Association Between Nonrandom X-Chromosome Inactivation and BRCA1 Mutation in Germline DNA of Patients With Ovarian Cancer

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Background: Most human female cells contain two X chromosomes, only one of which is active. The process of X-chromosome inactivation, which occurs early in development, is usually random, producing tissues with equal mixtures of cells having active X chromosomes of either maternal or paternal origin. However, nonrandom inactivation may occur in a subset of females. If a tumor suppressor gene were located on the X chromosome and if females with a germline mutation in one copy of that suppressor gene experienced nonrandom X-chromosome inactivation, then some or all of the tissues of such women might lack the wild-type suppressor gene function. This scenario could represent a previously unrecognized mechanism for development of hereditary cancers. We investigated whether such a mechanism might contribute to the development of hereditary ovarian cancers.

Methods: Patterns of X-chromosome inactivation were determined by means of polymerase chain reaction amplification of the CAG-nucleotide repeat of the androgen receptor (AR) gene after methylation-sensitive restriction endonuclease digestion of blood mononuclear cell DNA from patients with invasive (n = 213) or borderline (n = 44) ovarian cancer and control subjects without a personal or family history of cancer (n = 50). BRCA1 gene status was determined by means of single-strand conformational polymorphism analysis and DNA sequencing. All statistical tests were two-sided.

Results and Conclusions: Among individuals informative for the AR locus, nonrandom X-chromosome inactivation was found in the DNA of 53% of those with invasive cancer versus 28% of those with borderline cancer (P = .005) and 33% of healthy control subjects (P = .016). Nonrandom X-chromosome inactivation can be a heritable trait. Nine of 11 AR-informative carriers of germline BRCA1 mutations demonstrated nonrandom X-chromosome inactivation (.0002 < P < .008, for simultaneous occurrence of both).

Implications: Nonrandom X-chromosome inactivation may be a predisposing factor for the development of invasive, but not borderline, ovarian cancer. [J Natl Cancer Inst 1999;91:339–46]

Assessment of the clonal origin of human tumors in females is facilitated by random X-chromosome inactivation that occurs in somatic cells (1–4). This phenomenon, termed Lyonization, occurs during interphase early in embryogenesis when one X chromosome is randomly inactivated by condensation to form the sex chromatin body (5). The remaining, uncondensed X chromosome becomes the active X chromosome and is inherited by all daughter cells through subsequent mitotic divisions. Because this process is random, adult female tissues are cellular mosaics, wherein half of the cells contain an active maternal X chromosome and the other half contain an active paternal X chromosome. Cancers arising from a sequence of molecular ge-
etic changes to a single cell should demonstrate the presence of the same active X chromosome throughout the tumor. Conversely, when a series of carcinogenic events generate a polyclonal cancer, tumor samples should demonstrate mosaicism at informative X-chromosome loci.

Due to its highly informative nature, study of the CAG repeat of exon 1 of the androgen receptor (AR) has facilitated tumor clonality studies (6–10). Four distinct potential methylation sites located upstream of this repeat control expression of the AR locus. When these sites are methylated, as on the inactive X chromosome, the gene is not transcribed. When these sites are unmethylated, as on the active X chromosome or in males, the AR gene is transcribed. The methylation sites are sequences recognized and cut by the restriction endonucleases HhaI (GGCG) and HpaII (CCGG) only when they are unmethylated. Resistance to digestion by these enzymes thus identifies the inactive X chromosome (11). At least two laboratories studying tumor clonality with markers less informative than this CAG repeat have noted nonrandom X-chromosome inactivation in germline DNA of both healthy and cancer-affected individuals (12,13). Two large AR analyses of X-chromosome inactivation patterns in more than 400 healthy females have confirmed that while patterns generally follow a normal distribution, sizable segments of the population are skewed in their pattern of X-chromosome inactivation. (14,15)

