Research article

THE TUMOR SUPPRESSOR FUNCTION OF STGC3 AND ITS REDUCED EXPRESSION IN NASOPHARYNGEAL CARCINOMA

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Abstract: STGC3 is a novel candidate tumor suppressor gene that was found to be associated with nasopharyngeal carcinoma (NPC) via the cDNA cloning and RACE processes. The biological function of the STGC3 protein and its expression level in nasopharyngeal carcinoma remain unknown. This study aimed to evaluate the STGC3 protein expression level in NPC and to investigate the inhibitory function of STGC3 as a candidate tumor suppressor gene. We assessed the expression of the STGC3 protein in NPC biopsies and normal control specimens via Western blot and immunohistochemical analysis. The expression of STGC3 as induced by doxycycline (Dox) via a tetracycline (Tet)-regulated system in human nasopharyngeal carcinoma cell line CNE2 was also established, and the effect of STGC3 restoration on the biological behavior of CNE2 was observed. A reduced level of STGC3 expression (0.978 ± 0.213 versus 0.324 ± 0.185, \( P < 0.05 \)) was detected in NPC versus normal nasopharyngeal tissue by Western blot assay. Immunohistochemical assays for STGC3 detected positive staining in the nuclei and cytoplasm of epithelial cells, and the positive expression rate in NPC, 8 of 21 (38%), was lower than that in...
normal nasopharynx samples, 16 of 22 (72%). After STGC3 expression was restored, the growth capacity and clone formation potential of CNE2 cells in soft agar were significantly suppressed, and the cell percentage in G0/G1 phase increased, while the percentage of cells entering the S and G2 phases decreased. This indicates that an abnormality in STGC3 expression is associated with nasopharyngeal carcinogenesis and that it may play an important role in controlling cell growth and regulating the cell cycle.

**Key words:** Nasopharyngeal neoplasm, STGC3, Gene expression, Tet-on system

**INTRODUCTION**

Although only sporadic in the west, nasopharyngeal carcinoma (NPC) is endemic to southern China, where it is the third most common form of malignancy amongst men, with incidence rates between 15 and 50 per 100,000 [1]. The etiological factor is believed to be the interaction between genetic susceptibility and environmental factors, especially EBV infection [2-5]. Molecular studies of NPC have revealed genetic abnormalities such as the activation of various oncogenes (NF-κB, met, bcl-2 and Ras), the inactivation of tumor suppressor genes (p53, p16, p14, Rb and TSLC1), and the loss of heterozygosity at numerous chromosomal localities [6-17]. STGC3 (GenBank Accession No: AY078383) is a novel candidate tumor suppressor gene found to be associated with nasopharyngeal carcinoma by the processes of cDNA cloning and RACE [18, 19]. It is located on 3p21 and its full-length cDNA is composed of 1271 bp with a 443-bp open reading frame, which encodes a protein of approximately 16 kD. The predicted STGC3 protein consists of 146 aa that contain a N-glycosylation site, a CK2 phosphorylation site, three Myristyl N-myristoylation sites and a laminin G domain, which is involved in integrin-mediated cell adhesion and binding to heparin, sulfatides, and the α-dystroglycan receptor [20-24]. Our previous study indicated that the gene was down-regulated at the mRNA level in NPC tissues and NPC cell lines [18]. To elucidate whether the expression level of the STGC3 protein correlates with NPC tumorgenesis, we monitored STGC3 protein expression in normal tissue and tumor biopsies using the polyclonal antibody against STGC3, and compared the differences between groups. Our data showed that the expression of STGC3 is reduced in NPC.

