YB-1 Provokes Breast Cancer through the Induction of Chromosomal Instability That Emerges from Mitotic Failure and Centrosome Amplification

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Abstract

YB-1 protein levels are elevated in most human breast cancers, and high YB-1 levels have been correlated with drug resistance and poor clinical outcome. YB-1 is a stress-responsive, cell cycle–regulated transcription factor with additional functions in RNA metabolism and translation. In this study, we show in a novel transgenic mouse model that human hemagglutinin-tagged YB-1 provokes remarkably diverse breast carcinomas through the induction of genetic instability that emerges from mitotic failure and centrosome amplification. The increase of centrosome numbers proceeds during breast cancer development and explanted tumor cell cultures show the phenotype of ongoing numerical chromosomal instability. These data illustrate a mechanism that might contribute to human breast cancer development. (Cancer Res 2005; 65(10): 4078-87)

Introduction

Breast cancer is the most frequent malignancy among women in Western countries, and there are an estimated 1 million new cases per year worldwide. Breast cancer results from genetic and environmental factors, and several genes involved in the hereditary and familial forms of breast cancer have been identified, which account for ~5% of breast cancer. In sporadic breast cancer, the most common type of cytogenetic alteration is the amplification of genes, such as ErbB2, c-MYC, and cyclin D1 (reviewed in ref. 1). The c-ErbB2 proto-oncogene is located on chromosome 17q21 and encodes a receptor tyrosine kinase that is a member of a growth factor receptor family, which includes the epidermal growth factor receptor family, which includes the epidermal growth factor receptor family. c-ErbB2 is overexpressed in 20% to 30% of human breast tumors (2). The c-MYC gene, located on chromosome 8q24, is amplified in ~20% to 30% of breast cancers, and cyclin D1, located on chromosome 11q13, is amplified in up to 20% of human breast cancers (3). However, at the level of protein, cyclin D1 is overexpressed in ~50% of human mammary carcinomas and is seen in all histologic types of human breast cancers (4). The ability of these genes to induce mammary gland transformation has been shown by using transgenic mice (reviewed in ref. 5). We have originally reported that the Y-box binding protein YB-1 is overexpressed in ~75% of human breast carcinomas (6). The YB-1 gene is located on chromosome 1 at position 1p34 and 80% of primary breast tumors show increased copy numbers of chromosome 1 (7). In human breast cancer, nuclear localization of YB-1 is associated with intrinsic MDR1 gene expression (6), and the MDR1 gene product P-glycoprotein plays a major role in the development of a multidrug-resistant tumor phenotype (8). A clinical study revealed that YB-1 predicts drug resistance and patient outcome in breast cancer independent of clinically relevant tumor biological factors HER2, urokinase-type plasminogen activator, and plasminogen activator inhibitor-1 (9). To investigate the consequence of aberrant YB-1 expression in the mammary gland and to determine its role in breast cancer, we created transgenic mice where expression of a human hemagglutinin (HA)–tagged YB-1 cDNA was controlled by the ovine β-lactoglobulin promoter (BLG). We find that transgene-derived YB-1 protein expression in epithelial cells of the mammary gland induces cell proliferation with mitotic failure and centrosome amplification. All multiparous transgenic animals developed multiple mammary tumors within 12 to 15 months that were diagnosed as invasive breast carcinomas with remarkably different histologic types. The data we present illustrate a mechanism that might contribute to human breast cancer development. Thus, the BLG/YB-1 transgenic mouse model enables us to address fundamental aspects of YB-1-induced breast cancer and to test therapeutic strategies.

Materials and Methods

Generation of BLG/YB-1 transgenic mice. The BLG promoter expression vector pBJ41 was a gift from B. Binas (Edinburgh Research Station, Scotland, United Kingdom). This vector contains 4.3 kb of 5′ flanking sequence, exons 1, 7, and 8, and 1.9 kb of 3′ flanking sequence of the BLG gene. For cloning a HA-tagged YB-1 cDNA into pBJ41, an EcoRV site was used, which was generated from a PvuII site in the untranslated region of exon 1 (10). The cDNA encoding a HA tag was a gift from J. Madruga (MDC, Berlin, Germany). YB-1/HA was constructed by PCR amplification of the HA sequence, thereby generating an EcoRI site at the 5′ end of the HA sequence. An EcoRI/EcoRI fragment of the human YB-1 cDNA was fused to the HA tag. BLG/YB-1/H4 was constructed by subcloning the 1.4-kb YB-1/H4 fragment into the EcoRV site of pBJ41. Digestion with XhoI/XbaI released the 7.9 kb long BLG-YB-1/H4 fragment from the vector. This DNA fragment was separated from the vector by electrophoresis through a 1% low-melt agarose gel in 1× Tris-borate EDTA. Gel slices containing BLG-YB-1/H4 DNA were digested with QiaEX (Qiagen, Hilden,
Germany) according to the manufacturer’s recommendations. The DNA was suspended in microinjection buffer (10 mmol/L Tris-HCl, 0.1 mmol/L EDTA (pH 7.4)) to a final concentration of 10 ng/μL. Microinjection of NMRI mouse embryos was described previously (11).