Tumor suppressor genes exert their effects according to the Knudson hypotheses in one of two ways (16,17). For most sporadic cancers, loss of one allele (loss of heterozygosity = LOH) is followed by mutation within the remaining allele. This mechanism explains the role of classic tumor suppressor genes, such as p53 (also known as TP53) and retinoblastoma (also known as Rb) (18). Additionally, germline carriers of a tumor suppressor gene mutation are predisposed to cancer on the basis of the need only to lose the wild-type allele because the remaining allele already carries a mutation. This mechanism explains the predisposition to a variety of cancers seen in individuals with Li–Fraumeni syndrome carrying germline p53 mutations (19). It also explains early onset, bilateral retinoblastomas in individuals carrying germline Rb mutations. Hereditary, early onset, breast, and ovarian cancers have been attributed to germline mutations of the BRCA1 and BRCA2 tumor suppressor genes (20–24). All four of these genes are autosomal in location so that normal human cells carry two copies of each gene. In contrast, for putative X-linked tumor suppressor genes, nonrandom X-chromosome inactivation is equivalent to a functional LOH in all affected cells. This equivalency is because each cell carries only a single active X chromosome. Therefore, either a mutation or a loss of the active allele from the preferentially active X chromosome would render the cell without a functional copy of putative X-linked tumor suppressor genes in a single step.

Several authors have demonstrated that in ovarian cancer, frequent LOH at several regions of the X chromosome suggests that this chromosome may indeed harbor one or more tumor suppressor genes (25–28). Nonrandom X-chromosome inactivation has been shown to play a role in the development of X-linked recessive disorders such as Wiskott–Aldrich syndrome (29). Thus, Mendelian inheritance of nonrandom X-chromosome inactivation associated with a germline putative X-chromosome-linked tumor suppressor gene mutation could contribute to some cases of familial cancers by short-circuiting the traditional Knudson model (16,17). To test this hypothesis as a mechanism of hereditary ovarian cancer, we evaluated germline DNA from a cohort of ovarian cancer patients and, where possible, from their affected first- and second-degree relatives. The frequency of nonrandom X-chromosome inactivation was also determined for healthy, unrelated control female subjects without a family history of breast, ovarian, or colon cancer.

Materials and Methods

Source and handling of tissue specimens—human tissue approvals. All samples were procured after obtaining a signed informed consent in accordance with the Institutional Committee for the Protection of Human Subjects. High-molecular-weight DNA was extracted from specimens of peripheral blood mononuclear cells obtained from patients with epithelial ovarian cancer (n = 257), including 44 patients with borderline ovarian carcinoma. Control subjects (n = 50) were established by recruiting paid, unrelated, adult females without a personal or family history of breast, ovarian, or colon cancer. Standard procedures of DNA preparation from either blood mononuclear cells (germline DNA) or tumor cells (somatic DNA) involved protease digestion and phenol/chloroform/isoamyl alcohol extraction. The details of the procedures used by our laboratory have been described elsewhere (30,31). The final concentration of DNA ranged from 10 to 40 ng/µL.

X-Chromosome inactivation studies. The highly polymorphic trinucleotide repeat (CAG) in the human AR gene was studied to determine X-chromosome inactivation (10). Germline DNA was subjected to restriction enzyme digestion at 37 °C by mixing 50–200 ng of DNA and 20 U of HhaI restriction endonuclease (New England Biolabs, Inc.) in New England buffer #4 (New England Biolabs, Inc., Beverly, MA) in New England buffer #4 (New England Biolabs, Inc.) in a total volume of 10 µL for the times indicated, usually 12 hours. In parallel, each sample served as its own control by omitting the restriction enzyme and normalizing the volume by the addition of distilled water. Reactions were terminated by heat inactivation at 95 °C for 10 minutes. Alternatively, substitution of the restriction enzyme HpaII for HhaI was carried out to confirm results. Each digest sample (2 µL) was amplified in a 10-µL polymerase chain reaction (PCR) reaction containing 1 µM of [32P]end-labeled AR sense primer, 2 µM of unlabeled AR antisense primer, 100 mM deoxyadenosine triphosphate (dATP), 100 mM deoxythymidine triphosphate (dCTP), 100 mM deoxyguanosine triphosphate (dTTP), 100 mM deoxythymidine triphosphate (dTTP), 0.1 µU Taq polymerase (Promega Corp., Madison, WI), 2 mM MgCl₂, 50 mM KCl, 10 mM Tris–HCl buffer, pH 9.0, 0.1 mg/µL of gelatin. PCR amplification was performed with an initial denaturation step of 95 °C for 5 minutes followed by 29 cycles as follows: 95 °C for 45 seconds, 61.5 °C for 30 seconds, and 72 °C for 30 seconds. The PCR products were analyzed on 8% polyacrylamide sequencing gels. Labeling only one of the primers enhanced band resolution. Primers used in this reaction were: AR-sense: 5'-CTT CAA AGT GCT TGT TCC AGA GCG TGC-3' and AR-antisense: 5'-GCT GTT CCT CAT-3' (10). In an attempt to quantitate differences in methylation for informative DNA samples, we evaluated the ratio of AR amplifier band intensity on autoradiograms. The lower band (shorter CAG repeat) was taken as the reference band. For informative samples, calculation of a modified allelic cleavage ratio was performed:

\[
\text{modified allelic cleavage ratio} = \frac{\text{lower band intensity}}{\text{upper band intensity}}
\]

