Loss of the Inactive X Chromosome and Replication of the Active X in BRCA1-Defective and Wild-type Breast Cancer Cells

Silvia M. Sirchia,1 Lisetta Ramoscelfi,1 Francesca R. Grati,1 Floriana Barbera,3,4 Danila Coradini,3 Franca Rossella,1 Giovanni Porta,1 Elena Lesma,2 Anna Ruggeri,1 Paolo Radice,1 Giuseppe Simoni,1 and Monica Miozzo1

1Genetica Medica, Farmacologia, Dipartimento di Medicina, Chirurgia e Odontoiatria, Università di Milano; 2Dipartimento di Oncologia Sperimentale, Istituto Nazionale Tumori; 3Istituto Fondazione Italiana per la Ricerca sul Cancro di Oncologia Molecolare, Milan, Italy; 4Dipartimento di Scienze Biomediche, Sperimentali e Cliniche, Università dell’Insubria, Varese, Italy; and 5Unità di Citogenetica e Biologia Molecolare, Laboratorio TUMA, Busto Arsizio, Italy.

Abstract

In females, X chromosome inactivation (XCI) begins with the expression of the XIST gene from the X chromosome destined to be inactivated (Xi) and the coating of XIST RNA in cis. It has recently been reported that this process is supported by the product of the BRCA1 tumor suppressor gene and that BRCA1+/- cancers show X chromatin structure defects, thus suggesting a role of XI perturbation in BRCA1-mediated tumorigenesis. Using a combined genetic and epigenetic approach, we verified the occurrence of XCI in mediated tumorigenesis. Using a combined genetic and epigenetic approach, we verified the occurrence of XCI in

Introduction

In mammalian females, dosage compensation of X-linked genes is achieved by random inactivation of one of the two X chromosomes in somatic cells. X chromosome inactivation (XCI) takes place in a brief window in early embryogenesis and can be divided into a series of events: counting and choice, initiation, propagation, and maintenance of inactive state (1). Initiation begins with the expression of XIST and stabilization of its noncoding RNA transcript in cis, along the X chromosome that is destined for inactivation (1, 2). Subsequently, the inactive X (Xi) acquires the typical features of constitutive heterochromatin: late replication, histone H3 lysine 9 methylation (H3mK9), hypoacetylation of histones H3 and H4, methylation of DNA CpG islands, and concentration of the variant histone macroH2A1 (mH2A1; 3–5). These epigenetic modifications seem to act synergistically and the silenced state is clonally maintained through subsequent cell divisions (1, 4).

Recently, Ganesan et al. (6) showed that XIST RNA concentration on the Xi is dependent on the product of the BRCA1 tumor suppressor gene. This is a nuclear protein playing a role in several fundamental cellular processes, including DNA damage repair, transcription regulation, and cell cycle checkpoints (7, 8). Heterozygous carriers of BRCA1 mutations are particularly prone to breast and ovarian cancer development (9–11). Ganesan et al. (6) reported XIST localization failure and changes of epigenetic markers of Xi in BRCA1+/- ovary and breast cancer cells. In particular, the authors did not find focal staining of XIST, mH2A1, and H3mK9 on X chromosomes in a human breast cancer BRCA1+/- cell line (HCC1937) and four BRCA1+/- fresh cancers. They also investigated one human BRCA1-expressing sporadic breast cancer and found focal staining of XIST, H3mK9, and mH2A1. These findings are consistent with the hypothesis that BRCA1 dysfunction increases the risk of failure of the maintenance of XCI and suggest that BRCA1 contributes to the maintenance of the association of Xi with key partner proteins. Whether the effect of BRCA1 loss on Xi silencing would determine reactivation of Xi genes remains unclear, even if the results obtained abolishing XIST expression, by conditional deletions of the gene, showed that the mutant Xi remains transcriptionally silent (12).