PCR analysis of tail DNAs. DNA was isolated from 1 cm tail biopsies as described previously (11), and transgenic founders were identified by PCR analysis. The following primers were used: a HA antisense primer 5′-GCG-GCCGCTACAAGCTGAATCTGGAGGCTGTC-3′ and a YB-1 COOH-terminal sense primer 5′-ACCATGGAGGGATCGGAGAGTGCTCCC-3′. This primer set detects a 0.4 kb long product in BLG-YB-1/HA Tg founder lines. PCR was done under standard conditions: 30 seconds at 94°C followed by 36 cycles for 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C and finally for 10 minutes at 72°C in a volume of 50 μL. The PCR products (10 μL) were size fractionated on a 1% agarose gel. As size standard, the DNA molecular weight marker VI (Roche Diagnostics, Mannheim, Germany) was used.

Reverse transcription-PCR analysis. Tg expression was examined by reverse transcription-PCR (RT-PCR) analysis. RNA was prepared by a single-step method (12). For RT-PCR, total RNA (25 μg) was converted to cDNA by reverse transcriptase (Invitrogen Life Technologies, Karlsruhe, Germany). The PCR was done as described previously (11) using Taq DNA polymerase (Invitrogen Life Technologies).

Histopathologic analysis of Tg mice and immunohistochemistry. Lactating and nonlactating mammary glands obtained from animal necropsies were fixed in formalin and embedded in paraffin. For immunohistochemistry, 5 μm sections of formalin-fixed, paraffin-embedded tissue were used. Specimens were deparaffinized and pretreated in a steam pressure cooker. After incubation with the primary antibody, immunodetection was done with alkaline phosphatase–labeled streptavidin-biotin with new water. Finally, the slides were washed and dehydrated with increasing concentrations of ethanol. Finally, the slides were returned to the tissue dehydrator and finally washed in distilled water. The mammary glands were stained with a mixture of xylene and ethanol.

Whole mount preparation of mammary glands. Tissues were spread on glass slides and fixed in Carnoy’s fixative for 4 hours at room temperature. The fixative consisted of 6 parts 100% ethanol, 3 parts CHCl₃, and 1 part glacial acetic acid. The glands were washed in 70% ethanol, gradually rehydrated, and finally washed in distilled water. The mammary glands were stained with carmine alum stain overnight and subsequently washed and dehydrated with increasing concentrations of ethanol. Finally, the glands were cleared with xylene and stored in methyl salicylate. For the whole procedure, we followed a protocol that is available online (http://mammary.nih.gov/tools/histological/wholemounts).

Preparation of protein extracts and immunoblot analysis. Protein extracts were prepared from frozen mouse mammary glands using a Dounce homogenizer. For homogenization, 1 mL 2% lysis buffer, 1% SDS, 10 mmol/L Tris-HCl (pH 7.5), and 2 mmol/L EDTA (pH 8.0) were added per milligram of tissue. The extracts were incubated at 90°C for 15 minutes and centrifuged (15,000 rpm) at room temperature for 15 minutes. The supernatant was transferred to clean tubes, and protein concentration was determined using a protein assay (Bio-Rad Laboratories, Munich, Germany). To detect YB-1/HA protein, the extract (50 μg) was subjected to electrophoresis through a 8% SDS-polyacrylamide gel followed by electrophoretic transfer to a nitrocellulose membrane. Blots were incubated with polyclonal antibodies specific for the HA epitope tag and bound antibodies were visualized using the enhanced chemiluminescence detection system (Amersham Biosciences Europe, Freiburg, Germany). To detect YB-1/DNA binding, the extract (50 μg) was incubated with an antibody specific for YB-1/DNA. The monoclonal α-tubulin antibody (Sigma-Aldrich Chemie, Munich, Germany) was used at a dilution of 1:400, and the monoclonal γ-tubulin antibody (Santa Cruz Biotechnology) was used at a dilution of 1:200. Antibodies were diluted in blocking solution, the cells were incubated for 60 minutes at room temperature with the primary antibodies. The secondary antibodies anti-mouse FITC (Amersham Biosciences Europe) and anti-mouse Texas red (Amersham Biosciences Europe) were used at dilutions of 1:40 and 1:10, respectively.

