GSG2 knockdown suppresses cholangiocarcinoma progression by regulating cell proliferation, apoptosis and migration

Jun Zhou  
Department of Peking University Cancer Hospital & Institute

Ranglang Huang (✉ scihrlang@163.com)  
the Third Xiangya Hospital of the Central South University  https://orcid.org/0000-0003-0710-5203

Wanpin Nie  
Department of Hepatobiliary and Pancreatic Surgery, the Third Xiangya Hospital of the Central South University

Jiajia Yuan  
Department of Peking University Cancer Hospital & Institute

Zeyu Zhang  
Department of Hepatobiliary and Pancreatic Surgery, the Third Xiangya Hospital of the Central South University

Liangliang Mi  
Department of Hepatobiliary and Pancreatic Surgery, the Third Xiangya Hospital of the Central South University

Changfa Wang  
Department of Hepatobiliary and Pancreatic Surgery, the Third Xiangya Hospital of the Central South University

Primary research

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Abstract

Background: Cholangiocarcinoma (CCA) is the second most common type of hepatocellular carcinoma with high aggressiveness and extremely poor prognosis. Homo sapiens germ cell associated 2 (GSG2) is a histone H3 threonine-3 kinase required for normal mitosis. Nevertheless, the role and mechanism of GSG2 in progression and development of CCA remain elusive. Methods: In the present study, correlation between GSG2 and CCA was elaborated by IHC, Mann-Whitney U and Spearman grading correlation. GSG2 knockdown cell lines were successfully constructed and further used to construct mouse xenotransplantation models. Subsequently, cell proliferation, cell apoptosis, cell cycle and cell migration were detected by MTT assay, Flow Cytometry and wound-healing assay, respectively. Results: It was demonstrated that GSG2 was overexpressed in CCA specimens and relevant cell lines. The statistical analysis determined that high GSG2 expression was positively associated with more advanced tumor grade. Importantly, GSG2 knockdown inhibited cell proliferation, migration, promoted cell apoptosis and arrested cell cycle in G2 phase. Meanwhile, in vivo results also supported that GSG2 knockdown inhibit tumor growth. Additionally, GSG2 was involved in CCA progression via suppressing EMT. Conclusions: In summary, our findings suggested that GSG2 may be a potential therapeutic target for CCA patients.

Background

Cholangiocarcinoma (CCA) is originated from the epithelium lining the biliary tree and could be classified into intrahepatic cholangiocarcinoma (iCCA) and extra-hepatic cholangiocarcinoma (eCCA), which was further stratified into perihilar (pCCA) and distal (dCCA) [1, 2]. CCA is the second most common primary malignancy in hepatocellular carcinoma [3, 4]. Most patients with CCA are diagnosed in the advanced and metastatic stages of the disease due to lack of signs and symptoms in the early stage [3]. Unfortunately, CCA is an invasive malignancy with a median survival of less than 2 years from diagnosis [5]. This fact, as well as the adverse outcomes of the current use of local and systemic therapy, is the cause of poor prognosis in CCA patients and strongly supports the need for new therapeutic drugs and strategies [6]. The molecular mechanism of CCA have been partially identified in recent years, including isocitrate dehydrogenases (IDH1 and 2) mutations and fibroblast growth factor receptor 2 (FGFR2) fusions, as well as gene mutations involved in chromatin remodeling, such as AT-rich interaction domain 1A (ARID1A), protein poly-bromo1 (PBRM1), and BRCA1-associated protein 1 (BAP1) [7, 8]. Elucidation of key molecules involved in CCA development, inhibition of some mutated genes or inhibition of related signaling pathways through specific inhibitors opens new horizons for novel therapeutic approaches [9, 10]. Thus, a deeper understanding of CCA molecular mechanisms is needed to lay the foundation for targeted therapy.

