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Relationship Between Mammaglobin Expression and Estrogen Receptor Status in Breast Tumors

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Mammaglobin (SCGB2A2) is a breast-specific member of the secretoglobin (SCGB) gene family. SCGB2A2 has previously been found overexpressed in breast tumors but possible associations between its expression and established prognostic tumor characteristics such as the levels of estrogen and progesterone receptors have not yet been investigated. We evaluated SCGB2A2 expression at the mRNA and at the protein level by reverse-transcription polymerase chain reaction and immunocytochemistry in 52 and 32 breast tumors, respectively. Both SCGB2A2 mRNA and protein expression were significantly higher in estrogen-receptor-positive compared to estrogen-receptor-negative tumors (Mann–Whitney rank sum test, $p = 0.04$; chi-square test, $p = 0.01$; respectively). In contrast, SCGB2A2 expression did not correlate with progesterone receptor levels or Nottingham grade. As estrogen and antiestrogen treatment of estrogen-positive breast cancer cell lines does not modify SCGB2A2 expression we suggest that SCGB2A2 may be a new independent breast cancer prognostic marker.

Key Words: SCGB2A2; MGB1; estrogen receptor; progesterone receptor; Nottingham grade; breast cancer.

Introduction

Mammaglobin (MGB1, SCGB2A2) was first identified in 1996, using differential display analysis, as a breast-specific member of the secretoglobin (SCGB) gene family overexpressed in some breast tumors *(1,2)*. Today, a search for breast-specific expressed sequence tags (ESTs) performed using the Differential Gene Expression Displayer (DGED) tool at the Cancer Gene Anatomy Project (CGAP) website (http://cgap.nci.nih.gov/Tissues/GXS) shows that SCGB2A2-related ESTs have been found in nine different breast cDNA libraries but only two non-breast libraries, further confirming the relative breast specificity of SCGB2A2 expression. Using a subtractive hybridization approach, we previously identified SCGB2A2 mRNA as overexpressed in the *in situ* compared to the invasive element within an individual breast tumor *(3,4)*. Further *in situ* hybridization analysis, performed in breast tumors selected to include normal, *in situ*, and invasive primary tumor elements revealed that SCGB2A2 expression, restricted to epithelial cells, could be detected in all elements and was significantly increased in tumor cells compared to normal cells *(4)*. This higher SCGB2A2 expression in malignant versus nonmalignant breast epithelium has also been confirmed at the protein level by immunocytochemistry *(5)*. In this latter study, Watson et al. concluded that SCGB2A2 expression was independent of tumor grade and histological type.

It has recently been demonstrated that circulating mammary carcinoma cells can also be detected in the blood of breast cancer patients via PCR detection of SCGB2A2 mRNA *(6–9)*. Even though its biological function remains unknown, SCGB2A2 is now considered as a relatively specific marker of axillary lymph node breast metastases as well as of occult breast cancer *(10–13)*. Interestingly, Zach et al. detected SCGB2A2 mRNA expression by nested reverse-transcription PCR (RT-PCR) more frequently in the blood of patients with estrogen-receptor-positive (ER+) breast tumor than in the blood of estrogen-receptor-negative (ER–) breast cancer patients, suggesting a possible relationship between SCGB2A2 and ER levels in primary breast tumors *(6)*. In order to investigate further possible associations between SCGB2A2 expression and estrogen and progesterone receptors in primary breast tumors, we assessed SCGB2A2 expression at the mRNA and at the protein level in a cohort of breast tumors.

Results

Assessment of SCGB2A2 mRNA Expression in a Cohort of 52 Human Breast Tumor Samples

To establish whether SCGB2A2 mRNA expression paralleled established known prognostic parameters such as ER and PR levels, a cohort of 52 cases was selected from the

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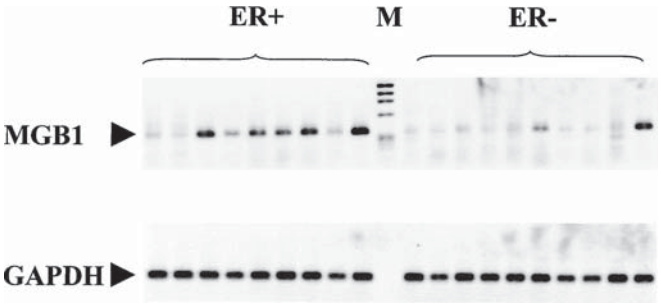

Fig. 1. RT-PCR analysis of SCGB2A2 and GAPDH mRNA expression in primary breast tumors. Total RNA was extracted from frozen tissue sections corresponding to ER positive (ER+) and ER negative (ER–) cases, reverse-transcribed and PCR amplified as described in the Materials and Methods section using SCGB2A2- or GAPDH-specific primers. PCR products were then separated on 2% agarose gels prestained with ethidium bromide. Black arrow: product corresponding to SCGB2A2, grey arrow: product corresponding to GAPDH. M: Molecular weight marker (Φx174 RF DNA/*Hae*III fragments, Gibco BRL, Grand Island, NY).