Confocal imaging and scoring of centrosomes in histologic sections of Tg hyperplasias and breast carcinomas. Mammary tumor tissue was embedded in cryogel and frozen 8 μm thick sections were generated using an IEC minitome cryostat (Diversified Equipment Co., Inc., Lorton, VA). The specimens were mounted onto positively charged slides, fixed with 4% formaldehyde in PBS for 30 minutes, and permeabilized by a treatment with 0.05% Triton X-100 in PBS for 5 minutes. Incubation with the primary antibody was done for 1 hour at 4°C in 1% bovine serum albumin in PBS. Subsequently, the preparations were washed for 1 hour in PBS at room temperature and incubated with the secondary antibody at 37°C for 3 hours. The tissue was washed and slides were mounted using Vecta shield as mounting medium. Centrosomes were detected with a human anti-γ-tubulin antibody (1:1,000) and nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI; 2 μg/mL). Immunofluorescence microscopy was done with a confocal laser scanning microscope (Leica Microsystems, Bensheim, Germany). Scoring was accomplished by counting the number of stained nuclei and the corresponding number of centrosomes in sections of the tumor tissue (13). Counts were made on cells in the terminal ducts, alveolar buds, and stroma. A minimum of 214 nuclei per sample were counted and displayed as number of centrosomes versus number of nuclei.

Spectral karyotyping. The cocktail of mouse chromosome paints was obtained from Applied Spectral Imaging (Carlsbad, CA). Hybridization and detection were carried out according to the manufacturer’s protocol. Chromosomes were counterstained with DAPI. Images were acquired with a SD200 Spectra cube (Applied Spectral Imaging) mounted on a Zeiss Axioplan II microscope using the Spectral Karyotyping View 1.2 software (Applied Spectral Imaging).

Results

Generation of mice expressing human YB-1 in the mammary gland. Y-box protein YB-1 is overexpressed in the majority of human breast cancers (6, 9). To determine whether YB-1 plays a role in mammary carcinogenesis, we engineered female mice that express human HA-tagged YB-1 protein in mammary epithelia under control of the ovine BLG promoter, which facilitates transgene expression during late pregnancy and lactation (10). Several YB-1/HA-expressing founder lines, termed Tg 2, Tg 5, and Tg 8, were generated using a BLG-YB-1/HA construct (Fig. 1A), and the corresponding lines were established and tested. The transgene was identified in genomic DNA by PCR using HA- and YB-1-specific primers (Fig. 1B). Transgene-derived mRNA expression was examined by RT-PCR (Fig. 1C) and Northern hybridization (data not shown). YB-1/HA protein expression levels in the mammary glands of primiparous lactating Tg 2, Tg 5, and Tg 8 mice were determined by immunoblotting using an antibody specific for the HA epitope tag (Fig. 1D, lane L). The blot reveals that the founder lines Tg 5 and Tg 8 express a high level of YB-1/HA protein in the mammary gland. In contrast, mammary glands of the Tg 2 line express a much lower level of YB-1/HA. In virgin BLG/YB-1 mice, YB-1/HA was not detectable in the mammary glands (Fig. 1D, lane V). The

Identification of YB-1 as a Novel Breast Cancer Oncogene
relative expression levels of YB-1/HA were determined (Fig. 1E). The Ponceau-stained nitrocellulose membrane shows equal amounts of protein in all lanes after transfer from the gel (Fig. 1D, bottom).

Transgenic YB-1 expression is associated with altered glandular morphology and abnormal proliferation of mammary epithelial cells. To investigate immediate effects of human YB-1 overexpression, parental and transgenic histologies of mammary glands were examined from primiparous (8-week-old) mice at day 11 of the lactation phase. Figure 2A and B shows low-power views of wild-type (WT) and transgenic mammary glands for comparison. The lactating mammary glands of WT mice consisted of lobuloalveolar structures with a single layer of secretory epithelial cells (Fig. 2A). In contrast, the lactating mammary glands of age-matched BLG/YB-1 mice (line Tg 5) showed a different morphology. BLG/YB-1 mammary glands are characterized by a persistence of large fat cells (arrows) and increased epithelial cell proliferation causing a hyperplastic phenotype (Fig. 2B). In these mammary glands, YB-1/HA protein was strongly expressed in all epithelial cells from collecting ducts and secretory cells from lobuloalveolar units (data not shown).

