Aberrant Expression of X-Linked Genes RbAp46, Rsk4, and Cldn2 in Breast Cancer

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Abstract
The consequence of activation status or gain/loss of an X-chromosome in terms of the expression of tumor suppressor genes or oncogenes in breast cancer has not been clearly addressed. In this study, we investigated the activation status of the X-chromosomes in a panel of human breast cancer cell lines, human breast carcinoma, and adjacent mammary tissues and a panel of murine mammary epithelial sublines ranging from low to high invasive potentials. Results show that most human breast cancer cell lines were homozgyzous, but both benign cell lines were heterozygous for highly polymorphic X-loci (IDS and G6PD). On the other hand, 60% of human breast carcinoma cases were heterozygous for either IDS or G6PD markers. Investigation of the activation status of heterozygous cell lines revealed the presence of only one active X-chromosome, whereas most heterozygous human breast carcinoma cases had two active X-chromosomes. Furthermore, we determined whether or not an additional active X-chromosome affects expression levels of tumor suppressor genes and oncogenes. Reverse transcription-PCR data show high expression of putative tumor suppressor genes Rsk4 and RbAp46 in 47% and 79% of breast carcinoma cases, respectively, whereas Cldn2 was down-regulated in 52% of breast cancer cases compared with normal adjacent tissues. Consistent with mRNA expression, immunostaining for these proteins also showed a similar pattern. In conclusion, our data suggest that high expression of RbAp46 is likely to have a role in the development or progression of human breast cancer. The activation status of the X-chromosome may influence the expression levels of X-linked oncogenes or tumor suppressor genes. (Mol Cancer Res 2007;5(2):171–81)

Introduction
In female mammalian cells, one of the two X-chromosomes is inactivated during a short window of embryonic life through heritable epigenetic modifications, thus achieving dosage compensation with males who have one active X-chromosome and a sex-determining Y-chromosome (1, 2). The epigenetic modifications render females mosaic for two cell types, either carrying an active maternal or paternal X-chromosome. These cells are distributed in a 50:50 ratio, and deviation from this distribution can lead to skewed X-chromosome inactivation (Xi).

Because female mammalian cells only have one active X-chromosome (aX), either loss of heterozygosity at the aX or skewed Xi may result in the loss of the function of an X-linked tumor suppressor gene in a single step and may lead to cancer predisposition. Buller et al. have proposed a concept that Xi in females is equivalent to a functional loss of heterozygosity for X-linked genes (3). This hypothesis is relevant to cancer formation given that X-linked tumor suppressor genes are also subjected to Xi, like most other X-linked genes. In this case, a preexisting functional loss of heterozygosity only requires single step for complete loss of function of a tumor suppressor gene either by a true loss of heterozygosity or a loss-of-function type mutation. This unique situation (i.e., physiologic loss of heterozygosity due to Xi) may render X-linked tumor suppressor genes more liable to lose their function than tumor suppressor genes on autosomal chromosomes (4). Loss of function of those genes that function to suppress tumor formation or cell proliferation, or to promote apoptosis, may predispose an individual to cancer formation.

Although nonrandom losses and gains of whole or chromosomal segments in tumors are often evidenced as a consequence of mutational selection for abnormal cell growth (5, 6), the consequence of gain of an additional aX or loss of an inactive X-chromosome (iX) in breast carcinogenesis is not clear. Earlier studies have reported loss of iX (bar body) and gain of an aX in breast and ovarian cancers (7-10), showing that loss of iX and subsequent gain of an additional aX is not an uncommon phenomenon. More recent studies showed that activation or inactivation of certain X-linked genes is a predisposing factor for breast cancer (11). Furthermore, skewed Xi was shown to be a predisposing factor for the development of invasive ovarian cancer and early onset of breast cancer (12, 13). The role of X-chromosome dosage in cancer is further supported with the observation that 47 XXY males have increased predisposition for breast cancer compared with 46 XY males (14, 15). These studies, thereby, underscore the significance of the X-chromosome or X-linked genes in cancer.

Received 3/14/06; revised 11/22/06; accepted 12/27/06.

Grant support: Elsa U. Pardee Cancer Foundation (KM Wahidur Rahman).
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Note: KM Wahidur Rahman and A. Thakur equally contributed to this work.

