Characterization of γ-Aminobutyric Acid Type A Receptor–Associated Protein, a Novel Tumor Suppressor, Showing Reduced Expression in Breast Cancer

Christiane Klebig,[1] Susanne Seitz,[2] Wolfgang Arnold,[3] Nicole Deutschmann,[1] Manuela Pacyna-Gengelbach,[1] Siegfried Scherneck,[2] and Iver Petersen[1]

[1]Institute of Pathology, University Hospital Charité; [2]Department of Tumour Genetics, Max Delbrueck Center for Molecular Medicine; and [3]atugen AG, Berlin, Germany

Abstract

Frequent allelic loss of the chromosomal region *17p13* in breast cancer has suggested that more tumor suppressor genes, besides *p53*, are located in this region. By doing suppression subtractive hybridization to detect differentially expressed genes between the breast cancer cell line CAL51 and a nontumorigenic microcell hybrid CAL/17-1, we identified the gene for the γ-aminobutyric acid type A ($GABA_A$) receptor associated protein (GABARAP), located on *17p13.1*. *GABARAP* displayed high expression levels in the microcell hybrid CAL/17-1 but only weak expression in CAL51 and other breast cancer cell lines tested. Furthermore, we observed large vesicles in CAL/17-1 by immunofluorescence staining, whereas no signal could be detected in the tumor cell line. GABARAP mRNA expression and protein expression were significantly down-regulated in invasive ductal and invasive lobular carcinomas compared with normal breast tissue measured by semiquantitative reverse transcription–PCR and immunohistochemistry, respectively. We assessed that neither mutations in the coding region of the gene nor hypermethylation of CpG islands in the promoter region are responsible for loss of gene expression in CAL51; however, 5-aza-2′-deoxycytidine treatment was effective in gene up-regulation, suggesting a methylation-dependent upstream effect. Stable transfection of *GABARAP* into CAL51 resulted in an increase of gene expression and remarkably influenced the ability of colony formation in soft agar and the growth rate *in vitro* and, moreover, suppressed the tumorigenicity of the cells in nude mice. In summary, our data suggest that GABARAP acts via a vesicle transport mechanism as a tumor suppressor in breast cancer. (Cancer Res 2005; 65(2): 394-400)

Introduction

Previous studies have revealed a high frequency of allelic loss on *17p* in human cancers (1–4). Recently, we showed that there are at least seven commonly deleted regions on chromosome *17p13.1-p13.3* in sporadic breast cancer (5). Furthermore, transfer of the short arm of chromosome 17 into a *p53* wild-type breast cancer cell line resulted in suppression of tumorigenicity in nude mice (6). These data indicate the presence of at least one more putative tumor suppressor gene in the chromosomal region *17p13,* besides the previously described genes *p53* (17p13.1), *Hic-1* (17p13.3), *OVCA1* (17p13.3), and *HCCS1* (17p13.3; refs. 7–10).

Trying to identify new breast cancer–associated genes, we did suppression subtractive hybridization (SSH) by comparing the mRNA expression levels between the breast cancer cell line CAL51 and the nontumorigenic microcell hybrid CAL/17-1 containing an additional short chromosomal arm and the distal part of 17q (17q24-25; ref. 6). We detected the gene for γ-aminobutyric acid type A ($GABA_A$) receptor–associated protein (GABARAP), which is located in the chromosomal region *17p13.1.*

GABARAP, a recently identified cytoplasmic protein of 14 kDa, shows sequence similarity to light-chain 3 of microtubule-associated proteins 1A and 1B at the NH_2 terminus, whereas the COOH-terminal part of the protein is thought to interact with the target protein (11). GABARAP was previously described acting as a trafficking molecule for different receptors like the $GABA_A$ receptor in cortical neurons or the transferrin receptor in HeLa cells (12). Nevertheless, the real biological function of this protein is still under debate.

In this report, we show that the GABARAP mRNA and protein expression are significantly down-regulated in breast tumors compared with normal tissue. Neither gene mutation nor methylation of the promoter was responsible for this decreased expression; however, 5-aza-2′-deoxycytidine (5-aza-dCyd) treatment was effective in gene up-regulation, suggesting a methylation-dependent upstream effect. Moreover, we show that stable *GABARAP* transfectants showed reduced growth rates and impaired colony-forming ability in soft agar in contrast to the tumor cells. Furthermore, stable transfection resulted in suppression of tumorigenicity in nude mice. Consequently, we suggest a potential role of *GABARAP* acting as a putative tumor suppressor gene in breast cancer.

Materials and Methods

Cell Culture

Human breast carcinoma cell lines (MCF7, MDA-MB231, T47D, BT20, and CAL51) were purchased from the American Type Culture Collection (Rockville, MD) and from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany). MCF7 and MDA-MB231 were cultured in Leibovitz 15 medium supplemented with 10% FCS and 1% glutamine. T47D and BT20 were grown as recommended. The CAL51 variant CAL/17-1 was generated by microcell-mediated chromosome 17 transfer as previously described. The A9-17neo cells were a kind gift of Dr. J.C. Barrett (Breast Cancer Research Laboratory, Fox Chase Cancer Center, Philadelphia, PA) (6, 13, 14). CAL51, CAL/17-1, and the transfectants CAL/*GABARAP*-2 and CAL/*GABARAP*-12 were grown in DMEM supplemented with 10% FCS. For the microcell hybrid and the transfectants, medium

Requests for reprints: Iver Petersen, Institute of Pathology, Charité Medical School, Humboldt-University, D-10098 Berlin, Germany. Phone: 49-30-450536050; Fax: 49-30-450536110; E-mail: iver.petersen@charite.de.

was supplemented with 800 g/mL G418. Primary human mammary epithelial cells (Clonetics, Apen, Germany) were grown according to the supplier's recommendations.

Tumor Tissues

Twelve normal breast tissues and 46 breast tumors used for reverse transcription–PCR (RT-PCR) were derived from surgical resections done in the Department of Surgery, University Hospital Charité, Berlin, Germany. All tissues were stored frozen at −80°C.