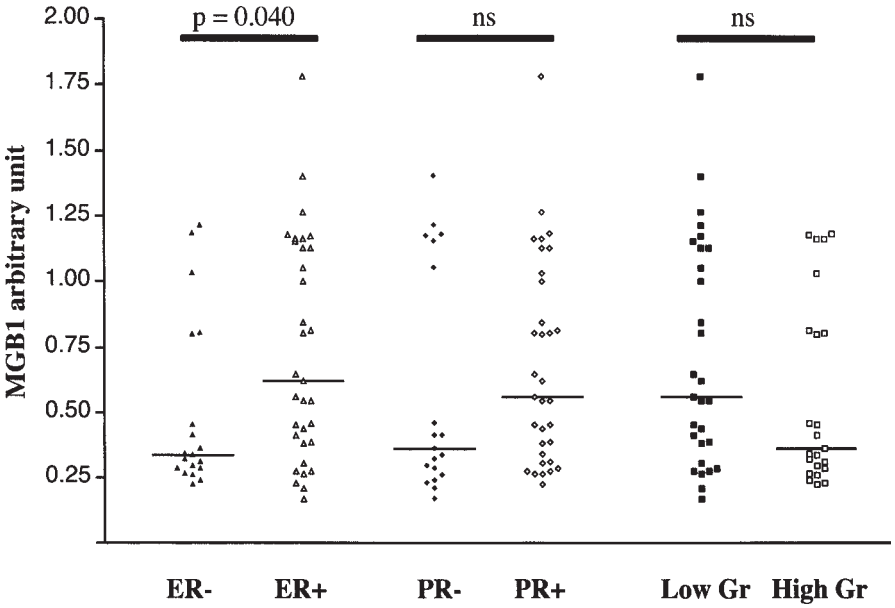

Fig. 2. Quantification of SCGB2A2 mRNA expression in different breast tumor subgroups. Total RNA was extracted from frozen tissue sections corresponding to 52 cases and analyzed as described in Fig. 1. SCGB2A2 mRNA expression was quantified relative to GAPDH mRNA as described in the Materials and Methods section. Tumors were grouped according to their ER status (ER+, ER–), their PR status (PR+, PR–) or their grade (Low Gr: Nottingham scores between 5 and 7; High Gr: Nottingham scores between 8 and 9). Difference between subgroups were tested using the Mann–Whitney rank sum test, two-sided.

NCIC-Manitoba Breast Tumor Bank. For each case, clinical characteristics of the tumor (i.e., ER and PR levels, Nottingham grade) were known (*see* Materials and Methods for a summary of tumor subgroup characteristics). Total RNA was extracted from frozen primary tumor sections, reverse-transcribed and analyzed by RT-PCR using primers recognizing specifically SCGB2A2 cDNA, and chosen to span intronic regions. As shown Fig. 1, SCGB2A2 corresponding signal can be detected in the majority of cases, even though levels of expression varied from one sample to another. Amplification of the ubiquitously expressed GAPDH cDNA in the same cDNA samples was performed in parallel and, for each case, a normalized SCGB2A2 mRNA expression value was calculated (*see* Materials and Methods). SCGB2A2 expression was found to strongly correlate with ER levels ($n = 52$, Spearmann coefficient $r = 0.282$, $p = 0.042$) but not with PR levels or grade (data not shown). Similarly (Fig. 2), using the established clinical cut-off of ER positivity (ER positive tumors have a binding higher than 3 fmol/mg of total protein), SCGB2A2 mRNA expression was significantly (Mann–Whitney rank sum test, two-sided, $p = 0.040$) higher in ER+ ($n = 33$, median value SCGB2A2 = 0.62) than in ER– ($n = 19$, median SCGB2A2 value = 0.33).

