

Inhibition of synuclein- γ expression increases the sensitivity of breast cancer cells to paclitaxel treatment

YUE ZHOU, SATORU INABA and JINGWEN LIU

Department of Veterans Affairs Palo Alto Health Care System, Palo Alto, CA, USA

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Abstract. Anti-microtubule drugs that cause mitotic arrest and subsequent apoptosis of cancer cells are frequently used to treat breast cancer patients with advanced or metastatic diseases. However, patient response rates to this class of chemotherapeutic agents vary significantly. Identification of cellular and genetic factors that are associated with the sensitivity to anti-microtubule drug treatment would have great clinical implications. Our previous studies have demonstrated that the neuronal protein, synuclein- γ (SNCG), plays oncogenic roles in breast carcinogenesis and is abnormally expressed at high levels in advanced and metastatic breast carcinomas but not expressed in normal or benign breast tissues. In this study, we show that responses of 12 breast cancer cell lines to paclitaxel-induced mitotic arrest and cytotoxicity highly correlate with SNCG expression status. SNCG-positive cells exhibit a significant higher resistance to paclitaxel-induced mitotic arrest than SNCG-negative cells ($p < 0.01$). Moreover, we demonstrate that down-regulation of SNCG expression directly increased the effectiveness of anti-microtubule drug-induced cytotoxicity in breast cancer cells without altering cell responses to doxorubicin. These new findings suggest that SNCG expression in breast carcinomas is probably a causal factor contributing to the poor patient response to paclitaxel treatment.

Introduction

Breast cancer (BC) is the leading cause of cancer-related deaths in women of the Western world. Many studies have demonstrated that the development and progression of this

disease involve abnormality of multiple genes through genetic and epigenetic alterations. By conducting differential DNA sequencing and *in situ* hybridization, the aberrant expression of synuclein- γ (SNCG) (1), also referred to as breast cancer specific gene 1 (BCSG1) (2), has been linked to the disease progression of BC. SNCG mRNA and protein are not expressed in normal breast tissue or tissues with benign breast diseases but abundantly expressed in a high percentage of invasive and metastatic breast carcinomas (2-4). A series of *in vitro* and *in vivo* functional studies performed in our laboratory and others have demonstrated that SNCG expression significantly stimulates proliferation (5-8), invasion, and metastasis of BC cells (9).

Our previous studies conducted through a yeast two-hybrid screening and co-immunoprecipitation revealed a specific interaction of SNCG with the mitotic checkpoint kinase, BubR1, in BC cells (10). BubR1 is an essential component of the mammalian checkpoint machinery that monitors the proper assembly of the mitotic spindle to ensure the accurate segregation of chromosomes by preventing cells with unaligned chromosomes from exiting the mitotic phase (11-14). By performing several different lines of functional studies, we have demonstrated that SNCG expression in BC cells induces chromosomal instability during normal cell cycle, overrides the mitotic checkpoint control upon spindle damage, and confers the cellular resistance to a microtubule inhibitor nocodazole-caused apoptosis (10). We further showed that inhibitory effects of SNCG on mitotic checkpoint could be overthrown by enforced overexpression of BubR1 in SNCG-expressing cells (15).

Chemotherapy has been widely used in BC post-surgery treatment. According to their working mechanisms, chemodrugs can be divided into several categories; one includes microtubule inhibitors such as paclitaxel (Taxol) (16), vincristine (17), and JIMB01 (18). These microtubule-disrupting agents are thought to arrest cells in mitosis by triggering mitotic checkpoint activation, resulting cells arrested in mitotic phase without entering anaphase (19). Prolonged treatments with these agents lead to cell death by apoptosis (20). Since the working mechanism of anti-microtubule drugs heavily relies on the normal function of the mitotic checkpoint machinery in which BubR1 is a critical component (20), the inhibitory effect of SNCG on BubR1 function may explain the induced resistance of BC cells to Taxol after exogenous expression of SNCG (21). Currently, anti-microtubule drugs are used as first-line chemotherapeutic agents to treat patients with advanced

Correspondence to: Dr Jingwen Liu, VA Palo Alto Health Care System (154P), 3801 Miranda Avenue, Palo Alto, CA 94304, USA
E-mail: jingwen.liu@med.va.gov

Abbreviations: BC, breast cancer; BCSG1, breast cancer specific gene 1; FBS, fetal bovine serum; IP, immunoprecipitation; OM, oncostatin M; PI, propidium iodide; Taxol, paclitaxel; SNCG, synuclein- γ

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or metastatic BC (22,23). However, the response rates to this class of drugs vary significantly. SNCG expression status could influence patient responses to the drug treatment and may even affect the survival rate after surgery.