A total of 119 formalin-fixed, paraffin-embedded breast tissue samples with different clinicopathologic features (93 tumors, 15 lymph node metastases, and 11 normal tissues) were used for microarray construction and were also obtained from the Department of Surgery, University Hospital Charité.

SSH and Expression Analysis

We constructed one cDNA library by doing SSH between CAL51 as driver and the microcell hybrid CAL/17-1 as tester as described previously (15). Similarly, expression analysis by Northern blotting and RT-PCR were essentially carried out as previously described (15, 16).

PCR was done in a 50-μL volume (100 ng of template cDNA, 80 μmol/L of each deoxynucleotide triphosphate, 10 pmol of gene-specific primers, and 2.5 units of Taq DNA polymerase). Initial denaturation was done at 94°C for 2 minutes followed by 26 cycles of 1 minute at 94°C, 30 seconds at optimal annealing temperature (52°C for *GABARAP* and 56°C for *ribosomal protein S9*), and 30 seconds at 72°C, with a final extension at 72°C for 5 minutes. The optimal cycle number was determined as described previously (17). Controls were done with primers for *ribosomal protein S9*. Primer sequences are available upon request. All PCR products were subjected to electrophoresis on a 1.5% agarose gel and visualized by staining with ethidium bromide. Intensities of PCR bands were densitometrically measured. The absorbance of PCR bands corrected with *ribosomal protein S9* was considered to represent the mRNA expression level of the target gene.

Tissue Microarray Construction

Tissue cylinders with a diameter of 1 or 0.6 mm were punched out of the paraffin block and transferred into a recipient array block using a manual tissue arrayer purchased from Beecher Instruments (Woodland, WI). After construction, 4-μmol/L sections were cut from the block and transferred to glass slides.

Immunohistochemistry

Immunohistochemistry was carried out as described previously (16). The primary GABARAP antibody (Alpha Diagnostic, San Antonio, TX) was applied at a concentration of 1:10. Protein expression was scored as follows: negative, 0; weak, 1; moderate, 2; and strong, 3.

Immunofluorescence

Immunostaining of different cell lines was carried out as described previously (18). Incubation with primary rabbit anti-GABARAP polyclonal antibody (1:25; Alpha Diagnostic) took place for 1 hour at room temperature. Secondary FITC-conjugated goat anti-rabbit immunoglobulin G (H + L) antibody was used at a concentration of 1:200 (Jackson ImmunoResearch Laboratories, Baltimore, MD) for 40 minutes.

Statistical Analysis

To evaluate associations between mRNA expression and the clinicopathologic features, Student's *t* test was used. To compare the protein expression of GABARAP with clinicopathologic parameters, 2 × 2 contingency tables (e.g., GABARAP score 0-1 versus 2-3 and G_1-G_2 versus G_3) were set up and the χ^2 test was applied. All analyses were done using the statistical software package SPSS; P values < 0.05 were considered statistically significant.

DNA Extraction and Mutational Analysis

Genomic DNA was extracted from cell lines following standard procedures (proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation).

For detection of point mutations, homozygous deletions, or genomic rearrangements, sequencing of PCR products and Southern blotting were carried out as previously described (16). All primer sequences are available upon request.

Treatment of Cells with 5-Aza-dCyd

Cells were seeded at a density of 1×10^6 in 100-mm dishes, cultured for 48 hours, and treated with 10, 25, 50, or 100 μmol/L 5-aza-dCyd (Sigma Chemical Co., St. Louis, MO) or left untreated. Forty-eight hours after treatment, cells were washed with PBS, fresh medium containing 5-aza-dCyd was added, and cells were incubated for another 48 hours before isolating total RNA. The gene-specific expression was measured by RT-PCR and Northern blot as described above.

Analysis of DNA Methylation by Sequencing of Sodium Bisulfite–Treated DNA

Treatment of genomic DNA with sodium bisulfite was done as described previously (16). Bisulfite-treated DNA was subjected to PCR, resulting in a PCR product that included the promoter region and exon 1. The PCR conditions were as follows: 94°C for 2 minutes, 35 cycles of 94°C for 45 seconds, 60°C for 60 seconds and 72°C for 90 seconds, and a final extension at 72°C for 10 minutes. Primer sequences are available upon request. The PCR product was subcloned into the T/A cloning vector pCR2.1 (Invitrogen, Karlsruhe, Germany) and then sequenced with M13 forward primer as described previously.

Construction of Expression Vector and Transfection

We amplified the full-length cDNA from *GABARAP* by PCR using *GABARAP*-specific primers (primer sequences are available upon request). The PCR conditions were as follows: initial denaturation at 95°C for 2 minutes, followed by 30 seconds at 95°C, 30 seconds at 55°C, and 45 seconds at 72°C for a total of 35 cycles and a terminal extension at 72°C for 10 minutes. The PCR product was digested with the restriction enzymes *Xba*I and *Hin*dIII and inserted into the *Xba*I/*Hin*dIII restriction sites of the mammalian expression vector pcDNA3.1 (−) (Invitrogen), resulting in the vector pcDNA3.1/*GABARAP*. To confirm that the vector contained a wild-type gene without any mutations, the construct was sequenced in both directions.

To obtain stable transfectants, CAL51 cells were seeded at a density of 1×10^5 cells in 35-mm dishes, cultured for 24 hours, and transfected with 3 μg of pcDNA3.1/*GABARAP* and 5 g of lipofectin (Invitrogen) according to the manufacturer's instructions. Selection was done with 800 g/mL G418, starting at 48 hours after transfection for 2 to 3 weeks until single colonies could be picked up.