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doi:10.1158/1541-7786.MCR-06-0071
To date, there are very few studies that have addressed the issue of whether known X-linked tumor suppressor genes are subjected to Xi or if they escape from Xi. Investigation of genetic changes that occur in breast tumors and their role in breast carcinogenesis will elucidate novel approaches for cancer treatment. In this study, we investigated the activation status of X-chromosome by allelotyping and reverse transcription-PCR analysis for two highly polymorphic X-linked loci [iduronate-2-sulfatase (IDS) and glucose-6 phosphate dehydrogenase (G6PD)] in human breast cancer cell lines and human breast cancer biopsies. Furthermore, we assessed human breast cancer cell lines, human breast cancer biopsies, and isogenic murine cell lines with different oncogenic potentials for the expression of X-linked tumor suppressor or oncogenes by reverse transcription-PCR and Western blot to examine whether the activation/inactivation status of X-chromosomes affects the expression levels of these genes.

**Results**

**Heterozygosity of Marker Genes in Breast Cancer Cell Lines, Breast Cancer Biopsies, and Microdissected Breast Cancer and Normal Samples**

IDS and G6PD are two highly polymorphic genes and thus were used to determine X-chromosome activation status. In human breast cancer cell lines, only one marker (i.e., IDS) was informative in 42.8% of cell lines (3 of 7 cell lines), whereas the G6PD locus was noninformative for breast cancer cell lines. Interestingly, we found that two normal cell lines (i.e., WSU15 and MCF10) as well as the malignant MCF10CA1a, which was derived from MCF10 by ectopic expression of Ras construct (16), were heterozygous for IDS. Four other malignant cell lines (i.e., MDA-MB-231, MCF7, BT20, and T47D) were homozygous for both IDS and G6PD. Genotyping data for cell lines are shown in Fig. 1A.

The heterozygosity of the IDS and G6PD genes was also determined in 20 breast carcinoma biopsies and their adjacent normal/benign (bulk) breast tissues. In human breast carcinoma cases, IDS was informative in 45% of cases (9 of 20 cases), and G6PD was informative in 27.3% of 11 remaining cases (3 of 11 remaining cases). Thus, at least one marker was informative in 60% of cases. The heterozygosity rate of IDS was similar to what has been reported for the normal healthy Caucasian population (17); however, no data have been reported for G6PD in a normal healthy population. A representative data for IDS genotyping in 18 cases are shown in Fig. 1B.

In microdissected breast cancer cells, IDS was informative in 56% of cases (14 of 25 cases), and G6PD was informative in

**FIGURE 1.** Genotyping of human breast cancer cell lines and breast carcinoma samples. **A.** Genotyping for two highly polymorphic genes (IDS and G6PD) in human breast cancer cell lines. Amplified PCR product was digested with HpaII for IDS gene and PvuII for G6PD gene. IDS marker was informative in 42.8% of cell lines (3 of 7 cell lines), whereas G6PD was noninformative in all cell lines. **B.** Representative data showing IDS gene polymorphism in 18 human breast cancer (T) and adjacent mammary tissues (N). Nine of 20 cases were heterozygous for the IDS gene (45%). **C.** Representative data showing IDS gene polymorphism in 14 microdissected human breast cancer and adjacent normal samples. Fourteen of 25 cases were heterozygous for the IDS gene (56%).
36% of cases (4 of 11 remaining cases). A representative data for IDS in 14 cases are shown in Fig. 1C.

**X-chromosome Status in Breast Cancer Cell Lines, Breast Cancer Biopsies, and Microdissected Breast Cancer and Normal Samples**

We used the transcribed polymorphisms of X-linked genes (IDS and G6PD) to characterize the activation/inactivation status of the X-chromosome in a panel of human breast cancer cell lines and human breast cancer biopsy samples. Our results show that all three heterozygous cell lines, including two benign cancer cell lines (WSU15 and MCF10NT) and one breast cancer cell line (MCF10CA1a), had only one amplified IDS cDNA band, which is indicative of one active X-chromosome. Representative data for IDS gene expression. Reverse transcription-PCR analysis of two highly polymorphic genes in bulk breast cancer and adjacent normal tissues (B) and microdissected breast cancer and adjacent normal samples (C) were used to detect the inactivation/activation status of the X-chromosome in heterozygous patients. Representative data for IDS gene expression. Heterozygous samples showed two bands after HpaII digestion, suggesting the presence of two active/partially active X-chromosomes.