Several evidences indicate the occurrence of XCI changes in breast cancer. In early studies, several authors noted that a few aggressive breast and ovarian tumors did not show a detectable Barr body (13–16). More recently, cytogenetic studies of breast carcinomas evidenced the gain of an additional active X chromosome (Xa), together with the lack of the inactive one (17–19). Extra X chromosome dosage seems to be an event also in male breast tumors, in which the lack of Y chromosome and the duplication of X were often reported (18, 20–24). The role of X chromosome dosage in breast tumor development is further strengthened by the evidence that males with a 47, XXY karyotype have a 50-fold increased risk to develop a breast cancer respect to 46,XY males (25, 26).

Taken together, the findings of a BRCA1 role on XIST localization and the cytologic, cytogenetic, and epidemiologic evidences of X chromosome alterations in breast cancer indicate that the lack of the Xi may be a key point in breast tumor development, both in BRCA1-deficient tumors and in sporadic tumors.
In the present study, we have analyzed the XCI pattern and the epigenetic status of XIST and of other X-linked genes, in a panel of BRCA1<sup>−/−</sup> and BRCA1<sup>wt</sup> breast cancer cell lines and noncultured primary breast carcinomas to (a) investigate the genetic and epigenetic changes of X chromosome in BRCA1<sup>−/−</sup> and BRCA1<sup>wt</sup> mammary cancer cells, (b) understand the mechanism and the effects of the lack of Xi chromosome, and (c) evaluate XIST expression respect to XCI status in breast cancer cell lines.

**Materials and Methods**

**Materials.** Breast cancer cell lines HCC1937, MCF7, HCC2185, T47D, MDA MB 231, and Hs578T were maintained in DMEM or RPMI 1640 (EuroClone, Paignton, United Kingdom) with 5% FCS. Human epithelial mammary cells (HMEC) from reduction mammoplasty were obtained from a commercial source (Clonetics, Walkersville, MD) and cultured according to the protocols using Clonetics reagents. For drug treatment, from a commercial source (Clonetics, Walkersville, MD) and cultured mammary cells (HMEC) from reduction mammoplasty were obtained MDA MB 231, and Hs578t were maintained in DMEM or RPMI 1640.

**Materials.** Breast cancer cell lines HCC1937, MCF7, HCC2185, T47D, MDA MB 231, and Hs578T were maintained in DMEM or RPMI 1640 (EuroClone, Paignton, United Kingdom) with 5% FCS. Human epithelial mammary cells (HMEC) from reduction mammoplasty were obtained from a commercial source (Clonetics, Walkersville, MD) and cultured according to the protocols using Clonetics reagents. For drug treatment, exponentially growing cells were seeded in 10-cm<sup>2</sup> plates at a density of 3 × 10<sup>5</sup> cells per plate and were allowed to attach to the plastic substrate before being treated for 24 hours with the histone deacetylase inhibitor Trichostatin A (Sigma, St. Louis, MO) at a final concentrations of 33 nmol/L.

Frozen samples form histologically confirmed breast carcinomas (cases 1-7) from seven patients and the corresponding peripheral blood lymphocytes were obtained from the biological repository of the Istituto Nazionale Tumori (Milan, Italy). In all instances patients were not treated with chemotherapy and/or radiotherapy before the surgery. Five patients displayed a family history suggestive of genetic predisposition to breast cancer.

**BRCA1 Mutation Analysis.** The BRCA1 status in cancer cell lines, HMEC, and peripheral blood lymphocytes from cases 1 to 7 was examined using a combination of different methods. All coding exons, except exon 11, and adjacent intronic sequences, as well as regions at the 5′ and 3′ end of exon 11, were amplified by PCR and examined by denaturing high performance liquid chromatography on a WAVE 3500 System (Transgenicomic, Omaha, NE), using previously published protocols (27) with minor modifications. Because the method is based on the detection of heteroduplex molecules that occur during PCR in the presence of heterozygous mutations, each amplified sample was examined both as such and after mixing, before the chromatography run, with the corresponding PCR products from wild-type human DNA at the ratio of 2:1, followed by a denaturing and reannealing step, to allow for the detection of homozygous mutations. The presence of nonsense and frameshift mutations introducing premature stop codons of protein translation in BRCA1 exon 11, was verified by protein truncation test, as previously described (28). Sequencing reactions were done on amplification products using the ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and examined on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), using the Sequencing Analysis 3.7 software (Applied Biosystems).