Western Blot Analysis

For isolation of total proteins from cell lines, cells were washed twice with PBS, lysed with lysis buffer [50 mmol/L Tris base, 5 mmol/L EGTA, 150 mmol/L NaCl, 1% Triton X-100 (pH 7.4), 1 tablet of protease inhibitor for 50 mL buffer, 500 μL trasylol] for 30 minutes on ice and centrifuged Han for 10 minutes at 4°C at 14000 rpm. SDS-PAGE and Western blot analysis were done according to standard protocols. Polyclonal anti-GABARAP antibody was used at 1:200 dilution. Signal was visualized with alkaline horseradish peroxidase–conjugated secondary antibody (1:1000; DAKO, Hamburg, Germany) and enhanced chemiluminescence detection system (Amersham, Freiburg, Germany) according to standard procedures. As loading control, we used rabbit anti-actin antibody at a concentration of 1:200.

In vitro Growth Assay and Tumorigenicity Test

Calculation of the growth rate and colony-forming ability in soft agar were determined as described previously (19). For testing tumorigenicity in nude mice, suspensions of 1×10^6 cells in a volume of 0.2 mL of serum-free culture medium were injected s.c. into 5- to 8-week-old female immune-deficient nude mice (Shoe:NMRI-*nu/nu*). Tumor volumes were estimated as described previously (19). Cell populations were considered to be nontumorigenic if no tumors were detected after 9 weeks postinjection.

Results

Expression Analysis of GABARAP in Breast Cancer Cell Lines

By performing SSH between the breast cancer cell line CAL51 and the nontumorigenic microcell hybrid CAL/17-1, we constructed one cDNA library representing the genes down-regulated in CAL51.[4] We selected one clone, containing the gene *GABARAP,* which is located on *17p13.1,* for further investigations. *GABARAP* showed overexpression in the microcell hybrid CAL/17-1 but was significantly down-regulated in CAL51 and all other breast cancer cell lines (T47D, MCF7, BT20, and MDA-MB231), as measured by Northern blot (Fig 1*A*).

Expression Analysis of GABARAP in Normal and Tumor Tissue

GABARAP was first identified as a protein that interacts with the γ2 subunit of $GABA_A$ receptors in neurons. However, we could reveal by immunohistochemistry that this protein was highly expressed not only in brain but also in other normal tissues such as breast, spleen, endometrium, smooth muscle, prostate, kidney, skeletal muscle, thyroid, lung, stomach, pancreas, gall bladder, tonsil, appendix, lung, placenta, colon, testis, bleb, dermis, liver, thymus, heart, fat tissue, salivary gland, parathyroid, small intestine, and endothelial cells (data not shown). This is in agreement with previous data concerning *GABARAP* mRNA distribution in multiple human tissues tested by Northern blot analysis (20).

To test whether the down-regulation of *GABARAP* mRNA expression observed in tumor cell lines is also reflected in primary breast cancers, we did semiquantitative RT-PCR for 46 breast tumors and 12 normal tissues. We obtained high expression levels in normal breast tissues but only weak or no expression of *GABARAP* in most of the tumor samples tested. Representative examples are shown in Fig. 1*B*. Statistical analysis of these data indicated that the transcript level was significantly decreased ($P < 0.001$) in invasive ductal and invasive lobular carcinoma compared with that in normal tissue [from 1.64 ± 0.79 to 0.53 ± 0.35 (in invasive lobular carcinoma) and from 0.38 ± 0.38 (in invasive ductal carcinoma); Fig. 1*C*.

Furthermore, GABARAP protein expression was examined by immunostaining of tissue microarrays with polyclonal anti-GABARAP antibody. We constructed two tissue microarrays with 119 tissue samples in total, representing 83 invasive ductal carcinomas, 10 invasive lobular carcinomas, 15 lymph node metastases, and 11 normal breast tissues. As shown in Fig. 2*A*, significant reduction in GABARAP protein expression is found to occur in a high proportion of breast cancer specimens. In contrast, most normal breast tissues exhibited a strong specific staining (Fig. 2*B*). Statistical analysis revealed a significant loss of protein expression in invasive ductal and invasive lobular carcinomas ($P = 0.022$ and 0.05, respectively; Table 1), but no correlation could be shown between loss of protein expression in these tumors and the clinicopathologic features like grading, tumor size, estrogen receptor status, Ki-67 staining, or age at diagnosis (data not shown). Moreover, a reduction in GABARAP expression could also be detected in lymph node metastases but without any statistical significance (data not shown).