In contrast to human breast cancer cell lines, nine heterozygous human breast cancer samples but activation of two X-chromosomes in adjacent breast tissues. These results suggest that reactivation of the inactive X-chromosome is a common phenomenon in breast cancer, and that this might occur at an early stage of tumor development because the adjacent tissue, which usually contains benign lesions, also showed reactivation of the inactive X-chromosome. Results from the investigation of X-chromosome activation status in heterozygous cases for IDS marker are shown in Fig. 2B.

We compared these data (bulk tumor samples) with microdissected samples. Of 14 IDS informative cases, 6 (43%) cases (cases 1, 2, 4, 7, 12, and 13) showed only one active X-chromosome in adjacent normal tissue but two active X-chromosomes in tumor cells. In two cases (cases 3 and 10), tumor cells showed gain of an additional active X-chromosome (note an amplified product of lower band) along with the reactivation of the inactive X-chromosome. In general, these data are in agreement with the data obtained from bulk tumor samples. Representative data from 14 IDS informative cases are shown in Fig. 2C and Table 1.

**Expression of Tumor Suppressor Genes and Oncogenes mRNA in Human Breast Cancer Cell Lines**

Next, we tested whether the gain of an additional active X-chromosome was reflected in the expression levels of tumor suppressor genes or oncogenes. For this purpose, we selected six
putative oncogenes (Gpc4, IL13R1a, Pdk3, Rsk2, Smc111, and Tm4sf6) and six putative tumor suppressor genes (Rsk4, Dkc1, Apx1, Cldn2, Fgf13, and RbAp46) based on our previous report of the microarray analysis of X-linked genes in mammary tumors from MMTV-c-myc transgenic mice (18). The expression patterns of the putative X-linked tumor suppressor genes Rsk4, Dkc1, Apx1, Cldn2, Fgf13, and RbAp46 as well as oncogenes Gpc4, IL13R1a, Pdk3, Rsk2, Smc111, and Tm4sf6 did not reveal any specific pattern representing activation of one or more X-chromosomes. These results suggest a cell line–specific regulation of the gene activation/inactivation mechanism. Of interest was the expression of a putative tumor suppressor gene Apx1, which was only found in the malignant cell lines and not in the WSU15 and MCF10NT benign cell lines and the MCF10-derived malignant clone MCF10CA1a. Results are shown in Fig. 3A and B.

Expression of Tumor Suppressor Genes and Oncogenes mRNA in Human Breast Cancer Samples

Similar to cell lines, the expression profiles of tumor suppressor genes and oncogenes were also tested in human breast cancer biopsies and adjacent mammary tissues. Surprisingly, we found that a large percentage of breast cancer cases had high expression of two putative tumor suppressor genes (Rsk4 and RbAp46), which have been shown to be growth inhibitory genes in in vitro studies (19-23). The RbAp46 gene showed increased expression in 79% of cases (15 of 19) compared with adjacent mammary tissues. Similarly, 47% of cases (9 of 19) showed higher expression of Rsk4 in breast cancer tissues compared with adjacent mammary tissues. Conversely, Cldn2 expression was reduced in 52.6% of cases (10 of 19) compared with adjacent normal tissues (Fig. 4A).

The expression of putative oncogenes, such as Gpc4, Pdk3, Smc111, and Rsk2, was also analyzed in the human cancer biopsies. All these genes showed increased expression in breast cancer biopsies compared with their adjacent normal tissue; the frequencies of aberrant expression for Gpc4, Pdk3, Smc111, and Rsk2 were 42% (5 of 12), 58% (7 of 12), 58% (7 of 12), and 25% (3 of 12), respectively. Results are shown in Fig. 4B.

Next, we determined whether activation status of X-chromosome influences the expression levels of some of the key X-linked genes, such as RbAp46, Rsk4, and Cldn2, in microdissected samples. Both RbAp46 and Rsk4 showed increased expression in 77% and 50% tumor cases, respectively, compared with adjacent normal tissues (Table 2). Although these data are surprisingly similar to the percentage of positive cases from bulk tumor and normal adjacent tissue samples but only one active X-chromosome in normal samples, showed increased expression of RbAp46 and Rsk4 genes (Table 2). Similarly, tumor samples from cases 3 and 10, which showed reactivation of the inactive X-chromosome as well as duplication of an active X-chromosome, also showed increased expression of RbAp46 and Rsk4 transcripts, suggesting that copy number may influence the expression levels of X-linked oncogenes or tumor suppressor genes.