The occurrence of intragenic rearrangements (deletions or duplications of one or more exons) was assessed by multiple ligation–dependent amplification assay with the SALSA P002 BRCA1 exon copy number test kit (Medical Research Council-Holland, Amsterdam, the Netherlands), following the manufacturer’s instructions. Separation and relative quantification of the amplification products were obtained by ABI PRISM 3100 Genetic Analyser, using the GeneScan Analysis 3.7 software (Applied Biosystems).

**Reverse Transcription-PCR.** RNA was isolated from cultured cells using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

To measure BRCA1 transcripts we used quantitative real-time reverse transcription-PCR (RT-PCR) based on Taqman methodology, using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Probe and specific primers for BRCA1 and RNasep (internal control) were provided by Applied Biosystems (BRCA1 assay on demand).

For XIST RT-PCR, 100 ng of DNase-treated total RNA were amplified with the Superscript One-Step RT-PCR System (Invitrogen) using specific primers previously described (29).

**Barr Body Staining.** Cells were grown in chamber slides for 4 to 5 days, rinsed with 0.9% NaCl, and fixed with ethanol/acetic acid (3:1, v/v). Barr body staining was done as previously reported (17).

**Fluorescence In situ Hybridization Analysis.** Fluorescence in situ hybridization (FISH) was done on chromosome preparations from cell lines and on tumor touch samples from breast carcinomas (30). FITC-conjugated alpha-satellite X probe (Q-Biogene, Illkirch, France) and FITC-conjugated whole chromosome X painting (Vysis, Inc., Downers Grove, IL) were used according manufacture’s instructions. Cells were counterstained using propidium iodide (Sigma) and 200 nuclei were scored in each sample.

**Interphase Nuclear Indirect Immunofluorescence and FISH Analyses.** Cells lines were grown in chamber slides for 2 to 3 days, whereas tumor touch samples were fixed on slides by treatment with formaldehyde (4%). The slides were rinsed for 7 minutes at room temperature in KCM buffer [120 mmol/L KCl, 20 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 8), 0.5 mmol/L EDTA, and 0.1% Triton X-100] and incubated in a humidified chamber with the primary antibody anti-dimethyl-Histone H3 Lys9 (Upstate Biotechnology, Lake Placid, NY; 1:500) for 1 hour at 4°C and subsequently washed twice for 5 minutes in KCM. A biotinylated anti-rabbit second antibody (Pierce, Rockford, IL; 1:100) was then applied for 1 hour at 4°C, the slides washed in KCM and then fixed for 10 minutes in ice-cold 3:1 methanol/acidic AC. After fixation, the slides were air dried and used for in situ hybridization using FITC-conjugated alpha-satellite X probe (Q-Biogene). The biotinylated second antibody was detected by Texas Red–labeled avidin (Q-Biogene).

**X Chromosome Genotyping.** DNA was isolated from cultured cells, frozen tumor specimens, and peripheral blood lymphocytes using standard phenol-chloroform-isooamylic alcohol procedure. The analysis was done using a panel of primers specific for the following X-linked short tandem repeat markers, obtained from Genome Database (http://www.ncbi.nlm.nih.gov/genomes/static/euk_ght.html): DXS8105, DXS8051, MAOA, PRC1, HUMARA, DXS6673E, DXS6683D, DXS6809, DXS11153, DXS178, DXS94, DXS1348, DXS8057, HPRT, DXS8043, DXS8577, DXS8011, and X22.

After PCR using one fluorescent-labeled primer, the products were run on the Fluorescent Capillary System ABI PRISM 310 (Applied Biosystems) and analyzed with GeneScan software.

**Methylation Assay.** The methylation status of promoter regions of AR, PGK1, POIA, OCLR, and ZFX X-linked genes was analyzed by PCR using as template the genomic DNA previously digested with the methylation sensitive enzyme HpaII (New England Biolabs, Beverly, MA). The primers used the amplifications spanned several methylation-sensitive restriction sites in the 5′ of the tested genes. Primers and methods are fully described in Allen et al. (31) and Gilbert and Sharp (32).

