CBFA2T3 (MTG16) Is a Putative Breast Tumor Suppressor Gene from the Breast Cancer Loss of Heterozygosity Region at 16q24.3

Marina Kochetkova, Olivia L. D. McKenzie, Anthony J. Bais, Julie M. Martin, Genevieve A. Secker, Ram Seshadri, Jason A. Powell, Susan J. Hinze, Alison E. Gardner, Hayley E. Spendlove, Nathan J. O’Callaghan, Anne-Marie Cleton-Jansen, Cees Cornelisse, Scott A. Whitmore, Joanna Crawford, Gabriel Kremmidiotis, Grant R. Sutherland and David F. Callen

Department of Cytogenetics and Molecular Genetics, Women’s and Children’s Hospital, North Adelaide 5006, South Australia, Australia [M. K., O. L. D. M., A. B., J. M. M., J. A. P., A. E. G., H. E. S., N. J. O., G. R. S.]; Bionomics Ltd, Thebarton, South Australia, Australia [G. A. S., S. J. H., S. A. W., J. C., G. K., D. F. C.]; Department of Hematology/Genetic Pathology, Flinders Medical Centre, Flinders University, Bedford Park, South Australia, Australia [R. S.]; and Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands [A-M. C-J, C. C.]

ABSTRACT

Numerous cytogenetic and molecular studies of breast cancer have identified frequent loss of heterozygosity (LOH) of the long arm of human chromosome 16. On the basis of these data, the likely locations of breast cancer tumor suppressor genes are bands 16q22.1 and 16q24.3. We have mapped the CBFA2T3 (MTG16) gene, previously cloned as a fusion partner of the AML1 protein from a rare (16;21) leukemia translocation, to the 16q24.3 breast cancer LOH region. The expression of CBFA2T3 was significantly reduced in a number of breast cancer cell lines and in primary breast tumors, including early ductal carcinomas in situ, when compared with nontransformed breast epithelial cell lines and normal breast tissue. Reintroduction of CBFA2T3 into different breast tumor derived cell lines with decreased expression of this gene reduced colony growth on plastic and in soft agar. CBFA2T3 was shown to function as a transcriptional repressor when tethered to the GAL4 DNA-binding domain in a reporter gene assay and, therefore, has the potential to be a transcriptional repressor in normal breast epithelial cells. Taken together, these findings suggest that CBFA2T3 is a likely candidate for the breast cancer tumor suppressor gene that is the target for the frequent 16q24 LOH in breast neoplasms.

INTRODUCTION

Extensive analyses of breast tumors using cytogenetic and molecular genetic techniques have identified 16q and 17p as the most frequently changed chromosomes in human breast cancer (1). Loss of 16q has been suggested to be an early event in breast tumorigenesis because 16q copy number alterations were found in atypical ductal hyperplasia (2) which represents the first clonal transformation event of ductal epithelial cells, and in about 45% of preinvasive DCIS2 (3). Overall, LOH on human chromosome 16q is reported to range from 36 to 67% in breast tumors of various subtypes and progression stages.

In breast cancer, loss of the entire chromosome 16 long arm is the most commonly observed LOH event. A recent study of LOH in 712 breast tumors concluded that there were three regions likely to harbor tumor suppressor genes, one at 16q22.1 and two at 16q24.3 (4). The E-cadherin gene, CDH1, has been identified as the likely LOH target at 16q22.1 in the less frequent subgroup of lobular breast carcinomas because truncating mutations of CDH1 are found in ~60% of cases (References cited in Ref. 5). However, in the more frequent ductal breast carcinomas, mutations in CDH1 have been described only in cell lines (6), and a role of this gene as a tumor suppressor in ductal carcinoma is unclear (5).

We have constructed a physical map of the 3-Mb LOH region at 16q24.3 and generated a complete detailed annotated transcript map.4 The expression of 103 identified transcripts was assayed in a panel of breast cancer cell lines, and from this analysis, CBFA2T3 (MTG16) emerged as a candidate tumor suppressor gene.