Next, we examined H&E-stained histologic sections of BLG/YB-1 mammary glands (line Tg 5) at higher magnification (Fig. 2C). Several pathologic features are evident in an area of increased proliferation. We noted a high frequency of binucleate cells where the nuclei are attached to each other (arrowheads). The nuclei of these proliferating cells are pale, pleomorphic with coarse chromatin and large prominent nucleoli. In addition, islands of persistent fat cells were seen (arrows). In contrast, the nuclei of age-matched WT lactating mammary glands were inconspicuous (Fig. 2D, top). These nuclei are small, homogeneous in size, and more intensely stained by H&E (Fig. 2D, top, arrows). Next, we examined H&E-stained histologic sections of 8-week-old lactating mammary glands of Tg 8 mice. This mouse line has a lower level of transgenic YB-1 protein than Tg 5 (Fig. 1D). The lobuloalveolar units of Tg 8 mammary glands of these mice are quite different (Fig. 2D, Tg 8) from the WT lactating mammary glands. The secretary cells are bloated and the nuclei are pale, pleomorphic with multiple prominent nucleoli (Fig. 2D, open arrows). Frequently, secretary cells contain two nuclei (Tg 8, arrowheads). We noted that in binucleate cells the nuclei are often arranged in pairs along the apicobasal axis (Fig. 2D, middle and bottom left, arrowheads). We conclude that YB-1 has the capacity to induce proliferation of lactating mammary epithelial cells but at the expense of mitotic or cytokinesis failure. Thus, aberrant YB-1 expression has two immediate effects on the mammary epithelium: (a) induction of cell proliferation and (b) generation of binucleate cells that are tetraploid. Tetraploidy is a condition that can lead to centrosome amplification and chromosomal instability (14).

Development of focal hyperplasias and premalignant lesions in mammary glands of BLG/YB-1 mice. We found that proliferative abnormalities in mammary glands of BLG/YB-1 mice progressed in relation to the number of pregnancies. At the age of...
8 months, whole mount preparations of mammary glands of WT and BLG/YB-1 females were examined. The multiparous BLG/YB-1 female mice passed through 5 to 7 lactations. The ductal components of WT mammary glands form a simple tree-like structure with some side buds and small terminal end buds but without distinct alveoli (Fig. 3A, WT). In contrast, the mammary glands of BLG/YB-1 mice were grossly abnormal and contained dilated lactiferous ducts (Fig. 3A, bottom right, arrow) with a persistence of enlarged lobuloalveolar units (Fig. 3A, bottom left, arrows). In addition, all mammary glands from these 8-month-old BLG/YB-1 mice (lines Tg 5 and Tg 8) had developed large hyperplastic alveolar nodules (HAN; Fig. 3A, top right, arrow) that are a preneoplastic condition (15). Next, we examined H&E-stained mammary gland tissue sections of 8-month-old multiparous WT, Tg 5, and Tg 8 female mice. The mammary glands of WT mice consist of resting ducts and large fat cells (Fig. 3B). In contrast, multiple discrete foci were identified in Tg 2, Tg 5, and Tg 8 mammary tissues. The degree of focal hyperplasias in BLG/YB-1 mice correlates with the level of YB-1/HA protein. Tg 2 mice express a low level of YB-1/HA and have evidently fewer and smaller lesions (Fig. 3C, arrows) than Tg 5 mice, which express a high level of YB-1/HA (Fig. 3D). Our data show that YB-1 overexpression in the mammary gland causes focal proliferations and HANs that eventually may emerge as autonomous tumors.