**Chromatin Immunoprecipitation.** Chromatin immunoprecipitation assay was done with the chromatin immunoprecipitation assay kit using anti-acetyl-histone H3, anti-acetyl-histone H4, anti-dimethyl-histone H3 Lys4, and anti-dimethyl-histone H3 Lys 9 antibodies (Upstate Biotechnology), according to the manufacturer’s instructions with minor modifications. Chromatin was immunoprecipitated from 2 × 10<sup>6</sup> cells. Primers sequences were PKG1 sense 5′-AGCGGGCGTCGCTTGCCC-3′ and antisense 5′-TATAGGGCGGAGGAGAAG-3′.

**Combined Bisulfite Restriction Analysis.** Combined bisulfite restriction analysis consists of a standard sodium bisulfite PCR treatment followed by restriction digestion (33). PCR primers were designed to be complementary to the converted DNA sequence in a region in which CpG dinucleotides are not located. The PCR product was then digested with BstUI restriction enzyme with a recognition sequence GGCG. Cleavage can occur only if the CpGs have been retained during the bisulfite conversion by a methylated status of the cytosine residue. The primers for the modified sequence used to analyze XIST promoter were sense (262-285) 5′-GAGGTA ATTTTTAATTTATAGG-3′ and antisense (866-888) 5′-ACTAACAAT-CATTCTTAC-3′. After denaturation at 95°C for 5 minutes, 35 cycles of PCR were done; each cycle consisted of 1 minute at 95°C, 1 minute at 50°C, and 1 minute at 72°C.
Results

**BRCA1 Characterization.** BRCA1 mutation analysis of breast cancer cell lines, HMEC, and peripheral blood lymphocytes of cases 1 to 7 revealed a frame-shift mutation (c.5382insC) in HCC1937, thus confirming previous findings (34), and two nonsense mutations in cases 1 and 2 (p.Gln1135X and p.Glu1302X). No sequence variation of pathogenic significance or intragenic rearrangements was detected in the other samples examined.

We quantified BRCA1 mRNA by real-time RT-PCR and we found enhanced levels in all cancer cell lines, with respect to HMEC (Fig. 1).

Western analysis showed that the BRCA1 protein was present and correctly localized within the nucleus in all tumor cell lines and in the normal HMEC, whereas in HCC1937 the specific product was absent (data not shown).

**Absence of the Inactive X Chromosome in Breast Cancer Cell Lines.** To characterize the XCI status in our panel of breast cancer cell lines and normal HMEC, we did Barr body staining and interphase cell indirect immunofluorescence with antibody against H3 Lysine 4 methylation (H3mK4) associated with FISH using alpha-satellite X probe (immuno-FISH). A detectable Barr body was absent in all cancer cell lines but not in the normal HMEC (Fig. 1).

To exclude the monosomy X condition, we assessed the X chromosome numbering by FISH and observed that all breast cancer cell lines displayed two major subpopulations carrying two or three X chromosomes (Fig. 2A).

Similarly to Barr body staining, the immuno-FISH experiment displayed the absence of Xi epigenetic features only in tumor cells. In fact, in HMEC nuclei one of the two specific spots of X chromosome was located into the dark region resulting from hypomethylation of K4-H3, which is typically associated with the X inactive chromatin. On the contrary, in HCC1937, MCF7 and MDA MB 231 nuclei, all X spots colocalized with the K4-H3 light-stained hypermethylated region, a specific epigenetic marker of active chromatin (Fig. 2C).

These results indicate that, irrespective of the BRCA1 genetic status, the breast cancer cell lines did not show a detectable Xi.

**High Level of Homozygosity of X-Linked Loci in the Presence of More than One X Chromosome in the Breast Cell Lines.** To investigate the mechanisms underlying Xi absence in the breast cell lines, we assessed the origin of X chromosomes, genotyping a panel of highly polymorphic X-linked loci showing at least 75% of heterozygosity and spanning p and q arms (Fig. 3A). The results indicated a high level of homozygosity (>90%) in all tumor cell lines, whereas in HMEC it did not exceed 38% (Fig. 3A).