CBFA2T3 was recently cloned from a rare, but recurrent, therapy-related leukemia translocation (t(16;21)(q24;q22)). The fusion partner on chromosome 21 was the transcription factor RUNX1 (AML1; Ref. 7), the most frequent target of chromosomal translocations in acute myeloid leukemia. CBFA2T3 belongs to the ETO family of proteins that also includes CBFA2T1 (MTG8, ETO) and CBFA2T2 (MTGR1) in mammalian cells, and nervy in Drosophila. All of the mammalian members of the ETO protein family are highly conserved and share four common nervy homology regions and three PST regions with the Drosophila protein. CBFA2T1 is the best-characterized member of this family and is commonly involved in the acute leukemia translocation (t(8;21)) that also results in a fusion protein with RUNX1. Recent data suggested that the CBFA2T1 protein is a member of a high-molecular-weight transcriptional repressor complex containing histone deacetylases and that this protein can also independently function to repress transcription in a reporter-based assay (8). Although the precise physiological role for CBFA2T1 has not yet been assigned, its function and properties have been extensively studied (Refs. 9 and 10 and References therein), in contrast to the very limited data available for the CBFA2T3 protein.

In this report, we show that the expression of the CBFA2T3 gene is significantly reduced in a number of breast cancer cell lines and primary tumor tissues when compared with normal breast epithelial cell lines and tissues. Reexpression of the CBFA2T3 gene in breast tumor cell lines with low or undetectable levels of CBFA2T3 mRNA resulted in a significant inhibition of colony growth on plastic and in soft agar. These data show that genetic and functional properties of CBFA2T3 are consistent with those of a tumor suppressor gene and suggest a potential role for this gene in breast cancer tumorigenesis.

MATERIALS AND METHODS

Plasmids. The full-length CBFA2T3b coding region was amplified from fetal spleen total cDNA using myc-tag containing forward primer 5’-ATG GAG CAG CAG ATG ATG CTG AGC GAC GCC GAG GCC CCA GCC GA-3’ and reverse primer 5’-TCA GGG GGG CAC GGT

Received 12/6/01; accepted 6/2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported in part by the National Health and Medical Research Council of Australia.

2 To whom requests for reprints should be addressed, at Department of Cytogenetics and Medical Genetics, Women’s and Children’s Hospital, 28 Dalgleish Street, Thebarton, South Australia, Australia. Phone: 618-8354-6132; Fax: 618-8354-6160; E-mail: marinak@wch.bionomics.com.au.

3 The abbreviations used are: DCIS, ductal carcinoma(s) in situ; LOH, loss of heterozygosity; HMEC, human mammary epithelial cell; Dig, digoxigenin; DAPI, 4’,6-diamidino-2-phenylindole; PST, proline-serine-threonine rich (region); CAT, chloramphenicol acetyl transferase; TK, thymidine kinase.


4599
GTC CA-3. The construct pLNCX2-CBFA2T3 was made by subcloning this cDNA into the SalI/ClaI sites of the pLNCX2 retroviral expression vector (Clontech). For transfection studies, the complete open reading frame of CBFA2T3 was fused to the GAL4 DNA-binding domain of the expression vector pM (Clontech) to generate the pMCFB expression construct. A control construct pMCFB-AGAL4 contained the CBFA2T3 open reading frame in the absence of the GAL4 DNA-binding domain. To generate various segments of the CBFA2T3 protein the relevant fragment of the cDNA was generated by PCR amplification and cloned in-frame downstream of the GAL4 DNA binding domain of the pM vector. Positions of the NH2- and COOH-terminal amino acids of these fragments are indicated in Fig. 4B. A positive control expression plasmid pMKN10 contained the KRAB domain of the mouse NK10 protein (amino acids 1-112) fused to the GAL4 DNA-binding domain of the pM plasmid. The CAT reporter construct pBLCAT2 was modified by subcloning five copies of the GAL4 DNA-binding site directly upstream of the thymidine kinase promoter to produce the pGAL4CAT2 construct. pGAL-CAT3 is a promoter-less control CAT reporter construct used as a reference. Construct pcDNA3.1βGal, expressing β-galactosidase activity, was used as an internal control.

