Short Communication

Stage-specific Expression of Breast Cancer-specific Gene γ-Synuclein[1]

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Abstract

γ-Synuclein (SNCG), also referred as breast cancer-specific gene 1, is the third member of a neuronal protein family synuclein. SNCG is highly expressed in human-infiltrating breast carcinomas but not expressed in normal or benign breast tissues. To evaluate the clinical relevance of SNCG expression in breast cancer progression and its correlation with clinical parameters, we analyzed SNCG expression in 79 clinical breast specimens from primary breast cancer, hyperplasia, and fibroadenoma patients by reverse transcription-PCR. The status of estrogen receptor, progesterone receptor, proliferating cell nuclear antigen, and C-erBb2 was also analyzed by immunohistochemistry. Overall SNCG mRNA expression was detectable in 38.8% of breast cancers. However, 79% of stage III/IV breast cancers were positive for SNCG expression, whereas only 15% of stage I/II breast cancers were positive for SNCG expression. In contrast, the expression of SNCG was undetectable in all benign breast lesions. The expression of SNCG was strongly correlated to the stage of breast cancer ($P = 0.000$). This study suggests that the expression of SNCG is stage specific for breast cancer. SNCG is expected to be a useful marker for breast cancer progression and a potential target for breast cancer treatment.

Introduction

Using the differential cDNA sequencing (1–3), we undertook a search for isolation of differentially expressed genes in the cDNA libraries from normal breast and breast carcinoma. Of many putative differentially expressed genes, a breast cancer-specific gene, BCSG1,[3] was identified as a putative breast cancer marker (1). This gene was highly expressed in the advanced breast cancers cDNA library but scarce in a normal breast cDNA library. BCSG1 is not homologous to any other known growth factors or oncogenes. Rather, there is extensive sequence homology to the neural protein synuclein. Subsequent to the isolation of BCSG1, SNCG (4) and persyn (5) were independently cloned from a brain genomic library and a brain cDNA library. The sequences of these two brain proteins were found to be identical to BCSG1. Thus, BCSG1 is now also named as SNCG or persyn and is considered to be the third member of the synuclein family (6).

Synucleins are a family of small proteins consisting of three known members, SNCA, SNCB, and SNCG. The previously identified BCSG1 shares 54 and 56% amino acid sequence identity with SNCA and SNCB, respectively. The NH_2-terminal halves of SNCA, SNCB, and SNCG are highly conserved. However, although the residues near the COOH terminus of SNCA are similar to those of SNCB, those of SNCG diverge greatly from the SNCA counterpart (7). Although they are homologous, each synuclein is encoded by a different gene on chromosomes 4q21.3-q22 (SNCA), 5q35 (SNCB), and 10q23 (SNCG; Ref. 8). Synucleins are predominantly present in brain and thought to be involved in neuronal plasticity and the formation of depositions in brain tissues (6). Synucleins has been specifically implicated in neurodegenerative diseases such as AD and PD. Mutations in SNCA is genetically linked to several independent familial cases of PD (9). More importantly, wild type of SNCA is the major component of Lewy bodies in sporadic PD and in a subtype of AD known as Lewy body variant AD (10, 11). SNCA peptide known as nonamyloid component of plaques has been implicated in amyloidogenesis in AD (12, 13). SNCB and SNCG have also been recognized to play a role in the pathogenesis of PD and Lewy bodies cases (14, 15).

Although synucleins have been specifically implicated in neurodegenerative diseases and serve as a hallmark for AD and PD, studies indicate a potential role of synucleins, particularly SNCG, in the pathogenesis of steroid-responsive tumors of breast and ovary (1, 4, 16–20). Identified as a breast cancer-specific gene, we first demonstrated by *in situ* hybridization that SNCG was undetectable in normal or benign breast lesions, partially expressed in DCIS, and was expressed at a high level in advanced infiltrating breast cancer (1). Others have also demonstrated the similar findings in breast and ovary cancer (4, 18). In this study, we used RT-PCR analysis to detect SNCG expression in the clinic samples of breast cancer and its correlation with clinical parameters such as tumor size, stage, status of ER, PR, PCNA, and C-erBb2.