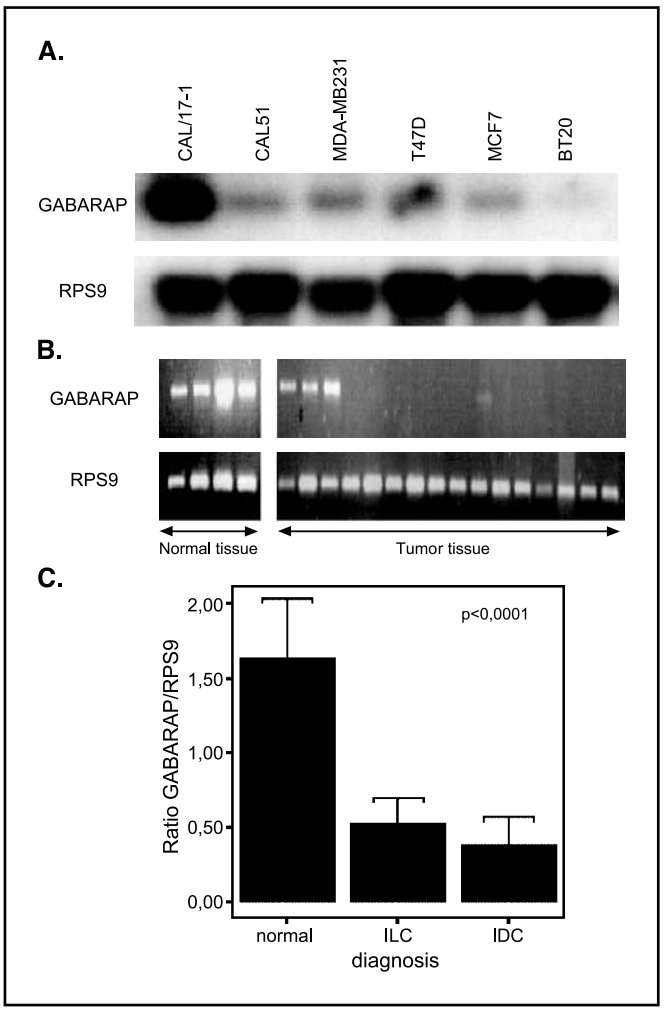

Figure 1. *A,* Northern blot analysis of *GABARAP* from different breast cancer cell lines. Control hybridization was done with *ribosomal protein S9* as housekeeping gene. *B*, RT-PCR analysis of *GABARAP* expression in primary breast cancers and normal tissue. *C*, Diagram of *GABARAP* mRNA expression in normal breast tissue and invasive lobular (*ILC*) and invasive ductal (*IDC*) carcinoma.

Subcellular Localization of GABARAP

For further testing of the subcellular localization of GABARAP in different cell lines, immunostaining with polyclonal anti-GABARAP antibody and fluorescence microscopy were done. Neither in the tumor cell line CAL51 nor in other breast cancer cell lines could GABARAP-specific immunoreactivity be detected. In contrast, large perinuclear vesicles could be observed in the microcell hybrid CAL/17-1 (Fig. 2*C*). In the primary human mammary epithelial cells showing moderate GABARAP mRNA expression in Northern blot analysis, small cytoplasmic vesicles were noted (Fig. 2*D*).

Mutational Analysis

To determine whether mutations within the *GABARAP* gene were responsible for reduced mRNA levels in the tumor cell lines, we analyzed the whole coding region and all exon-intron boundaries by sequencing of PCR products with flanking exon-specific primers. Furthermore, genomic DNA from tumor cell lines and the microcell hybrid was isolated and Southern blotting was done to detect potential genomic rearrangements. Neither

[4] In preparation.

Table 1. Correlation between GABARAP underexpression and breast tumor specimen detected by immunohistochemistry

	Total	GABARAP-negative, *n* (%)	GABARAP-positive, *n* (%)	*P*
Normal	11	3 (27%)	8 (73%)	
IDC	83 (89%)	53 (57%)	30 (32%)	0.022
ILC	10 (11%)	7 (8%)	3 (3%)	0.050
All tumors	93 (100%)	60 (65%)	33 (35%)	0.017

mutations nor large interstitial deletions could be observed in the tested tumor cell lines by these investigations (data not shown) indicating that other factors are responsible for transcriptional silencing of *GABARAP* in tumor cells.

Analysis of Methylation Status

Bioinformative analysis of the genomic sequence of *GABARAP* revealed the existence of CpG islands spanning the promoter region and exon 1, from −248 to +268 bp relative to the transcriptional start site. To investigate whether the lack of expression in tumor cells is associated with hypermethylation of these CpGs, we treated CAL51 cells with increasing concentrations of the DNA methyltransferase inhibitor 5-aza-dCyd and measured *GABARAP* mRNA expression by RT-PCR and Northern blotting. As shown in Fig. 3, only a low *GABARAP* mRNA expression level was detected in CAL51 cells without 5-aza-dCyd treatment, whereas the expression could be increased in a dose-dependent manner of the demethylating agent.

To determine the exact methylation status of the *GABARAP* promoter and exon 1 in CAL51, genomic DNA from CAL51 and CAL/17-1 as control were treated with sodium bisulfite and analyzed by sequencing. Treatment of DNA with sodium bisulfite allows one to distinguish between methylated and unmethylated cytosines due to conversion of unmethylated cytosine to uracil by bisulfite-dependent deamination, whereas methylated cytosines are resistant to this modification and remain as cytosine. Sequence analysis of modified DNA did not reveal any differences between the tumor cell line and the microcell hybrid (data not shown),