Assessment of SCGB2A2 Protein Expression in a Cohort of 32 Human Breast Tumor Samples

In order to determine whether SCGB2A2 protein expression correlated with SCGB2A2 mRNA expression and whether a similar association between ER status and SCGB2A2 expression could be observed at the protein level, paraffin

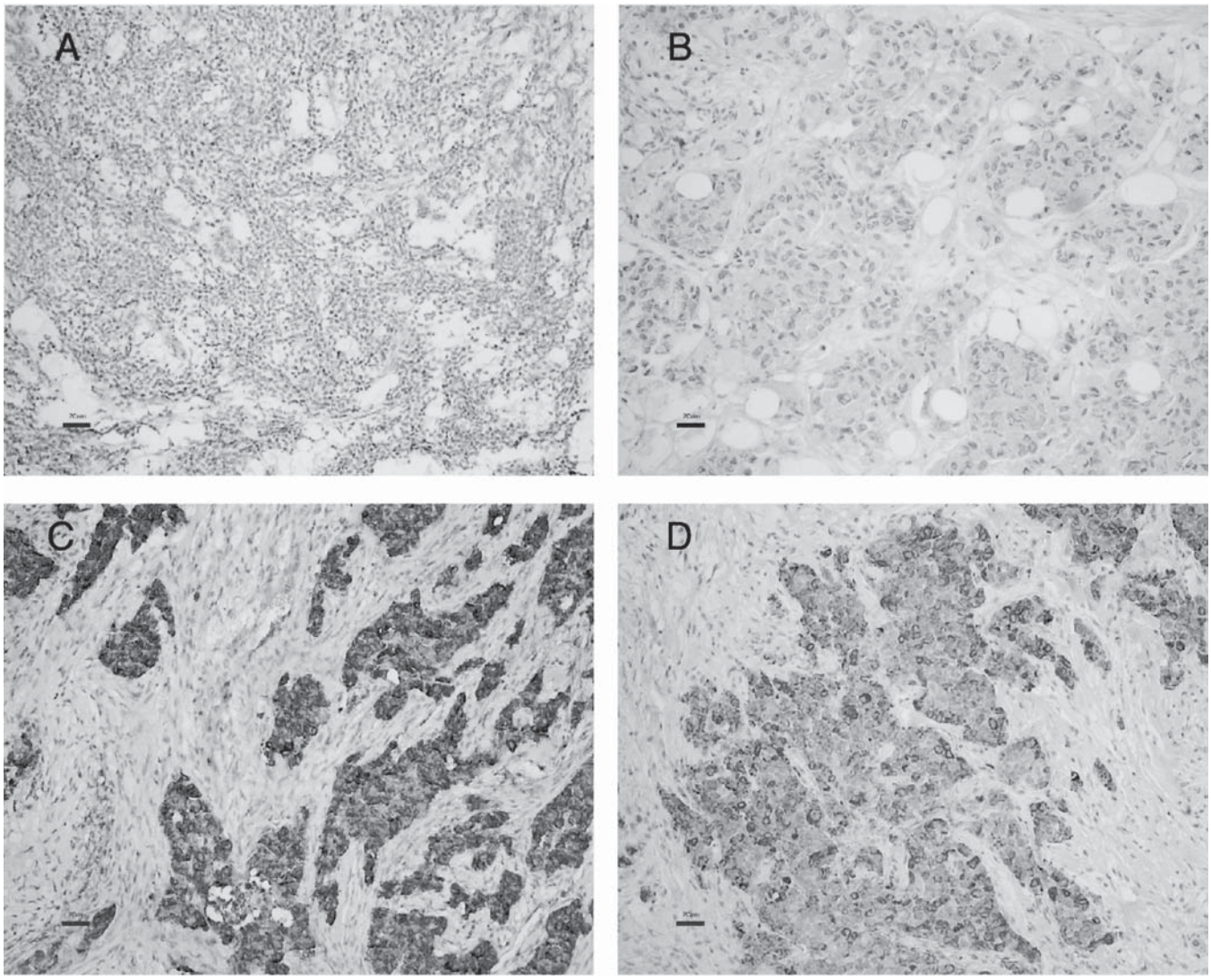

Fig. 3. Detection of SCGB2A2 protein in breast tumors by immunohistochemistry. SCGB2A2 protein was detected on paraffin-embedded breast tumor tissue sections using a rabbit polyclonal primary anti-SCGB2A2 antibody (Kindly provided by Dr. T Fleming) and the Ventana-Discovery system as described in the Materials and Methods section. Panel A and B: Two independent ER– cases showing no (A) or low (B) SCGB2A2 protein expression in tumor epithelial cells. Panel C and D: two independent ER+ cases presenting a strong SCGB2A2 signal. Blue bar: 20 μm.