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[3] The abbreviations used are: BCSG1, breast cancer specific gene 1; AD, Alzheimer's disease; ER, estrogen receptor; PD, Parkinson's disease; PR, progesterone receptor; SNCA, α synuclein; SNCB, β synuclein; SNCG, γ synuclein; RT-PCR, reverse transcription-PCR; PCNA, proliferating cell nuclear antigen; DCIS, ductal carcinoma *in situ*.

Materials and Methods

Patients and Tissue Specimens. Breast tissue specimens were obtained from 79 Chinese women accepting operation in Department of Surgery, XinHua Hospital affiliated with Shanghai Second Medical University from September 1999 to July 2002. These patients were randomly selected, including 67 primary breast cancers, 7 breast hyperplasias, and 5 fibroadenomas. The mean age of 67 breast cancer patients was 58.2 years old; among them, 47 cases were older than 50 and 45 cases from the women with menopause. There were 64 cases with tumor size $\geq$ 2 cm and only 3 cases with tumor size $<$ 2 cm. According to the WHO histological classification, 67 breast cancers were composed of 55 infiltrating ductal carcinomas, 4 medullary carcinomas, 3 mucous carcinomas, 2 infiltrating lobular carcinomas, 2 DCISs, and 1 papillary carcinoma. The tumors were staged based on Union International Contre Cancer Tumor-Node-Metastasis classification, including 2 DCISs, 41 stage I/II, and 24 stage III/IV. The issues samples were quickly frozen after surgical removal and stored in liquid nitrogen.

RNA Isolation. Tissue samples were homogenized in 1 ml of Trizol Reagent (Life Technologies, Inc.; 1 ml/35–45 mg of tissue). The homogenized samples were incubated for 5 min at room temperature. After addition of 0.2 ml of chloroform, the samples were vigorously shaken for 15 s and incubated at room temperature for another 3 min. The samples were then centrifuged at 13,500 rpm for 15 min at 4°C. After the centrifugation, the aqueous phase containing total RNA was transferred to a fresh tube, and the same volume of isopropyl alcohol was added to the tube. The samples were incubated for 10 min at room temperature, and the RNA was precipitated by centrifugation. The RNA pellets were washed with 1 ml of 75% ethanol and dissolved in 40 μl of RNase-free water.

RT-PCR Analyses. RT-PCR analysis was performed by using a standard RT-PCR with the primers specific for human SNCG and β-actin. Three μg of total RNAs were used for reverse transcription reaction using oligo$(dT)_{15}$ primers. The samples were heated to 70°C for 10 min and then quickly chilled on ice. Then, 4 μl of 5$\times$ first strand buffer, 2 μl of 0.1 M DTT, 1 μl of 10 mM deoxynucleoside triphosphate, and 200 units of reverse transcriptase were added to the samples. The mixtures were incubated at 42°C for 60 min, and one-fifth of this reaction was amplified by PCR using PCR kit. Each reaction consisted of 30 cycles in the GeneAmp PCR System 2400 (Perkin-Elmer). The parameters for PCR were: denaturation at 95°C for 30 s; annealing at 55°C for 30 s; and elongation at 72°C for 60 s. One-third of the PCR products were electrophoresed through 1% agarose gel. After primers were synthesized and used for amplifying the corresponding genes: (*a*) human SNCG: 5′-ATGGATGTCTTCAAGAAGGG-3′ and 5′-CTAGTCTCCCCCACTCTGGG-3′; and (*b*) human β-actin: 5′-GCTGTGCTATCCCTGTACGC-3′ and 5′-TGCCTCAGGGCAGCGGAACC-3′.

Immunohistochemical Staining. As we previously described (21), deparaffinized, rehydrated, and acid-treated human breast sections (5-μm thick) were treated with H_2O_2, trypsin, and blocked with normal goat serum. Sections were incubated with specific mouse monoclonal antibodies against with ER, PR, C-erBb2, and PCNA (Antibody Diagnostica, Inc.) at 25°C for 30 min followed by the incubation with the biotin-conjugated secondary antirabbit antibodies (Dako). The colorimetric detection was performed by using a standard indirect streptavidin-biotin immunoreaction method by Dako's Universal LSAB kit according to the manufacturer's instructions. There were some variations in staining intensity for ER, PR, PCNA, and C-erbB2 expression among the specimens. The positive case was regarded as >20% of tumor cells with immunostaining. Eight fields were randomly selected in each slide were counted under a Nikon microscope at $\times$200 amplification. The negative cases were confirmed with two independent experiments. All stainings were reviewed by two pathologists.