**Development of invasive mammary carcinomas in BLG/YB-1 mice.** To investigate the further evolution of YB-1-induced lesions in the mammary glands of BLG/YB-1 animals, we surveyed cohorts of Tg 5 and Tg 8 females over an extended period. After 12 months, protrusions of the nipples, lumps, deformations, and tumors of the mammary glands were detected. After 15 months, large tumors appeared in the mammary glands of all BLG/YB-1 female mice. Thus, the incidence of breast tumor formation is 100%. In contrast, the mammary glands of age-matched WT mice were inconspicuous. The Tg 5 cohort comprised 13, the Tg 8 cohort 11, and the WT cohort 15 animals. Tumors from 11 individual BLG/YB-1 mice were fixed embedded in paraffin and H&E-stained sections were prepared. The histopathologic examination identified breast carcinomas in all 11 cases with remarkably different histologic types. Table 1 delineates the histopathologic features of these tumors. Selected examples of different tumor types are shown in Fig. 4. Tumor number 2 has a solid nodular growth pattern with a high degree of cytologic atypia (Fig. 4A). In contrast, tumor number 7 has a tubular, papillary growth pattern with a marked increase in dense fibrous tissue stroma (arrows) and intermediate cytologic atypia (Fig. 4B). Tumor number 9 has a heterogeneous growth pattern with areas of solid and areas of tubular growth. This tumor is also characterized by inflammatory infiltrates, stromal hyalinization, and intermediate cytologic atypia. Figure 4C displays an area of tumor growth surrounded by large fat cells. The tumor cell nuclei are small and intensely stained by H&E. Tumor number 10 has morphologic similarities with an...
adenocarcinoma of the secretory type and is characterized by vacuolar cytoplasmic degeneration and inflammatory infiltrate. In this tumor, the cytoplasm and the nuclei are weakly stained by H&E (Fig. 4D). Our data show that aberrant YB-1 expression in the mammary gland is an initiating event in the process of multistage breast cancer development. We have thus identified YB-1 as a novel breast cancer oncogene.

Our data have shown a causal link between YB-1 expression and the development of breast carcinomas. However, the tumor-bearing mice we have analyzed (Table 1) were not pregnant and it was thus interesting to examine the status of transgene-derived YB-1/HA protein in tumor tissue. We analyzed expression of YB-1/HA protein in several breast carcinomas by immunohistochemistry using an antibody specific for the HA tag of the YB-1 transgene (Fig. 5). Figure 5 shows that transgene-derived YB-1/HA protein is present in all tumors examined. However, like in human breast carcinomas, we found diverse expression patterns in different tumors. In tumor number 5, we found strong nuclear YB-1/HA expression in most nuclei. However, cytoplasmic YB/HA levels were variable in different regions of the tumor (Fig. 5A). In tumor number 7, nuclear and cytoplasmic YB-1/HA expression was very heterogeneous and varied from high to very low levels of YB-1/HA protein. The dense fibrous tissue stroma of this tumor is indicated by arrows (Fig. 5B). Tumor number 9 has areas of solid and areas of tubular growth pattern. YB-1/HA is expressed at very high levels in both the cytoplasm and the nucleus of all tumor cells (Fig. 5C). In tumor number 10, YB-1/HA protein expression is variable. We found cytoplasmic and nuclear expression, whereas in other areas YB-1/HA expression was confined to the cytoplasm. In other regions of this tumor, we detected a complete loss of YB-1/HA protein expression in an undifferentiated region of the tumor (Fig. 5D, arrows). Our data show that BLG promoter activity is uncoupled from pregnancy and lactation in transgenic breast carcinomas. It will be interesting to identify the mechanisms responsible for the diverse YB-1/HA protein expression patterns in our transgenic breast carcinomas.

Centrosome amplification precedes breast cancer development in BLG/YB-1 mice. Human breast carcinomas are characterized by centrosome abnormalities, and frequently, malignant cells contain increased numbers of centrosomes (16). Our discovery of binucleate lobuloalveolar cells in the mammary glands of primiparous BLG/YB-1 mice (Fig. 2) unveiled a mitotic defect that is a precondition for centrosome amplification (16). To investigate whether centrosome amplification occurs in BLG/YB-1 mammary glands, we analyzed the centrosomes in BLG/YB-1 mammary glands during breast cancer development by immunohistochemistry. To detect centrosomes, we used an antibody...
specific for γ-tubulin and examined breast tissues by confocal laser scanning microscopy. We found evidence for centrosome amplification in focal hyperplasias, which strongly increased in breast carcinomas. These semiquantitative results are displayed in a block diagram (Fig. 6A). We conclude that centrosome amplification occurs in premalignant lesions and proceeds during YB-1-dependent breast carcinoma development. Our data show that centrosome amplification is an early event in YB-1-dependent breast cancer development. The question whether centrosome aberrations are a cause or consequence of cancer progression is an important issue in cancer research (17).

