

Original Article

Lentivirus-carrying glypican-6 shRNA inhibits viability and colony formation of human nasopharyngeal carcinoma cells

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Abstract: Glypican-6 (GPC6) is a cell surface co-receptor to transduce growth factor and protease signaling and regulate cell proliferation and differentiation. Altered GPC6 expression was associated with human tumorigenesis. This study investigated the effects of GPC6 knockdown on regulation of nasopharyngeal carcinoma cell proliferation, cell cycle distribution, and colony formation *in vitro*. A lentivirus-carrying GPC6 shRNA or negative control was used to infect nasopharyngeal carcinoma CNE-2Z cells. Levels of GPC6 mRNA and protein were assayed using qRT-PCR and Western blot, respectively. Cell proliferation was detected using Celigo cell counting, while cell cycle distribution was assessed using flow cytometry. Colony formation was assayed using PI-FACS. GPC6 mRNA was expressed in CNE-2Z cells, and lentivirus-carrying GPC6 shRNA decreased levels of GPC6 mRNA and protein in CNE-2Z cells. GPC6 knockdown reduced tumor cell proliferation and colony formation as well as number of cells at G1 phase of the cell cycle, but increased the number of cells at the S and G2/M phases. This study demonstrated that GPC6 knockdown has a critical effect on proliferation and colony formation of nasopharyngeal carcinoma cells; thus, GPC6 may be a potential target in treatment of nasopharyngeal carcinoma.

Keywords: Nasopharyngeal carcinoma, glypican-6, shRNA, cell proliferation, colony formation

Introduction

Nasopharyngeal carcinoma occurs most commonly in southern China and Southeast Asia, compared to other parts of the world, with an incidence of 15-50 cases per 100,000 annually [1]. Although the precise etiology and pathogenesis remain poorly understood, nasopharyngeal carcinoma most likely results from a combination of factors, including viral infection, such as Epstein-Barr virus (EBV), and dietary and genetic factors [1, 2]. Epidemiological studies revealed that environmental factors, including EBV, consumption of salted and pickled vegetables, and tobacco smoke, are risk factors in the development and progression of nasopharyngeal carcinoma [3-5]. Nasopharyngeal carcinoma significantly affects the quality and length of life of patients. Currently, treatment of nasopharyngeal carcinoma, like most other cancers, includes surgery, radiotherapy,

or combined chemo-radiotherapy, leading to a cure rate of over 90% in early-stage patients [6]. However, 5% to 15% of such patients suffer local recurrence, and 16% and 34% of patients will develop distant metastases [7]. Patients with distant metastases have been shown to have worse survival with a progression-free survival rate of 7.3 to 10 months [8]. Recently, molecular targeted therapy has become a novel approach and is demonstrated to be a useful option in treating nasopharyngeal carcinoma [9]. Nevertheless, further investigation of the etiology, pathogenesis, progression, and recurrence rates in nasopharyngeal carcinoma could result in higher cure rates, alongside improvements in quality of life and, even, prevention.

Glypican is a family of heparin sulfate proteoglycans and syndecans and recent evidence has implicated its role in cell growth and division through the manipulation of growth factor-relat-