Statistical Analysis. The data were tested by χ^2 in SPSS10.0. $P < 0.05$ was consider to be significant.

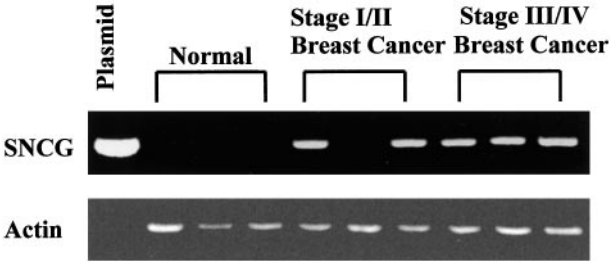

Fig. 1. Expression of SNCG in human breast tissue. Total RNA was isolated from frozen human breast specimens. RT-PCR analysis of SNCG expression was conducted using primers within SNCG-cording sequence as described in "Materials and Methods." The 384-bp PCR product is a specific indication of the presence of SNCG. The integrity and the loading control of the RNA samples were ascertained by actin expression with a set of primers for 314-bp β-actin. A representative RT-PCR analysis was shown here with 3 benign breast lesions, 3 stage I/II breast cancer cases, and 3 stage III/IV breast cancer cases. The SNCG cDNA plasmid was used as a positive control.

Table 1 Stage-specific expression of SNCG on breast cancer[a]

Stage	Benign (n = 12)	Stage I/II (n = 41)	Stage III/IV (n = 24)
Expression	0 (0%)	6 (15%)	19 (79%)

[a] Expression of SNCG in total 79 clinical breast specimens were investigated by four RT-PCR analyses. For each round of reaction, we used SNCG cDNA plasmid as a template for the positive control. The integrity and the loading control of the RNA samples were ascertained by actin expression. Negative cases were confirmed with at least two independent experiments. These patients were randomly selected with the mean age of 58.2 years old, including 67 breast cancers, 7 breast hyperplasias, and 5 fibroadenomas.

Results

Expression of SNCG in Breast Tissue. Previously, our *in situ* hybridization analysis has demonstrated an association between SNCG expression and breast cancer progression (1). To further evaluate the clinical relevance of SNCG expression to breast cancer progression in a large set of clinical specimens, we performed a semiquantitative RT-PCR analysis on 79 clinical breast specimens, including 12 normal or benign lesions, 2 DCISs, 41 stage I/II breast cancers, and 24 stage III/IV breast cancers. As shown in Fig. 1 and summarized in Table 1, although no SNCG mRNA was detectable in 12 benign breast specimens, SNCG was expressed in most of advanced infiltrating breast cancers. The expression of SNCG mRNA was detectable in 26 of 67 breast cancer cases (38.8%). Among the 26 of SNCG-positive cases, 19 cases were detected in the cancer with stage III/IV, only 6 cases were detected in the cancer with stage I/II, 1 case was detected in DCIS. There was no detectable SNCG mRNA in 3 cases with tumor size $<$ 2 cm. Therefore, SNCG expression is stage specific for breast cancer with no detectable expression in benign lesion, 15% (6 of 41) expres-