To further investigate the tumor suppressor function of STGC3 in NPC, we transfected an STGC3-deficient NPC cell line, CNE2, with a wild-type STGC3 expression construct, and observed the biological behaviors of the cells expressing exogenous STGC3. The results suggested that restoring STGC3 expression in CNE2 cells suppressed growth by arresting tumor cells at the G1 phase.
MATERIALS AND METHODS

Reagents
The regulator plasmid pRevTet-on and response plasmid pRevTRE were provided by Dr. Z.M. He. The ECL reagent was obtained from Pierce (USA) and DAB and anti-β-actin monoclonal antibody were from Santa Cruz Biotechnology (USA). The Ultra Sensitive SP Kit for immunohistochemistry was from Maixin.Bio (China). The polyvinylidene difluoride (PVDF) membrane was from Millipore (USA). TRIzol reagent, T4 DNA ligase and AMV RT kit were from Promega (USA). Lipofectamine 2000, G418 and RPMI1640 medium were purchased from Invitrogen (USA). Doxycycline (Dox) and hygromycin were from Roche (Switzerland). The DL2000 marker was from Takara. The λDNA/HindIII marker and restriction enzymes were from New England Biolabs (USA). The prime oligonucleotides were synthesized by Sangon (China).

Tissue samples
20 NPC and 20 normal nasopharynx biopsy samples were obtained from the University of South China affiliated hospital. Each biopsy sample was divided into two sections. One half was submitted for routine histological diagnosis, and the other was immediately stored at -80°C for Western blot analysis. As the patient specimens were too small to allow further sectioning, for immunohistochemical staining, a set of archival paraffin-embedded specimens (21 NPC and 22 nasopharynx biopsies) unrelated to the frozen samples were selected. Informed consent was obtained from all of the patients, and the research was permitted by the Ethics Committee of the Medical College in the University of South China.

STGC3-specific antibody
Anti-STGC3 antiserum was produced by immunizing rabbits with a synthetic peptide derived from the COOH-terminal end of the STGC3 protein (KIRKPHNG LVLSS PSQTDYRPAG). This STGC3-specific antibody was purified by peptide affinity chromatography.

Western blot analysis
Protein extracts were prepared using a standard extraction protocol [25]. Samples containing 50 µg total protein were electrophoresed on 15% SDS-polyacrylamide gel and electrophoretically transferred to PVDF membrane. The blots were blocked with phosphate-buffered saline (PBS) containing 5% non-fat milk and 0.1% Tween-20. STGC3 and β-actin were detected using the polyclonal antibody against human STGC3 and the monoclonal antibody against β-actin. The STGC3 and β-actin protein expression levels were quantitatively estimated by densitometric scanning performed with an Imaginer2200. The STGC3 protein concentration was normalized to the β-actin level and expressed as a densitometric ratio.
Immunohistochemical staining

The immunohistochemical analysis was performed on paraffin-embedded biopsies with an Ultra Sensitive SP Kit. Sections were deparaffinized, ethanol-rehydrated, and rinsed with PBS. The endogenous peroxidase activity was blocked with 0.35% hydrogen peroxide for 10 min at room temperature. The sections were rehydrated and washed in PBS. After blocking the nonspecific binding sites with 2% normal horse serum in PBS for 10 min at room temperature, the sections were incubated with polyclonal anti-STGC3 antibody in PBS overnight at 4°C. After rinsing with PBS, the sections were incubated with a biotin-conjugated secondary antibody for 10 min at room temperature, followed by washing with PBS. The sections were incubated with streptavidin-peroxidase (S-P) complex for 10 min at room temperature. The reaction was visualized via diaminobenzidine (DAB) reaction. The sections incubated with PBS instead of the corresponding primary antibody were used as negative controls. The sections were assessed by a pathologist and designated positive or negative staining, where positive meant any specimen with >10% positive cells in the mucosa. To quantitatively analyze the STGC3 expression, the integral optical densities (IOD) were obtained using the Imageproplus image analysis software.

Plasmid construction and identification

To construct the recombinant plasmid pRevTRE-STGC3, the open reading frame of STGC3 encoding the full-length protein was amplified by PCR using the primers containing BamHI and HindIII restriction sites (sense 5'-CGGGATCCATGGTTCTTGTTTAT-3', antisense 5'-GCCCCAAGCTTTTAGAGTAATAAAAGATTTC-3'). The PCR product was subcloned into the expression vector pRevTRE, which contains the hygromycin-resistance gene. The recombinant plasmid was confirmed by BamHI/HindIII digestion and DNA sequencing.