The expected value of the modified allelic cleavage ratio is 1.0 because germline tissues are predicted to contain equal populations of active maternal and paternal AR alleles, according to the Lyon hypothesis. Several investigators have noted some degree of asymmetry or skewing in the distribution of this parameter and have defined nonrandom X-chromosome inactivation variously as twofold (32), threefold (12), or fourfold (13) differences in the band intensities of the X-linked gene following appropriate restriction digest. Initially, we quantitated this ratio for all DNA samples from control subjects, both digested and undigested (see the “Results” section), by optical scans on a laser densitometer (Protein & DNA Image Wave System, Huntington Station, NY). This analysis allowed us to confirm the published observation of Mutter et al. (32) that differences of this magnitude are readily detectable visually. We found significant variability in the calculated modified allelic cleavage ratio for a given sample. Parameters influencing this variability included the observer, band resolution on a given gel, whether 32P or 3H-adenosine triphosphate (ATP) was used, and the scan technique such as adjustment of baseline and whether peak height or area
under the curve was used. Accordingly, we arbitrarily chose to utilize a visual difference of greater than 3:1 between band intensities to denote nonrandom X-chromosome inactivation for our analysis of the germline DNA samples from patients with ovarian cancer. This corresponds to a modified allelic cleavage ratio of less than 0.33 or greater than 3.0 and resulted in a minimum difference between band intensities of at least twofold, independent of observer, and scan technique determined by repetitive scans made by several observers. Furthermore, as shown below in the “Results” section, this difference exceeds any potential systematic error. Substitution of 7-deaza-2′-dGTP for dGTP was performed on selected samples exhibiting nonrandom X-chromosome inactivation to rule out PCR bias, which may occur when amplifying GC-rich targets (33).

Determination of loss of heterozygosity. LOH at various loci in available paired ovarian cancer (somatic) and germline DNA samples was determined using standardized PCR methodology. p53 LOH was determined by allelotyping the tumor on the basis of the codon 72 polymorphism. Alu repeats, and mutational analysis as we described (31). BRCA1 LOH was determined using the microsatellite repeats D17S855, D17S1322, and D17S1323 (34). The D17S1322 and D17S1323 markers can be multiplexed. BRCA2 LOH was based on allelic loss of either D13S1700, D13S1701, or D13SBR2. In each case, approximately 20 ng of DNA was added to a reaction mix that contained 20[μ]M end-labeled primer, 100mM dATP, 100mM dCTP, 100mM dGTP, 100mM dTTP, and 1 U of Tag polymerase for amplification as we have described (31,35–36). For this analysis, we selected 102 probands from our 213 case patient cohort for which paired germline and ovarian cancer (somatic) DNA samples were available and required the tumor DNA samples to be informative at a minimum of two of the three study loci (BRCA1, BRCA2, or p53). The samples were also selected to render as many informative (100 of 102) at the AR locus as possible to minimize the power of the LOH analysis.

Family history, pedigree, pathologic confirmation of cancers, and follow-up. A complete pedigree was obtained on each individual studied with pathologic follow-up of reported breast or ovarian cancers among family members when possible. Family history of breast or ovarian cancer was determined by reviewing the proband’s pedigree to determine the number of relatives affected by these cancers. For this analysis, we counted only first-, second-, and third-degree relatives. A positive family history was noted if one additional ovarian cancer or two additional breast cancers were documented by pathologic review or by death certificate.

Statistical analysis. Statistical analyses included population sample statistics, skewness, kurtosis, Pearson chi-squared testing of categorical or t tests of continuous variables. The binomial distribution was used to calculate the probability of a specific combination of BRCA1 mutations in association with nonrandom X-chromosome inactivation (14). These calculations were performed by the BMDP Statistical Software (Biostatistical Data Package Statistical Software, Inc., Los Angeles, CA) package on a desktop computer. P values less than .05 were considered significant. All statistical tests were two-sided.