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ed cell signal transduction [10]. To date, six types of glypicans have been identified in mammals (i.e., GPC1 to GPC6), and genes encoding these glypicans belong to the family of heparan sulfate proteoglycans (HSPGs) that associate with or link to the cell membrane and the extracellular matrix [11] and possess important biological functions in cell adhesion, growth, migration, and differentiation [11]. Glypicans are predominantly expressed in a spatiotemporal manner during embryogenesis; thus, they are believed to be involved in morphogenesis and tissue differentiation [12, 13]. Furthermore, glypicans have been linked to human tumorigenesis through manipulation of growth factor signaling [14]. For example, GPC1 is overexpressed in pancreatic cancer and gliomas, suggesting that GPC1 plays a role in the response of tumor cells to various mitogenic agents such as heparin-binding epidermal growth factor (EGF)-like growth factor or fibroblast growth factor (FGF) [15, 16]. GPC3 expression has also been demonstrated to be upregulated in hepatocellular carcinoma (HCC) and promotes HCC cell proliferation by inhibition of bone morphogenetic protein-7 and FGF signaling, as well as increasing canonical Wnt signaling [12]. Conversely, GPC5 overexpression or knockdown in isolated rhabdomyosarcoma cells induced and decreased tumor cell proliferation, respectively [17]. Thus, manipulation of glypicans may affect the secretion of different growth factors and their signals, and therefore, could be considered as novel targets in the treatment of various carcinomas [10]. To date, the role of GPC6 in cancer, in particular, in nasopharyngeal carcinoma, are limited. However, previous studies have demonstrated that glypican 6 (GPC6) is a cell surface co-receptor protein for the transduction of growth factor, extracellular matrix protein, protease and anti-protease signaling [18-20]. Thus, in this study, we utilized RNA interference (RNAi) as a tool [21] to knockdown GPC6 expression in human nasopharyngeal carcinoma using the lentivirus-carrying target gene shRNA. We then assessed the effect of GPC6 knockdown on nasopharyngeal carcinoma cell proliferation, colony formation, and cell cycle progression *in vitro*.

Materials and methods

Cell lines and culture

Human nasopharyngeal carcinoma cell lines CNE-2Z and 5-8F were obtained from the Cell

Bank of Chinese Academy of Sciences (Shanghai, China). CNE-2Z cells were cultured in RPMI-1640 (Corning, Corning, NY, USA) containing 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma Chemicals, St Louis, MO, USA), while 5-8F cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Corning) containing 10% FCS and 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified incubator with 5% CO₂ at 37°C.

Construction of recombinant lentivirus and cell infection

Human GPC6 cDNA sequences (5'-ACAGCAAGCCAGATACTT-3') were selected from the full-length GPC6 (GenBank number: NM005708, GeneChem Co. Ltd. Shanghai, China). The stem-loop oligonucleotides were then synthesized, aligned, and then inserted into the lentiviral vector GV115 (GeneChem Co. Ltd.) at AgeI/EcoRI cloning sites. After plasmid amplification and DNA sequence confirmation, the GPC6-shRNA and negative control vectors together with pHelper 1.0 and pHelper 2.0 vectors (Qiagen, Shanghai, China) were co-transfected into embryonic kidney 293T cells (Invitrogen, Shanghai, China) for production of lentivirus using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. In brief, after 48 h of transfection, supernatants containing lentivirus were collected from cell culture and centrifuged and then filtered using 0.45 µm polyvinylidene fluoride membranes.

CNE-2Z cells were thereafter infected with GPC6-shRNA or control lentivirus at a multiplicity of infection (MOI) of 2 and the infected cells were inspected under a fluorescence microscope 2009 (CKX41, Olympus, Tokyo, Japan) to determine the infection efficiency. The cells were then collected for qRT-PCR and Western blot analyses of gene expression.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cultured cells using Trizol reagent (Shanghai Universal Biological Technology Co., Ltd., Shanghai, China) and then converted into cDNA using the M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturers' instructions. cDNA samples were amplified using qPCR with a SYBR master mixture (TAKARA,

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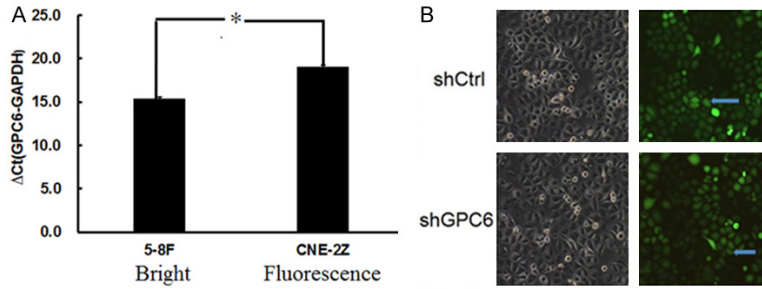


Figure 1. Establishment of GPC6 knockdown in CNE-2Z cells. A. Level of GPC6 mRNA in CNE-2Z cells and 5-8F cells using RT-PCR. B. Fluorescence microscopy assessment of the infection efficiency lentivirus vector in 293T cells (approximately 80%). $\times 100$ magnification. There was no significant difference between the negative control and the GPC6 groups, indicating that change in cell proliferation was GPC6 shRNA-induced.