**Real-Time PCR.** Cell lines were grown to 80% confluence, and total RNA was extracted from ~1 × 10^6 cells and DNase I treated using the RNAqueous-4PCR kit (Ambion). Polyadenylated mRNA from normal human mammary gland was purchased from Clontech. First strand complementary cDNA synthesis was performed with oligo(DT)16 primers using Superscript RNase H- reverse transcriptase (Life Technologies, Inc.). A final 20-μl reaction contained 1 μg of total RNA, 1 μl of oligo(DT)16 (500 ng/μl), and 1 μl of Superscript reverse transcriptase (100 units/μl) and was incubated at 42°C for 2 h. Real-time PCR was performed on a Rotor-Gene 2000 (Corbett Research, Sydney, Australia); 25-μl reactions contained 12.5 μl of SYBR Green I PCR Master Mix (PE Biosystems), 0.2 μM forward primer 5'-GGG CCT GGT GAA GAG TGG TAC CTG GCC ATG GCC GCA GAG GAG GAT TGG T-3' and 30 ng of cDNA template. Fluorescent data were acquired at 510 nm during each 70°C extension phase. Product specificity was examined by melt curve analysis and agarose gel electrophoresis after each real-time PCR run. The results were normalized to cyclophilin A, a housekeeping gene showing uniform expression profile across all cell lines.4 Primers used for cyclophilin A were 5'-GGG AAA TTA TCG TGG ACC CAA CAC AAA-3' and 5'-CTA GCC GTG GAA GGG AAG A-3'. Quantification was performed using internal cDNA standard curves as described.4 Fold changes of CBFA2T3 gene expression in cell lines were calculated relative to the levels in normal breast tissue.

**Cell Lines and Cell Culture.** All of the breast cancer cell lines, HEK-293T, and untransformed immortalized breast epithelial cell lines MCF-12A and HBL-100 were obtained from the American Type Culture Collection (Manassas, VA) and were grown according to supplier instructions. HMECs were purchased from Clonetics (San Diego, CA) and cultured in serum-free defined media supplied by the manufacturer. NIH-3T3 and HEK-293T cells were maintained in DMEM (Life Technologies, Inc.), supplemented with 10% calf serum, 2 mM L-glutamine and 100 μg/ml penicillin and gentamicin. All of the cells were grown at 37°C and in 5% CO2.

**Retroviral Infection and Cell Growth Suppression Assays.** 293T packaging cells were transfected with 10 μg of pLNCX2 retroviral expression vectors, 8 μg of pVpack-VSV-G, 8 μg of pVpack-AP (Stratagene), and 60 μl of LipofectAMINE 2000 reagent (Life Technologies, Inc.) in 100-mm tissue culture dishes in Opti-MEM medium (Life Technologies, Inc.) without FCS and without antibiotics, essentially as recommended by the supplier. The medium was replaced 16 h later, and virus-containing supernatants were harvested at 48 h posttransfection. Supernatants were filtered through a 0.45-μm Minisart syringe filter (Sartorius AG, Gottingen, Germany), and polybrene (Sigma) was added to a final concentration of 8 μg/ml. Cells to be infected were plated in 6-well plates at ~40% confluency; and 24 h later, cell medium was removed, and 1 ml of specific and control viral supernatants were added, followed 3 h later by the addition of 2 ml of cell-growing medium. The infected cells were then incubated for an additional 24 h at 37°C before initiation of assays.

To assay monolayer colony formation, 3 × 10^4 of infected tumor cells were plated in 6-well plates in the presence of 500 ng/ml G418. After 2 weeks of selection, cells were fixed in 3% formaldehyde in PBS, stained with Giemsa (Sigma), and dried for subsequent quantification. The number of colonies visible in each well without magnification was determined. Anchorage-independent growth assay of colony formation by infected tumor cells in soft agar was performed as described elsewhere (11).