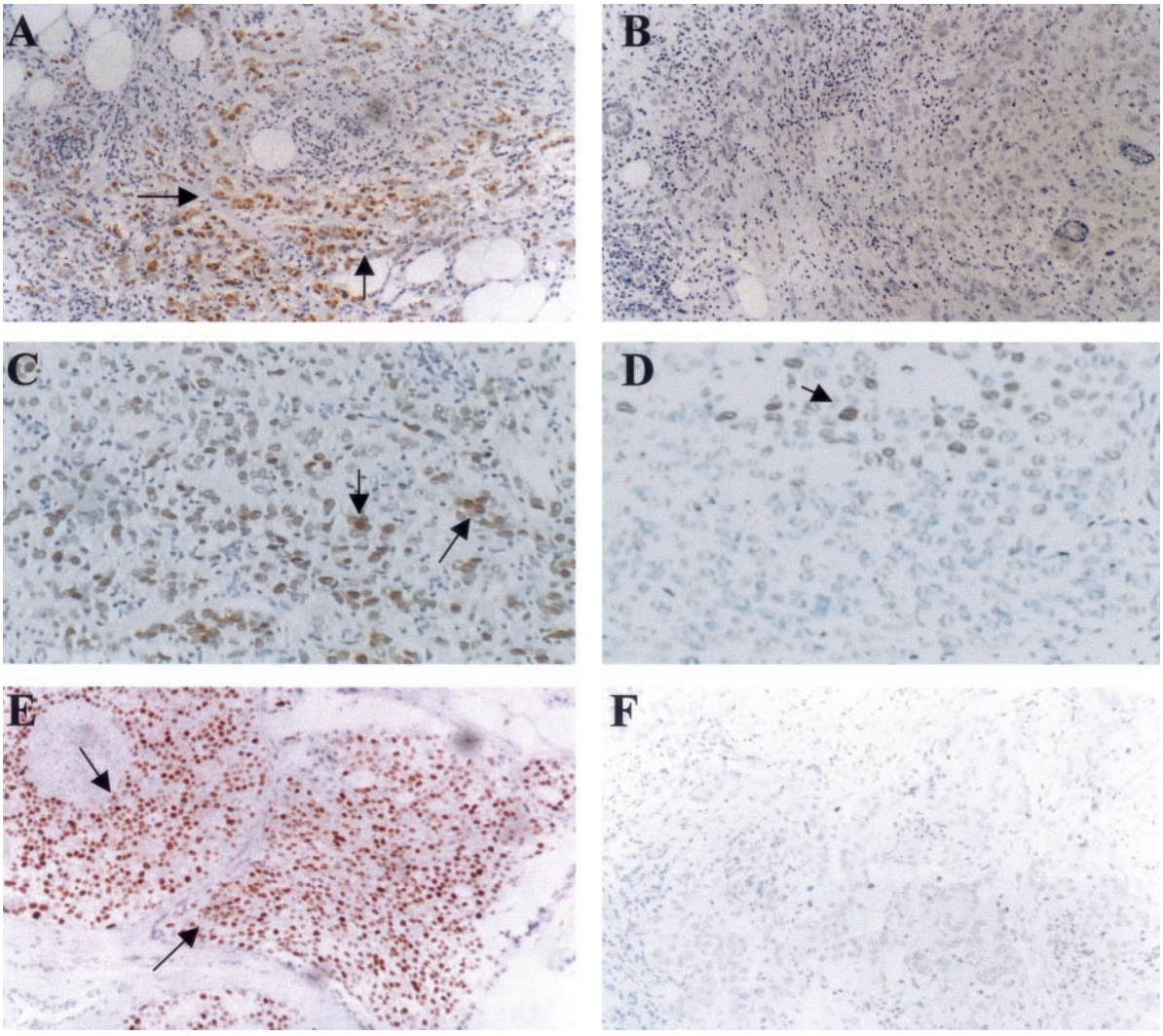

Fig. 2. Immunohistochemical analysis of ER, PR, and PCNA. Sections in *A–F* were stained immunohistochemically with specific antibodies against ER (*A* and *B*), PR (*C* and *D*), and PCNA (*E* and *F*), with *brown*, indicating ER, PR, and PCNA protein expression in mammary tumor cells. All sections were also counterstained lightly with hematoxylin for viewing nonstained negative cells. The tissue samples were classified into either positive or negative staining. The ER, PR, and PCNA positive sections were defined as >20% of the cells showing positive staining under a light microscope at 200-fold magnification. *A*, strong nuclear ER staining in almost all infiltrated breast cancer cells. *Arrows* indicate the positive ER cells. *B*, negative staining for ER. *C*, strong nuclear PR staining in >60% of cancer cells. *Arrows* indicate PR-positive cells. *D*, negative staining for PR (<10% of cancer cells were PR positive). An *arrow* indicates the PR-positive cells. *E*, strong PCNA staining in the highly proliferative cancer cells. *F*, negative staining for PCNA. For every sample, a serial slide from the same block was also incubated with nonimmunized control IgG, and no significant detectable background staining was observed at the same conditions for each specific antibodies (data not shown).

sion in stage I/II breast carcinomas, and 79% (19 of 24) expression in stage III/IV breast carcinomas.