blocks corresponding to 32 out of these 52 tumors were sectioned and processed for immunohistochemical analysis of SCGB2A2 expression (*see* Materials and Methods). Slides were scored blindly for SCGB2A2 protein expression by a pathologist as described in the Materials and Methods section. Some sections showed no (Fig. 3A, SCGB2A2 score = 0) or low (Fig. 3B, SCGB2A2 score = 1) SCGB2A2 expression, whereas others presented strong SCGB2A2 protein signal (Fig. 3C, SCGB2A2 score = 3; Fig. 3D, SCGB2A2 score = 2). Comparison of SCGB2A2 protein scores and previously obtained normalized SCGB2A2 mRNA levels revealed a strong correlation ($n = 32$, Spearman r coefficient $r = 0.575$, $p = 0.0006$) between protein and mRNA levels. Tumors were classified as low (scores between 0 and 1) and high (1.5 and 3) SCGB2A2 protein expressors, and differences between tumor subgroups (ER+/ER–, PR+/PR–, low grade/high grade) were assessed using chi-square test. As observed for SCGB2A2 mRNA, SCGB2A2 protein positivity was associated (chi-square test, $p = 0.017$) with ER status but not with PR status or grade (Fig. 4).

Absence of Estrogen Regulation of SCGB2A2 Expression

These data suggested that estrogen might regulate SCGB2A2 expression. In order to address the question of a possible regulation of SCGB2A2 expression in breast cancer cells, ZR-75 cells, known to express SCGB2A2 *(14)*, were treated by estradiol-17β 10^{-8} *M* or the antiestrogen ICI-182,780 10^{-6} *M* for 6, 24, and 48 h as described in the Materials and Methods section. Total RNA was extracted and analyzed by RT-PCR using primers recognizing GAPDH, SCGB2A2, or psoria-

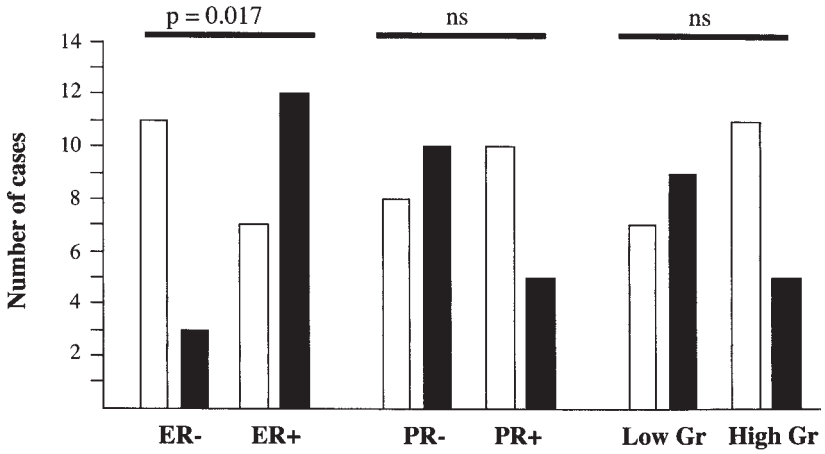

Fig. 4. Quantification of SCGB2A2 protein in breast tumor subgroups. Paraffin-embedded tissue section corresponding to 32 cases were processed as shown Fig. 3. Slides were independently reviewed and scored as described in the Materials and Methods section. For each tumor subgroup (ER–, ER+, PR+, PR–, low grade, and high grade), the number of cases negative (White columns) or positive (black columns) is shown. Differences between subgroups were tested using the chi-square test.

sin cDNAs. Psoriasin was chosen as its expression has previously been shown to be regulated by estrogen treatment (*15*, our unpublished data). SCGB2A2 mRNA expression was not changed under any treatment condition (data not shown), whereas, as expected, psoriasin signal was found to be increased by estradiol and decreased by antiestrogen treatment as soon as 6 h of treatment, with a maximum effect after 24 and 48 h of treatment (estradiol treatment: 1.5-, 2.8-, and 4.5-fold control and antiestrogen treatment 0.90-, 0.80-, and 0.70-fold control, respectively).

Discussion

Assessment of SCGB2A2 expression at the mRNA and the protein levels in a cohort of breast tissue samples showed a statistically significant relationship between SCGB2A2 levels and ER status. However, within the same cohort, no association was found between SCGB2A2 expression and other known prognostic marker such as PR levels or Notthingham grade.