**Tissue Sections and RNA in Situ Hybridization.** Breast tumor paraffin-embedded tissue blocks were obtained from patients operated on between 1987 and 1997 at the Flinders Medical Centre (Bedford Park, South Australia) after approval from the appropriate medical ethics committee. Serial 4-μm tissue sections were cut from formalin-fixed, paraffin-embedded normal breast and breast tumor specimens. The sections were mounted on 3-aminopropylmethoxysilane (sigma)-treated slides, and the *in situ* hybridization was performed essentially as described previously (12) with minor modifications. Sense- and antisense-oriented RNA probes labeled with DIG were generated by *in vitro* translation of DNA fragments cloned in pGEM-T vector (Promega) using a DIG RNA labeling kit (SP6/T7; Roche, Mannheim, Germany) according to the manufacturer’s instructions. CBFA2T3 probe template was a 483-bp fragment from the 3’ untranslated region that was amplified using 5’-GAC AGC AGA GCA GAT GCC GGC CTT GAC ATG-3’ forward and 5’-GCA AGG TAG TTC ACA AGT GAG-3’ reverse primers. A 202-bp fragment from the *β*-actin gene spanning the junction of exons 4 and 5 was used as a template for the positive control probe.

Microwaved sections were prehybridized in 4× SSC and 50% (v/v) deionized formamide in a humidified chamber for a minimum of 10 min at 37°C. Approximately 10 ng of DIG-labeled RNA probes in 30 μl of hybridization buffer [40% deionized formamide, 10% dextran sulfate, 1× Denhardt’s solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 10 μg/ml BSA)], 4× SSC, 10 mM DTT, 1 mg/ml yeast tRNA, and 1 mg/ml denatured sheared herring sperm DNA] was denatured at 80°C for 10 min and overlaid over tissue sections; slides were incubated at 52°C overnight in a humidified chamber. Unbound probe was removed with extensive washes, and bound RNA was detected using sheep anti-DIG Fab fragments coupled to alkaline phosphatase (1:500 dilution) and visualized using nitroblue tetrazolium chloride (sigma)/5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (sigma) and 1 M levamisole. Slides were counterstained in a 0.1% solution of methyl green and mounted in glycerol/PBS (9:1) for analysis.

**Immunostaining.** Cells expressing Myc-tagged CBFA2T3 were grown on glass coverslips, fixed in PBS containing 3.7% formaldehyde for 15 min at room temperature, and permeabilized with 0.4% Triton X-100 for 5 min at 4°C. Cells on coverslips were then incubated with a Myc monoclonal antibody 9E10 (1:500 dilution; Santa Cruz Biotechnology) for 1 h at room temperature followed by a 1-h incubation with a FITC-conjugated sheep antimouse IgG (1:600 dilution; Silenus, Victoria, Australia). Coverslips were mounted on glass slides with Vectashield mounting liquid (Vector Laboratories) containing DAPI for DNA staining, and cell images were obtained with a cooled CCD camera using the CytoVision Ultra image collection and enhancement system (Applied Imaging).

**Transcription Repression Assays.** In each well of a 6-well plate, 1 × 10^5 293T cells were transfected with 0.5 μg of pGAL4CAT2 (or promoter-less control pGAL4CAT3) reporter construct, up to 3 μg of specific and control GAL4 fusion expression vectors [pMCFB and derivatives containing deletions of the CBFA2T3 open reading frame (Fig. 4B), pMCFB-AGAL4 and pMKN10] and 0.2 μg of β-galactosidase expression plasmid pcDNA3.1βGal using LipofectAMINE 2000 reagent. After 24 h, cells were lysed and CAT concentration was estimated using a CAT ELISA kit (Roche). The β-galactosidase assay (Stratagene) was performed to normalize CAT values for the transfection efficiency.

**Single-stranded Conformation Analysis and Sequencing.** Hex-labeled primers were designed to amplify all 12 exons of CBFA2T3 (primer sequences available on application). Thirty ng of genomic DNA was PCR-amplified in a total volume of 10 μl. Products were separated on nondenaturing 4% polyacrylamide gels containing 2% glycerol using the GelScan 2000 (Corbett Research). PCR products showing a conformational change were reamplified from 100 ng of genomic DNA with unlabeled primers and sequenced using the BigDye Terminator ready reaction kit (Perkin-Elmer).