Status of ER, PR, and PCNA in Breast Cancer. In an attempt to evaluate the potential association between SNCG expression and other well-established clinical markers in breast cancer, we studied ER, PR, and PCNA nuclear protein expression in 67 formalin-fixed and paraffin-embedded tissue sections from the same clinical specimens used for RT-PCR analysis. In these experiments, we examined two aspects of ER, PR, and PCNA expression, including their correlation with the stage of breast cancer and the association with SNCG expression. Fig. 2 shows representative immunohistochemical staining for ER, PR, and PCNA. Expression of ER, PR, and PCNA were heterogeneous in these carcinomas. Because the colorimetric immunostaining is not quantitative, the tissue samples were clas-

Table 2 Analysis of ER, PR, PCNA and C-erbB2 protein expression on human breast tissues[a]

Stage	ER (%)	PR (%)	PCNA (%)	C-erbB2 (%)
Stage I/II (n = 41)	16 (39)	9 (22)	39 (95)	17 (41)
Stage III/IV (n = 24)	9 (37)	5 (21)	23 (96)	9 (38)

[a] Total 67 breast cancer specimens, including 41 cases of stage I/II breast cancer and 24 cases of stage III/IV breast cancer, were analyzed by immunohistochemical staining using specific antibodies against ER, PCNA, and C-erbB2. There was a wide variation in staining intensity for ER, PCNA, and C-erbB2 expression among the examined breast cancer specimens. Because of the nonquantitative nature, no attempt was made to address the levels of expression among different samples. Breast cancer samples were classified into either positive or negative staining for ER, PCNA, and C-erbB2 with positive staining representing the sections with >20% of the counted cells stained positive for the specific antigen. The negative cases were confirmed by two independent experiments. All stainings were reviewed by at least two pathologists.