RESULTS

Fig. 1 shows several examples of germline AR expression from three different control subjects: C1, C6, and C28. Traditional random X-chromosome inactivation, with a modified allelic cleavage ratio = 1.36, was seen in the DNA of subject C6, while visually complete nonrandom X-chromosome inactivation, with a modified allelic cleavage ratio = 4.81, was demonstrated for subject C28. The modified allelic cleavage ratio for subject C1 was 0.213, representing an intermediate degree of nonrandom X-chromosome inactivation. All amplimers from control subject undigested DNA samples prepared from peripheral blood mononuclear cells were scanned as described in the “Materials and Methods” section. A mean modified allelic cleavage ratio of 1.12 (median, 1.10; standard deviation, 0.187; skewness, 0.66) was obtained. The modified allelic cleavage ratio ranged between 0.66 and 1.76, with only two values outside two standard deviations from the mean (0.74–1.49). These data show that under ordinary circumstances, relatively symmetric unbiased amplification from both AR alleles was obtained. In contrast, AR amplimers from HhaI restriction digests demonstrated skewed amplification (skewness, 1.36), with a slightly greater tendency toward nonrandom X-chromosome inactivation of the lower allele (shorter AR repeat). These data are plotted in Fig. 2. For this dataset, 20 of 45 informative samples fall outside three standard deviations of the population mean defined from our analysis of the paired undigested samples. In 15 samples, greater than a threefold difference in band intensity was calculated. These samples were considered representative of nonrandom X-chromosome inactivation. Ten of these samples were reanalyzed by carrying out the PCR reaction with 7-deaza-2′-dGTP substituted for dGTP. This substitution has been reported to eliminate PCR bias that may occur when amplifying GC-rich targets (33). No significant shift in the calculated modified allelic cleavage ratio was detected for any sample (data not shown). Furthermore, retention of either the upper (C1, Fig. 1) or the lower band (C28, Fig. 1) as the inactive allele is taken as strong evidence against simple PCR bias as the cause of the skewed patterns of X-chromosome inactivation.

AR analysis of 213 germline DNA samples from ovarian cancer probands, 44 germline samples from patients with borderline ovarian cancer, and 50 healthy control subjects are summarized in Table 1. Two distinct AR alleles were identified in 83% (range, 82%–90%) of the DNA samples analyzed. Nonrandom X-chromosome inactivation was recorded for 33%, 28%, and 53% of informative germline DNA samples from control subjects, patients with borderline epithelial ovarian cancer, and patients with invasive epithelial ovarian cancer, respectively. Chi-squared analysis of informative DNA samples in 2 x 2 tables revealed that nonrandom X-chromosome inactivation was more common in invasive cancers than in borderline cancers ($\chi^2 = 7.87, P = .005$) or in DNA from control subjects without a family history of cancer ($\chi^2 = 5.79; P = .016$). The rate of random X-chromosome inactivation was the same for control subjects and for patients with borderline ovarian cancer ($\chi^2 = 0.59, P = .29$). Indeed, if the data from germline samples from control subject and borderline cancer patients are combined for comparison with the data from germline DNA from patients with invasive cancers, the difference in frequency of nonrandom X-chromosome inactivation becomes more impressive ($\chi^2 = 11.3, P = .0007$). Within the invasive cancer group, the frequency of nonrandom X-chromosome inactivation was the same when comparisons were carried out on the basis of histology, age at diagnosis (56.1 versus 57.4 years, $P = .90$), or stage of the disease. Stage of disease was determined by the criteria of
FIGO stage of borderline cancers was also independent of the X-chromosome inactivation pattern, suggesting that tumor initiation rather than progression is modified in some way by this process.

To rule out nonrandom X-chromosome inactivation as an artifact of incomplete digestion due to the ratio of restriction enzyme to DNA, we carried out a time course of digestion. The standard deviations were calculated on the basis of scans of the sham digest band intensities for control subject DNA samples as described in the “Results” section.

Table 1. Patterns of germline X chromosome inactivation in patients with ovarian cancer and healthy control subjects