Centrosome abnormalities in cultured breast cancer cells from BLG/YB-1 mice. In human breast cancer, centrosome abnormalities that are associated with abnormal mitoses are a common feature, and it was suggested that centrosome amplification drives chromosomal instability and aneuploidy in breast tumor development (16). To investigate the centrosomes of individual BLG/YB-1 breast tumor cells, we established short-term cultures and

Table 1. Histopathologic features of mammary tumors in BLG/YB-1 transgenic mice

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Solid growth pattern</th>
<th>Tubular growth pattern</th>
<th>Inflammatory infiltrates</th>
<th>Grade</th>
<th>Necrosis</th>
<th>Cytologic atypia</th>
<th>Tumor characteristics</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>++</td>
<td>++</td>
<td>@</td>
<td>2</td>
<td>+</td>
<td>Intermediate</td>
<td>Heterogenous differntiation</td>
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<tr>
<td>2</td>
<td>+++</td>
<td>+</td>
<td>—</td>
<td>3</td>
<td>++</td>
<td>High</td>
<td>Well-differentiated acinar structures</td>
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<td>3</td>
<td>—</td>
<td>++++</td>
<td>—</td>
<td>1</td>
<td>—</td>
<td>Low</td>
<td>Microcellular</td>
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<td>4</td>
<td>++++</td>
<td>—</td>
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<td>3</td>
<td>+</td>
<td>High</td>
<td>Microcellular</td>
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<td>5</td>
<td>+</td>
<td>+++</td>
<td>—</td>
<td>2</td>
<td>—</td>
<td>Intermediate-high</td>
<td>Intratubular secretion and cellular debris</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+++</td>
<td>@</td>
<td>3</td>
<td>—</td>
<td>Intermediate-high</td>
<td>Intratubular secretion and cellular debris</td>
</tr>
<tr>
<td>7</td>
<td>—</td>
<td>+</td>
<td>—</td>
<td>2</td>
<td>—</td>
<td>Intermediate-high</td>
<td>Microcellular</td>
</tr>
<tr>
<td>8</td>
<td>+++</td>
<td>+</td>
<td>@</td>
<td>3</td>
<td>++</td>
<td>Intermediate-high</td>
<td>Solid, partially cribriform growth pattern</td>
</tr>
<tr>
<td>9</td>
<td>++</td>
<td>++</td>
<td>@</td>
<td>2</td>
<td>+</td>
<td>Intermediate-high</td>
<td>Stromal hyalinization</td>
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<tr>
<td>10</td>
<td>++</td>
<td>++</td>
<td>@</td>
<td>2</td>
<td>—</td>
<td>High</td>
<td>Vacuolar cytoplasmic degeneration cribriform growth pattern</td>
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<tr>
<td>11</td>
<td>++</td>
<td>+</td>
<td>@</td>
<td>2</td>
<td>+</td>
<td>High</td>
<td>Small irregular glandular lumina</td>
</tr>
</tbody>
</table>

NOTE: +, degree of growth pattern; @, present.

Figure 4. Transgenic YB-1 provokes the development of breast carcinomas with remarkably different histologic types. Tumor samples were taken from BLG/YB-1 mice 12 to 15 months old, fixed with formaldehyde, and embedded in paraffin. All tumor sections were stained with H&E. Bar, 100 μm. Note that the histopathologic features of 11 mammary tumors are summarized in Table 1. A, tumor number 2 with a solid growth pattern. The nuclei are pleomorphic and nuclear H&E staining is heterogeneous. B, tumor number 7, adenocarcinoma with tubular growth pattern, extensive fibrous tissue stroma (arrows), and sporadic necrotic areas. C, tumor number 9 with heterogeneous solid and tubular growth patterns, pink cytoplasm, small nuclei with pronounced atypia, and hyperchromatic nuclei. D, tumor number 10 with similarity to an adenocarcinoma of the secretory type. Well-defined glands lined by an epithelium with marked vacuolar degeneration and pale nuclei.
The nuclei of explanted BLG/YB-1 of cultivated BLG/YB-1 which is an adequate number for the detection of numerical chromosomal instability, we did spectral karyotyping (18). We breast cancer cells are profoundly aneuploid. To investigate the issue, tumor cells from another breast carcinoma cells were investigated with an antibody specific for α-tubulin. In 12.6%, the spindles were unipolar (Fig. 6C, top, white arrow), in 84.9% dipolar, and in 2.5% hyperpolar with three to seven spindle poles. Fig. 6C (bottom) shows a cell with four spindle poles (white arrows). In this case, free chromosomes not aligned in a metaphase plate will be lost in subsequent cell divisions and thus lead to numerical chromosomal instability in BLG/YB-1 breast carcinoma cells.