Homo sapiens germ cell associated 2 (GSG2) (also known as Haspin) gene encodes serine/threonine protein kinases, which are mainly expressed in haploid germ cells [11, 12]. GSG2 has been shown to be weakly expressed in proliferating normal somatic cells but plays a crucial role in mitosis, where it specifically phosphorylates Thr-3 in histone H3 (H3T3) [12-14]. GSG2 RNAi in tumor cells prevents chromosome alignment and normal mitosis, suggesting that GSG2 inhibitors may be a novel anti-mitotic
agent that prevents cancer cell proliferation [15, 16]. On the other hand, GSG2 does not belong to family of eukaryotic protein kinase, which is a structurally unique kinase and may result in fewer off-target effects [17]. Ample evidence suggested that identifying specific GSG2 inhibitors may be feasible and useful for basic biological studies and as candidates for cancer therapy [18-21]. Therefore, we were committed to exploring the molecular mechanisms of GSG2 in CCA to determine whether GSG2 inhibitors have the potential to be molecular anticancer drugs against CCA.

In this study, we explored the role and mechanism of GSG2 in the regulation of CCA progression and development. First, we found that GSG2 was abundantly expressed in CCA and its expression was positively correlated with pathological grade. Additionally, it was revealed that GSG2 knockdown inhibited cell proliferation, migration, promoted cell apoptosis and arrested cell cycle in G2 phase. These findings highlighted the significance of GSG2 in CCA and evaluated its therapeutic potential.

**Materials And Methods**

**Tissue Microarray Chip**

Total 80 cases/80 point of microarray chips of CCA were purchased from Xi’an Alina Biotechnology Co., Ltd (Xi’an, China). These included 48 cases of eCCA, 27 cases of iCCA, and 5 cases of intrahepatic bile duct tissue. This paraffin embedded human tissue chips were 1.5 mm in diameter and 5 µm in thickness and stored immediately at -4°C for later use.

**Cell Culture**

The human CCA cell lines including HCCC-9810, QBC939 and HUCCT1, which were provided by Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultivated applying RPMI-1640 medium (Gibco, life technologies, California, USA) and added with 10% fetal bovine serum and 100 mg/mL streptomycin plus 100 UI/mL of penicillin (Gibco, life technologies, California, USA). The incubation atmosphere was at 37°C and contained 5% CO2.

**Immunohistochemical (IHC) Staining**

Antigen of the chip was recovered by boiling citric acid buffer for 30 min. After that they were blocked with 3% H2O2 and rabbit serum and then incubated with anti-GSG2 (1:200, Bioss, Cat. # bs-15413R) at 4°C overnight. Subsequently, secondary antibody goat anti-rabbit (1:200, Beyotime, Cat. # A0208) was added and immersed for 2 h at room temperature. These chips were stained with DAB solution and then with hematoxylin. All tissue chips were photographed with microscopic, viewed with ImageScope and CaseViewer. IHC total scores were determined by staining percentage scores (classified as: 1 (1%-24%), 2 (25%-49%), 3 (50%-74%), 4 (75%-100%)) and staining intensity scores (scored as 0: less color, 1: brown, 2: light yellow, 3: dark brown). Finally, high or low expression of GSG2 were determined by the median of IHC experimental scores of all tissues.
Cell Transfection, Lentivirus Production and Infection

For knockdown of GSG2, small interfering RNAs specifically targeting GSG2 (shGSG2) (RNAi-10055, RNAi-00176, RNAi-00177) were designed by Shanghai Yibeirui Biomedical science and Technology Co., Ltd. and negative controls were scramble siRNAs (shCtrl) (sequence were detailed in following Table). The shGSG2 sequences were inserted into BR-V108 vectors (Shanghai Yibeirui, China) containing green fluorescent protein (GFP) which acted as a detectable marker.

HCCC-9810, QBC939 and HUCCT1 cells were seeded into 96-well plates at an approximate density of 2×10^5 cells per well. Subsequent to 24 h cultivation, cells were infected with 100 μL lentiviral vectors (1×10^7 TU/mL) additive with ENI.S and polybrene (10 μg/mL, Sigma-Aldrich). Next, reconstructed vectors were introduced into 293T cells for the generation of lentiviruses, together with pHelper 1.0 and pHelper 2.0 as packing vectors. 72 h following infection, supernatants containing lentivirus expressing shGSG2 or shCtrl were harvested. Subsequently, qPCR analysis and Western Blot were used to evaluate the GSG2 knockdown efficiency. Finally, these successfully infected cells were subjected to following function assays.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>RNAi-Pbr10055</td>
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</tr>
<tr>
<td>RNAi-Pbr00176</td>
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<tr>
<td>RNAi-Pbr00177</td>
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<tr>
<td>Scramble</td>
<td>TTCTCCGAACGTGTCACGT</td>
</tr>
<tr>
<td>BR-V108</td>
<td>CCGGTTCTCCGAACGTGTCACGTTTCAAGAGAA</td>
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</table>