**RESULTS**

**Quantitation of CBFA2T3 RNA Expression.** Quantitative real-time PCR was used to analyze the expression of the CBFA2T3 gene in a panel of 22 breast cancer cell lines, including HBL-100 and...
MCF-12A that are immortalized but untransformed, and primary finite life span HMECs. CBFA2T3 was aberrantly expressed in this cell group with one-half of the analyzed breast cancer cell lines showing ≥50-fold down-regulation of mRNA expression relative to the expression in normal breast tissue (Fig. 1). The expression of CBFA2T3 was also reduced in the cell line MCF12A, derived from fibrocystic breast disease with focal areas of intraductal hyperplasia, but not in the milk-derived HBL-100 cells nor in primary HMECs.

RNA in situ hybridization analysis was used to assess the levels of CBFA2T3 gene expression in primary breast tumors. CBFA2T3 was expressed in normal breast ductal epithelium in five assayed samples from different individuals. Four of six primary breast carcinomas with previously determined LOH of 16q24.3 were shown not to express CBFA2T3. In addition, CBFA2T3 RNA was not detected in 5 of 10 primary breast tumors (LOH of chromosome 16q was unknown because of the unavailability of normal DNA samples from the same individuals). Typical examples of breast tumor tissues found negative and positive for the CBFA2T3 transcript are represented in Fig. 2. Of particular interest was the observation that, in the same paraffin-embedded sections of several tumors, normal unaffected breast ductal epithelial cells showed expression of CBFA2T3, whereas areas containing both DCIS and more advanced invasive carcinoma did not have detectable CBFA2T3 mRNA (Fig. 2). All normal and tumor tissue sections were positive for β-actin (data not shown), used as a control probe.

Single-stranded conformation polymorphism analysis (SSCA) of CBFA2T3 was used to detect the possible presence of tumor-specific mutations. The screened samples consisted of DNA from 23 breast cancer cell lines, 55 pairs of tumor and normal paired DNA samples with known LOH of 16q24.3, and 50 blood bank donor DNAs. Four rare DNA variants in exons 2, 4, and 9 were detected that did not cause an amino acid change, together with a common polymorphism in the intronic sequence adjacent to exon 5. No cancer-specific mutations were found in primary tumor DNAs. However, the cell line MDA-MB-175 was heterozygous for a nonconservative amino acid change (Pro→Thr). Although the significance of this mutation is unknown, CBFA2T3 RNA levels were reduced in this cell line.

Suppression of Tumor Cell Growth by CBFA2T3. Suppression of cancer cell growth is a hallmark of the majority of well-characterized tumor suppressor genes (13). The effect of forced expression of the CBFA2T3 gene on the cell growth of four breast cancer cell lines was investigated. SK-BR-3, MDA-MB-231, and MDA-MB-468 have reduced CBFA2T3 expression, whereas the expression in MCF-7 was similar, when compared with normal breast epithelial cells (see Fig. 1). Infection of these cell lines with Myc-tagged CBFA2T3 expressing retroviruses, followed by 2 weeks of selection in G418-containing growth media, showed that the reintroduction of CBFA2T3 significantly inhibited colony growth (up to 96%) of SK-BR-3, MDA-MB-231, and MDA-MB-468 relative to vector-only controls (Fig. 3). In contrast, CBFA2T3 retroviral expression in the breast cancer cell line MCF-7 with nonreduced CBFA2T3 expression resulted in a small reduction in the number of colonies that averaged 22%.