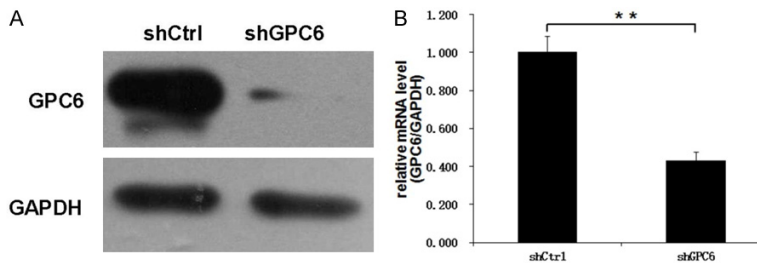


Figure 2. shGPC6 downregulation of GPC6 expression in nasopharyngeal carcinoma cells. A. Western blot. Levels of GPC6 protein was assayed using Western blot in the negative control and shGPC6-infected CNE-2Z cells. B. qRT-PCR. Level of GPC6 mRNA was assessed in the negative control and shGPC6 infected CNE-2Z cells using qRT-PCR. The data show that level of GPC6 mRNA was reduced to 56.8% after shGPC6 infection. The data are presented as means \pm SEM with significant differences indicated (** $P < 0.05$) as compared to the control group. GAPDH was used as internal control.

Dalian, China) for GPC6 expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for quantification. GPC6 primers were 5'-GTCCACGGAGTTT GAGTTT-3' and 5'-ATGGTCCATTGTCCAAGAGT-3', while GAPDH primers were 5'-TGACTTCAACAGCGACACCCA-3' and 5'-CAC CCTGTTGCTGTAGCCAAA-3'. The qPCR was set to be an initial cycle of 95°C for 5 s and 45 cycles of 95°C for 15 s, and 60°C for 30 s). Relative levels of GPC6 mRNA in each sample were summarized using the $2^{-\Delta\Delta Ct}$ method against GAPDH mRNA.

Protein extraction and Western blot

Cells after 36-48 h viral transfection were scraped on ice, centrifuged, and lysed in a 2x lysis buffer containing 100 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 20% glyce-

rol, and 2% mercaptoethanol and centrifuged to collect supernatants. Protein concentration was then assessed with a BCA Protein Assay Kit (Beyotime, Shanghai, China) and these protein samples were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred on to polyvinylidene fluoride membranes using an electrophoresis apparatus from Bio-Rad (Hercules, CA, USA) at 300 mA at 4°C for 120 min. The membranes were then incubated in 5% skim milk solution in phosphate buffered saline (PBS)-Tween 20 (PBS-T) at room temperature for 1 h and further incubated with a primary antibody for 2 h or overnight at 4°C. The primary antibodies were mouse anti-flag (F1804, 1:2000; Sigma) and mouse anti-GAPDH (Sc-32233, 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following this, the membranes were washed with PBS-T briefly thrice and then incubated with a second

goat anti-mouse IgG (Sc-2005, 1:2000; Santa Cruz Biotechnology). The level of protein in the membranes was detected by using the enhanced chemiluminescence (ECL) Western blotting system (Thermo, Waltham, MA, USA).

Cell viability determined by MTT assay

After viral infection, CNE-2Z cells were seeded in 96-well plates at a density of 2000 cell/well and cultured for up to 5 days. At the end of each experiment, each well was replaced with 20 μ L 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Genview, FL, USA) and cells were further incubated at 37°C for 4 h. After, cell culture medium was replaced with 100 μ L dimethyl sulfoxide (DMSO, Shanghai Testing Chemical Reagent Co., Ltd., Shanghai, China) to dissolve MTT-formed formazan. The absorbance of each well was mea-

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sured by using a microplate reader (M2009PR, Tecan Infinite, Männedorf, Switzerland) at 490 nm. The data were expressed as mean \pm SD and summarized as % of control. The experiments were carried out in triplicate and repeated three times.