qPCR

RNA of HCCC-9810 and QBC939 cells was isolated with TRIZOL reagent (Invitrogen, Carlsbad, USA) with DNase I according to the manufacturer with a standard procedure. RNA was converted into cDNA using M-MLV RT kit (Promega). cDNA was amplified with SYBR Green mastermixs Kit (Vazyme) and BioRad CFX96 sequence detection system (Bio Rad company, berkeley, CA). Sequence were detailed in following Table and GAPDH as an internal reference. Results of qPCR were evaluated with 2^−ΔΔCt method and converted into the fold change.

<table>
<thead>
<tr>
<th>Primer</th>
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<th>Downstream Sequence (5’-3’)</th>
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<tr>
<td>GAPDH</td>
<td>TGACTTCAACACGCGACACCCA</td>
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Western Blot
HCCC-9810 and QBC939 cells were fully lysed in ice-cold RIPA buffer (Millipore) to obtain protein. The protein concentration detection was performed by BCA Protein Assay Kit (HyClone-Pierce, Cat. # 23225). 20 μg protein from per group was separated by 10% SDS-PAGE, transferred onto PVDF membranes, and analyzed with required antibodies (antibodies were detailed in following Table). The blots were visualized by Amersham ECL plus TM Western Blot system and the density of the protein band was analyzed.

<table>
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<tr>
<th>Antibody Name</th>
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<td>1:3000</td>
<td>Rabbit</td>
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<td>AP0063</td>
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</table>

**MTT Assay**

HCCC-9810 and QBC939 cells were cultured in 96-well plates at 2×10^3 per milliliter. MTT solution (Cat. # JT343, GenView) 5 mg/mL was added. After incubation for 4 h, 100 μL dimethyl sulfoxide (DMSO) were added to each well. Following that, formazan was quantified at 24 h, 48 h, 72 h, 96 h and 120 h by measuring the 490 nm absorbance with microplate reader. Notably, OD490 indirectly reflected the number of viable cells.

**Cell Apoptosis Analysis by Flow Cytometry**

HCCC-9810 and QBC939 cells were collected, washed and stained by working solution (500 μL binding buffer with 5 μL annexin V-FITC) for 15 min at room temperature in the dark. The FACSCanto II Flow Cytometry (BD Bioscience) was used to analyze apoptotic cells. Cell apoptosis rate was calculated in 3 randomly selected visual fields. Notably, apoptosis rate = (number of positive cells/number of all counted cells) ×100%.

**Cell Cycle Analysis by Flow Cytometry**

HCCC-9810 and QBC939 cells were inoculated in 96-well plates until cell density reached 85%. Afterwards, these cells were harvested, centrifuged (1200 × g), and resuspended. Cells were then washed with PBS and labeled with 500 μL PI (BD Biosciences, Franklin Lakes, NJ, USA). The FACSCanto II Flow Cytometry was used to analyze the ratio of cells in the G1, S and G2 phases distribution.
Wound-healing Assay

HCCC-9810 and QBC939 cells were cultivated into 96-well plates. Next day, the 10 μL pipette was performed to scratch a wound at the middle of each well. Then, the medium was substituted with the 1% FBS contained fresh medium. Photographs of the wound were captured at pre-set time points (4 h, 8 h, 24 h and 48 h). The percentage of migration was evaluated utilizing Image Pro Plus.

Human Apoptosis Antibody Array

For signal pathway gene detecting, Human Apoptosis Antibody Array (abcam, Cat. # ab134001) were applied following the manufacturer’s instructions. Briefly, QBC939 cells were lysed in cold RIPA buffer (Millipore) and protein concentration was detected by BCA Protein Assay Kit (HyClone-Pierce). Proteins were incubated with blocked array antibody membrane overnight at 4°C. After washing, Detection Antibody Cocktail (1:100) was added incubating for 1 h, followed by incubated with HRP linked streptavidin conjugate for 1 h. All spots were visualized by enhanced ECL and the signal densities were analyzed with Image J software (National Institute of Health).