Cell culture and transfection

CNE2 is a poorly differentiated NPC cell line. CNE2 cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO2. To establish a stable cell line that expresses the reverse tetracycline/doxycycline-responsive transcriptional activator (rtTA), CNE2 cells were first transfected with the pRevTet-on regulator plasmid encoding rtTA by using lipofectamine 2000. Resistant cell clones grown in the presence of 400 µg/ml G418 were isolated. One cell clone in which the rtTA expression was found to be high via the RT-PCR method was selected. This cell clone was subsequently transfected with either the pRevTRE-STGC3 plasmid or the pRevTRE plasmid as a control. After transfection, individual cell clones were selected and isolated in the presence of 400 µg/ml G418 and 200 µg/ml hygromycin in the culture medium. To examine the expression of STGC3, cell clones were cultured at high cell density in the presence and absence of 1 µg/ml Dox for 24 h and then subjected to RT-PCR and Western blot analyses.
RT-PCR analysis
Total cellular RNA was prepared from $1 \times 10^6$ cells, using TRIzol Reagent according to the manufacturer’s instructions. Full-length first strand cDNA was synthesized with ImProm-II TM Reverse Transcription System. 2 µg of RNA were transferred into a microfuge tube. After heating at 70°C for 10 min, the sample of mRNA was chilled on ice for 5 min. Subsequently, the following materials were added in the RNA reactive tube: 4 µl of 25 mM MgCl₂, 2 µl of 10×RT-PCR buffer, 2 µl of 10 mM dNTPs mixture, 2 µl of oligo(dT)₁₅, 0.5 µl of RNase inhibitor, and 15 u of AMV Reverse Transcriptase, and the volume was adjusted up to 20 µl with RNase-free water. The reactive solution was incubated at 42°C for 1 h, then degenerated at 95°C for 5 min. Fragments of interest were amplified by PCR with special primers.

Cell growth analysis
Growth curves were constructed by cell counting over a set period of cultivation. Cells were resuspended in RPMI1640 medium and seeded into 24-well plates at $1 \times 10^4$ cells per well. A total of 3 µg/ml Dox were added to the medium. The culture medium was changed every two days and the number of cells was counted consecutively for seven days. Each experiment was done in triplicate.

Soft-agar colony formation assay
Single cells were plated in 0.3% semi-solid agar in growth medium containing 3 µg/ml Dox. Aliquots containing 1000 cells were plated in triplicate on a basal layer of 0.5% semi-solid agar in growth medium onto 6-well plates. Colonies containing more than 100 cells in soft agar were scored under an inverted microscope after two weeks of incubation at 37°C, and the rate of colony formation was represented by the mean percentage of colonies.

Cell cycle analysis
Cell cycle distribution was analyzed by flow cytometry (FCM) after the cells had been stained with propidium iodide (PI). Briefly, $5 \times 10^5$ cells were seeded and allowed to attach overnight. The medium was replaced with fresh complete medium containing 3 µg/ml Dox. After 24 h incubation at 37°C, cells were harvested by trypsinization, fixed in 70% ethanol, and stored in fixative at -20°C until prepared for cell cycle analysis. Fixed cells were washed with PBS and incubated with PI (50 µg/ml in PBS) for 30 min at room temperature in the dark. The analysis of the DNA content in the propidium iodide-stained cells was performed by FCM.

Statistical analysis
The data is given as mean ± SEM. The SPSS11.0 software package was used for all the statistical analyses. For the immunohistochemistry, differences in STGC3 expression between the groups were analyzed by the $\chi^2$ test. For Western blot analysis, differences between the groups were checked via the independent t test. One-way analysis of variance (ANOVA) was used to evaluate the statistical
significance between the three cell groups for growth rate, anchorage-independent growth, and cell cycle distribution. $P < 0.05$ was considered to be statistically significant.

RESULTS

STGC3 expression detected by Western blot analysis

Typical immunoblots of representative samples from both groups are shown in Fig. 1. Immunoblot detection of STGC3 revealed down-regulation of STGC3 expression in NPC. An image analysis of the Western blots showed that the average STGC3 level of the NPC specimens was significantly less ($0.978 \pm 0.213$ versus $0.324 \pm 0.185$, $P < 0.01$) than that of the normal nasopharynx specimens.