To evaluate the potential use of SNCG as a biomarker to predict the effectiveness of anti-microtubule chemotherapy in BC patients, we used various BC cell lines that are either SNCG-positive or SNCG-negative as an *in vitro* working model to extensively examine the correlation between SNCG expression and responses of cancer cells to anti-microtubule drug treatment. The inverse relationship between SNCG expression and sensitivity to Taxol treatment was further examined by manipulating the endogenous expression of SNCG.

Materials and methods

Reagents and antibody. Paclitaxel (Taxol), etoposide, and doxorubicin were purchased from Sigma Chemical Co. Mouse anti-Cdc2 (sc-54) mAb and goat anti-SNCG (sc-10698) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti- α actin monoclonal antibody was purchased from Chemicon (Temecula, CA).

Cells and culture conditions. BC cell lines, MCF-7, MDA-MB435, BT-20, MDA-MB231, T47D, and the normal mammary epithelial-derived cell line, MCF10A, were obtained from ATCC and cultured according to the supplier's instructions. BC cell lines H3922 and H3396 were grown in IMDM in the presence of 10% FBS (34). Establishment of SNCG stable transfected cell lines MCF7-SNCG and MDAMB435-SNCG as well as the corresponding control cell lines (MCF7-neo and MDAMB435-neo) have been previously described (9,15) and cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 300 μ g/ml of G418. The T47D-derived cell clone (T47D-As) that stably expresses the antisense mRNA of SNCG was cultured in RPMI medium supplemented with 10% FBS and 300 μ g/ml of G418 (6).

Determination of mitotic index. Cells grown on 13-mm round-glass coverslips inserted into wells of 24-well-culture plates were untreated or treated with an anti-microtubule drug at a 0.5- μ M concentration for the specific indicated times. Thereafter, cells were fixed with 100% methanol for 20 min at -20°C and then stained with 1 μ g/ml of DAPI. For each sample, 200-300 cells randomly chosen from 5 different views under a Nikon fluorescent microscope were scored for interphase, mitotic, or apoptotic based upon nuclei morphology.

Measurement of cell survival rate by MTT assay. Cell survival rates under drug treatments and EC₅₀s of different chemoreagents were determined by Cell Proliferation Kit I (MTT) obtained from Roche Applied Sciences (Indianapolis, IN). Cells were seeded in 96-well plates at a density of 5x10⁴ cells/well in 100 μ l medium supplemented with 10% FBS at 37°C and 5% CO₂. After 24 h, cells were incubated with fresh medium containing different concentrations of drugs for 24 h. At the end of drug treatment, 10 μ l of MTT labeling reagent per well was added to cells to reach a concentration of 0.5 mg/ml.

After a 4 h-reaction, 100 μ l of solubilization solution was added to each well and the plate was incubated at 37°C overnight. The sample spectrophotometrical absorbance was measured by a microplate reader at a wavelength of 550 nm. The reading at wavelength 690 nm as the background was subtracted from the reading of 550 nm. The reading of sample without drug treatment was defined as 100% survival and readings from drug-treated samples were plotted relative to that value. Triplicate wells were used in each culture condition and 3-5 separate assays were conducted for each drug. EC₅₀ was defined as the concentration of drug eliciting 50% cell killing.

Soft agar colony assays. Anchorage-independent growth was carried out in 24-well culture plates. The bottom layer consists of 375 μ l of 5% FBS and 0.5% agar in RPMI medium. The top layer consists of 375 μ l of 5% FBS, 0.33% agar, 2x10⁴ cells without or with Taxol at indicated concentrations. Cells were cultured in an atmosphere of 5% CO₂/95% air under saturating humidity at 37°C. After 12 days, the number of colonies was counted under a Nikon microscope at 200x amplification. Eight fields were randomly selected in each well using a Whipple glass ring with 10x10 grid and colonies at the sizes of >10 cells were counted.

Cell cycle analysis. MDAMB435-SNCG and MDAMB435-neo cells were first synchronized by a double thymidine block using a protocol for HeLa cells (35). Cells were grown in the presence of 2 mM thymidine for 20 h, washed with PBS, and grown in fresh medium for 8 h without thymidine. Thymidine was added to cells again at 2-mM concentration to block cells at the G1/S boundary. After another 20 h, cells were washed with PBS and cultured in fresh medium for 6 h before the addition of 0.1 μ M of Taxol to prevent spindle assembly. Cells were subsequently harvested by centrifugation at 6-h intervals for a total period of 48 h. Cells were fixed in 70% ethanol at 4°C for 2 h, washed with PBS and stained with 5 μ g/ml propidium iodide (PI) along with 50 μ g/ml RNaseA, and subjected to flow cytometry. The histogram of DNA distribution was modeled as a sum of G1, G2-M, and S phase by using ModFitLT software.