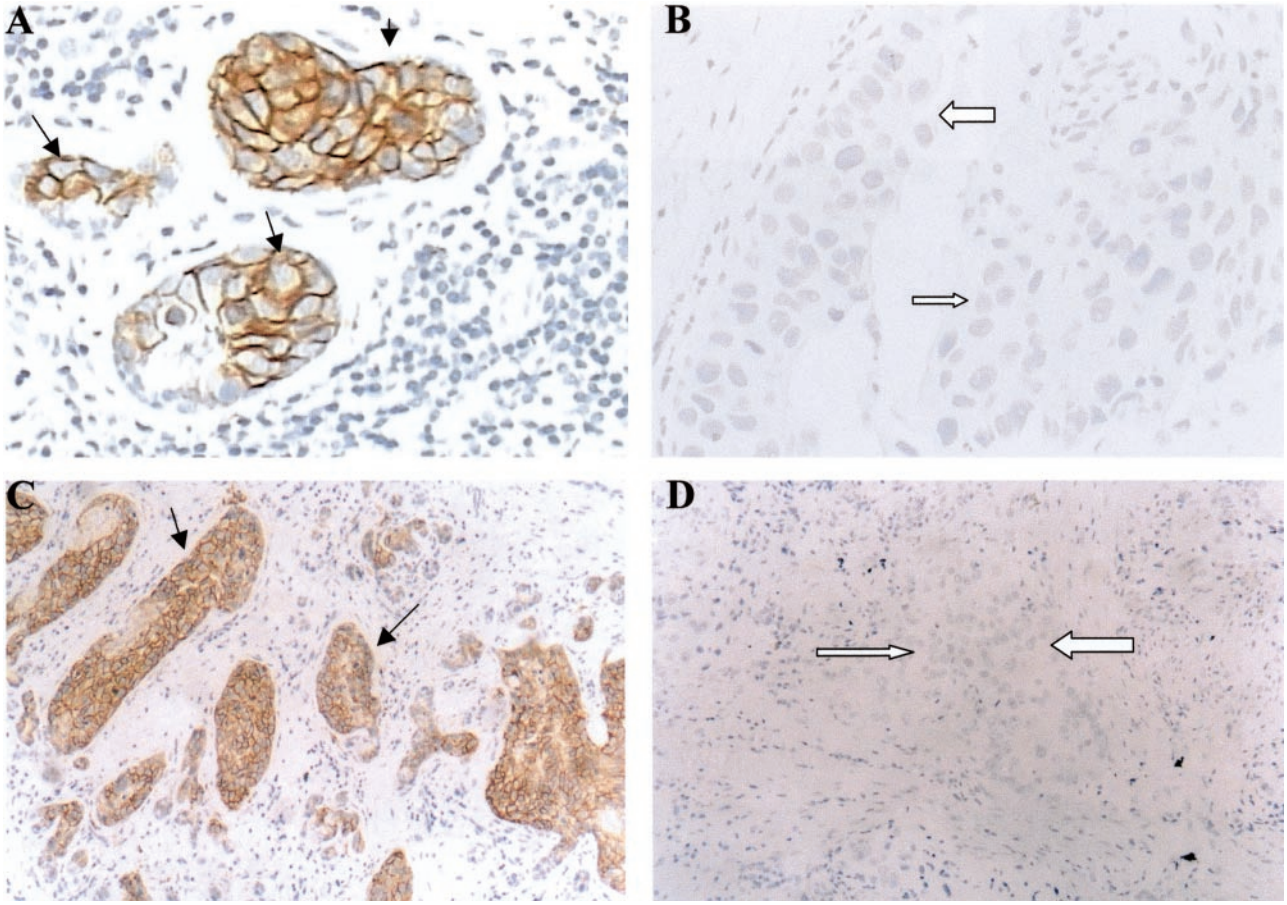

Fig. 3. Immunohistochemical analysis of C-erbB2. Sections were stained immunohistochemically with specific antibodies against C-erbB2 with *brown* indicating C-erbB2 protein expression in tumor cell membrane. All sections were also counterstained lightly with hematoxylin for viewing nonstained negative cells. *A*, higher magnification showing (×20) positive C-erbB2 membrane staining (*arrows*). *B*, higher magnification showing (×20) negative C-erbB2 membrane staining (*open arrows*). *C*, lower magnification showing (×10) positive C-erbB2 membrane staining (arrows). *D*, lower magnification showing (×10) negative C-erbB2 membrane staining (*open arrows*). Sections with C-erbB2-positive staining were defined as >20% of counted cells showing positive staining.

sified into either positive or negative staining; no attempt was made to differentiate the levels of expression of ER, PR, and PCNA among positive staining specimens. Immunoreactivity was scored as positive when >20% of the examined cells showing the positive staining. As summarized in Table 2, we found that the status of these markers was clearly not related with the stage of breast cancer. PCNA, which shows the proliferative ability of cell, was expressed almost in all breast cancer cases with 95% expression in stage I/II breast cancer cases and 96% expression in stage III/IV breast cancer cases. Expression of ER was detected 39% in stage I/II breast cancer cases and 37% in stage III/IV breast cancer cases. Expression of PR was also detected both in state I/II breast cancer and stage III/IV breast cancer cases with the similar percentages. There were no significantly different expression percentages of ER, PR, and PCNA in stage I/II *versus* stage III/IV breast cancer. Therefore, unlike SNCG, the expression of ER, PR, and PCNA appeared having no correlation to the stages of breast cancer.

Status of C-erbB2 in Breast Cancer. We also analyzed C-erbB2 expression in 67 breast cancer specimens by immunohistochemical staining. Because of the expression variation and different staining intensities, a positive C-erbB2 immunostaining was defined as >20% of tumor cells showing immunoreactivity to C-erbB2 antibody. Expression of C-erbB2 was not significantly different in stage I/II and stage III/IV breast cancers. As shown in Fig. 3 and Table 2, C-erbB2 expression was detectable in 41% of stage I/II breast cancer cases and 38% of stage III/IV breast cancer cases; 1 of the 2 DCISs was also positive for C-erbB2 expression. These data indicate that C-erbB2 expression was not associated with the stage of breast cancer.