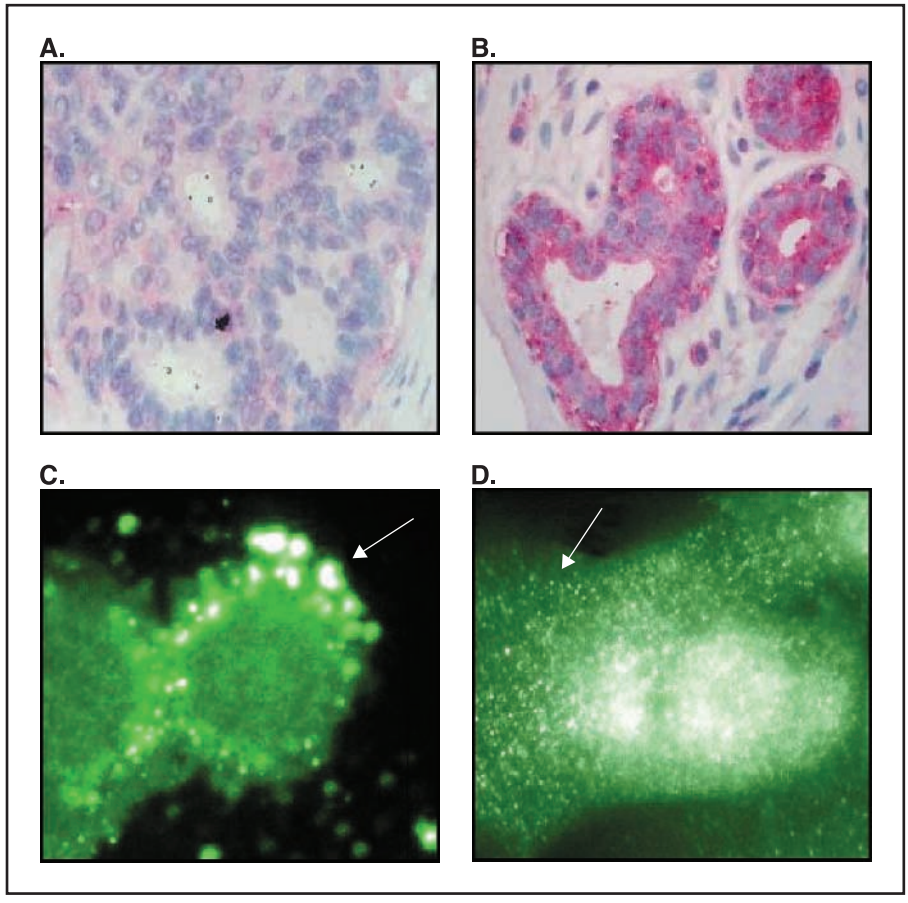

Figure 2. *A,* Representative sections with immunohistochemical staining of GABARAP in formalin-fixed, paraffin-embedded, primary breast cancer tissue (*A*) and normal breast tissue (*B*), with polyclonal anti-GABARAP antibody showing decreased expression in tumor tissue. *C,* Subcellular localization of GABARAP determined by using immunofluorescent-labeled polyclonal anti-GABARAP antibody. In the microcell hybrid CAL/17-1 with high mRNA expression levels large cytoplasmic vesicles could be detected (*C*), whereas normal mammary epithelial cells with moderate expression levels showed smaller vesicles (*D*, *arrow*).

suggesting that no hypermethylation occurred in the promoter region of *GABARAP* in tumor cells.

Characterization of Stable Transfectants

To further examine the possible biological role of GABARAP, we transfected the tumor cell line CAL51 with the mammalian expression vector pcDNA3.1/*GABARAP* containing the whole coding region of the gene. Colonies showing resistance against G418 were selected and further characterized. GABARAP expression in different clones was measured by Northern and Western blotting. As shown in Fig. 4*A*, we obtained high protein levels in the transfectants CAL/*GABARAP*-2 and CAL/*GABARAP*-12 compared with those in CAL51. In contrast, the transfectant containing the control plasmid pcDNA3.1 (CAL/pcDNA3.1) without the gene for *GABARAP* displayed no increase in gene expression or protein amount.

We selected these transfectants to elucidate if GABARAP has a potential function in terms of cell growth. As seen in Fig. 4*B*, the *GABARAP*-transfected cells CAL/*GABARAP*-2 and CAL/*GABARAP*-12 showed reduced growth rates as compared with CAL51 and CAL/pcDNA3.1.

Furthermore, we also assessed a reduced capacity of the *GABARAP* transfectants to form colonies in semisolid agar medium (Fig. 4*C*). Moreover, the *GABARAP*-transfected clones were analyzed for tumorigenicity in immune-deficient nude mice. As shown in Fig. 4*D*, the tumor growth rate was slightly reduced in the mock-transfected cells, whereas tumorigenicity of CAL/*GABARAP*2 and CAL/*GABARAP*12 was remarkably suppressed compared with that of the parental cell line CAL51.

Discussion

In this study, we analyzed the gene for *GABARAP* which we found in our SSH screening for down-regulated genes between the breast cancer cell line CAL51 and its nontumorigenic microcell hybrid CAL/17-1. It mapped to the chromosomal region *17p13.1. GABARAP* was highly expressed in the microcell hybrid but was markedly down-regulated in CAL51 and all other breast cancer cell lines tested.

GABARAP was originally identified as a putative $GABA_A$ receptor clustering protein through its interaction with the cytoskeleton and the γ2 subunit of $GABA_A$ receptor (11, 21, 22). It was thought to promote trafficking of $GABA_A$ receptor from intracellular pools to the cell surface, finally acting as an anchor between the receptor and the cytoskeleton at synaptic membranes. Although GABARAP was found to colocalize with the receptor in cultured cortical neurons (11), further experiments revealed that no colocalization between the microtubule-binding protein gephyrin, for which an important role in $GABA_A$ receptor clustering has been described (23), and GABARAP could be found at the synapse (24, 25). Moreover, the majority of $GABA_A$ receptor clusters did not contain GABARAP, confirming the hypothesis that GABARAP is solely involved in receptor trafficking but not in receptor clustering (25).

We detected the subcellular localization of GABARAP in the differentially expressing microcell hybrid CAL/17-1, normal mammary epithelial cells, and the breast cancer cell line CAL51. Only in CAL/17-1 could large vesicles be observed; however, in human mammary epithelial cells, small cytoplasmic vesicles could be detected, whereas CAL51 did not show any specific signal. These results are concordant with previous findings in HeLa cells, displaying a perinuclear and scattered distribution of GABARAP, whereas overexpression of exogenous protein also resulted in the formation of large vesicles (12). In cultured cortical and hippocampal neurons, a punctate distribution of GABARAP was seen throughout the cytoplasm, predominantly concentrated in intracellular membrane compartments, such as the Golgi apparatus, endoplasmic reticulum, and synaptic cisternae (24, 25). Interestingly, GABARAP has strong homology to a Golgi-associated transport factor, Golgi-associated ATPase enhancer of 16 kDa (GATE-16), also called ganglioside expression factor 2 (26), and to yeast Aut7p, which are both involved in intracellular trafficking events (27). Furthermore, GABARAP interacts with *N*-ethylmaleimide–sensitive factor, a transport factor participating in membrane fusion events (25, 28). These data indicate that GABARAP may have a potential role in the transport of different receptor-containing Golgi vesicles before vesicle fusion with the plasma membrane.

We also confirmed previous findings that GABARAP is not restricted to the nervous system but is expressed in a wide range of different tissues (20, 29). This suggests that GABARAP is playing a more generalized function in cells than binding exclusively to neuronal $GABA_A$ receptor.

Recently, a decrease of gene expression in cancerous tissues versus normal tissues has been reported by investigating the differences in expression levels between *GABARAP* and *GEC1/GABARAPL1*, a family member having strong identity and similarity (87% and 94%) with *GABARAP*, by using human multiple tissue Northern blot (29).