Immunoprecipitation (IP) and Cdc2 kinase assay. One mg of total cell lysate from each sample was incubated with 8 μ g of mouse anti-Cdc2 monoclonal antibody for 3 h at 4°C with slow rotation. Protein A sepharose (15 μ l of protein) was then added to the lysate and the mixture was diluted with 2X IP buffer and further incubated for 1 h at 4°C. After centrifugation, the IP complex was washed twice with 1X IP buffer (1% Triton X-100, 10 mM Tris, pH 7.4, 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA pH 8.0) containing protease inhibitor cocktail. Pellets were washed once with kinase buffer (10 mM Tris pH 7.4, 150 mM NaCl, 10 mM MgCl₂, and 0.5 mM DTT) and centrifuged. The pellets were incubated for 30 min with 30 μ l of the kinase buffer containing 15 μ M ATP, 5 μ Ci [³²P] γ ATP, and 8 μ g histone H1. The kinase reaction was terminated by addition of SDS sample buffer and boiling. The phosphorylated H1 was separated by SDS-PAGE and detected by exposing to a screen of PhosphoImager.

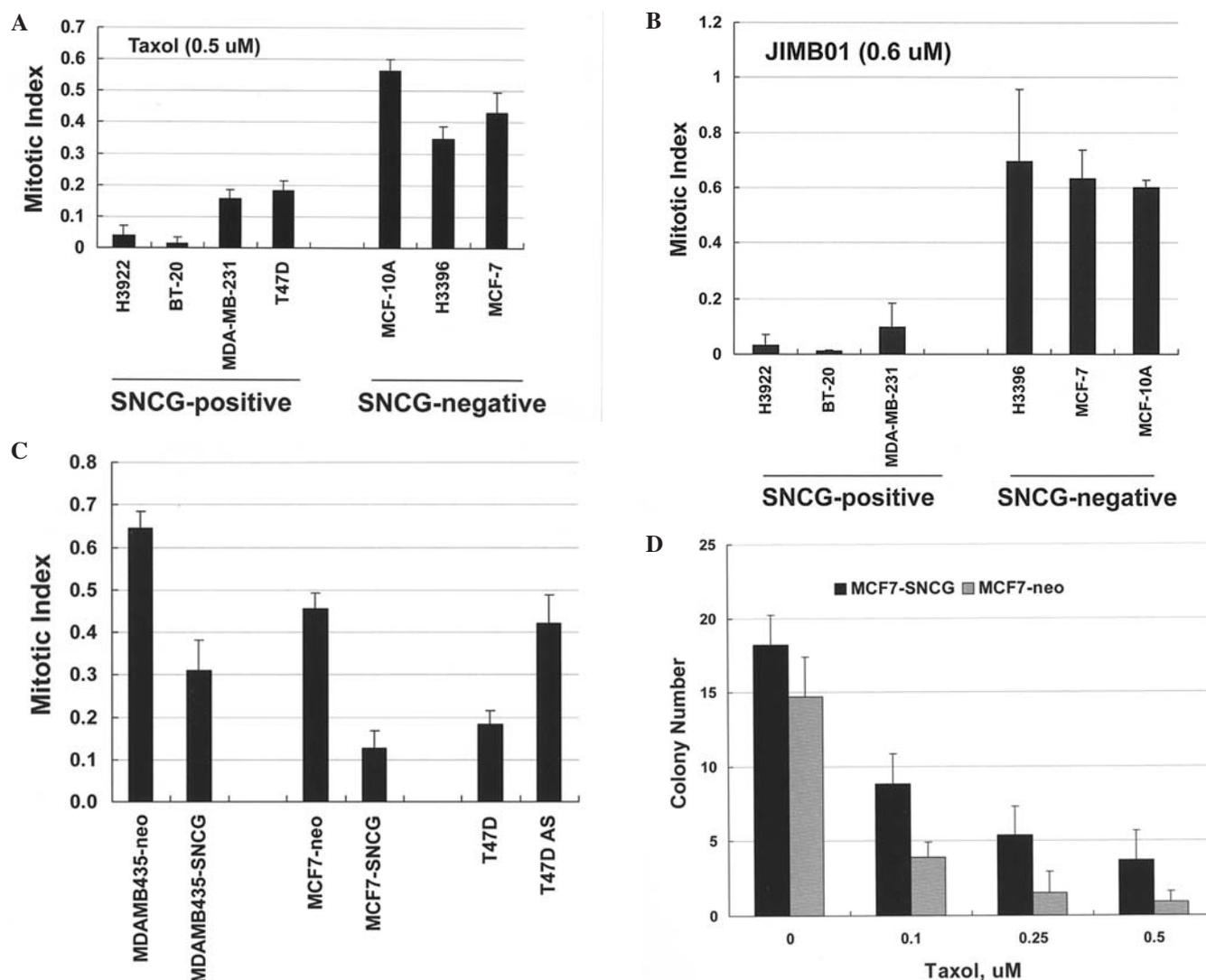


Figure 1. Association of SNCG expression with lower mitotic index. Different BC cell lines were cultured in cover slips inserted into wells of 24-well culture plates overnight. Cells were treated with 0.5 μ M Taxol for 18 h (A and C) or 0.6 μ M JIMB01 (B) for 20 h before fixing and staining with DAPI. For each cell line, 200-300 cells randomly chosen from 5 different views were scored for interphase or mitotic, based upon nuclear DNA morphology. The difference in mitotic indices between SNCG-positive and negative cell lines were also observed in cells treated with different concentrations of drugs and for different lengths of time. The p-value for different mitotic indices between SNCG-positive and negative cell lines treated with Taxol is 0.004 and is 0.007 for JIMB01 treatment. In D, cells at a density of 2×10^4 cells/well were plated in a 24-well plate in medium containing 5% FBS, 0.33% agar, and the indicated concentrations of Taxol. After 12 days, colonies were counted using a Whipple Glass Ring with a 10x10 grid. Numbers of colonies at sizes large than 10 cells were counted.