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Informative (%)*</th>
<th>Nonrandom X-chromosome inactivation (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control subjects</td>
<td>50</td>
<td>45 (90)</td>
<td>15 (33)</td>
</tr>
<tr>
<td>Borderline cancers</td>
<td>44</td>
<td>36 (82)</td>
<td>10 (28)</td>
</tr>
<tr>
<td>Invasive cancers</td>
<td>213</td>
<td>174 (82)</td>
<td>93 (53)</td>
</tr>
<tr>
<td>FIGO stage‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>48</td>
<td>39 (81)</td>
<td>23 (59)</td>
</tr>
<tr>
<td>II</td>
<td>16</td>
<td>13 (81)</td>
<td>6 (46)</td>
</tr>
<tr>
<td>III</td>
<td>112</td>
<td>89 (79)</td>
<td>43 (37)</td>
</tr>
<tr>
<td>IV</td>
<td>34</td>
<td>30 (88)</td>
<td>14 (47)</td>
</tr>
<tr>
<td>X</td>
<td>3</td>
<td>3 (100)</td>
<td>2 (67)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>adenocarcinoma not otherwise specified</td>
<td>50</td>
<td>44 (88)</td>
<td>25 (57)</td>
</tr>
<tr>
<td>clear cell</td>
<td>11</td>
<td>9 (82)</td>
<td>5 (56)</td>
</tr>
<tr>
<td>endometrioid</td>
<td>32</td>
<td>27 (84)</td>
<td>11 (41)</td>
</tr>
<tr>
<td>mucinous</td>
<td>23</td>
<td>21 (91)</td>
<td>12 (57)</td>
</tr>
<tr>
<td>papillary serous</td>
<td>94</td>
<td>70 (74)</td>
<td>37 (53)</td>
</tr>
<tr>
<td>transitional</td>
<td>3</td>
<td>3 (100)</td>
<td>3 (100)</td>
</tr>
</tbody>
</table>

*For androgen receptor locus.
†This can be determined only for informative subjects.
‡International Federation of Gynecology and Obstetrics (FIGO) staging corresponds to tumor confined to the ovary (stage I), confined to the pelvis (stage II), confined to the abdomen (stage III), spread to the liver parenchyma or outside the abdomen (stage IV), or insufficient surgical–pathologic data to assign a stage (stage X) when the proband is initially diagnosed (36).

Male DNA has only one X chromosome and it is active, complete digests yield no bands when amplified by PCR.

HpaII enzymatic digestion performed on 141 samples was used to compare differences in methylation determined from the HhaI digest. Fourteen (10%) samples revealed inconsistent patterns between the two different restriction enzymes, suggesting some degree of differential methylation. However, since this difference was not statistically significant and because we were interested in methylation at either locus, we subsequently carried out digests using both HhaI (10 U) and HpaII (10 U) with Buffer 4 plus BSA in a 25-μL volume that precluded the need for dual reactions.

The pedigrees of many ovarian cancer probands with nonrandom X-chromosome inactivation were striking in that a family history of breast and/or ovarian cancer was commonly reported. Pedigrees of four such individuals are shown in Fig. 4. Fig. 5 represents a gel analysis of available germline DNA samples from three of the families from Fig. 4. In these figures, a Roman numeral represents the generation and an Arabic subscript serves to differentiate between individuals within each generation. When two bands appear on PCR amplification of the control (undigested) DNA sample, but only one from the PCR

![Fig. 2. Distribution of pattern of modified allelic cleavage ratios. Autoradiograms similar to those in Fig. 1 were scanned by use of a laser densitometer as described in the “Materials and Methods” section. The ratio of the lower band divided by the upper band intensity was calculated and plotted as a frequency distribution histogram for restriction digests of control subject DNA samples. Intervals for modified allelic cleavage ratios were calculated on the basis of standard deviations from the sample mean, found to be 1.1 in the sham digests. The standard deviations were calculated on the basis of scans of the sham digest band intensities for control subject DNA samples as described in the “Results” section.](https://academic.oup.com/jnci/article-abstract/91/4/339/2543931)

![Fig. 3. Results of the time course of restriction enzyme digestion to ascertain that nonrandom X-chromosome inactivation is not an artifact of incomplete digestion due to the ratio of restriction enzyme to DNA. Mononuclear cell DNA samples were subjected either to sham (C) showing nonrandom X-chromosome inactivation or restriction endonuclease HhaI digestion (D) as described in the “Materials and Methods” section. Aliquots were removed at the indicated times for PCR amplification. The digestion reaction was essentially complete by 1 hour for the DNA sample from C28, showing nonrandom X-chromosome inactivation. Digestion of the DNA sample C25 was unchanged over a 12-hour time period, demonstrating traditional random X-chromosome inactivation.](https://academic.oup.com/jnci/article-abstract/91/4/339/2543931)
amplified restriction nuclease digested germline DNA sample, one is able to determine if the same X chromosome is active (disappears with digest) in the germline of multiple family members and the association of this relationship to cancers in the families. For a given family, the presence of a band at the same position in the gel for two different family members indicates a shared AR allelotype. Several important observations emerge from a careful study of these two figures. Individuals in different generations of the same family can demonstrate germline non-random X-chromosome inactivation associated with either ovarian or breast cancer or both. This association did not hold for all individuals in a given family who developed one of these cancers. The potential for mother-daughter (families 8, 23, and 26) as well as father–daughter transmission (Family 15) exists. Finally, not all affected individuals share the same active or inactive AR allelotypes. Thus nonrandom X-chromosome inactivation may play a complex role in some hereditary breast and ovarian cancer families.