Expression of Tumor Suppressor Genes and Oncogenes in Murine Mammary Epithelial Cell Lines

For an additional study of tumor suppressor and oncogenes expression in cells with different oncogenic potentials, we used murine sublines generated from the benign mouse mammary epithelial cell line NMuMG. These sublines showed variable oncogenic potential ranging from low to highly invasive cells in a soft agar assay and in a severe combined immunodeficient mouse model. Interestingly, two highly invasive sublines, transfected with either cyclin D1 (ND5) or cyclin D1/c-Myc (ND5/C23), showed a differential expression pattern compared with a low-oncogenic c-myc (NC2)–transfected cell line and vector control cells (NN2 or NH2). Both ND5 and ND5/C23 cell lines showed very low levels, or no expression of tumor suppressor genes and high expression of most of oncogenes.

The expression of Cldn2, a putative tumor suppressor gene, was almost undetectable in the highly malignant ND5/C23 cell clone, whereas it was weakly expressed in ND5 cells that are less invasive than ND5/C23 cells. These data are in line with our finding in human breast cancer biopsies and suggest that decreased expression of Cldn2 may occur in more advanced stages of breast cancer.

The expression of RbAp46 was higher in ND5 and ND5/C23 malignant cells as well as in c-myc–expressing NC2 cells than in the vector-expressing cells, thus suggesting that RbAp46 may be an oncogene in malignant cells. Interestingly, Rsk4 expression seems to be regulated by c-Myc as the NC2 clone showed high expression of Rsk4. It is likely that Rsk4 may mediate the dual functions of c-Myc under certain cellular context. However, it is yet to be determined whether or not this gene also has dual roles like c-Myc in growth inhibition and proliferation. The expression of Apx1 was lost in the ND5 and ND5/C23 malignant cells and highly expressed in the benign cell clones; yet, in the human cell lines, it was highly expressed in malignant cell lines. On the other hand, Apx1 expression could not be detected in any of the human breast carcinoma or adjacent mammary tissues. The reason for this controversy is unclear and remains to be addressed. Expression levels of putative oncogenes (Gpc4, IL13R1a, and Pdk3) were increased in ND5 and ND5/C23

Table 1. Activation Status of the X-chromosome (Xa) Using IDS and G6PD Markers in Microdissected Cases

<table>
<thead>
<tr>
<th>Markers</th>
<th>Cases</th>
<th>Informative cases</th>
<th>Two Xa in N + T</th>
<th>One Xa in N + T</th>
<th>One Xa in N and two Xa in T</th>
<th>Two Xa in N and one Xa in T</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDS</td>
<td>25</td>
<td>14</td>
<td>6</td>
<td>2</td>
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<tr>
<td>G6PD</td>
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<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
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<td>18</td>
<td>8</td>
<td>2</td>
<td>6</td>
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</table>
cell clones compared with benign cell clones NC2, NN2, and NH2. Results are shown in Fig. 5A and B.

Protein Expression of Tumor Suppressor Genes and Oncogenes in Human Breast Cancer Samples

We performed the immunostaining to confirm whether protein expression is also increased or decreased in a similar manner as mRNA expression levels in human breast cancer samples. The immunostaining for Rsk4, RbAp46, and Cldn2 proteins was carried in a series of 20 paraffin-embedded sections of breast cancer biopsies (Table 3). Some of the human breast cancer sections contained areas of ductal carcinoma in situ and invasive ductal carcinoma as well as nonneoplastic or benign tissue, therefore allowing us to assess these proteins during breast cancer progression. Consistent with mRNA expression data, Rsk4 protein was positively stained in both nucleus and cytoplasm of 35% of invasive breast carcinoma cases. Interestingly, normal mammary ducts also showed strong expression of Rsk4, which was completely or partially lost in benign breast lesions (papilloma) on the same section (Fig. 6A, e and f). Our data show that RbAp46 was strongly expressed in the nucleus and cytoplasm in 40% of invasive ductal carcinoma lesions, whereas the remaining 20% of cases showed moderate to weak expression of the RbAp46 protein (Fig. 6B). Serial sections stained with anti-RbAp46 and anti-Rsk4 antibodies showed that ~ 50% of Rsk4-positive cases were also positively stained for the RbAp46 protein in human breast cancers. The Cldn2 protein was weak to moderately expressed in 20% of the cases and localized in both nucleus and cytoplasm of invasive ductal cancer cells (Fig. 6C).

Discussion

This study shows two important findings: (a) RbAp46 and Rsk4 genes may play roles in the development or progression of human breast cancer. (b) The expression of X-linked tumor suppressor genes and oncogenes seems to be regulated at the individual gene level, and overexpression may be associated with the X-chromosome copy numbers.