In this work, for the first time a comprehensive expression analysis of the mRNA and protein level of GABARAP in breast cancer was done. We revealed a significant down-regulation of gene expression and protein expression in a high proportion of breast cancer tissues compared with normal breast tissue. To test whether GABARAP has a putative function in cancer progression, we stably transfected the breast cancer cell line CAL51 with full-length cDNA of this gene. The obtained transfectants displayed high GABARAP expression levels, which correlated with reduced growth rates and impaired ability of anchorage-independent growth *in vitro* and suppression of tumorigenicity in nude mice compared with the cancer cells.

Thus, expression analysis as well as *in vitro* and *in vivo* growth characteristics are compatible with a role of GABARAP as a tumor suppressor. However, the precise role of GABARAP in tumorigenesis is not yet clear. Hanahan and Weinberg (30) postulated that a manifestation of six essential alterations in cell physiology collectively dictate malignant growth. These hallmarks of cancer are self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis,

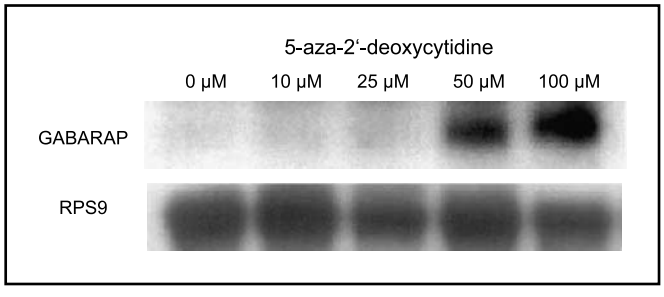

Figure 3. *GABARAP* expression analysis by Northern blot after treatment with 5-aza-dCyd. *Ribosomal protein S9* was used as housekeeping gene.

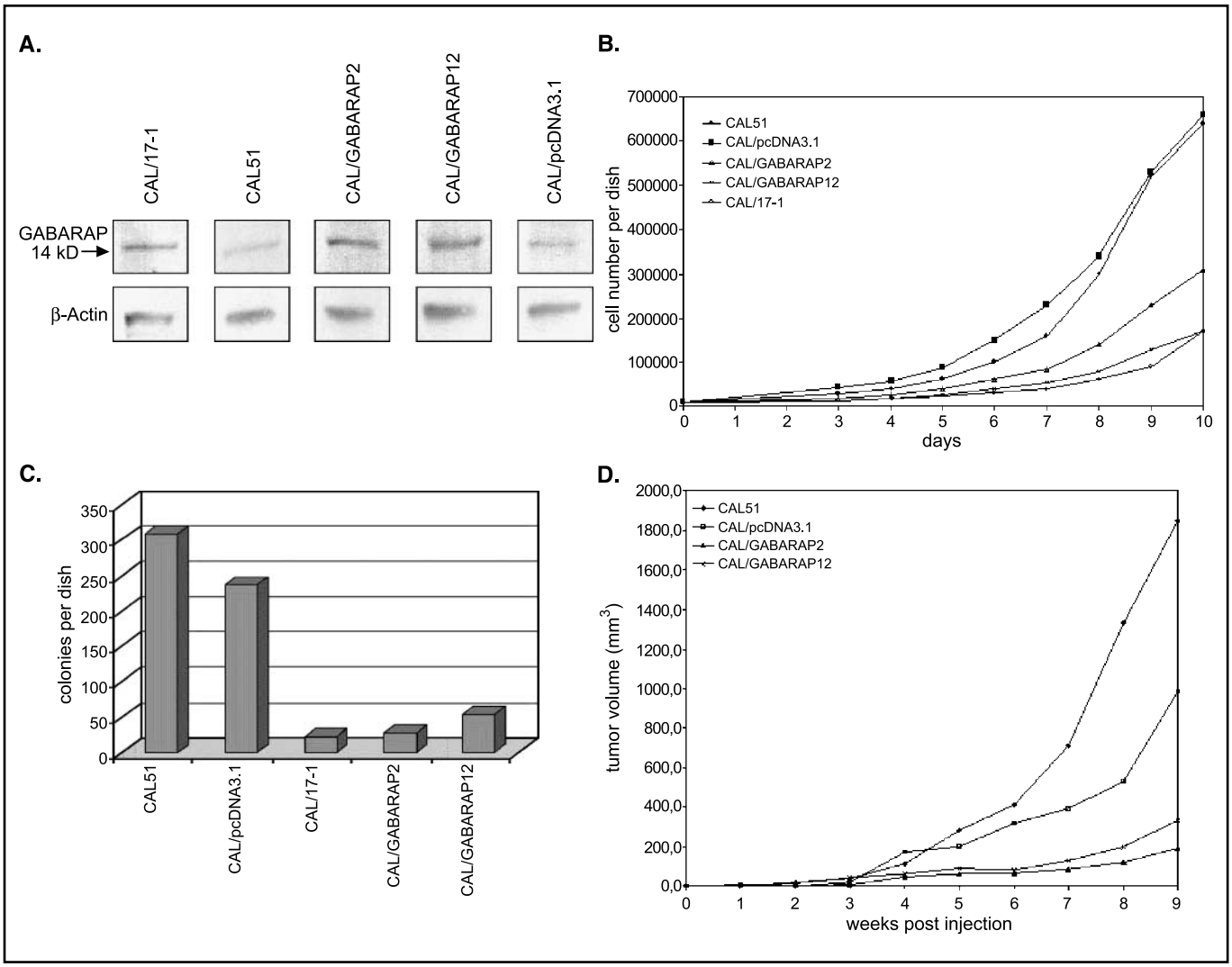

Figure 4. Western blot analysis of GABARAP protein expression in cells stably transfected with full-length cDNA of *GABARAP* showing increased expression of the protein in the *GABARAP* transfectants compared with the tumor cell line CAL51 and mock-transfected cells CAL/pcDNA3.1. Equal loading of the blot was confirmed by staining with rabbit anti-actin antibody (*A*). Analysis of the growth rate *in vitro* (*B*), colony-forming ability in soft agar (*C*), and tumorigenicity in immune-deficient nude mice (*D*) indicated reduced malignant phenotypes of the CAL/GABARAP transfectants.