Results

Expression of SNCG in breast cancer cells confers resistance specifically to anti-microtubule agents. Our previous studies using SNCG stable transfected BC cell lines MCF7-SNCG and MDAMB435-SNCG have shown that exogenous expression of SNCG increased cell resistance to nocodazole-induced mitotic arrest and subsequent apoptotic cell death (10,15). To determine whether endogenous expression of SNCG in BC cells also affects cell responses to anti-microtubule drugs, we determined mitotic indices of 4 SNCG-positive BC cell lines, H3922, BT-20, MDA-MB-231, and T47D; 2 SNCG-negative BC cell lines, H3396, and MCF-7; and 1 SNCG-negative normal breast epithelium derived cell line, MCF-10A, under Taxol treatment. The SNCG expression status of these cell lines had been previously determined by RT-PCR and Western

blot analysis (10,24). Cells were treated with 0.5 μ M Taxol for 18 h, fixed, stained with DAPI, and mitotic arrested cells were counted. Fig. 1A shows that all SNCG-positive cells were more resistant to Taxol as compared to SNCG-negative cells. Under the same treatment condition, <20% of SNCG-expressing cells were arrested at the mitotic phase whereas cells that do not express SNCG protein were predominantly in the mitotic stages. The difference of mitotic index between SNCG-positive and -negative cell lines is highly significant ($p < 0.01$). Similar observations were made by treating cells with JIMB01, another inhibitor of microtubule assembly ($p < 0.01$, Fig. 1B).

We also compared responses to Taxol treatment in SNCG-stable transfected cells or in T47D-As in that the level of SNCG protein is markedly diminished by the stable expression of SNCG antisense mRNA (6). Fig. 1C shows