Small regions of heterozygosity were found in HCC1937, MCF7, and MDA MD 231, possibly due to mitotic recombination events or complex chromosomal rearrangements, by which the presence of homologous X chromosome segments could be maintained, as shown by chromosome X painting done on the MDA MB 231 (Fig. 3B).

The presence of a high level of homozygosity together with the observation of two (or more) X chromosomes support the duplication of the Xa, replacing the loss of the inactive one in all breast cancer cell lines.

**X-Chromosome Status in Breast Carcinomas.** FISH experiments showed that more than one X chromosome was present in the vast majority of cancer cells in all tumor samples (Fig. 3C). The comparison of X-linked alleles between normal and tumor DNAs showed high levels of reduction to homozygosity (>50%) at the informative polymorphic loci in one BRCA1-associated tumors (case 2) and in two (cases 6 and 7) of the remaining tumors (Fig. 3A). The reduction to homozygosity was assessed by calculation of alleles ratio as previously reported by Canzian et al. (35). The above findings are consistent with the results of the analysis of cell lines and indicated that the reduction to homozygosity in presence of more than one X chromosome occurs also in noncultured breast carcinomas.

Interphase cell indirect immunofluorescence with antibody against H3 Lysine 4 methylation (H3mK4) associated with FISH using alpha-satellite X probe was done in two breast carcinomas, one with and the other without BRCA1 germ line mutation (cases 1 and 4, respectively), with low-level allele losses. In both tumors, all X spots colocalized with the specific epigenetic marker of Xa in the majority (>60%) of nuclei. This data, together with the maintenance of heterozygosity, suggests the Xi is reactivated. The remaining nuclei exhibiting an immunofluorescence pattern consistent with the presence of an Xi, could be explained assuming the presence of different subclones of tumor cells or of normal cells in the surgical specimens.

**Transcriptionally Competent Chromatin State of X-Linked Genes Subjected to XCI.** H3mK9 and the CG methylation occur in heterochromatic regions (36) and are largely concentrated on X (3, 37), whereas acetylation of histones H3 and H4 and H3 lysine 4 methylation correlate with the Xa chromosome (5, 32). These features were also described at the promoter regions of X-linked genes (5, 32).

To assess the epigenetic status of the X-linked genes in cancer cell lines and HMEC, we analyzed the DNA methylation of the CpG islands and chromatin modifications within the promoter regions of four genes known to be silenced on the Xi (AR, PGK1, OCRL, and POLA) and of a gene escaping X inactivation (ZFX). The position of these genes is shown in Fig. 3A. Standard RT-PCR analysis confirmed the presence of the expected expression of them in all cell lines (data not shown). Using methylation-sensitive digestion with HpaII enzyme, the CpG islands of AR, PGK1, OCRL, and POLA gene promoters displayed a generalized demethylation in all tumor cell lines (Fig. 4A), whereas in HMEC the typical methylation pattern of normal female cells was evidenced. As expected, ZFX promoter was demethylated in all cells (Fig. 4A).

Chromatin immunoprecipitation assay allowed us to characterize the chromatin epigenetic status of PGK1 promoter region. We tested four different modifications on the histone tails: H3, H4 acetylation, and H3 methylation on lysine 4 associated with active chromatin state, and H3mK9 specific for transcriptionally silenced chromatin. Immunoprecipitates were subjected to PCR amplification to assay

![Figure 1](image-url)
for the presence of PGK1 promoter. Although the breast cancer cell lines contained at least two X chromosomes (Fig. 2B), using antibody against H3mK9, the PGK1 promoter specific immunoprecipitation was never revealed. Differently, this product was recovered from the HMEC normal cells (Fig. 4B). The immunoprecipitates obtained with antibodies recognizing active chromatin were positive for PGK1 promoter in all samples (Fig. 4B). These results underline that in breast tumor cells, only transcriptionally competent chromatin of X-linked gene promoters is present.

These findings show that the absence of XCI implies lack of epigenetically repressed X-linked genes in presence of more than one copy of the same X chromosome, originated by duplication events.

