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14. 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 be of great clinic implications. Our previous studies have demonstrated that the neuronal protein synuclein-gamma (SNCG), previously named BCSG1, 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 are highly correlated 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 likely one causal factor contributing to the poor patient response to paclitaxel treatment.					
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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-gamma (SNCG) (1), also referred 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 existing 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, chemo drugs can be divided into several categories. One class is microtubule inhibitor including paclitaxel (Taxol) (16), vincristine (17), and JIMB01 (18). These microtubule-disrupting agents are thought to arrest cells in mitosis by triggering the mitotic checkpoint activation, resulting cells arrested in mitotic phase without entering anaphase (19). Prolonged treatments with these agents lead to cell death by undergoing 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 the first-line chemotherapeutic agents to treat patients with advanced or metastatic BC (22-23). However, the response rates to this class of drugs vary significantly. SNCG expression status could have influenced 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, in this current study 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.

BODY

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 expressions of SNCG in BC cells also affect cell responses to anti-microtubule drugs, we determined mitotic indices of 4 SNCG-positive BC cell lines H3922, BT-20, MDA-MB-231, T47D, and 2 SNCG-negative BC cell lines H3396, 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. Figure 1A shows that all SNCG-positive cells were more resistant to Taxol as compared to SNCG-negative cells. Under the same treatment condition, less than 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 ($p < 0.01$, Figure 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). Figure 1C shows 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 be arrested at the mitotic phase by anti-microtubule agents is generally impaired in SNCG expressing 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 a 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. Figure 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 approximately 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 was already in G1 phase as compared to 12% of neo cells in G1. By 30 h, 35% of SNCG-expressing cells moved to S phase and only about 20% of neo cells were in S phase. These results indicate that SNCG expression has impaired the ability of cells to stay in the mitotic phase and allows 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 SNCG 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 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 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 SNCG expression level by western blotting, and another portion was treated with JIMB01 for various time points for a total period of 40 h. Figure 4A shows that SNCG protein level was decreased by 70% after OM treatment. Subsequent treatment with JIMB01 at 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 prominent mitotic arrest in H3396 cells regardless of OM treatment (Fig. 4B, lower panel).

It has been shown when cells are exposed to microtubule inhibitors, the mitotic checkpoint activates, resulting in cells arrest in pro-metaphase with persisting Cdc2 kinase activity (26,28). To obtain additional evidence for SNCG-inhibited mitotic checkpoint function, the Cdc2 kinase activities on histone H1 phosphorylation in H3922 and H3396 cells without and with OM treatment were 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 [γ ³²P]-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 PhosphorImager. As shown in Fig. 5 JIMB01 did not activate Cdc2 kinase activity in SNCG-positive H3922 cells that was not treated with OM, but elicited a strong kinase activity after OM treatment. In contrast, JIMB01 treatment resulted in strong activation of Cdc2 kinase activity in SNCG-negative H3396 cells without or with OM treatment.

To further link SNCG expression with the specific response to anti-microtubule chemo agent, 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.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of an inverse correlation between SNCG expression and resistance to anti-microtubule chemo drugs.

REPORTABLE OUTCOMES

- The manuscript describing this work has been published in The International Journal of Oncology (2006) 28:1081-1088.

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FIGURES

Figure 1. Association of SNCG expression with lower mitotic index. Different BC cell lines were cultured in cover slips inserted in wells of 24-well culture plates for 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.

Figure 2. Cell cycle analysis of SNCG and neo clones. SNCG stably transfected clone of MDA-MB435 (435-SNCG) and the muck-transfected clone (435-neo) were synchronized by a double-thymidine block. After a 6 hr release from G1/S, Taxol at 0.1 μ M 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.

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 muck-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.

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 wells plates and treated with JIMB01 for the indicated times. Mitotic arrested cells were counted after fixing and DAPI staining.

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 activities.

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. The asterisk signs indicate the statistically significant differences between OM treated and control ($p < 0.0001$).