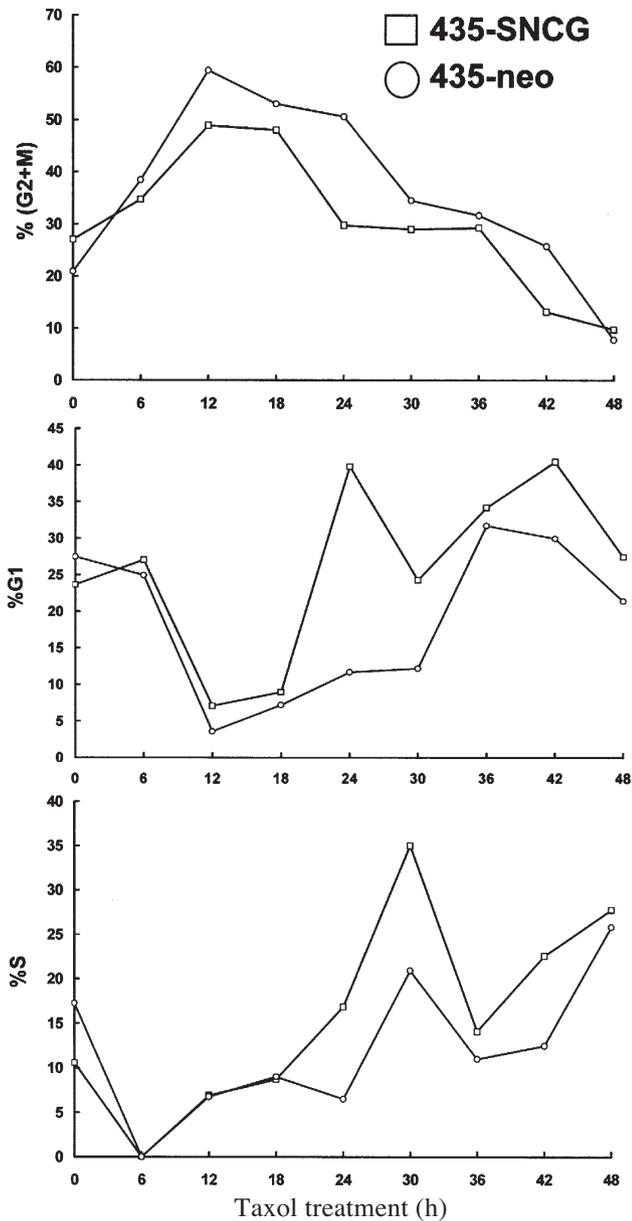


Figure 2. Cell cycle analysis of SNCG and neo clones. SNCG stably transfected clone of MDA-MB435 (435-SNCG) and the mock-transfected clone (435-neo) were synchronized by a double-thymidine block. After a 6-h release from G1/S, Taxol at 0.1 μM was added and cells were harvested at indicated time points for fixing and staining with PI. Percentages of cells in G1, S, and G2/M phases were plotted as a function of time. The data shown is representative of 3 different assays with consistent results.

that exogenous expression of SNCG in MCF-7 or MDAMB-435 cells markedly decreased the number of mitotic arrested cells as compared to their respective control neo clones. Conversely, knocking down the endogenous expression of SNCG in T47D cells increased the percentage of mitotic arrested cells. Soft agar colony assays further demonstrate that MCF7-SNCG cells were less sensitive to Taxol-induced cytotoxicity as compared to MCF7-neo cells (Fig. 1D). These results together clearly demonstrate that the normal mitotic checkpoint function which is required for cells to be arrested at the mitotic phase by anti-microtubule agents is generally impaired in SNCG expressing cells.

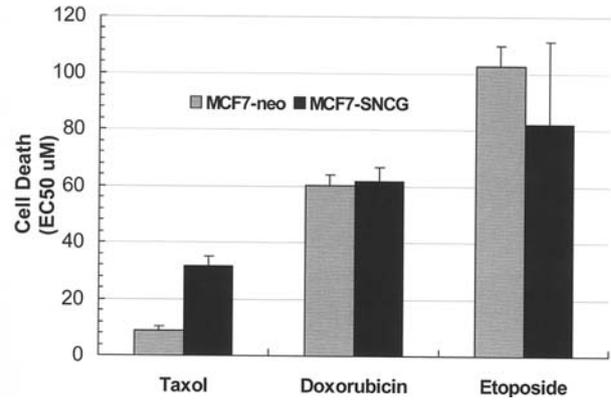


Figure 3. Overexpression of SNCG in MCF-7 cells increases the resistance to Taxol but not to doxorubicin or etoposide. SNCG stably transfected clone of MCF-7 (MCF7-SNCG) and the mock-transfected clone (MCF7-neo) were seeded in wells of 96-well plates with medium containing 10% FBS and different concentrations of Taxol, doxorubicin, or etoposide for 24 h. Cell survival rates were determined by MTT assay. EC₅₀ was defined as the concentration of drug eliciting 50% cell killing. Similar results were also obtained by trypan blue staining to count viable cells.