and tissue invasion and metastasis. According to this postulation, GABARAP function has to be associated with one of these six capabilities of cancer cells. Because of the findings by us and other groups that (*a*) GABARAP is predominantly localized in large vesicles or intracellular membrane compartments, (*b*) GABARAP seems to be responsible for trafficking of different receptor molecules through the cytoplasm to their target place, and (*c*) stable expression of this protein resulted in impaired growth abilities, we suggest a potential role of GABARAP in endocytosis of either activated membrane-bound growth factor receptors or soluble macromolecules like growth-inhibitory signals resulting in lysosomal degradation of the receptors or triggering signal cascades with growth-inhibitory effects. In normal tissues, activated membrane-bound receptors are removed from the cell surface via receptor-mediated endocytosis and then sorted to the degradation pathways, which seems to be the major process in regulating the kinetics of signal transduction by epidermal growth factor receptors (31). Recent studies have shown that trafficking defects in epidermal growth factor receptors can facilitate cell transformation (32). Furthermore, abnormal expression or mutation of some endocytotic proteins, such as BIN1 or CALM, has been reported in human cancer (33, 34). Because no proteins playing a role in signal transduction by growth factor receptors or growth-inhibitory signals during carcinogenesis have yet been described to interact with GABARAP, this seems to be an auspicious topic for a better understanding of how GABARAP functions in normal and tumor cells.

Screening of CAL51 for alterations within the coding region and the exon-intron boundaries did not detect any somatic mutations. We also assessed the breast cancer cell lines for large deletions by Southern blotting, but no genomic rearrangements could be observed. This suggests that *GABARAP* might act as a class II tumor suppressor gene, which is characterized by a down-regulation in its expression by epigenetic events rather than by mutations or deletions (35).

Bioinformative analysis revealed that a CpG island is spanning the promoter region and the first exon of *GABARAP*, raising the possibility of epigenetic regulation of *GABARAP* expression by CpG

methylation. Although treatment of cells with high concentrations of the demethylating agent 5-aza-dCyd resulted in increasing expression levels in CAL51, there was no indication for methylation by sequencing of sodium bisulfite–treated genomic DNA. These results indicate that 5-aza-dCyd–dependent increase of *GABARAP* gene expression occurs indirectly, most likely through effects at other loci, in a way that *GABARAP* gene expression is regulated by another protein that itself is methylated in its promoter region and thus responds positively to 5-aza-dCyd treatment. Furthermore, recent studies have shown that altered DNA methylation is not the only epigenetic mechanism for down-regulation of genes (36–39). Differential acetylation status of histones seems to be another important regulator of gene expression, which has already been described for $p16^{INK4A}$ in colorectal cancer cells and *gelsolin* and *maspin* in breast cancer cells (37–39).

Taken together, our data strengthen the suggestion that GABARAP functions as a putative tumor suppressor gene class II in breast cancer. However, the regulatory pathways of GABARAP gene expression, which lead to down-regulation in tumors, and the precise function of this protein remain to be elucidated.

Acknowledgments

Received 4/14/2004; revised 9/23/2004; accepted 11/11/2004.

Grant support: Wilhelm Sander Stiftung.

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.

We thank Dr. Karsten Schlüns for helpful suggestions in bioinformative analysis, Britta Beyer for performing immunohistochemical staining, and Dr. Glen Ole Kristiansen for construction of TMA photography.