To rule out the possibility that the observed low numbers of surviving colonies from CBFA2T3-expressing cells were caused by
CBFA2T3: A PUTATIVE TUMOR SUPPRESSOR

Fig. 3. CBFA2T3 suppresses growth of breast cancer cells. SK-BR-3, MDA-MB-231, and MDA-MB-468 breast cancer cell lines were infected with recombinant retroviruses expressing Myc-tagged CBFA2T3 or Neo only (empty vector) RNA. Two days after infection, G418 was added to the cell medium, and 2 weeks later, surviving colonies were fixed, stained with Giemsa, and counted. A, infected cells of the cell lines MDA-MB-231 and MDA-MB-468 were also suspended in soft agar for assay of anchor-independent growth. Colonies were counted after 3 weeks growth in the presence of G418. Photographs of representative plates for the cell lines expressing recombinant CBFA2T3 and empty vector are shown in B and C, respectively. Myc monoclonal antibody was used to visualize cells infected with myc-tagged CBFA2T3 containing retroviruses 2 days postinfection (D). Green fluorescence (FITC) indicates CBFA2T3-expressing cells and blue fluorescence (DAPI) uninfected cells. Data represent results from at least two independent experiments. The values shown are the mean and SD of triplicate samples.

Low retroviral infection efficiency, the cells were stained with anti-Myc monoclonal antibodies 48 h postinfection to visualize the extent of CBFA2T3 transduction. In all of the cell lines, at least 50–70% of the infected cells expressed Myc-tagged CBFA2T3 protein (Fig. 3).

It is interesting to note that attempts to expand surviving colonies into cell lines stably producing CBFA2T3 have failed with both SK-BR-3 and MDA-MB-468 cells. MDA-MB-231-selected clones did survive expansion; however, a large proportion of cells rapidly lost CBFA2T3 protein expression within an additional few weeks of culture in selective medium (data not shown). This observation further suggests that forced CBFA2T3 expression is detrimental to the growth of at least some breast cancer cell lines.

The effect of CBFA2T3 on the ability of MDA-MB-231 and MDA-MB-468 breast cancer cell lines to form colonies in an anchorage-independent manner was then examined. SK-BR-3 was not included in these assays because this is a nontumorigenic cell line (14), and these cells did not form well-defined colonies in soft agar. Cells infected with CBFA2T3-expressing or control (Neo only) recombinant retroviruses were suspended in soft agar containing G418, and colony numbers were scored after 2–3 weeks of incubation. Results paralleled the assays of colony formation on plastic with forced expression of CBFA2T3 specifically inhibiting colony formation by 90% in MDA-MB-468 cells and by 70% in MDA-MB-231 cells while reducing MCF-7 cells colony growth in soft agar by an average of only 19% relative to vector-only controls (Fig. 3).

CBFA2T3 Functions as a Transcriptional Repressor. A GAL4-based TK CAT reporter assay was used to study the transcriptional regulatory properties of CBFA2T3. The CAT reporter construct, pGAL4CAT2, containing five GAL4 DNA-binding sites upstream of the TK promoter, was cotransfected into HEK-293T cells together with increasing amounts of pMCFB (a construct containing the complete open reading frame of CBFA2T3 fused to the heterologous GAL4 DNA-binding domain). Results from this assay suggested that CBFA2T3 can act as a transcriptional repressor (Fig. 4A), because the TK promoter-driven CAT reporter activity was reduced in a dose-dependent manner to a maximum of 10-fold with increasing concentrations of cotransfected pMCFB. Because cotransfection of pMCFB:GAL4 (a construct without GAL4 binding sites) and the CAT reporter construct did not result in repression of CAT activity, the ability of CBFA2T3 to repress transcription was strictly dependent on GAL4 binding to its specific DNA-binding sites.

To functionally map the repressor domains of CBFA2T3, a number of NH2- and/or COOH-terminal deletion mutants fused to the GAL4 DNA binding domain were constructed (Fig. 4B) and tested for their ability to repress transcription from the CAT reporter gene, relative to the construct pMCFB with the complete open reading frame of CBFA2T3. The correct expression and similar transfection efficiency for all pMCFB constructs was confirmed by Western blot analysis (data not shown). The construct coding for the first 213 amino acids of CBFA2T3 did not significantly contribute to repressor activity, which suggested that this portion of the gene is involved in alternative functions. The construct coding for the central 254 amino acids of CBFA2T3 (pMCFBΔ2, amino acids 141–405) showed more than 80% of the repressor activity of the full-length construct. Therefore, the most essential domains of CBFA2T3 for the ability to repress transcription correspond to the second PST and second nerry homology regions.