Celigo cell counting

After viral infection, CNE-2Z cells were cultured in 96-well plates in RPMI-1640 (Corning) containing 10% FBS at a density of 2000 cells/well in a humidified incubator with 5% CO₂ at 37°C for up to five days. Cells were then subjected to Celigo cell counting on each day. The number of cells with green fluorescence staining was calculated by adjusting the input parameters of each measurement and the cell proliferation curves were then plotted.

Tumor cell colony formation assay

After three days of viral infection, CNE-2Z cells were seeded in 6-well plates at a density of 500 cells/well in 2 mL/well RPMI-1640, containing 10% FBS, and cultured for 15 days to form tumor cell colonies. Cell culture medium was refreshed every three days. Following this, cells were fixed with 4% paraformaldehyde for 1 h and washed with PBS and then stained with Giemsa-PBS solution. Colonies with 50 cells or more on the plates were counted under an inverted phase contrast microscope (Cai Kang Optical Instrument Co., Ltd., Shanghai, China). The data were expressed as mean \pm SD and summarized as % of control. The experiments were carried out in triplicate and repeated three times.

Flow cytometric cell cycle assay

After viral infection, CNE-2Z cells were cultured in 6 cm dishes for three days and harvested and washed with an ice-cold D-Hanks solution at 4°C and then fixed in 75% ice-cold ethanol for 1 h at 4°C. After that, cells were washed again with D-Hank's solution and stained with 2 mg/mL propidium iodide (PI, Sigma, USA) in D-Hank's solution containing 10 μ g/mL RNase (Fermentas, MA, USA) at room temperature for 30 min and then incubated at 37°C for additional 30 min. DNA contents in cells were determined using a FACS flow cytometer (Guava easyCyte HT, Millipore, Billerica, MA, USA) and analyzed with the Cell Quest software. The data were expressed as mean \pm SD of each cell

cycle and the experiments were set in triplicate and repeated three times.

Statistical analysis

SPSS version 21.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze all data statistically. Data were expressed as mean \pm standard deviation (SD) of three independent experiments and subjected to Student *t*-test for statistically significant differences between two groups. A *p* value of equal to or < 0.05 was considered statistically significant.

Results

GPC6 expression and knockdown in nasopharyngeal carcinoma cells

In the current study, we first assayed the level of GPC6 in nasopharyngeal carcinoma cell lines CNE-2Z and 5-8F using qRT-PCR. As shown in **Figure 1A**, GPC6 mRNA was expressed at higher levels in CNE-2Z cells (Δ Ct = 19.09 \pm 0.199) than in 5-8F cells (Δ Ct = 15.41 \pm 0.134). Data on lentivirus-transfected 293T cells are shown in **Figure 1B** and the transfection efficiency reached up to 80%. We then selected CNE-2Z cells for GPC6 knockdown and determined the effects of GPC6 knockdown on proliferation, colony formation, and cell cycle alterations *in vitro* using a lentivirus-carrying GPC6 shRNA.

Lentivirus-mediated GPC6 silence in nasopharyngeal carcinoma CNE-2Z cells

As shown in **Figure 2A**, expression of GPC6 protein was greatly reduced in the GPC6-shRNA infected cell cultures, indicating effective knockdown of the target gene, while levels of GPC6 mRNA were also significantly decreased in shGPC6-infected cells compared to the control group (0.432 \pm 0.043 to 1.002 \pm 0.081, *P* < 0.05; **Figure 2B**) and the efficiency of GPC6 knockdown reached 56.8%. These data indicate that the lentivirus-carrying GPC6 shRNA significantly knocked down the levels of GPC6 mRNA and protein expression in nasopharyngeal carcinoma CNE-2Z cells *in vitro*.