We found that 60% human breast cancer samples showed heterozygosity for two highly polymorphic markers tested in this study, whereas most breast cancer cell lines (4 of 5) were homozygous for both these markers. These data are consistent with the recently published report by Sirchia et al. (24), which also showed a high percentage of heterozygosity in human breast carcinoma cases and homozygosity in breast cancer cell

FIGURE 3. X-linked gene expression in human breast cancer cell lines. Reverse transcription-PCR analysis for the expression of selected putative tumor suppressor (A) and oncogenes (B) showed variable expression levels among breast cancer cell lines with more than one active X-chromosomes, suggesting cell line–specific activation or inactivation of tumor suppressor genes or oncogenes.

Cell clones compared with benign cell clones NC2, NN2, and NH2. Results are shown in Fig. 5A and B.

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FIGURE 4. X-linked gene expression pattern of putative tumor suppressor genes and oncogenes in human breast cancer and adjacent mammary tissue biopsies with one or more active X-chromosomes. Reverse transcription-PCR analysis for the expression of selected putative tumor suppressor genes (A) and oncogenes (B) showed variable expression levels among different breast cancer samples and their adjacent benign mammary tissues.

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Two Xa in N and one Xa in T
One Xa in N and two Xa in T

Similarly, a high frequency of X-chromosome gains was not associated with any specific genetic abnormality. This showed that in spite of the presence of two or more X-chromosomes, none of them was functionally inactivated, irrespective of BRCA1 status and Xist expression in a panel of wild-type BRCA1 and BRCA1-deficient breast cancer cell lines and non-cultured primary tumors. The expression profiles of tumor suppressor genes or oncogenes in human breast cancer samples showed surprisingly higher expression levels of two putative tumor suppressor genes (RbAp46 and Rsk4). A higher expression of these two putative tumor suppressor genes in breast cancer biopsy samples compared with adjacent normal mammary tissues conflicts with previous in vitro studies that suggest that these two genes have growth inhibitory activity (19-23). However, this is the first study to screen the mRNA and protein expression of these two genes (RbAp46 and Rsk4) and other putative tumor suppressor gene (Cldn2) in human breast cancer and adjacent normal tissues. The Rsk4 protein was strongly expressed in normal mammary ducts but was completely or partially lost in benign mammary tissue and reappeared in ductal carcinoma in situ and invasive ductal carcinomas. It is likely that suppression of Rsk4 may provide a growth advantage and thus may play a role in the early stages, such as initiation of tumorigenesis. The expression pattern of RbAp46 in cancer versus adjacent tissue suggests that RbAp46 might act as an oncogene reported in cases of hepatoblastomas (26). Because inactivation of X-chromosomes only occurs during a short window of embryonic life, additional gains of X-chromosome through the duplication of aX are not likely to be inactivated. Two separate studies by Van Dyke et al. (27) and Wang et al. (11) have shown that postembryonic nondisjunction of the active X-chromosome is not followed by inactivation in tumors. Similar results were shown by Sirchia et al. (24) such that in spite of the presence of two or more X-chromosomes, none of them was functionally inactivated, irrespective of BRCA1 status and Xist expression in a panel of wild-type BRCA1 and BRCA1-deficient breast cancer cell lines and non-cultured primary tumors.

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in breast cancer, although we cannot rule out that it may have a growth-suppressive activity or a cell-specific role in other tissues or cell line models. Interestingly, we found that the expression of Cldn2, a putative tumor suppressor protein, was weak in most of the cases, and only 20% of breast cancer cases showed positive staining, suggesting that Cldn2 may be a tumor suppressor protein. Strikingly, the expression of Cldn2 was also lost in the more malignant ND5/C23 cell clone of NMuMG, suggesting that loss or reduced expression of Cldn2 may provide a growth advantage. Further studies in more breast cancer biopsies with regard to histologic patterns and tumor stages are needed to confirm the involvement of Rsk4, RbAp46, and Cldn2 in breast carcinogenesis. The expression levels of tumor suppressor genes and oncogenes also varied among breast cancer cell lines, suggesting that there may be several hierarchical levels of control that regulate X-linked gene expression.