Ongoing numerical chromosomal instability in breast cancer cells from BLG/YB-1 mice. Next, we investigated the chromosomes of cultivated BLG/YB-1 breast cancer cells. We examined 282 metaphases and found that 4.2% were near diploid containing 30 to 35 chromosomes, 76.2% were near tetraploid containing 65 to 82 chromosomes, and the remaining 19.5% had a higher ploidy containing 102 to 158 chromosomes. Tumor cells from another BLG/YB-1 breast carcinoma showed a similar result. Thus, BLG/YB-1 breast cancer cells are profoundly aneuploid. To investigate the issue of chromosomal instability, we did spectral karyotyping (18). We analyzed the metaphase plates of nine individual breast cancer cells, which is an adequate number for the detection of numerical chromosomal aberrations (19). The basic chromosome content was near tetraploid, but the numbers of individual chromosomes varied from metaphase to metaphase, directly demonstrating an ongoing numerical chromosomal instability in BLG/YB-1 breast cancer cells. The spectral karyotypes of two metaphases are shown for comparison (Fig. 6D). All numerical aberrations for each chromosome are summarized in Table 2. We conclude that overexpression of YB-1 in the mammary glands of transgenic mice initiates a chain of events that leads to the development of chromosomal instability and aneuploidy in vivo. It is evident that these alterations precede the formation of breast carcinomas in BLG/YB-1 mice.

**Discussion**

The level of YB-1 protein expression has been shown to predict for the overall survival of patients with breast cancer as well as for resistance to chemotherapeutic agents (9). Overexpression of YB-1 mRNA and protein is a hallmark of scores of human malignant diseases. For example, increased levels of YB-1 mRNA were identified in pancreatic adenocarcinoma, metastatic prostate carcinoma, ovarian carcinoma, medulloblastoma (20), and malignant melanoma (21). Increased levels of YB-1 protein were detected in breast cancer (6, 9), osteosarcomas, colorectal carcinomas, lung cancer (reviewed in ref. 22), thyroid neoplasms (23), and prostate cancer (24). Several clinical studies revealed that increased YB-1 protein levels negatively affect clinical outcome of breast cancer (9), ovarian cancer (25), non–small cell lung cancer (26), and synovial sarcoma (reviewed in ref. 22). Thus, YB-1 is a clinically important molecule that may be causally linked to the development of many human malignant diseases.

To investigate whether YB-1 has an oncogenic potential, we created transgenic mice with YB-1 overexpression in mammary epithelial cells under control of the ovine BLG promoter. Our
study revealed that YB-1 has strong oncogenic potential as all BLG/YB-1 mice developed breast tumors. We have thus identified YB-1 as novel breast cancer oncogene with a genetic penetrance of 100%. The BLG/YB-1 breast tumors were diagnosed as breast carcinomas with remarkably different histologic types. A comparison of the histologic types of BLG/YB-1 breast tumors with the histologic types of well-established transgenic mouse models for breast cancer identifies YB-1-specific biological attributes. For example, c-ErbB2 transgenic mice develop poorly differentiated solid and nodular tumors with slightly atypical nuclei, and this phenotype is transgene specific (27). In contrast, c-MYC transgenic mice develop distinctly different tumors that have relatively large cells with large, pleomorphic nuclei with a coarse chromatin and prominent nucleoli; in addition, this tumor phenotype is transgene specific (27). Cyclin D1 transgenic mice develop papillary adenocarcinomas that could mimic human cancer, with a mean onset time of 18 months (28). Mammary tumors in genetically engineered mice with a unique phenotype have been designated as signature tumors (5, 27). As shown here, all BLG/YB-1 mice develop breast tumors with multiple phenotypes and this shows that the oncogenic activity of YB-1 is different in comparison with the well-established breast cancer oncogenes. Human breast carcinomas are characterized by extensive histologic diversity (29) and the BLG/YB-1 tumors reflect this heterogeneity.