Effect of GPC6 knockdown on suppression of tumor cell proliferation

After, we assessed the effect of GPC6 knockdown on the proliferation of CNE-2Z cells using

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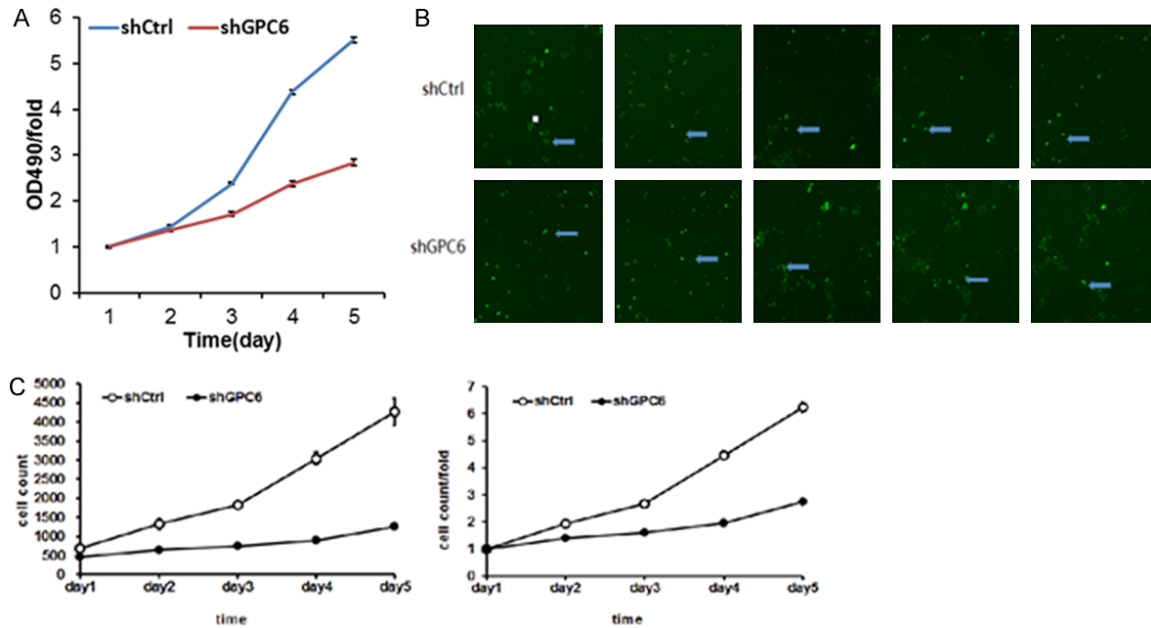


Figure 3. GPC6 knockdown in suppression of nasopharyngeal carcinoma cell proliferation. A. MTT assay. The growth curve of GPC6 shRNA-infected CNE-2Z cells compared with control group was determined by the MTT assay. Data are presented as means \pm SEM with significant differences indicated (* $P < 0.05$) as compared to the control group. B. Representative images of Celigo cell counting. Scale bar, 25 μ m (shown the arrows). C. The similar trend was observed in Celigo cell counting. Cell growth rate was monitored on the 2nd, 3rd, 4th and 5th days by the assay. Data are presented as means \pm SEM with significant differences indicated *($P < 0.01$) as compared to the control group.

cell viability MTT and Celigo cell counting assays. As shown in **Figure 3A**, cell viability of the shGPC6 group was remarkably reduced compared to the negative control lentivirus-infected cells at day 4 and day 5 (day 4: 0.332 ± 0.0095 to 0.649 ± 0.0069 , $P < 0.05$; day 5: 0.397 ± 0.0105 to 0.815 ± 0.0085 , $P < 0.05$). Similarly, compared to the control cells, the number of shGPC6-infected cells, determined by Celigo cell counting assay, was significantly lower. The data also showed differences after the multiple comparisons at day 4 and day 5 (day 4: 1.963 ± 0.147 to 4.456 ± 0.163 , $P < 0.05$; day 5: 2.745 ± 0.142 to 6.233 ± 0.183 , $P < 0.05$; **Figure 3B** and **3C**).