Furthermore, we dissected the expression pattern of tumor suppressor genes and oncogenes in murine NMuMG sublines. We used these sublines for two important reasons. First, this series of cell lines, which were generated from a benign mouse mammary epithelial cell line, have different oncogenic potentials ranging from nonmalignant to highly malignant cell clones. Second, and most importantly, these cell lines have recently been generated in our lab and thus may not have accumulated additional genetic defects due to continuous culturing conditions. Although isogenic human cell lines with different oncogenic potentials, such as the MCF10 series, are widely used, these cell lines have been established for decades and cultured under various conditions and thus are likely to have accumulated many more genetic defects than breast cancer in patients. Our results in these sublines clearly show that the expression of transcripts for most of the tumor suppressor genes is either lost or decreased, as there is a progression from less malignant to more malignant phenotype. Intriguingly, the expression pattern of RbAp46 and Cldn2 in NMuMG clones corroborate our results in human carcinoma cases; for example, RbAp46 expression was higher in more malignant cell clones, whereas Cldn2 expression was lost in a more malignant cell clone, similar to our findings in human breast cancer cases.

Surprisingly, in microdissected tumor samples, we observed difference between samples with reactivation or partial reactivation of inactive X-chromosomes (heterozygous for two markers) or duplication of an active X-chromosome (homozygous for two markers) in the expression pattern of tumor suppressor genes/oncogenes. However, it is not clear whether gain of an additional aX is a consequence rather than a cause of breast carcinogenesis, or whether this consequence contributes to the disease progression. Alternatively, it is likely that the heterogeneous nature of breast cancer and its microenvironment dictates the selective up-regulation or down-regulation of certain X-linked genes at the gene level regardless of chromosomal copy number or activation status. Gilbert et al. (28) also suggested that the promoter-specific hypoacetylation may be a key component of the X-inactivation machinery that operates at the level of individual genes. Nevertheless, we do not exclude the possibility of aneuploidy-associated aberrant gene expression. To this end, Sirchia et al. (24) suggested that overexpression of X-linked genes may be a key point of breast tumorigenesis in the absence of gene silencing on all of the X-chromosomes, which is based on the finding that most human tumor cell lines or human breast carcinoma cases have more than one aX.

In summary, for the first time, our data show higher expression of RbAp46 in breast carcinoma cases compared with adjacent normal tissues, suggesting that this gene may play a role in the progression of breast cancer. On the other hand, expression pattern of Rsk4 and decreased expression of Cldn2 in most of the breast cancer cases suggest that these genes may have potential growth inhibitory effects in breast cancer, although it is likely that cellular microenvironment may influence the activity of these genes. Exploration of these

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**Table 3. Immunostaining in 20 Breast Cancer and Adjacent Benign or Normal Mammary Tissues**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cancer cells</th>
<th>Adjacent normal</th>
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<tbody>
<tr>
<td>RbAp46</td>
<td>12 (60)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>RSK4</td>
<td>7 (35)</td>
<td>5 (30)</td>
</tr>
<tr>
<td>Cldn2</td>
<td>4 (20)</td>
<td>5 (30)</td>
</tr>
</tbody>
</table>

- a, weak expression of cytoplasmic Rsk4 protein in infiltrative invasive ductal cancer.
- b, moderate cytoplasmic and nuclear staining in a representative example of invasive ductal carcinoma.
- c, strong Rsk4 immunoreactivity in invasive ductal carcinoma-colloid type.
- d, representative case showing a strong positive staining for Rsk4 in ductal carcinoma in situ.
- e, no staining was detected in a benign breast lesion (papilloma).
- f, strong nuclear (inset) and cytoplasmic staining in normal mammary ducts.


- a and b, strong expression of both nuclear and cytoplasmic RbAp46 protein in invasive ductal cancer.
- e, representative example showing no detectable RbAp46 protein in invasive ductal cancer.
- f, representative example showing no detectable RbAp46 protein in benign tissue.

C. Immunostaining for Cldn2 protein in breast cancer biopsies. 

- a and b, strong nuclear and cytoplasmic (inset) staining in invasive ductal carcinoma-colloid type.
- b and c, representative examples showing weak cytoplasmic and nuclear staining (inset) in invasive ductal carcinomas.
- d and e, negative staining in invasive ductal carcinoma.
- f, benign tissue showing a weak immunostaining for Cldn2 protein. High-power magnification, ×400.
oncogenes and tumor suppressor genes may elucidate novel approaches for cancer treatment.