**XIST Expression and Promoter Epigenetic Characterization.**

XIST, the major gene involved in the XCI process, is the only one known to be expressed exclusively from the Xi (2). On Xi, XIST promoter shows highly acetylated histone tails and demethylated CG sites, whereas on Xa it displays methylation and weak levels of acetylation (32).

XIST expression and DNA methylation status of its promoter region were studied in the following cell lines: HCC1937, MCF7, HCC2185, T47D, and MDA MB 231. RT-PCR analysis revealed the presence of the specific XIST transcript only in HCC1937 and MCF7 tumor cell lines but not in the others (Fig. 5A). This implies that XIST can be transcribed from the native Xa in cancer cell, irrespective of genetic status and expression levels of BRCA1.

XIST RT-PCR results correlate with epigenetic status of XIST promoter. We did combined bisulfite restriction analysis on XIST promoter to evaluate the methylation status of its CpG island. In Fig. 5B is shown the restriction map of the methylation-sensitive enzymes of XIST CpG island, HhaI, AvaI, and SstII that are unmethylated on the Xi only (38). After bisulfite DNA modification, converting unmethylated cytosine residues to thymine and methylated cytosine residues to cytosine, the SstII sites (CCGCGG) switch in BstUI sites (TCGCGG) only in the presence of methylated CpGs. XIST-expressing HCC1937 and MCF7 tumor cell lines showed a BstUI-undigested PCR product derived from an unmethylated allele (Fig. 5C). After the histone deacetylase inhibitor Trichostatin A treatment of the XIST-negative MDA MB 231 cell line, displaying only a methylated XIST promoter, we observed XIST transcriptional reactivation (Fig. 5D) but not a detectable Barr body (data not shown).

**Discussion**

It has been identified that a new function for BRCA1 protein in the epigenetic phenomenon of heterochromatin formation on Xi. Ganesan et al. (6) discovered that BRCA1−/− tumor cells lack focal...
XIST staining that is restored by ectopic expression of wild-type BRCA1. This BRCA1 role could explain the high predisposition to female cancers associated with germinal BRCA1 mutations. A link between X chromosome epigenetic changes and female cancers was already evidenced several years ago, when a few authors reported that, in a subset of breast and ovarian cancers, the Barr body was absent (13–16). In the light of the latest information about the involvement of BRCA1 in XCI, those observations become very intriguing. Nevertheless, the following aspects remain to be investigated: the frequency of XCI defects in breast tumors, the mechanism at the basis of these alterations, and their effects on breast tumorigenesis.

Figure 3. X chromosome genotyping (A), structural rearrangements (B), and X chromosome numbering (C) on breast cancer cell lines, breast carcinomas, and normal control (HMEC). A, genotyping results of 18 STRs mapping to chromosome X with heterozygosity of >75%. Homozygosity is >90% in cancer cell lines, whereas in HMEC is 38%. Reduction to homozygosity respect to control tissue is present in breast carcinomas 2, 6, and 7. Chromosome X ideogram indicates the localization of the STRs and of the genes epigenetically studied. B, X chromosome painting on MDA MB 231 of an interchromosomal structural rearrangement. C, FISH results on primary breast carcinomas using alpha-satellite X probe of the presence of two or three X specific spots in all cancers.
In the present work, we found in an in vitro model that breast cancer cells, irrespective of the BRCA1 status, lack the Xi and show two or more Xa chromosomes. These findings, together with the almost complete homozygous allelic pattern of X-linked short tandem repeats markers, indicate that the mechanism causing the XCI lack is the Xa replication combined with the Xi loss and not the erasing of the epigenetic silencing of Xi. This genetic defect represented a shared feature in both BRCA1−/− (HCC 1937) and BRCA1+/− (HCC2185, T47D, MDA MB231, MCF7, and Hs578t) breast cancer cell lines. The overall data obtained in BRCA1-associated and BRCA1−not associated noncultured primary breast cancers suggest that an excess of Xa is also present in primary tumors, although the results are not homogeneous among the analyzed tumors. This can be due to the tumor heterogeneity and/or to different genetic mechanisms leading to XCI lacking. Among them, the reactivation of the native Xi could be compatible with the results obtained in cases 1 and 4, in which we failed to detect high levels of reduction to homozygosity and found by immuno-FISH that X chromosomes were active in the majority of the nuclei. Differently, an acquired homozygosity of ≥50% in presence of more than one X, detected in cases 2, 6, and 7, mirrored that shown in the in vitro model. The preliminary results suggest that XCI disturbances are frequent events in primary breast cancer, as well as in cell lines and they occur independently of BRCA1 status.