Studies (20–24) have shown that germline mutations of both BRCA1 and BRCA2 are associated with hereditary breast and ovarian cancers. Both genes are predicted to be tumor suppressor genes and in each case wild-type LOH at the appropriate locus contributes to expression of mutant germline BRCA product from the remaining allele. BRCA1 LOH is common in sporadic as well as familial ovarian cancers. p53 mutation is also frequently associated with ovarian and breast cancers, but unlike BRCA1 does not appear to be related to hereditary cancers other than those of the Li–Fraumeni syndrome (19,30). To further understand the relationship of nonrandom X-chromosome inactivation to hereditary breast and ovarian cancer, we investigated LOH at the BRCA1, BRCA2, and p53 loci. The LOH data was then related to nonrandom X-chromosome inactivation. These results are shown in Table 2. BRCA1 LOH, but not BRCA2 LOH or p53 LOH was significantly associated with nonrandom X-chromosome inactivation (P = .04).

All four families whose pedigrees and AR allelotypes are

Table 2. Association of germline nonrandom X-chromosome inactivation with tumor loss of heterozygosity (LOH) at the BRCA1, BRCA2, and p53 loci in patients with ovarian cancer

<table>
<thead>
<tr>
<th>Marker</th>
<th>LOH</th>
<th>No</th>
<th>Yes</th>
<th>χ²</th>
<th>P (two-sided)</th>
</tr>
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<tbody>
<tr>
<td>BRCA1</td>
<td>No</td>
<td>26</td>
<td>15</td>
<td></td>
<td>.04</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>22</td>
<td>30</td>
<td>4.09</td>
<td></td>
</tr>
<tr>
<td>BRCA2</td>
<td>No</td>
<td>35</td>
<td>28</td>
<td></td>
<td>.42</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>13</td>
<td>16</td>
<td>.65</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>No</td>
<td>17</td>
<td>13</td>
<td></td>
<td>.59</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>35</td>
<td>34</td>
<td>.30</td>
<td></td>
</tr>
</tbody>
</table>

*To qualify for this analysis, case patients had to be informative for the X-chromosome-linked androgen receptor gene (n = 100). Simultaneous paired comparisons were not possible for all informative patients for all three markers (BRCA1, BRCA2, and p53); however, patients who were informative for any two of the three markers were included in the analysis.
shown in Figs. 4 and 5 are candidate hereditary breast and ovarian cancer families. In concurrent and ongoing studies, our laboratory has carried out BRCA1 mutation screening and sequenced candidate mutations (35,37). To date, we have identified 19 BRCA1 mutations among a 102 case patient subset of the DNA samples, and we also evaluated for patterns of X-chromosome inactivation. The specifics of our initial analysis have been submitted for publication elsewhere. The mutations sequenced include six somatic and 13 candidate germline mutations, of which eight are classic frameshift or nonsense mutations. Among this 102 patient subset, one hundred (98%) germline samples were informative at the AR locus and, of these, 50 (49%) demonstrated nonrandom X-chromosome of the DNA inactivation. Eleven (85%) of 13 candidate germline BRCA1 mutation carriers were informative at the AR locus for X-chromosome inactivation studies. Nine (82%) of these 11 displayed nonrandom X-chromosome inactivation patterns including both members of Family 15 (Fig. 5, middle panel). Using the 33% prevalence rate of nonrandom X-chromosome inactivation in control subjects as a lower limit and the 53% prevalence of nonrandom X-chromosome inactivation in ovarian cancer probands as an upper limit, the probability that nine of 11 germline BRCA1 carriers would simultaneously demonstrate nonrandom X-chromosome inactivation can be estimated from the binomial distribution: \(11(0.33)^9(0.67)^2 = 0.0002 < P < 0.008 = 11(0.53)^9(0.47)^2\).