Effect of GPC6 knockdown on CNE-2Z cell cycle progression

As the reduction in nasopharyngeal carcinoma cell proliferation may be affected by changes in cell cycle distribution, we performed flow cytometric analysis of cell cycle markers. As shown in **Figure 4**, we found that the shGPC6 group cells exhibited a lower proportion of cells in the G1 phase (53.28 ± 1.11 to 60.48 ± 1.37 , $P < 0.05$), but a higher proportion of cells in the S phase (41.33 ± 1.51 to 37.18 ± 1.38 , $P < 0.05$)

and G2/M phases (5.39 ± 0.40 to 2.34 ± 0.02 , $P < 0.05$), indicating that GPC6 knockdown could promote tumor cells to undergo apoptosis. These results indicated that GPC6 is essential for G1-S transition in the nasopharyngeal carcinoma cell line CNE-2Z.

GPC6 knockdown on suppression of CNE-2Z cell colony formation

To investigate the effect of GPC6 knockdown on regulation of cell colony formation, we performed the colony formation assay. After cultured for 15 days, as shown in **Figure 5**, we found that compared to controls, the colony formation rate of shGPC6 cells was significantly reduced (23 ± 5 to 70 ± 6 , $P < 0.01$).

Discussion

Nasopharyngeal carcinoma in Southern China and Southeastern Asia shows a distinct morphology arising from epithelial cells of the head and neck region [22], with a strong geographical preference, and an overall high prevalence in the aforementioned areas [23]. Our current study determined the effects of GPC6 knockdown on cell growth, cell cycle distribution, and

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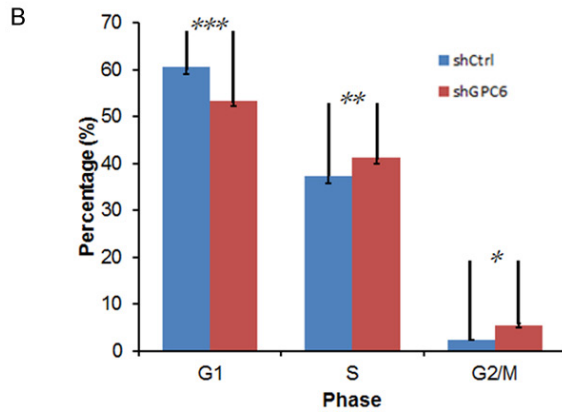
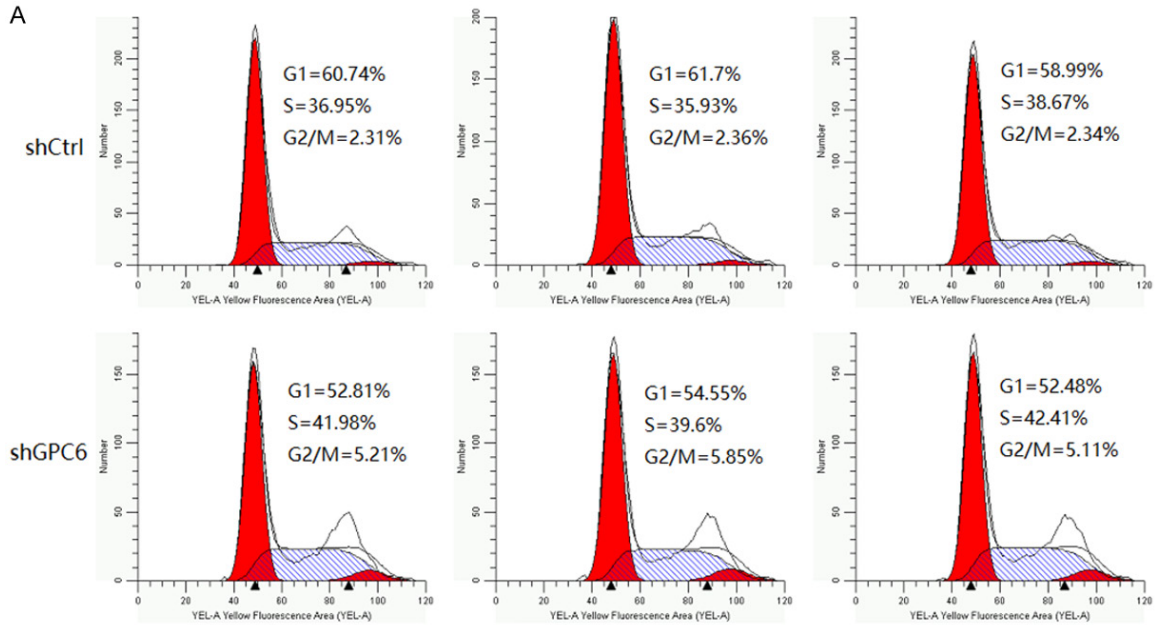