To further characterize different responses of SNCG-positive and negative cells to Taxol treatment, we performed cell cycle analysis. SNCG and neo clones were first synchronized at the G1/S boundary using a double thymidine block. Following 6-h release from G1/S by adding fresh growth medium, Taxol was added to prevent spindle assembly. Cells were harvested at 6-h intervals for FACS analysis to determine DNA content. Fig. 2 shows that for the first 18 h of Taxol treatment, both SNCG and neo cells progressed through S phase and entered G2 and mitosis with similar kinetics. However, after 18 h, the behaviors and fates of these two populations were dramatically different. The SNCG-negative cells (neo) continued to accumulate in mitosis for the next 6 h, and subsequently exited mitosis after ~24 h despite the presence of microtubule inhibitor. In contrast, SNCG-positive cells exited mitosis after 18 h of Taxol treatment; and by 24 h, 40% of SNCG cells were already in G1 phase as compared to 12% of neo cells. By 30 h, 35% of SNCG-expressing cells and only ~20% of neo cells were in S phase. These results indicate that SNCG expression impaired the ability of cells to stay in the mitotic phase and allowed cells to exit from Taxol-exerted mitotic arrest earlier than control cells.

To further demonstrate that SNCG-induced resistance is specific to microtubule-disrupting agents, MCF7-SNCG and MCF7-neo were treated with different concentrations of doxorubicin and etoposide along with Taxol. After a period of 24 h, cell survival rates were measured by MTT assay and EC₅₀ for each drug was determined. The results show that SNCG expression only increased the cellular resistance to Taxol, whereas effective doses for doxorubicin and etoposide that kill cells by DNA damaging were not affected (Fig. 3). The data shown represent results with identical trends in experiments to compare survival rates of additional SNCG-positive and -negative BC cell lines treated with doxorubicin, etoposide, and Taxol.

Down-regulation of SNCG increases cell sensitivity to JIMB01 treatment. Our previous study has shown that the

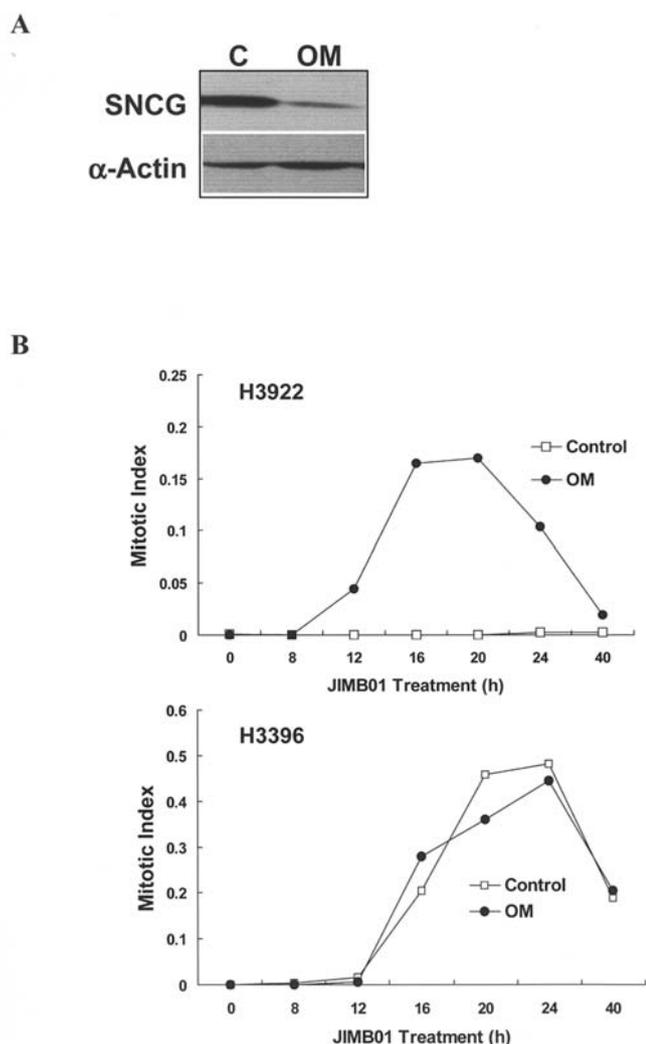


Figure 4. Down-regulation of SNCG expression by OM partially restores mitotic checkpoint function in H3922 cells. (A) Western blotting of SNCG. H3922 cells were untreated or treated with OM at a dose of 50 ng/ml for two days. Cell lysates were collected and were analyzed for SNCG protein expression by Western blotting. (B) H3922 cells and H3396 cells were treated with OM for 2 days. At the end of treatment, cells were trypsinized and reseeded in 24-well plates and treated with JIMB01 for the indicated times. Mitotic arrested cells were counted after fixing and DAPI staining.