To the clinician, a factor is considered a prognostic factor when it is associated with the outcome of the disease, i.e., predicts how the disease would evolve if not treated, whereas a predictive factor is associated to the degree of response to therapy, i.e., predicts the likelihood of response to a particular treatment. A high level of ER in tumor tissue has a good prognostic value and also predicts a good likelihood of responding to hormonal adjuvant therapy such as tamoxifen *(16,17)*. As PR expression is positively regulated by estrogens, higher PR levels in ER+ tumors support the hypothesis of an operational ER signaling pathway and is therefore also considered as a good prognostic and predictive parameter. Whereas the parallel between SCGB2A2 and ER expression suggested that SCGB2A2 could be a new ER target gene, the lack of association with a known regulated gene such as PR suggested that SCGB2A2 expression was independent of ER signaling pathway. This latter hypothesis was further supported by the absence of estrogen and antiestrogen regulation of SCGB2A2 expression in ZR-75 cells, even though ER signaling pathway appears functional, as shown by the induction of a known ER-regulated gene, psoriasin. It should be noted that a similar absence of regulation was also observed in another ER+ breast cancer cell line MCF-7 cells (our unpublished results; *18*). However, even though the SCGB2A2 gene was not grossly rearranged in MCF-7 cells *(18)*, these cells do not express endogenous SCGB2A2 (our unpublished results; 2). It might therefore be hypothesized that SCGB2A2 expression in MCF-7 cells is negatively regulated by other factors, resulting in an absence of estrogen regulation in these cells. Further experiments performed on other breast cancer cell lines and primary cells *(19)* are needed to confirm these preliminary results.

Interestingly, the general expression of SCGB2A2 as well its association with ER levels observed in vivo in breast tissue contrasts with in vitro observations made on mammary epithelial cancer cell lines. Indeed, looking at a panel of different breast cancer cell lines, Watson et al. reported the detection of SCGB2A2 transcripts only in few cell lines (MB361, MB415, MB468, BT474, MB175) with no expression in MCF7, MB134, MB231, or MCF10A cells *(2)*. Similarly, we did not detect SCGB2A2 expression in breast cell lines such as BT20, T47D, or MCF10AT1 even though a strong signal was seen in ZR-75 (our unpublished observation). As cells such as MB468 and MB361 are ER– and cells such as ZR-75 or BT474 are ER+, SCGB2A2 expression does not appear related to ER status in cells grown in vitro. Overall, this suggests that most of cell lines, through

selection, medium conditions, and/or dedifferentiation lost their ability to express SCGB2A2 in vitro. Presently, no data are available regarding the possible biological function of SCGB2A2. It has however recently been reported that SCGB2A2 existed in a tetrameric complex with BU101 (lipophilin B), another member of the secretoglobin family, the expression of which correlated with SCGB2A2 expression in breast tissue *(20)*. The role of this complex as well as the possible regulation of its components remains to be determined.

In conclusion, we found that SCGB2A2 expression correlated with ER levels in breast tumor tissue. As ER is considered as a good prognostic factor and as SCGB2A2 does not appear to be directly regulated by the ER signaling pathway, we hypothesize that SCGB2A2 expression may be a new independent prognostic marker in breast cancer. Further experiments performed on a larger cohort of patients and completed with follow up studies are needed to test this hypothesis.

Materials and Methods

Human Breast Tissues and Cell Lines

All breast tumor cases used for this study were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As it has been previously described *(21)*, tissues are accrued to the Bank from cases at multiple centers within Manitoba, rapidly collected and processed to create matched formalin-fixed embedded and frozen tissue blocks for each case with the mirror image surfaces oriented by colored inks. The histology of every sample in the Bank is uniformly interpreted by a pathologist in hematoxylin and eosin (H&E)-stained sections from the face of the paraffin tissue block. This information is available in a computerized database along with relevant pathological and clinical information and was used as a guide for selection of specific paraffin and frozen blocks. Fifty two tumors were selected, spanning a wide range of estrogen and progesterone receptor levels, as determined by ligand binding assay. Within these tumors, 9 were ER–/PR– (ER < 3 fmol/mg total protein; PR < 10 fmol/mg), 10 were ER–/PR+ (ER < 3 fmol/mg; PR > 10 fmol/mg), 10 were ER+/PR– (ER > 3 fmol/mg; PR < 10 fmol/mg), and 23 were ER+/PR+ (ER > 3 fmol/mg, PR > 10 fmol/mg). These tumors also spanned a wide range of Nottingham grade for ER– (n = 19, grade ranging from 5 to 9, median 8) and ER+ (n = 33, grade ranging from 5 to 9, median 6) tumors. SCGB2A2 mRNA expression was assessed by RT-PCR on total RNA extracted from frozen tissue sections. Paraffin blocks corresponding to 32 out of these 52 tumors were sectioned and processed for immunohistochemical analysis of SCGB2A2 expression.