**DISCUSSION**

There is a lack of consensus on the best method to measure patterns of X-chromosome inactivation (38–40). The mathematical model used to quantitate skewing and indeed the cutoff point used to define a significant difference in band intensities changes depending on the investigator carrying out the study (12–15,32,41). We initially used a modified allelic cleavage ratio based on the band intensity of the lower allele amplified from the HhaI restriction digest of the control subject DNA samples. This ratio is biased neither by differences in DNA content between undigested DNA samples and those DNA samples digested with restriction endonuclease nor the amount of sample loaded on the gel for electrophoresis. It would not detect a reversal of asymmetric amplification between control DNA samples and digests. We were troubled by modified allelic cleavage ratio variations of 25%–30% routinely generated. The variations were dependent on the person performing the scan, day-to-day variation for the same person scanning the same gel, scatter from \(^{32}P\) incorporated into the AR amplifier, band spread on a given gel as well as between gels, and the particular software options used to calculate band intensities. At this time, we are unable to assign a biological significance to modified allelic cleavage ratios of 1.1, 1.4, or 1.8. After a careful analysis of all DNA samples from control subjects, we adopted the approach (visual evaluation of electrophoretic bands) of Mutter et al. (32) and the stringency level proposed by Gale et al. (12) requiring a visual threefold difference in band intensities to make a call of nonrandom X-chromosome inactivation.

The original reports of skewed patterns of X-chromosome inactivation in normal tissue emanated from tumor clonality studies (12,13). There are no previous reports of this phenomenon related to ovarian cancer. A 33% incidence of nonrandom X-chromosome inactivation among healthy control subjects in the present study is higher than the 23% found in the hemato-logic normal individuals studied by Gale et al. (12) in England and much higher than the 3/81 (4%) reported by Vogelstein et al. (13). However, if we apply the same 3:1 difference in relative band intensities to the data from 365 normal females reported by Naumova et al. (14), we calculate a nonrandom X-chromosome inactivation rate of approximately 32%. This rate is precisely in agreement with the rate among the control individuals in our study. In marked contrast, nonrandom X-chromosome inactivation was found in 53% of germline DNA from probands we have studied with invasive ovarian cancer. This frequency is a remarkable finding that is statistically significantly different from: 1) our healthy control subjects without a family history of breast or ovarian cancer, 2) individuals with borderline ovarian cancers, and 3) the frequency noted among a large series of healthy females previously reported in the literature (14). Clearly, the use of the AR polymorphism to determine X-chromosome inactivation in tumor clonality studies should be very carefully controlled.

Occasionally, we have drawn blood samples during chemotherapy. Because profound neutropenia and lymphocytopenia can occur after chemotherapy, it is theoretically possible that we sampled clonal expansions following pancytopenic nadirs for a small subset of patients (12). This explanation is unlikely because we have repeated blood draws more than a year following completion of chemotherapy and the same X-chromosome was inactivated in the follow-up specimens (data not shown). In addition, we have retrieved several normal tissue samples such as large and small bowel removed as part of the cytoreductive ovarian cancer surgery for three individuals who demonstrated skewed patterns of X-chromosome inactivation. In each case, the same skewed pattern of X-chromosome inactivation was present in the non-hematologic tissue as well. These studies require expansion. However, on a preliminary basis it appears that nonrandom X-chromosome inactivation is not tissue specific, but rather can be a characteristic of a given individual. This finding argues in favor of nonrandom X-chromosome inactivation occurring due to selection for a gene that offers proliferative advantage not only to lymphoid cells, but to precursor cells for any organ of the body as well. The association of nonrandom X-chromosome inactivation with germline BRCA1 mutation could in part explain why there are increases in prostate cancer, and colon cancer in addition to breast and ovarian cancer in hereditary breast or ovarian cancer families (42).

Gale et al. (43) have observed both similar and dissimilar patterns of skewed X-chromosome inactivation between normal hematologic and nonhematologic tissues. These authors offer an alternative explanation for skewed X-chromosome inactivation arguing that this phenomenon is still random. Specifically, they support the hypothesis of McLaren (44) that the number of cells present in the embryo at the time of inactivation or, the number of cells that generate a given organ, modifies the pattern of X-chromosome inactivation.

The pedigrees and AR allelotype analyses we have presented suggest that nonrandom X-chromosome inactivation can be inherited. By studying additional family members, the development of more cancers in the study families, and the use of other highly informative X-chromosome markers to render some of our noninformative AR samples informative, we hope to determine in future studies whether or not the inheritance pattern is Mendelian.