Considering the low proportion of breast cancers with BRCA1 mutation, our data fit with the previous cytologic (13–16) and cytogenetic (17–24) data, showing Xi absence on a large number of breast carcinomas.

To further investigate the epigenetic characteristics of the X chromosomes in cancer cell lines, we studied chromatin modification of H3mK9 and CpGs methylation, typical features of silenced promoters, in a group of X-linked genes subjected to inactivation. Loss of XCI condition was associated with a complete absence of epigenetically repressed X-linked genes, as shown by the absence of H3mK9 and CpG methylation of X-linked promoters. The absence of gene silencing on all the X chromosomes suggests that overexpression of X-linked gene(s) is a key point of breast tumorigenesis. It is intriguing that loss of heterozygosity of X-linked loci was frequently described in breast cancers (39–42). Piao and Malkhosyan (41) found that the allelic loss occurred preferentially on the Xi, thus suggesting the occurrence of a tumor suppressor gene escaping XCI. However, in the light of our findings, loss of heterozygosity would seem to lead to the overexpression of X-linked loci, rather than to the inactivation of tumor suppressor gene(s).

XCI is mediated by the XIST RNA that is specifically expressed from the Xi (2). Consequently, the lack of XIST expression would be expected in our panel of breast cancer cell lines. Indeed, XIST RNA was absent in four cell lines (HCC2185, T47D, and MDA MB231) and present in HCC1937 and MCF7; this implies that XIST can be expressed from the native Xi. Based on BRCA1 mutational analysis and real-time RT-PCR, we can argue that XIST expression is not related to BRCA1 genetic status and expression levels. Although MCF7 had been previously found to express BRCA1 mRNA at a lower level compared with other tumor cell lines (43, 44), our quantitative RT-PCR results indicate that, as well as for the other cancer cell lines, it showed an increased BRCA1 mRNA level, compared with normal epithelial mammary cells (HMEC).

The expression of XIST is epigenetically controlled (38) and in XIST-expressing cell lines we found a methylation status of XIST promoter consistent with the presence of both active and inactive alleles; whereas it was always methylated in those not expressing it. In addition, a chromatin remodeling experiment using HDAC inhibitor Trichostatin A, restores XIST expression in MDA MB 231 cells; nevertheless, in the same experiment the specific Barr body staining failed to revealed a detectable Xi. Taken together, these findings indicate that XIST expression from the native X is not sufficient to trigger the XCI pathway.

Although XCI perturbation remains to be extensively shown in primary tumors, our data suggest that XCI absence is a common event in breast cancer cells, irrespective of their BRCA1 status. In breast cancer cell lines, Xi loss and Xa duplication seem to be a shared pathogenetic mechanism, whereas in noncultured primary breast cancers this event was evidenced in three out of seven cases. The reactivation of Xi could be an alternative event leading to XCI perturbation, this is suggested by results of cases 1 and 4. The overall data obtained in noncultured breast carcinomas indicate that XCI defects characterize all the tumor samples extensively analyzed (case 1, 2, 4, 6, and 7).

The evaluation in cell lines of X-linked genes epigenetic status showed that genes normally subjected to XCI were always active,
thus suggesting that Xa replication may determine an overexpression of all X-linked genes. In addition, we provided evidence that XIST can be expressed in breast cancer cells in the absence of a detectable Xi, and that the restoring of XIST expression did not lead to XCI in agreement with the notion that XCI is a temporarily restricted phenomenon occurring in the developing embryo (45).

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