References

1. Hoff C, Mollenhauer J, Waldau B, Hamann U, Poustka A. Allelic imbalance and fine mapping of the 17p13.3 subregion in sporadic breast carcinomas. Cancer Genet Cytogenet 2001;129:145–9.
2. Phillips N, Ziegler M, Saha B, Xynos F. Allelic loss on chromosome 17 in human ovarian cancer. Int J Cancer 1993;54:85–91.
3. Konishi H, Takahashi T, Kozaki K, et al. Detailed deletion mapping suggests the involvement of a tumour suppressor gene at 17p13.3, distal to p53, in the pathogenesis of lung cancer. Oncogene 1998;17: 2095–100.
4. Piao Z, Park C, Park JH, Kim H. Allelotype analysis of hepatocellular carcinoma. Int J Cancer 1998;75:29–33.
5. Seitz S, Poppe K, Fischer J, et al. Detailed deletion mapping in sporadic breast cancer at chromosomal region 17p13 distal to the TP53 gene: association with clinicopathological parameters. J Pathol 2001; 194:318–26.
6. Theile M, Hartmann S, Scherthan H, et al. Suppressor of tumorigenicity of breast cancer cells by transfer of human chromosome 17 does not require transferred BRCA1 and p53 genes. Oncogene 1995;10:439–47.
7. Bálint É, Vousden KH. Activation and activities of the p53 tumour suppressor protein. Br J Cancer 2001;85: 1813–23.
8. Makos Wales M, Biel MA, El Deiry W, et al. p53 activates expression of HIC-1, a new candidate tumour suppressor gene on 17p13.3. Nat Med 1995;1:570–7.
9. Bruening W, Prowse AH, Schultz DC, Holgado-Madruga M, Wong A, Godwin AK. Expression of OVCA1, a candidate tumor suppressor, is reduced in tumors and inhibits growth of ovarian cancer cells. Cancer Res 1999;59:4973–83.
10. Zhao X, Li J, He Y, et al. A novel growth suppressor gene on chromosome 17p13.3 with a high frequency of mutation in human hepatocellular carcinoma. Cancer Res 2001;61:7383–7.
11. Wang H, Bedford FK, Brandon NJ, Moss SJ, Olsen RW. $GABA_A$-receptor-associated protein links $GABA_A$ receptors and the cytoskeleton. Nature 1999;397:69–72.
12. Green F, O'Hare T, Blackwell A, Enns CA. Association of human transferrin receptor with GABARAP. FEBS Lett 2002;518:101–6.
13. Saxon PJ, Srivatsan ES, Leipzig GV, Sameshima JH, Stanbridge EJ. Selective transfer of individual human chromosomes to recipient cells. Mol Cell Biol 1985;5: 140–6.
14. Yang X, Tahin Q, Hu YF, et al. Functional roles of chromosomes 11 and 17 in the transformation of human breast epithelial cells *in vitro*. Int J Oncol 1999; 15:629–38.
15. Petersen S, Heckert C, Rudolf J, et al. Gene expression profiling of advanced lung cancer. Int J Cancer 2000; 86:512–7.
16. Chen Y, Hühn D, Knösel T, Pacyna-Gengelbach M, Deutschmann N, Petersen I. Downregulation of connexin 26 in human lung cancer is related to promoter methylation. Int J Cancer. In press 2004.
17. Chen Y, Petersen S, Pacyna-Gengelbach M, Pietas A, Petersen I. Identification of a novel homeobox-containing gene, LAGY, which is downregulated in lung cancer. Oncology 2003;64:450–8.
18. Pietas A, Schlüns K, Marenholz I, Schafer BW, Heizmann CW, Petersen I. Molecular cloning and characterization of the human S100A14 gene encoding a novel member of the S100 family. Genomics 2002; 79:513–22.
19. Theile M, Hartmann S, Naundorf H, et al. Wild-type p53 is not involved in reversion of the tumorigenic phenotype of breast cancer cells after transfer of normal chromosome 17. Int J Oncol 1994;4:1067–75.
20. Xin Y, Yu L, Chen Z, et al. Cloning, expression patterns, and chromosome localization of three human and two mouse homologues of $GABA_A$ receptor-associated protein. Genomics 2001;74:408–13.
21. Wang H, Olsen RW. Binding of the $GABA_A$ receptor-associated protein (GABARAP) to microtubules and microfilaments suggests involvement of the cytoskeleton in GABARAP-$GABA_A$ receptor interaction. J Neurochem 2000;75:644–55.
22. Chen L, Wang H, Vicini S, Olsen RW. The γ-aminobutyric acid type A ($GABA_A$) receptor-associated protein (GABARAP) promotes $GABA_A$ receptor clustering and modulates the channel kinetics. Proc Natl Acad Sci U S A 2000;97:11557–62.
23. Kneussel M, Brandstatter JH, Laube B, Stahl S, Muller U, Betz H. Loss of postsynaptic GABA(A) receptor clustering in gephyrin-deficient mice. J Neurosci 1999; 19:9289–97.
24. Kneussel M, Haverkamp S, Fuhrmann JC, et al. The γ-aminobutyric acid type A receptor ($GABA_AR$)-associated protein GABARAP interacts with gephyrin but is not involved in receptor anchoring at the synapse. Proc Natl Acad Sci U S A 2000;97:8594–9.
25. Kittler JT, Rostaing P, Schiavo G, et al. The subcellular distribution of GABARAP and its ability to interact with NSF suggest a role for this protein in the intracellular transport of $GABA_A$ receptors. Mol Cell Neurosci 2001; 18:13–25.
26. Paz Y, Elazar Z, Fass D. Structure of GATE-16, membrane transport modulator and mammalian ortholog of autophagocytosis factor Aut7p. J Biol Chem 2000;275:25445–50.
27. Lang T, Schaeffeler E, Bernreuther D, Bredschneider M, Wolf DH, Thumm M. Aut2p and Aut7p, two novel microtubule-associated proteins are essential for delivery of autophagic vesicles to the vacuole. EMBO J 1998;17:3597–607.
28. Rothman JE. Mechanisms of intracellular protein transport. Nature 1994;372:55–63.
29. Nemos C, Mansuy V, Vernier-Magnin S, Fraichard A, Jouvenot M, Delage-Mourroux R. Expression of *gec1/GABARAPL1* versus *GABARAP* mRNAs in human: predominance of *gec1/GABARAPL1* in the central nervous system. Brain Res Mol Brain Res 2003;119: 216–9.
30. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
31. Sorkin A, Waters CM. Endocytosis of growth factor receptors. BioEssays 1993;15:375–82.
32. Wells A, Welsh JB, Lazar CS, Wiley HS, Gill GN, Rosenfeld MG. Ligand-induced transformation by a noninternalizing epidermal growth factor receptor. Science 1990;247:962–4.
33. Sakamuro D, Elliot KJ, Wechsler-Reya R, Prendergast GC. BIN1 is a novel MYC-interacting protein with features of a tumour suppressor. Nat Genet 1996;14:69–77.
34. Dreyling MH, Martinez-Climent JA, Zheng M, Mao J, Rowley JD, Bohlander SK. The t(10;11)(p13;q14) in the U937 cell line results in the fusion of the AF10 gene and CALM, encoding a new member of the AP-3 clathrin assembly protein family. Proc Natl Acad Sci U S A 1996;93:4804–9.
35. Sager R. Expression genetics in cancer: shifting the focus from DNA to RNA. Proc Natl Acad Sci U S A 1997; 94:952–5.
36. Wiechen K, Diatchenko L, Agoulnik A, et al. Caveolin-1 is downregulated in human ovarian carcinoma and acts as a candidate tumor suppressor gene. Am J Pathol 2001;159:1635–43.
37. Bachmann KE, Park BH, Rhee I, et al. Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. Cancer Cell 2003;3:89–95.
38. Mielnicki LM, Ying AM, Head KL, Asch HL, Asch BB. Epigenetic regulation of gelsolin expression in human breast cancer cells. Exp Cell Res 1999;249:161–76.
39. Maass N, Biallek M, Rosel F, et al. Hypermethylation and histone deacetylation lead to silencing of the maspin gene in human breast cancer. Biochem Biophys Res Commun 2002;297:125–8.