DISCUSSION

Tumorigenic transformation of the breast epithelium is believed to result from a sequence of various genetic and/or epigenetic changes with as yet no universal pathway or pathways identified. Loss of well-characterized tumor suppressors, such as TP53, PTEN, and others, or amplification of known oncogenes such as MYC and ERBB2 (15) do not occur in all breast cancers, which indicates the existence of other molecular lesions involved in the initiation and progression of the disease. The long arm of chromosome 16 at bands q22.1 and q24.3 have been suggested as locations of breast cancer tumor suppressor genes with numerous studies demonstrating LOH for this region in more than 50% of both breast neoplasms and preneoplastic lesions. There were two minimal LOH regions of overlap on 16q24.3 (4), and we present evidence that the gene CBFA2T3 is a putative breast tumor suppressor in the more proximal of these two regions.

CBFA2T3 was aberrantly expressed in both breast cancer cell lines and sporadic breast tumors. CBFA2T3 mRNA levels were reduced at least 50-fold in a large proportion of the tested breast cancer cell lines compared with the expression levels in normal breast tissue or normal HMEC lines. The MCF-12A cell line is derived from nonmalignant fibrocystic breast tissue and also showed reduced CBFA2T3 expres-
presented in reporter gene activity by the indicated CBFA2T3 constructs, which were calculated using the KRAB repressor domain of the mouse DNA-binding domain. A plasmid containing the GAL4 DNA-binding domain only and normalized to cells were harvested 24 h posttransfection. CAT concentration was determined by ELISA described in internal control for transfection efficiency.

B

Fig. 4. CBFA2T3 represses transcription from CAT reporter gene in a transient transfection assay. A. 293T cells were cotransfected with 0.5 μg of CAT reporter plasmid and increasing amounts (0.3–3 μg) of pMCBF-expressing CBFA2T3 fused to the GAL4 DNA-binding domain. A plasmid containing the GAL4 DNA-binding domain only and pMCBF/GAL4 were used as negative controls, promoter-less GAL4CAT3 plasmid was used as a reference for the TK promoter activation, and the pMNK10 plasmid containing the KKAB repressor domain of the mouse NK10 gene was used as a positive control. The cells were harvested 24 h posttransfection. CAT concentration was determined by ELISA and normalized to β-galactosidase activity from the pcDNA3.1βgal vector used as an internal control for transfection efficiency. B, a schematic diagram of pMCBF deletion mutant constructs with indicated amino acid positions. Experimental conditions were as described in A. Results shown are percentage of transcriptional repression of the CAT reporter gene activity by the indicated CBFA2T3 constructs, which were calculated relative to the activity of the construct pMCBF containing the full-length protein. The data presented in A and B are means ± SD from triplicate samples representative of at least two independent experiments.

sion. This finding may indicate that CBFA2T3 down-regulation can be a preneoplastic event. This is consistent with the suggestion that chromosomal aberrations of the long arm of chromosome 16 are early events in breast carcinogenesis (2). Previous work has inferred the 16q LOH status of breast cancer cell lines (16). CBFA2T3 gene expression was reduced in the cell line MDA-MB-157 with inferred restricted 16q24.3 LOH, whereas expression was unaffected in the cell line MDA-MB-436 with inferred restricted LOH at 16q22.1. However, overall there was no significant correlation detected between the inferred LOH status of the breast cancer cell lines and CBFA2T3 expression. A number of factors are likely to contribute to this lack of correlation, a phenomenon that has been documented for previously well-characterized tumor suppressor genes, e.g., the retinoblastoma gene in cutaneous squamous cell carcinomas (17). LOH of the long arm of chromosome 16 usually encompasses the large part of 16q and will include the locations of LOH smallest regions of overlap both at 16q22.1 and at the more distal 16q24.3 regions. LOH studies of large numbers of sporadic tumors are required to identify the rare cases that will allow these minimal regions of overlap to be defined (4). When the LOH region is large, it is postulated that several alternative tumor suppressor genes could be targeted. It is also possible that some instances of LOH are unrelated stochastic events that occur because of the frequent chromosome aberrations in cancer. Furthermore, multiple tumor suppressors may exist in an LOH region and function simultaneously, as has been described for the 3p21.3 region in lung cancer (18).