Correlation between the SNCG Gene Expression and Clinical Parameters. To further investigating the correlation between the SNCG gene expression and clinical parameters, we analysis the data by χ^2 test (Table 3). It showed that the expression of SNCG was strongly correlated to the stage of breast cancer ($P = 0.000$). However, there was no significant correlation between SNCG gene expression and the age, menstruation, and tumor expression status of PCNA and C-erbB2. As to the tumor expression of ER and PR, there were more SNCG-positive cases in the tumors expressing both ER and PR compared with the tumors with negative ER and PR expression. Although 54% of ER- and PR-positive tumors expressed SNCG, only 33% of ER- and PR-negative tumors expressed SNCG. However, the difference was not significant because of the limited numbers of case analyzed.

Table 3 Correlation of SNCG gene expression with clinical parameters

	Case	SNCG expression		Positive rate %	*P*
		+	−		
Age (yr)					
<50	20	6	14	30.0	
≥50	47	20	27	42.6	.417
Menopause					
Yes	45	17	28	37.8	
No	22	9	13	40.9	1.000
Size					
<2 cm	3	0	3	0	
≥2 cm	64	26	38	40.6	—
Stage					
DCIS, I/II	43	7	36	16.3	
III/IV	24	19	5	79.2	.000
ER and PR status					
ER+, PR+	13	7	6	53.8	
ER+, PR−	12	5	7	41.6	
ER−, PR−	42	14	28	33.3	.207
PCNA					
+	63	24	39	38.1	
−	4	2	2	50.0	.638
C-erbB_2					
+	48	19	29	39.6	
−	19	7	12	36.8	1.000

Discussion

Breast cancer development and progression is accompanied by multiple genetic changes that lead to qualitative and quantitative alternations in individual gene expression. Consequently, the altered levels of these gene products and their cellular functions will disturb the normal physiological homeostasis of the cells and result in cancer formation. Identification of genes that are overexpressed or underexpressed in tumors and subsequent evaluation of their biological functions will help to understand the process of malignant transformation. We have used a differential cDNA-sequencing approach to isolate differentially expressed genes in the cDNA libraries from normal breast and breast carcinoma. Of many putative differentially expressed genes, a breast cancer-specific gene, BCSG1, also named as SNCG, which was highly expressed in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified (1). Identified as a breast cancer specific gene, SNCG mRNA was detected in neoplastic breast epithelial cells but not in normal mammary epithelial cells (1). Northern blot analysis detected a 1-kb transcript corresponding to SNCG mRNA in 2 of 4 human breast cancer cell lines derived from pleural effusions and 4 of 4 breast cancer cell lines derived from ductal-infiltrating carcinomas (1). *In situ* hybridization analysis has demonstrated a specific SNCG expression pattern varying from virtually no detectable expression in normal or benign breast tissue to low level and partial expression in low-grade breast carcinoma *in situ* to high expression in advanced infiltrating carcinomas (1). The association between SNCG expression and the progression of breast cancer led us to investigate the expression pattern of SNCG in relatively large-scale clinical breast specimens. Here, we reported a stage-specific SNCG expression in breast cancer based on RT-PCR analysis of SNCG expression in 79 human mammary tissues, including 67 breast cancers, 7 breast hyperplasias, and 5 fibroadenomas. Although the expression of SNCG was undetectable in all 12 benign breast lesions, 15% (6 of 41) of stage I/II breast cancer expressed SNCG, and 79% (19 of 24) of stage III/IV breast cancer expressed SNCG. The false-positive rate of SNCG is 0.

Previously, Western analysis of SNCG protein expression in human breast tissues showed a similar pattern in that it was not detected in normal breast tissues and stage I/II ductal breast carcinomas but was detected in 70% of stage III/IV ductal breast carcinomas (18). Ninkina *et al.* (5) were also able to confirm by using Northern and Western blotting that some breast tumors and breast tumor cell lines expressed SNCG, whereas normal breast tissue did not. Therefore, the stage-specific SNCG expression in breast tissue has been demonstrated, thus far, in three different assays: *in situ* hybridization analysis (1); Western analysis (18); and RT-PCR analysis of 79 clinical specimens shown here.