We have shown that a common AR allelotype and the same
active X chromosome can be shared among individuals with early onset breast and ovarian cancers. The two probands from Family 15, however, relate the independence of nonrandom X-chromosome inactivation and the AR allelotype. Thus, a factor other than the AR itself is responsible for this process. Naumova et al. (14) have proposed a model of nonrandom X-chromosome inactivation that results from an X-chromosome associated factor, X-inactive specific transcript (XIST), distinct from the X-chromosome inactivation center. These authors used their Family K1362 to demonstrate that nonrandom X-chromosome inactivation was unlikely to be due to an autosomal factor since there is a low probability that such a factor could be transferred from the grandmother to her son, and to all seven granddaughters. None the less, we have observed two distant relatives in our Family 15 with autosomal germline 17q21 BRCA1 germline mutation who both demonstrate nonrandom X-chromosome inactivation. Overall, 9 of 11 informative ovarian cancer probands carrying a germline BRCA1 mutation also demonstrated a skewed pattern of X-chromosome inactivation. We estimated that the probability of this association happening by chance alone ranged between .0002<P<0.008. In addition, downregulation of BRCA1 independent of BRCA1 mutation is a common occurrence in ovarian cancer (45). Therefore, it seems likely that some X-chromosome factor may modify the expression or action of the BRCA1 gene product. With only one functionally active X chromosome, inheritance of a germline mutation in such a factor on the active X chromosome, but associated with a skewed X-chromosome inactivation pattern could provide an explanation for some cases of hereditary breast and ovarian cancer independent of BRCA1 and BRCA2 mutation.

Female expression of at least two X-linked recessive genetic disorders has now been reported to result from nonrandom X-chromosome inactivation. These include Snyder-Robinson syndrome, a form of X-linked mental retardation, linked to Xp21.3–22.12 (15), and Wiskott-Aldrich syndrome linked to mutation of the WASP protein encoded at Xp11.22–23 (29). In the former, a mutation in the minimal promoter of the XIST gene at Xq13.2 was implicated as causal of nonrandom X-chromosome inactivation, while in the latter, a germline mutation in exon 4 of WASP coupled with nonrandom X-chromosome inactivation was responsible for disease. Since, the XIST promoter mutation was found to be sufficiently rare in the general population (less than 1 in 1166 independent chromosomes), we must hypothesize that other factors contribute to nonrandom X-chromosome inactivation associated with ovarian cancer. Indeed, nonrandom X-chromosome inactivation in general is much more prevalent than are XIST promoter mutations. Several authors have proposed that a candidate lies in the vicinity of Xq27 (46–49).

Another mechanism by which nonrandom X-chromosome inactivation could occur is via disruption of the normal methylation process. Methylation abnormalities, including both hypomethylation and hypermethylation, are frequently seen in cancer (50–52). Indeed, hypermethylation appears to be an important mechanism of gene suppression in cancer. The degree to which germline nonrandom X-chromosome inactivation was found in ovarian cancer probands tempts one to speculate that these individuals might carry a genetic predisposition to methylation abnormalities in general. If this is the case, nonrandom X-chromosome inactivation could represent an independent risk factor for the development of many cancers.

Cheng et al. (27) performed an analysis of borderline ovarian cancer tumor DNA samples that supports the presence of at least one tumor suppressor gene on the X chromosome. Other tumor suppressor genes are certain to be found based upon existing LOH analyses (25–26). Recognition of the importance of the X chromosome in cancer is emerging (53). X-chromosome tumor suppressor genes found in concert with nonrandom X-chromosome inactivation could provide a powerful carcinogenic stimulus. This combination is equivalent to a functional LOH. This combination could contribute to a one-step mechanism of carcinogenesis that contrasts with the classical Knudson two-step model (16,17).

In conclusion, we have begun to characterize an unusual and unpredicted phenomenon that departs from the classic Lyon hypotheses of random X-chromosome inactivation. We found that nonrandom germline X-chromosome inactivation is prevalent among individuals who develop invasive ovarian cancer, but not borderline ovarian cancer. Failure of this phenomenon to associate with borderline ovarian cancer provides additional evidence along with genomic patterns of LOH (27,54), microsatellite instability (55), and K-ras mutation (56,57) that borderline cancers do not evolve into invasive ovarian cancer. It may have important molecular epidemiological ramifications as an alternative mechanism for the development of hereditary breast and ovarian cancer independent of BRCA1 mutation.

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NOTES
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