ZR-75 cells, ER+ breast cancer cells known to express SCGB2A2, were grown and treated with estradiol-17β 10^{-8} M in charcoal-stripped medium or with the antiestrogen ICI 182,780 (10^{-6} M) in regular medium for 6, 24, or 48 h, as previously described *(22)*. Total RNA was extracted from frozen tissue sections or cell lines using Tri-reagent (MRCI, Cincinnati, OH).

RT-PCR Analysis

One microgram of total RNA was reverse transcribed in a final volume of 20 μL and 1 μL of the reaction mixture subsequently amplified by PCR as previously described *(23, 24)*. Primers used corresponded to SCGB2A2 (sense 5'-CCGACAGCAGCAGCCTCAC-3', located in SCGB2A2 sequence between bases 41 and 59, and antisense 5'-TCCG TAGTTGGTTTCTCAC-3', located between bases 401 and 383) *(2)*; to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (sense 5'-ACCCACTCCTCCACCT TTG-3' and antisense 5'-CTCTTGTGCTCTTGCTGGG-3'); and to psoriasin *(24)* gene (sense 5'-AAGAAAGATGA GCAACAC-3' and antisense 5'-CCAGCAAGGACAGA AACT-3'). To amplify cDNA corresponding to SCGB2A2, GAPDH, and psoriasin, 30 cycles (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C) of PCR were used. Ten microliters of PCR products were loaded on prestained (15 μg/mL ethidium bromide) 2% agarose gels. Identity of fragments corresponding to SCGB2A2, GAPDH, and psoriasin had previously been confirmed by sequencing.

Three independent PCRs were performed using SCGB2A2, psoriasin, and GAPDH primers and signals, visualized with UV irradiation on a GelDoc2000/ChemiDoc System (Biorad), were quantified by densitometry using the Quantity One software (Version 4.2, Biorad). SCGB2A2 and psoriasin expression were expressed relative to GAPDH expression as previously described *(25)*. Briefly, three independent PCRs were performed using each set of primers. In order to control for variations between experiments, a value of 1 was arbitrarily assigned to the signal of one particular tumor measured in each set of PCR experiments (always the same tumor) and all signals were expressed relative to this signal. Levels of SCGB2A2 were then expressed relative to the GAPDH signal corresponding to each individual tumor sample. Correlation between normalized SCGB2A2 expression and tumor characteristics was tested by calculation of the Spearman coefficient, r. Comparison between tumor subgroups was performed using the Mann–Whitney rank sum test, two-sided.

Immunohistochemical Analysis of SCGB2A2 Expression

Detection of SCGB2A2 protein was performed using an antibody previously characterized and kindly provided by Dr. Timothy Fleming *(1,2,5,18)*. Paraffin-embedded breast tissue sections were processed using the automated Discovery Staining Module, Ventana System (Tucson, Arizona) and the Research IHC DAB paraffin protocol according to the manufacturer's instructions. All steps were performed automatically: briefly, following deparaffination of tissue

sections, slides were incubated 60 min at 42°C in the presence of rabbit anti-SCGB2A2 antibody (1/1000 final concentration), washed, incubated with biotinylated secondary anti-rabbit antibody (14 minutes 42°C), washed, incubated 8 min with avidin–HRPO complex subsequently detected with DAB-H_2O_2 solution. Counterstaining was also performed automatically by the Ventana apparatus (hematoxylin/bluing reagent).

Levels of mammaglobin expression were assessed by bright-field microscopic examination at low-power magnification and using a previously described semiquantitative approach *(25)*. Scores were obtained by estimating average signal intensity (on a scale of 0 to 3) and the proportion of epithelial cells showing a positive signal (0, none; 0.1, less than one-tenth; 0.5, less than one-half; 1.0 greater than one-half). The intensity and proportion scores were then multiplied to give an overall score. Cases with a score lower than or equal to 1 were considered negative or weakly positive, whereas tumors with scores higher than 1.0 were classified as positive for SCGB2A2 expression. Statistical comparisons between tumor subgroups have been performed using the chi-square test.

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