It is a goal of our work to identify the underlying molecular mechanisms that cause breast cancer. It is incompletely understood how transformation of the mammary gland in well-established transgenic mouse models is brought about. However, pathways that lead to transformation have been delineated and the cooperating oncogenes were identified. c-MYC induces mammary tumorigenesis by a preferred pathway involving spontaneous Kras2 mutations (30), and the oncogenic activity of ErbB2 in mammary epithelium is absolutely dependent on the presence of cyclin D1 protein, demonstrating that the ErbB2 pathway is connected to the cell cycle machinery by cyclin D1 (28).

Our work delineates a novel mechanism that explains how mammary gland transformation by YB-1 is brought about. Aberrant YB-1 expression induces defective proliferation of mammary epithelial cells and the development of chromosomal instability. YB-1 may directly activate cell proliferation by controlling cyclin A and cyclin B1 gene expression (31). It is well established that cell cycle progression is regulated by the activities of several cyclin-dependent kinases, including cyclin A and cyclin B1 (32). The mammary glands of primiparous 6-week-old BLG/YB-1 mice showed the presence of a large number of binucleate breast epithelial cells and this indicates defective cell proliferation. Binucleate cells may arise due to cell fusion or mitotic failure (33). Cell division failure can have several distinct primary causes, including the persistence of...
unrepaired DNA damage or the deregulation of pathways that coordinate mitotic progression and cytokinesis (reviewed in ref. 17). YB-1 might provoke cell division failure because it specifically interacts with actin (34), and actin filaments form the contractile ring, which helps to cleave the cell during cytokinesis (35). YB-1 induces strongly elevated levels of cyclin B1 protein (31), which is subject to proteasome-dependent degradation before the exit of mitosis (36), and studies in numerous organisms have shown that stabilization of cyclin B blocks mitotic exit events, including cytokinesis (37). YB-1 might directly affect centrosome function as it binds to centrosomes in mitosis (38). The centrosome is an essential component, controlling exit of cytokinesis in animal cells (39). Cytokinesis failure invariably leads to tetraploidy, which is a precondition for centrosome amplification, chromosomal instability, and aneuploidy (16, 17). Aneuploidy and an underlying chromosomal instability are nearly ubiquitous in cancers (14). Sixty percent to 80% of human breast tumors are aneuploid, and 80% exhibit amplified centrosomes that occur together in early pre-invasive carcinomas (40). The cultured breast carcinoma cells from BLG/YB-1 breast cancer cells are also aneuploid and display the phenotype of ongoing numerical chromosomal instability in tissue culture. This is clearly different from cell cultures derived from signature tumors induced by activated c-ErbB2 gene or c-MYC. Cells from c-ErbB2 tumors are diploid, and except for a deletion of chromosome 4 D-E, recurring structural chromosomal alterations do not occur (41). In contrast, cells derived from MMTV/c-MYC tumors are predominantly triploid and show a recurring pattern of chromosomal aberrations (42). Although many malignant tumors are highly aneuploid, this is by no means an indication that chromosomal instability is still ongoing. For example, hypertriploid HeLa cells with a large number of abnormal chromosomes are exceptionally stable (43), and excessive centrosome abnormalities in a Burkitt’s lymphoma are not associated with chromosome instability (44).

We conclude that the BLG/YB-1 transgenic mouse model recapitulates major features of human breast cancer and shows that centrosome amplification, chromosomal instability, and aneuploidy are early events in breast cancer development. Thus, overexpression of YB-1 might be an initiating event and it will be a challenge to work out the pathways that lead to breast cancer in BLG/YB-1 transgenic mice. The transgenic mouse model will be essential in the evaluation of potential therapies, such as compounds designed to inhibit the activities of YB-1.

Acknowledgments

Received 11/11/2004; revised 2/14/2005; accepted 3/7/2005.

Grant support: Berliner Krebsgesellschaft, Interdisciplinary Research Project: Molecular Biology and Clinic of Breast and Ovarian Cancers (H-D. Royer); Deutsche Forschungsgemeinschaft grant Ba 1596/1-1 (R. Bargou); European Commission contract BMH4-CT96-1133 (J-C. Claude); Deutsche Krebshilfe grant 70-2633-R 5 (B. Royer-Pokora); State of Nordrhein-Westfalen (H-D. Royer); and Studienstiftung des deutschen Volkes (S. Bergmann).

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Table 2. Numerical chromosomal instability in BLG/YB-1 breast cancer cells

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References

Identification of YB-1 as a Novel Breast Cancer Oncogene


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Stephan Bergmann, Brigitte Royer-Pokora, Ellen Fietze, et al.


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