Figure 4. GPC6 knockdown in regulation of cell cycle distribution in CNE-2Z cells after 5-day culture. A. Representative graphs of flow cytometry analysis of CNE-2Z cell cycle using the PI staining. B. FI-FACS analysis of cell cycle reveals that GPC6-siRNA-infected cells showed the proportion in G1 phase ($53.28\% \pm 1.11\%$ vs. $60.48\% \pm 1.37\%$, $P=0.002$, $***P < 0.05$), S phase ($41.33\% \pm 1.51\%$ vs. $37.18\% \pm 1.38\%$, $P=0.02$, $**P < 0.05$) and G2/M phase ($5.39\% \pm 0.40\%$ vs. $2.34\% \pm 0.02\%$, $P=0.005$, $*P < 0.05$).

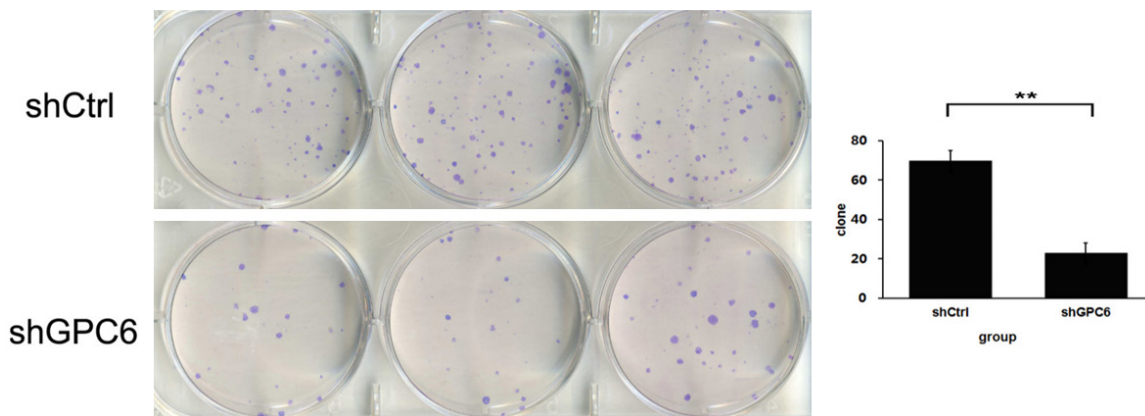


Figure 5. GPC6 knockdown in suppression of CNE-2Z cell colony formation. The colony number of shGPC6-infected tumor cells was lower after colony counting under a microscope compared with control group. $**P < 0.01$ as compared to the control group.

colony formation. We found that GPC6 was expressed in nasopharyngeal carcinoma CNE-2Z and 5-8F cell lines, whereas the lentivirus-

mediated GPC6 knockdown suppressed tumor cell growth, cell cycle re-distribution, and colony formation *in vitro*. In addition, we found that

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there was up to a 100-fold decrease in GPC6 protein level in Western blot images, but the quantitation of qRT-PCR data only showed approximately 2-fold changes in GPC6 mRNA level, suggesting that GPC6 shRNA primarily blocked GPC6 protein translation. Our current study demonstrated that GPC6 knockdown possessed a critical effect on the inhibition of nasopharyngeal carcinoma progression, and further study should assess the *in vivo* effects of GPC6 knockdown and related molecular therapies on nasopharyngeal carcinoma.