RNA in situ hybridization was used to determine expression of CBFA2T3 in paraffin sections of tissues from normal breast and breast tumors. This showed that in normal breast, CBFA2T3 was specifically expressed in the ductal epithelial cells but not in the surrounding stromal or adipose tissue. However, CBFA2T3 expression was reduced or absent in 59% of examined paraffin-embedded sections from primary breast tumors. CBFA2T3 expression did not appear to coincide with a particular stage of tumor progression because negative staining was observed in both preinvasive DCIS and invasive carcinoma specimens. Although this would support the notion that CBFA2T3 gene inactivation is an early event in breast tumorigenesis, larger sample numbers are needed to confirm this finding. However, aberrant expression of CBFA2T3, together with the finding that the forced expression of this gene in breast cancer cells inhibits colony growth on both plastic and soft agar, is consistent with a function as a breast cancer tumor suppressor.

In search of further evidence for the tumor suppressor function of CBFA2T3, we screened for the presence of tumor-restricted genetic changes. No deletions or mutations were identified that were restricted to the cancer samples. A heterozygous change (Pro→Thr) detected in one breast cancer cell line is of unknown significance. If mutation is a rare cause of CBFA2T3 inactivation, it will be necessary to use a more sensitive mutation detection procedure and screen additional tumors. The absence of genetic changes suggests that the lack or decrease of CBFA2T3 expression in breast cancer cells may arise from an alternative mechanism of gene inactivation, such as a breakdown in the regulatory pathway (loss of critical transcription factors, which are required for expression) or transformation-related stable promoter methylation. Although the frequency with which promoter methylation contributes to gene inactivation in cancer cells varies significantly (19), there are examples of tumor suppressor genes in which this is the major mechanism of down-regulation (e.g., Ref. 20). Studies are under way to establish whether there is a link between CBFA2T3 promoter methylation and the expression of this gene.

Using the TK promoter-driven CAT reporter system, we have shown that CBFA2T3 can function as a transcriptional repressor, mimicking its close homologue CBFA2T1 (7). These results were supported by a study on the Eto2 protein, the murine orthologue of human CBFA2T3 (21). Recent observations have proposed a link between transcriptional repression, chromatin remodeling, and cancer, which suggests that potential role of CBFA2T3 in tumor cell growth regulation. We have also mapped the major transcriptional repression activity of CBFA2T3 to the central region of the protein (amino acids 4603.
141–405). This parallels previous CBFA2T1 findings (22) and is consistent with the high sequence homology between the two proteins.

In conclusion, the identification of CBFA2T3 as a putative breast cancer tumor suppressor may lead to the elucidation of novel pathways in breast neoplastic transformation and, therefore, may allow the development of new targets for therapy, diagnosis, and treatment of this most common female cancer. Because the LOH at the 16q24 chromosomal region has been identified in other cancers, including prostate, liver, and gastric tumors, more studies are warranted to investigate the potential role of CBFA2T3 in various malignancies.

ACKNOWLEDGMENTS

We thank Drs. Tom Gonda and Frances Shannon for critical review of the manuscript and Dr. L. Coles (Hanson Centre for Cancer Research, Adelaide, South Australia) for supplying two constructs for the transcriptional repressor studies.

REFERENCES

CBFA2T3 (MTG16) Is a Putative Breast Tumor Suppressor Gene from the Breast Cancer Loss of Heterozygosity Region at 16q24.3


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/16/4599

Cited articles
This article cites 21 articles, 9 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/16/4599.full.html#ref-list-1

Citing articles
This article has been cited by 15 HighWire-hosted articles. Access the articles at:
/content/62/16/4599.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.