BC cell line, H3922, expresses a high level of SNCG mRNA and protein and SNCG expression in these cells could be down-regulated by cytokine oncostatin M (OM) (5,24). To determine whether the inhibited expression of SNCG in these cells by OM correlates with increased sensitivity to an anti-microtubule agent, H3922 cells were untreated or treated with OM at a dose of 50 ng/ml for two days. At the end of OM treatment, a portion of cells was used to determine the SNCG expression level by Western blotting, and another portion was treated with JIMB01 for various time points for a total period of 40 h. Fig. 4A shows that the SNCG protein level was decreased by 70% after OM treatment. Subsequent treatment with JIMB01 at a 0.5- μ M concentration did not induce mitotic arrest in control cells but significantly increased the mitotic index of OM-treated cells (Fig. 4B, upper panel). Identical experiments were conducted in parallel using another BC cell line, H3396. H3396 cells express OM receptor (25) but do not express SNCG (10). JIMB01 treatment resulted in

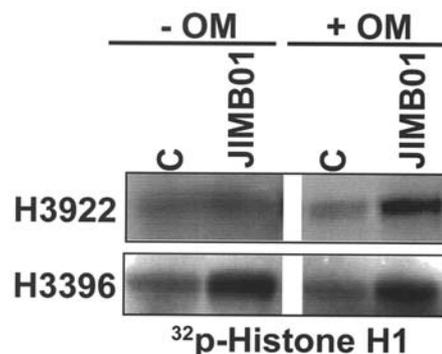


Figure 5. Examination of Cdc2 kinase activities in control and OM-treated cells. H3922 and H3396 cells without OM treatment or with 2 days of OM treatment were incubated with 0.5 μ M JIMB01 for 20 h. Cells were lysed simultaneously and 1 mg total lysate was used to measure Cdc2 kinase activity.

prominent mitotic arrest in H3396 cells regardless of OM treatment (Fig. 4B, lower panel).

It has been shown that when cells are exposed to microtubule inhibitors, the mitotic checkpoint activates, resulting in cell arrest in pro-metaphase with persisting Cdc2 kinase activity (26,27). To obtain additional evidence for SNCG-inhibited mitotic checkpoint function, the Cdc2 kinase activity on histone H1 phosphorylation in H3922 and H3396 cells without and with OM treatment was determined after exposing cells to JIMB01. Control or OM treated cells were incubated with 0.5 μ M JIMB01 for 20 h and cell lysates were harvested for IP with anti-Cdc2 antibody. Cdc2 kinase assay was conducted by incubation of the IP complex in kinase buffer with histone H1 in the presence of [α - 32 P]-ATP for 30 min at 37°C. SDS sample buffer was added to the reaction mixture and the phosphorylated histone H1 was detected after SDS-PAGE separation and exposure to a PhosphoImager. As shown in Fig. 5, JIMB01 did not activate Cdc2 kinase in SNCG-positive H3922 cells that were not treated with OM, but elicited a strong kinase activity after OM treatment. In contrast, JIMB01 treatment resulted in strong activation of Cdc2 kinase in SNCG-negative H3396 cells without or with OM treatment.

To further link SNCG expression with the specific response to anti-microtubule chemo-agent, the cell survival rates of H3922 cells without and with OM treatment under different drug exposures were compared. Fig. 6 shows that OM treatment did not alter cell responses to doxorubicin but significantly increased the effectiveness of JIMB01-induced cytotoxicity in H3922 cells.

Discussion

The exogenous expression of oncogenic protein, synuclein- γ , in breast cancer cells has been linked to stimulated proliferation, increased cell invasion and metastasis, and resistance to nocodazole-elicited apoptosis (5-8,10,15). One of the molecular mechanisms underlying the oncogenic functions of SNCG is its interaction with BubR1 and the consequential inhibition of BubR1-mediated mitotic checkpoint functions (10,15). The goals of the present study were to use a variety of breast cancer cell lines as *in vitro* working models to explore the clinical

