannot be measured accurately in cultured lymphoblastoid cell lines (Pearson product-moment correlation coefficient = −0.54; F. Lose, A.B. Spurdle, unpublished observations, 2007). Kristiansen et al. (19) reported a non–statistically significant approximately twofold increase in the frequency of skewed X inactivation in BRCA1 or BRCA2 mutation–positive breast cancer case subjects compared with control subjects. Two of 35 mutation carriers were found to display skewed X inactivation, but the breakdown by specific mutation status (BRCA1 or BRCA2) was not reported. In our study, BRCA1 mutation carriers were approximately threefold more likely than control subjects to display skewed X inactivation after adjustment for age, indicating that BRCA1 mutation status was associated with skewed X inactivation. When this BRCA1 mutation carrier group was stratified according to cancer status, we observed a borderline statistically significant twofold increase in the frequency of skewed X inactivation for BRCA1–mutant breast cancer–affected individuals only, similar to the magnitude of the non–statistically significant association observed in the dataset of Kristiansen et al. (19). This finding supports the possibility that BRCA1 mutations contribute to skewed X inactivation, because assessing BRCA1 mutation carriers without breast cancer is an indirect assessment of the association between BRCA1 mutation status and skewed X inactivation.

It is somewhat surprising that BRCA1 mutation carriers who were unaffected by breast cancer exhibited a statistically significant fivefold increase in skewed X inactivation compared with control subjects. This finding was corroborated by the Cox proportional hazards regression analysis of the association between skewed X inactivation and age at diagnosis of breast cancer, which revealed that age at diagnosis of breast or ovarian cancer was statistically significantly later in subjects with skewed X inactivation than in subjects with an X inactivation ratio of <90%. There was also suggestive evidence that a similar association, albeit of smaller magnitude, may exist for BRCA2 mutation carriers.

The mechanism responsible for skewed X inactivation in BRCA1 mutation carriers remains unclear. However, published data suggest several possibilities. First, candidate loci for genes involved in the choice of X chromosome for inactivation in humans have been identified at Xq13 and Xq25–26 (9–12). Xq13 contains the locus for the X inactivation–specific transcript gene (XIST), which encodes an RNA transcript that is a key player in initiating inactivation of one X chromosome and may also be involved in choosing which X chromosome will be inactivated (12,45). Another candidate gene for X chromosome choice is the human homolog of the mouse DXPas34 gene, which has been shown to be involved in X inactivation (46). Second, there is evidence to suggest that BRCA1 is involved in regulation of expression of X-linked genes. BRCA1 has been reported to colocalize with XIST RNA on the inactive X chromosome, and mutant BRCA1 has been shown to disrupt localization of XIST RNA (24,25). Although there is some speculation about whether BRCA1 is directly involved in X inactivation (26–29), three microarray studies have demonstrated that BRCA1 gene mutations are associated with the overexpression of clusters of genes located at Xp22, Xp11, Xq13, and Xq26–28 (22,23,47).

We hypothesize that the deregulated expression of X-linked genes that results from mutation of BRCA1 may have contributed to the observed results for BRCA1 mutation carriers. For example, it is possible that overexpression of an X-linked gene involved in X chromosome choice may lead to skewed X inactivation if an allele of this gene predisposed cells to undergo such skewing. Another possibility is that an X-linked gene that is overexpressed as a consequence of a BRCA1 mutation may possess a common allele that marginally improves genomic stability in cells carrying a BRCA1 mutation, which could lead to skewed X inactivation as a protective mechanism in germline cells of BRCA1 mutation carriers. Overexpression of such an allele in BRCA1 mutation carriers would also explain why skewed X inactivation was more common in unaffected BRCA1 mutation carriers than in affected BRCA1 mutation carriers and was associated with later age at cancer diagnosis. In addition, we observed a similar relationship between skewed X inactivation and age at cancer diagnosis for the much smaller sample of BRCA2 mutation carriers, although the association was not statistically significant, at least at the most stringent cut point of ≥90%. Given the lack of published evidence for any involvement of BRCA2 with the X chromosome (47), if this association is verified, we suggest that the expression of an X-linked allelic variant protective for cancer is also favored in unaffected BRCA2 mutation carriers. Because both BRCA1 and BRCA2 are involved in the same repair pathway (48), it is not unreasonable to assume that an X-linked genetic modifier of BRCA1 might also modify BRCA2.

Our study has several limitations. Despite the relatively large sample sizes examined for skewed X inactivation, our interpretations are based on the small number of individuals who displayed skewed X inactivation. Similarly, although we found no evidence to suggest that the treatment regimen of affected individuals was associated with the observed patterns of?
X chromosome inactivation, the numbers of individuals in each stratum were small. A much larger study would better assess the possibility that treatment regimen influences patterns of X chromosome inactivation. Such a study would also be useful to investigate the marginally statistically significant association we observed between skewed X inactivation and BRCA2 mutation status to clarify the underlying mechanism.

An alternative and more direct approach to identify X-linked modifiers would be to carry out a comprehensive study to assess the association between X-linked polymorphisms and cancer risk in a large sample of BRCA1 and BRCA2 mutation carriers. In this regard, it is interesting that several X-linked genes have been shown to predispose individuals to Fanconi anemia, an autosomal recessive syndrome that is characterized by diverse clinical symptoms, hypersensitivity to DNA cross-linking agents, chromosomal instability, and susceptibility to cancer (15,49), characteristics associated with individuals homozygous for deleterious BRCA2 mutations (50), and that the X-linked gene FOXF3 has recently been identified as a breast cancer suppressor gene (51).

In conclusion, our data show that skewed X inactivation occurs at increased frequency in BRCA1 mutation carriers compared with control subjects and is associated with a statistically significantly older age at breast cancer diagnosis. Skewed X inactivation may also occur at increased frequency in unaffected BRCA2 mutation carriers, although the evidence to support this possibility is much weaker. We propose that skewed X inactivation arises as a mechanism to favor expression of an X-linked allele that protects against cancer and that skewed X inactivation may be exacerbated in BRCA1 mutation carriers due to BRCA1 mutation–related overexpression of X-linked genes.

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