![Fig. 1. The expression of STGC3 in human NPC and normal nasopharyngeal biopsies. A – Western blot analysis of STGC3 expression. Total protein extracts were prepared from 20 normal nasopharynx biopsies and 20 NPC biopsies. 50 µg of total protein was separated by 15% SDS-PAGE for Western blot with the antibody against STGC3 and the antibody against β-actin. Representative Western blots are shown. B – A comparision of the average STGC3 expression levels in human normal nasopharyngeal (N) and tumor (T) tissues.]

![Fig. 2. Immunohistochemical STGC3 staining from representative benign and malignant nasopharyngeal tissue sections. Immunostaining was performed as described in the Materials and Methods section. Positive brown staining was shown both in the nuclei and cytoplasm of the epithelial cells. SP×200.]


Immunohistochemical analysis of STGC3 expression in human NPC

In order to examine the expression of STGC3 at the histological level, we performed an immunohistochemical analysis. STGC3 was expressed in both the cytoplasm and nuclei of epithelial cells in the normal nasopharyngeal biopsy specimens, but chiefly in the cytoplasm of tumor cells from NPC specimens. Sixteen of 22 (72%) normal nasopharynx specimens and 8 of 21 NPC specimens (38%) showed STGC3-positive staining (Fig. 2). There was a significant decrease in STGC3 expression in the NPC compared with the normal nasopharynx epithelia, and the average IODs were 0.0767 for NPC and 0.2711 for normal nasopharynx epithelia (P < 0.05).

Establishing a stable CNE2 cell line permitting the inducible expression of STGC3

We used a two-step selection procedure to isolate the stable cell clones. In the first step, pRevTet-on plasmid was transfected into CNE2 cells, and resistant cell clones were isolated via a G418 screen. RT-PCR analysis demonstrated that five clones expressed Neo' and rtTA, but the rtTA expression of clone 1 was higher than that of the other clones (the densitometric ratios of clones 1-5 for Neo' expression were respectively: 1.07, 1.03, 1.01, 1.05, 1.03; the densitometric ratios of clones 1-5 for rtTA expression were respectively: 0.42, 0.37, 0.41, 0.45, 0.43; Fig. 3). In the second step, this cell clone was amplified and transfected with the pRevTRE-STGC3 plasmid. The STGC3 expression in the cell clones was then detected by RT-PCR and Western blot analyses. One clone, termed CNE2/Tet/TRE-STGC3-4, which actually had undetectable background expression under non-induced conditions, showed remarkable expression of STGC3 mRNA after 1 µg/ml Dox induction (the densitometric ratios for the
STGC3 expression of CNE2, CNE2/Tet/TRE and clone 1-4 cells treated with Dox were respectively: 0.25, 0.32, 0.43, 0.61, 0.58, 0.62; Fig. 4). CNE2/Tet/TRE-STGC3-4 cells were amplified and treated with varying amounts of Dox for 24 h. STGC3 expression significantly increased in a dose-dependent manner after the cells were exposed to Dox for 24 h over a range of Dox concentrations from 0.01 µg/ml to 3 µg/ml (Fig. 5). Therefore, CNE2/Tet/TRE-STGC3-4 was selected and amplified for further study.

Fig. 4. The expression of STGC3 as detected by RT-PCR. All the cell clones showed expression of STGC3 mRNA with 1 µg/ml Dox induction for 24 h. Of these clones, CNE2/Tet/TRE-STGC3-4 showed the highest expression with Dox induction, but no expression without Dox induction. CNE2 and CNE2/Tet/TRE cells were found to have a weak STGC3 mRNA signal after 1 µg/ml Dox induction for 24 h. M – DL2000 Marker, 1 – CNE2 cells (Dox+), 2 – CNE2/Tet/TRE cells (Dox+), 3, 4 – CNE2/Tet/TRE-STGC3-1 clone (Dox-, Dox+), 5, 6 – CNE2/Tet/TRE-STGC3-2 clone (Dox-, Dox+), 7, 8 – CNE2/Tet/TRE-STGC3-3 clone (Dox-, Dox+), 9, 10 – CNE2/Tet/TRE-STGC3-4 clone (Dox-, Dox+).