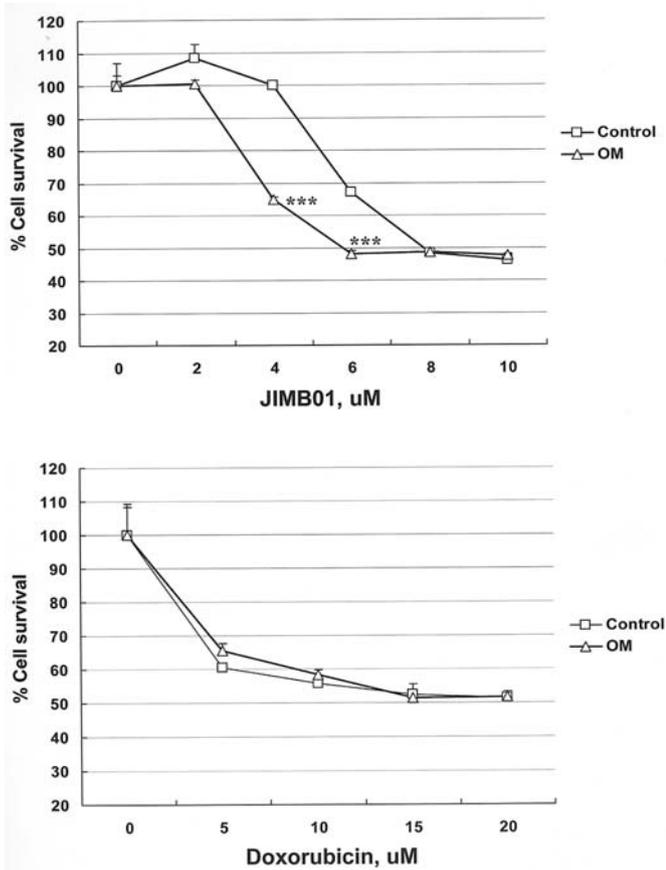


Figure 6. Inhibition of SNCG expression increases cytotoxicity of JIMB01. H3922 cells without OM or with 2 days of OM treatment were incubated with indicated concentrations of JIMB01 or doxorubicin for 24 h and the cell survival rates were measured by MTT assay. *Statistically significant differences between OM-treated and control cells ($p < 0.0001$).

implications of SNCG expression status as a biomarker to predict the effectiveness of anti-microtubule chemotherapy in breast cancer patients. From two different lines of investigation, we provide strong evidence of a clear inverse relationship between SNCG expression and lower sensitivity to anti-microtubule chemo-agents.

First, we show that endogenous expression of SNCG in different BC cell lines strongly correlates with lower mitotic index as compared to BC cell lines that do not express this oncogene. These results corroborate the findings obtained from BC cell lines in which SNCG was introduced by stable transfection, including MCF7-SNCG and MDAMB435-SNCG. In our previous studies, we developed T47D-derived clones that express an antisense mRNA of SNCG (T47D-As) (6). The cellular abundance of SNCG protein was reduced by more than 70% in T47D-As cells as compared to the parental cells. In this study, we consistently observed a higher mitotic index in the T47D-As stable clone than in parental T47D cells. T47D cells are known to harbor a mitotic defect by lacking functional Mad2, another component of mitotic checkpoint machinery (28,29). However, the fact that lowering SNCG expression increased the mitotic index upon Taxol treatment suggests that SNCG expression in T47D cells partially contributes to the poor response of this breast cancer cell line to Taxol-induced mitotic arrest.

The human SNCG gene is a tissue-specific gene primarily expressed in brain tissue and is abundant in presynaptic terminals (1). The exon 1 region of the SNCG gene contains a CpG island that is heavily methylated in tissues outside the neuronal system, resulting in the transcriptional silent (30). However, during malignant transformation, the CpG island of the SNCG gene became demethylated and led to the aberrant expression of this neuronal protein in breast cancer as well as in other types of cancer cells (24,30-32). Our previous studies have demonstrated that SNCG expression in H3922 cells can be specifically down-regulated by OM, a growth-inhibitory cytokine (5). The CpG island of SNCG became methylated after OM treatment, consequently leading to a strong inhibition of SNCG expression (24). Although the underlying mechanisms by which OM alters the methylation status of the SNCG CpG island have not been elucidated, H3922 cells provide another useful model to examine the correlation between SNCG expression and the response to anti-microtubule drug treatment. In this study, by measuring numbers of mitotic arrested cells as well as the activity of Cdc2 kinase, we showed that down-regulation of SNCG by OM resulted in an increased sensitivity of H3922 cells to JIMB01, another microtubule inhibitor, while the drug cytotoxicity of doxorubicin was not altered by OM treatment. Our data also indicated that the effect of OM was directly related to SNCG expression because OM treatment did not change JIMB01-induced Cdc2 kinase activity or the mitotic index of H3396 BC cells that do not express SNCG. These results provide additional evidence to support the notion that SNCG expression inhibits mitotic checkpoint function, thereby leading to cell resistance to anti-microtubule drugs.

In conclusion, we have provided strong evidence that SNCG expression confers resistance of breast cancer cells to anti-microtubule chemo-agents. Therefore, the expression status of SNCG on primary breast carcinomas could be used as a biomarker to predict the effectiveness of paclitaxel treatment. In light of our recent new findings showing prominent expression of SNCG in 8 different types of human cancer (31), we conclude that SNCG expression status may have a broad effect in patient responses to anti-microtubule chemotherapy.

Acknowledgements

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