Materials and Methods

Human and Mouse Cell Lines and Culture Conditions

Breast cancer cell lines MCF10CA1a, MCF7, BT20, T47D, and MDA-MB-231 (MB-231) and benign breast cancer cell lines MCF10NT and MCF12 were maintained in DMEM, DMEM/F12, or RPMI 1640 (Invitrogen, San Diego, CA) with 10% FCS. For the determination of inactivation status, DNA and RNA were extracted from ~80% confluent 10-cm² plates.

The mouse mammary epithelial cell line NMuMG obtained from the American Type Culture Collection (Manassas, MD) was used to generate the stably transfected cell clones over-expressing c-myc (NC2), cyclin D1 (ND5), or both cyclin D1/c-myc (ND5/C23) along with vector control clones [NN2 (vector control for G418) and NH2 (vector control for hygromycin)]. NMuMG is a benign cell line, suitable to investigate the mechanism of carcinogenesis of breast cancer as a normal mammary epithelial cell line. Cells were cultured in DMEM (Invitrogen) with 10% fetal bovine serum either in the presence of G418 or hygromycin (Invitrogen) at 37°C and 5% CO₂.

Tissue Samples and Laser-Assisted Microdissection

Forty-five histologically confirmed frozen or paraffin-embedded tissues of breast cancer cases and corresponding normal/benign tissues were obtained from the repository at the Pathology Department of Harper University Hospital of Wayne State University. We excluded those cases, which received any major treatment before surgery and biopsy sample collection. We used 20 frozen bulk tumor and adjacent normal tissues in this study. For laser-assisted microdissection, histologic sections were prepared from 25 formalin-fixed, paraffin-embedded tissues and were stained with H&E for microscopic evaluation. In these 25 samples, tumor tissues were identified and microdissected from the 5-μm histologic sections. Adjacent normal tissues from each case were microdissected as a control.

DNA and RNA Extraction

We used the 25 microdissected tumor and adjacent normal samples and 20 bulk frozen tumor and adjacent normal tissues as well as human and murine breast cancer or mammary epithelial cell lines for RNA and genomic DNA extraction.

Genomic DNA was extracted by proteinase K digestion, phenol/chloroform purification, and dehydration. Purified DNA from cancer and adjacent normal/benign tissues was PCR amplified for IDS and G6PD, two X-linked genes known to be highly polymorphic (29). Total RNA was extracted by using Trizol reagent (Invitrogen) according to the manufacturer’s instruction and immediately converted to cDNA using a Taqman reverse-transcription kit (Applied Systems, Foster City, CA) and stored at −20°C until further use.

Genotyping of IDS and G6PD Polymorphic X-Linked Loci

Genomic DNA extracted from human breast cancer cell lines and human breast cancer and adjacent tissues was used for genotyping of IDS and G6PD polymorphisms as described previously (8, 29). IDS and G6PD alleles were differentiated by PCR amplification with allele-specific primers. The primers used for the detection of polymorphic regions of these genes were described previously (29). Primers and PCR conditions are shown in Table 4. Briefly, a mismatch was introduced in the sequence of one primer to create a restriction site specifically in PCR products from one of the two alleles (Table 4A). Restriction fragments were separated by nondenaturing acrylamide gel electrophoresis after digestion with the specific enzymes and visualized by UV illumination. In heterozygous females, two bands were observed (151 and 126 bp for IDS and 183 and 162 bp for G6PD), whereas only one band was found in homozygous females.

Determination of Inactivation Status of the X-chromosomes

Similar to genomic DNA amplification, cDNA from human breast cancer cell lines and human breast cancer and adjacent normal/benign tissues was amplified, and the resulting products were digested and analyzed on nondenaturing acrylamide gel to detect the activation/inactivation status of the X-chromosomes. Samples that showed two bands (158 and 133 for IDS and 79 and 58 for G6PD) were identified to possess two active X-chromosomes through the reactivation of an inactive X-chromosome, whereas a single band may represent either two active X-chromosomes through duplication or only one active X-chromosome depending on the band density relative to undigested PCR product. Primer sequence and conditions are described previously (29) and shown in Table 4B.