Fig. 5. STGC3 expression of CNE2/Tet/TRE-STGC3 after induction with different concentrations of Dox. STGC3 expression was detected by both RT-PCR and Western blot analysis with a variety of concentrations of Dox treatment for 24 h. CNE2/Tet/pTRE-STGC3 cells expressed exogenous STGC3 in a dose-dependent manner. A – RT-PCR analysis of STGC3 expression. B – Western blot analysis of STGC3 expression. M – DL2000 Marker, 1 – 0 µg/ml, 2 – 0.01 µg/ml, 3 – 0.1 µg/ml, 4 – 1 µg/ml, 5 – 3 µg/ml.
STGC3 expression inhibited CNE2 cell growth

To evaluate the effects of STGC3 expression on growth, we examined the growth rates of the STGC3-transfected cells, parental CNE2 cells and vector-transfected cells with 3 µg/ml Dox induction. There was no significant difference in the growth rate between the CNE2 parental and empty vector-transfected cells. However, the CNE2/Tet/pTRE-STGC3 cells grew significantly more slowly than the CNE2/Tet/pTRE and CNE2 cells (P < 0.05, Fig. 6).

Fig. 6. The effect of STGC3 exogenous expression on CNE2 cell proliferation. Cells were resuspended in RPMI1640 medium by the addition of 3 µg/ml Dox, and seeded in 24-well plates in triplicate at 37°C. After every 24 h, the cell number was scored under a microscope. The CNE2/Tet/TRE-STGC3 cells grew significantly more slowly than the CNE2 parental and empty vector-transfected cells.

The expression of exogenous STGC3 reduces colony formation in soft agar

Further experiments were conducted to test whether STGC3 correlated with anchorage-independent growth in soft agar. The results showed that the colony formation rate was comparable in the CNE2 and CNE2/Tet/pTRE cells. However, CNE2/Tet/TRE-STGC3 with Dox induction had a reduced ability to grow in soft agar compared with CNE2/Tet/TRE and CNE2 (Fig. 7, P < 0.01).

The effect of STGC3 up-regulation on cell cycle distribution

The effect of up-regulating STGC3 expression on the cell cycle distribution of CNE2 cells was analyzed by FCM (Fig. 8). Tab. 1 shows the increase in G0/G1 phase cells (P < 0.05) and decrease in S and G2 phase cells (P < 0.05) that occurred when STGC3 was up-regulated by Dox induction. Our results indicate that the restoration of functional STGC3 in NPC cells suppressed cell growth by arresting cells in the G1 phase.

Tab. 1. The effect of STGC3 expression on the cell cycle of CNE2 cells (n = 3, mean ± SEM, %).

| Group                  | G0/G1 | S     | G2/M  |
|------------------------|-------|-------|-------|
| CNE2                   | 38.4 ± 1.5 | 35.8 ± 2.1 | 23.9 ± 2.5 |
| CNE2/TRE/Tet           | 36.7 ± 0.9 | 38.2 ± 1.2 | 25.4 ± 2.3 |
| CNE2/Tet/TRE-STGC3    | 62.5 ± 2.7 | 29.9 ± 1.6 | 7.3 ± 0.8 |
Fig. 7. The colony-forming ability of CNE2 cells in soft agar. The colony-forming ability in soft agar in the presence of Dox was examined as a measure of anchorage-independent growth as described in the Materials and Methods section. Two weeks after plating, positive colonies were photographed under 40× magnification. The values represent the mean percentage of colonies formed. A – CNE2, B – CNE2/Tet/TRE, C – CNE2/Tet/TRE-STGC3, D – The colony-forming rate for each group.

Fig. 8. Cell cycle analysis by flow cytometry. Cells were treated with 3 µg/ml Dox for 24 h. The cells were harvested and processed for analysis of the cell cycle distribution as described in the Materials and Methods section. A – CNE2, B – CNE2/Tet/TRE, C – CNE2/Tet/TRE-STGC3.