Glypicans attach to the outer cell membrane through the glycosylphosphatidylinositol anchor and can be released into the extracellular matrix space [18, 24], therefore, playing a role in cell growth and survival. Glypican-6 is a heparan sulfate proteoglycan with a molecular mass of 62.7 kDa, one of the six mammalian members of the glypican family of proteoglycans. A previous study demonstrated that GPC6 appeared to be a putative target gene in the amplified region of chromosome 13q21-q32 in gastric cancer [14]. Another study suggested that induced levels of GPC6 mRNA could serve as a biomarker for the detection of CD8+ T-cell infiltration and prediction of survival of early stage ovarian cancer patients [25]. Our current study revealed that GPC6 expression in nasopharyngeal carcinoma cell lines and GPC6 knockdown could suppress nasopharyngeal carcinoma tumor progression *in vitro*, further supporting the suggestion that GPC6 may be a target for cancer therapy. Indeed, lentivirus-carrying RNAi, which silences gene expression with a high specificity and low toxicity, may be a promising gene therapy to target cancer in the near future [26]. Our current data on lentivirus-carrying RNAi to silence gene expression was consistent with a previous study reported by Liu et al., showing that lentivirus-mediated small interfering RNAs were successfully knocked down RPL34 expression in human gastric cancer SGC-7901 cells [27]. Knockdown of RPL34 expression using siRNA technique significantly reduced tumor cell growth and arrested tumor cells at the S phase of cell cycle, whilst increasing apoptosis [27]. Together, these studies demonstrated that lentivirus is a useful gene delivery vehicle, due to the ability to deliver siRNA molecules into the host cells and to target specific genes, even in quiescent cells [27]. Furthermore, cell growth

and colony formation are two important events in monitoring tumor progression *in vitro*. In our current study, we first demonstrated that GPC6 was expressed in nasopharyngeal carcinoma cell lines and lentivirus-mediated GPC6 knockdown markedly reduced nasopharyngeal carcinoma cell proliferation and colony formation *in vitro*. We also found that GPC6-siRNA-treated cells displayed a lower proportion of cells in the G1 phase, but significantly increased the proportion of cells in the S and G2/M phases, indicating the occurrence of tumor cell apoptosis. These data demonstrate that GPC6 may play an important role in the progression of nasopharyngeal cancer. Future studies will validate the anti-apoptotic role of GP6 in nasopharyngeal carcinoma cell survival and association with poor survival in nasopharyngeal carcinoma patients.

It has been well documented in the literature that glypicans regulate signaling of Wnt, Hedgehog, FGF, and BMP genes [24]. GPC6 was reported to promote invasiveness of breast cancer cells through a non-canonical Wnt5A signaling pathway [28], while nuclear factor of activated T-cells (NFAT) was able to induce GPC6 expression to inhibit canonical Wnt and β -catenin activities, but promote Wnt5A activity, resulting in JNK and p38 α kinase activation [29]. Nevertheless, future studies will help understand the role of GPC6 and the underlying molecular mechanisms of GPC6 action in nasopharyngeal carcinoma, although it is reasonable to speculate that GPC6 regulates Wnt, Hedgehog, FGF, and BMP gene signaling to mediate cellular function in nasopharyngeal carcinoma.

Nevertheless, our current study is a proof-of-principle study and requires further exploration prior to the clinical use of GPC6 shRNA in the treatment of nasopharyngeal carcinoma. In summary, to the best of knowledge, our current study is the first to report GPC6 expression and knockdown in nasopharyngeal carcinoma. We found that GPC6 is expressed in nasopharyngeal carcinoma cells and that downregulation of GPC6 expression using lentivirus-driven GPC6 RNAi in CNE-2Z cells suppressed the malignant phenotype of tumor cells. Hence, these results suggest that GPC6 may be a potential novel strategy for the future treatment of nasopharyngeal carcinoma. Further

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study of GPC6 regulatory mechanisms in nasopharyngeal tumorigenesis should be undertaken to ensure findings are clinically transferrable.

Disclosure of conflict of interest

None.

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