Table 4. Primer Sequences for Genotyping and for the Determination of Activation Status

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences for genotyping and PCR product size</th>
<th>Sequence 5’-3’</th>
<th>Exon</th>
<th>PCR product size</th>
<th>Enzymes</th>
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<tr>
<td><strong>A. Primer sequences for genotyping</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDS</td>
<td>IDS 3b (sense)</td>
<td>CCAAAAGAAGGGAGGGTCCAC</td>
<td>Intron 3</td>
<td>151</td>
<td>HpaII</td>
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<tr>
<td></td>
<td>IDS 4 (antisense)</td>
<td>AGACCAGCTATACGGAGATAC©C</td>
<td>4</td>
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<td></td>
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<tr>
<td>G6PD</td>
<td>G6PD-E10 (sense)</td>
<td>GCTGACCTGACTCACGCAACA</td>
<td>10</td>
<td>183</td>
<td>PvuI</td>
</tr>
<tr>
<td></td>
<td>G6VPDPDVU1(antisense)</td>
<td>GAAAGCTCCAGGATGAGGCGA*Tc</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B. Primer sequence for reverse transcription-PCR analysis</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDS</td>
<td>IDS 3a (sense)</td>
<td>TGTCGACTCCAACCTCCTGGA</td>
<td>3</td>
<td>158</td>
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<tr>
<td></td>
<td>IDS (antisense)</td>
<td>AGACCACTATACGGAGATAC©C</td>
<td>4</td>
<td></td>
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</tr>
<tr>
<td>G6PD</td>
<td>G6PD-E10 (sense)</td>
<td>GCTGACCTGACTCACGCAACA</td>
<td>10</td>
<td>79</td>
<td>PvuI</td>
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<tr>
<td></td>
<td>G6VPDPDVU1 (antisense)</td>
<td>GAAAGCTCCAGGATGAGGCGA*Tc</td>
<td>11</td>
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</tbody>
</table>

Reverse Transcription-PCR for the Expression of Putative Tumor Suppressor Genes and Oncogenes

RNA extracted from human breast cancer cell lines, human breast cancer and adjacent normal tissues, and mouse NMuMG sublines was reverse transcribed to prepare cDNA. For subsequent PCR amplification, a maximum of 2 µL of each cDNA sample was used per 25-µL PCR reactions. The specific primers and PCR product size are listed in Table 5A and B. The amplified products were electrophoretically separated on ethidium bromide containing 1.2% agarose gels.

Immunostaining for Putative Tumor Suppressor Gene and Oncogene Proteins

Formalin-fixed, paraffin-embedded human breast cancer sections (5 µm thick) were deparaffinized in xylene and rehydrated in graded ethanol following antigen retrieval and peroxidase blocking. Primary antibody to RbAp46, Rsk4, or Cldn2 (1:50 dilution) was applied for 90 min then sections were incubated with biotinylated goat anti-rabbit secondary antibody (1:1,000) for 60 min at room temperature. Antigen was detected by using a complex of avidin and horseradish peroxidase with diaminobenzidine staining system. Sections were then lightly counterstained with hematoxylin, dehydrated, and mounted. Omitting the primary antibody from the immunohistochemical procedure and replacing it with non-immune rabbit serum acted as negative controls. Besides, different primary antibodies also served as mutual controls. Immunoreactivity for each antigen was evaluated by examination of the sections under bright-field light microscopy independently by two experienced observers (H.N. and A.T.).

Table 5. Human and Mouse Primer Sequences

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession no.</th>
<th>Primer sequence</th>
<th>Size of PCR product (bp)</th>
<th>Tm (°C)</th>
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<tbody>
<tr>
<td>A. Human primer sequences and conditions for reverse transcription-PCR</td>
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<tr>
<td>APXL</td>
<td>X83543</td>
<td>Upstream, 5'-CCGAGGTCCATTGGAAGACC-3'</td>
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<td>Downstream, 5'-TTCAACGGTGCTACTTAGTC-3'</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Downstream, 5'-CAGGATCAGAATGGAGGGAGG-3'</td>
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<tr>
<td>Downstream, 5'-GCACTTCAAGGGCTTTTCAT-3'</td>
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<tr>
<td>Downstream, 5'-GACATCAGATTGAAAGAAGG-3'</td>
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<td></td>
<td></td>
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<tr>
<td>Downstream, 5'-AGCACTTCCATTCTGTAC-3'</td>
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<td></td>
<td></td>
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<tr>
<td>B. Mouse primer sequences and conditions for reverse transcription-PCR</td>
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<td>APXL</td>
<td>NM_486797</td>
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<td>58</td>
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<tr>
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<tr>
<td>Downstream, 5'-AGCACTTCCATTCTGTAC-3'</td>
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References

Aberrant Expression of X-Linked Genes *RbAp46*, *Rsk4*, and *Cldn2* in Breast Cancer


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