DISCUSSION

Tumorigenesis results from the accumulation of genetic events that give rise to inappropriate cell behavior. This has been substantiated through the identification of both inherited and somatic mutations in human cancers. Mutations which lead to cancer progression are generally classified into two major categories: oncogenes and tumor suppressor genes. Cellular oncogenes
may be considered as genes whose products normally regulate growth and differentiation in a positive fashion, while tumor suppressor genes transduce negative growth regulatory signals in the cell. The mutational inactivation of tumor suppressor genes coupled with the activation of oncogenes can result in a loss of growth control and subsequent tumor formation. As a general rule, it was observed that the accumulation ultimately leads to tumor development. A multi-step model of carcinogenesis has been postulated, describing the accumulations and interaction of the multiple genetic alterations present in some cancers. Studies indicated that the nasopharyngeal carcinoma commonly found in South China was due to the interaction between genetic susceptibility and environmental factors. Many genes associated with NPC were cloned and their biological functions were studied. However, the precise molecular mechanism of NPC remains unknown.

We previously showed that STGC3 was expressed ubiquitously in many human healthy tissues such as the heart, brain, spleen, skeletal muscle and nasopharyngeal tissues, but down-regulated in various human tumor tissues or cell lines, including NPC biopsies and NPC cell lines (HONE1, HNE1, CNE2) [18, 19]. In this study, we found a significant difference in STGC3 protein expression between NPC and normal tissues, which was consistent with the results of the previous study. The amount of STGC3 protein was nearly 2-fold higher in normal nasopharyngeal tissue than in NPC, according to the Western blot assay. The results of the immunohistochemical analyses showed the localization of STGC3 protein expression. The normal nasopharyngeal mucosa showed granular staining localized in the nuclei and cytoplasm of the epithelial cells, but in NPC, it was chiefly found in the cytoplasm of the tumor cells. The positive expression rate for NPC, 8 of 21 (38%), was lower than that for the normal nasopharynx mucosa, 16 of 22 (72%). We again provided evidence for the involvement of STGC3 down-regulation in NPC. Tumor suppressors were shown to exercise their functions in various parts of the cell cycle. Some genes exert their effect in the nucleus, acting at control points of the cell cycle, while others play a role at the plasma membrane in cell-cell interactions. The localization of STGC3 protein and its alteration between normal nasopharyngeal mucosa and NPC tissues indicated that STGC3 probably acted chiefly in the nucleus and was associated with regulation in the cell cycle and gene transcription.

To investigate its inhibitory function in NPC further, the STGC3 gene expression level was up-regulated by lipofectin transfection of STGC3 into CNE2 cells without detectable STGC3 expression, and the biological behavior was observed.

The Tet-on gene expression system was developed by Gossen, et al. in 1992 [26, 27], and has been found to have several advantages [28-30]. Gene expression is easily regulated via the administration of Dox, which is minimally toxic, and Dox acts specifically on the target gene instead of activating other cellular genes. In this study, we successfully established a cell line CNE2/Tet/TRE-STGC3 in
which the STGC3 gene could be conditionally expressed with Dox treatment by utilizing the Tet-on gene expression system. Dox could modulate the level of STGC3 expression in a dose-dependent manner in this cell line, with a low background and a high-fold Dox-induced expression of STGC3. We found that restoring STGC3 expression inhibited the growth of CNE2 cells and suppressed the anchorage-independent cell growth in soft agar, indicating a tumor-suppression role of STGC3 in the development of NPC. Furthermore, FCM analysis experiments were performed to characterize its effect on the distribution of cells in different phases of the cell cycle. The population of cells entering the S and G2 phases was substantially reduced by STGC3 up-regulation, with an increase in the percentage of resting cells in the G0/G1 phase. Obviously, this growth inhibition was attributable to the significant proportion of STGC3-expressing cells arrested at G1 phase in the cell cycle. In summary, our study demonstrated that reduced STGC3 expression is associated with NPC tumorgenesis, and that the STGC3 gene may act as a tumor suppressor gene. However, additional analyses are needed to elucidate the exact role of the STGC3 protein and the inactive mechanism underlying NPC.

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