Many predictive and prognostic factors have been proposed and studied for breast cancer. Among them, the most widely used in clinical assessment for breast cancer are ER, PR, PCNA, and C-erbB2. Although expression of SNCG was highly associated with the stage of breast cancer, the status of ER, PR, PCNA, and C-erbB2 were not associated with the stage of breast cancer. It will be interesting to investigate whether SNCG expression in stage I/II cancers may indicate a high risk of malignant progression to more advanced state III/IV tumors. There is cause for concern about the high proportion of breast cancer cases treated with unnecessary mastectomy (22). If SNCG expression can provide some prognostic information on distinguishing the benign lesions or tumors in early stage that are not likely to become invasive from those that are most likely to become invasive, this will help to direct the treatment strategies and to reduce some inappropriate or unnecessary treatments.

In addition to the link between SNCG and breast cancer progression, it has also been found that synucleins, especially SNCG and SNCB, are involved in ovarian cancer. After our identification of SNCG, Lavedan *et al.* (4) first suggested that SNCG may be abnormally expressed in ovarian tumors as well as in breast tumors based on the discovery of some SNCG expressed sequence tags in the libraries derived from an ovarian tumor. This suggestion was additionally confirmed by Western and immunohistochemical analyses (18). Although synuclein (α, β, and γ) expression was not detectable by immunohistochemistry in normal ovarian epithelium, 87% (39 of 45) of ovarian carcinomas were found to express at least one type of synuclein, and 42% (19 of 45) expressed all three synucleins (α, β, and γ) simultaneously. Highly punctate SNCG expression was also observed in 20% of preneoplastic lesions of the ovary, including epithelial inclusion cysts, hyperplastic epithelium, and papillary structures, suggesting that SNCG up-regulation may occur early in the development of some ovarian tumors (18).

To elucidate the molecular mechanisms that convert SNCG from a silent gene in normal breast tissue to an actively transcribed gene in breast tumor and to identify upstream regulators of SNCG transcription in neoplastic mammary epithelial cells, we recently isolated a 2195-bp fragment of a human SNCG gene (19). This fragment includes 1 kb of 5′-flanking region, exon 1, and intron 1. By analyzing the promoter activity and the methylation status, we showed that exon 1 region of SNCG gene contains a CpG island that is unmethylated in SNCG-positive SKBR-3 and T47D cells but densely methylated in SNCG-negative MCF-7 cells. Treating MCF-7 cells with a demethylating agent 5-Aza-2′-deoxycytidine specifically activated SNCG transcription. Thus, our results suggest that aberrant expression of SNCG in breast carcinomas occurs at transcriptional activation by demethylation of exon 1.

The high-level expression of SNCG in the malignant-

infiltrating breast epithelial cells suggests that SNCG may play a positive role in breast cancer progression. What role SNCG has in breast and ovary and how it is implicated in breast and ovary cancer remains a mystery. On the basis of our data and others, SNCG has two major functions on the development and progression of mammary tumor: (*a*) stimulation of cell motility and metastasis; and (*b*) protection of tumor cells from apoptosis. We have demonstrated that overexpression of SNCG in MDA-MB 435 breast cancer cells led to a significant increase in motility and invasiveness in cell culture and a profound augmentation of metastasis in nude mice (16). The effect of SNCG on apoptosis in response to several chemotherapy drugs was investigated in ovarian cancer cells (20). SNCG-expressing cells are significantly more resistant to the chemotherapeutic drugs as compared with the parental cells. Consistent with its antiapoptotic effect, overexpression of SNCG leads to constitutive activation of extracellular signal-regulated protein kinases 1 and 2 and down-regulation of c-Jun NH_2-terminal kinase 1 in response to a host of environmental stress signals, including UV, arsenate, and heat shock. Taken together, these data indicate that oncogenic aberrant SNCG expression contributes to the development of breast and ovarian cancer by promoting tumor cell motility and invasion and tumor cell survival.

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