Animal Xenograft Model

Animal experiments were approved by the Ethics Committee of The IRB of Third Xiangya Hospital, Central South University and conducted in accordance with guidelines and protocols for animal care and protection. The four-week-old male BALB/c nude mice were obtained at Shanghai Lingchang Biotechnology Co., Ltd. for xenograft model. Mice were injected with 4× 10^6 HUCCT1 cell, which were randomly divided into two groups, shCtrl and shGSG2. Mice weight and tumor volume were detected twice a week after 10 days of subcutaneous injection. Tumor volume = \( \pi/6 \times L \times W \times W \) where L was long diameter and W was short diameter. Following, 0.7% pentobarbital sodium at a dose of 10 μL/g was injected into the abdominal cavity to anesthetize the mice and to observe fluorescence using bioluminescence imaging (IVIS spectral imaging system, emission wavelength of 510 nm). After 32 days, mice were sacrificed, tumor was taken out and measured with a ruler as reference, the specific scale was read and then photographed.

Ki67 Staining

Mice tumor tissues were fixed in 10% formalin and then were paraffin-embedded. 5 μm slides were cut and immersed in xylene and ethanol. Tissue slides were blocked with 3% PBS-H2O2 and incubated with anti-Ki67 (1:200, abcam, Cat. # ab16667) and HRP goat anti-rabbit IgG (1:400, abcam, Cat. # ab6721). Slides were stained by Hematoxylin (Baso, Cat. # BA4041) and Eosin (Baso, Cat. # BA4022). Stained slides were examined at 100× and 200× objective lens microscopic.

Statistical Analysis

All experiments were accomplished in triplicate and data were shown as mean ± SDs. Statistical analyses and graphs were performed by GraphPad Prism 7.0 (Graphpad Software) and P value < 0.05 as
Results

GSG2 is related to the development of CCA

According to the results of IHC staining, expression of GSG2 in CCA tissues was significantly higher than that in normal tissues (P < 0.001) (Table 1, Figure 1A). Besides, we also found that mRNA levels of GSG2 were abundantly expressed in CCA cell lines detected by qPCR (Figure 1B). Subsequent Mann-Whitney U statistical analysis revealed a significant correlation between GSG2 expression and pathological grade (P < 0.001) (Table 2). Consistently, Spearman grade correlation analysis further confirmed that GSG2 expression was positively correlated with pathological grade (Table 3). More specifically, the increase in GSG2 expression was accompanied by CCA deterioration. Taken together, GSG2 was not only highly expressed in CCA, but also related to the development of CCA.

Construction of GSG2 knockdown CCA cell model

Firstly, qPCR analysis expressed that the transfection efficiency of GSG2 in shGSG2 (RNAi-10055) group was 99.6% and then it was used in following experiments (P < 0.01) (Figure 1C). Furthermore, the percentage of GFP positive cells infected with shCtrl or shGSG2 for 72 h observed under fluorescence microscope was more than 80% (Figure 1D). The results of qPCR showed that in HCCC-9810 and QBC939 cells, compared with shCtrl group, the knockdown efficiency of GSG2 in shGSG2 group was 62.4% (P < 0.001) and 43.6% (P < 0.001), respectively (Figure 1E). Not surprisingly, the results of the Western Blot analysis showed a consistent downregulation of protein expression in HCCC-9810 and QBC939 cells compared with controls (Figure 1F). The above results clearly revealed that CCA cell model of GSG2 knockdown was successfully constructed.

Knockdown of GSG2 inhibits CCA cell proliferation in vitro

The results of MTT assay were presented as Figure 2A, cell proliferation of HCCC-9810 and QBC939 cells in shGSG2 group was obviously slower compared with shCtrl group (P < 0.001). These indicated that viable cells were both reduced as time goes on after knockdown of GSG2. All in all, GSG2 knockdown has a certain inhibitory effect on CCA cell proliferation.

Knockdown of GSG2 promotes CCA cell apoptosis and arrests cell cycle in vitro

Cell apoptosis and cell cycle were assessed using Flow Cytometry. Ratio of apoptotic cells in shGSG2 group of HCCC-9810 and QBC939 cells was significantly higher than that in shCtrl group (P < 0.001) (Figure 2B). Moreover, results of cell cycle distribution detection showed that the percentage of cells in S
phase sharply decreased whereas that in G2 phase obviously increased in shGSG2 group, compared with shCtrl group (P < 0.001) (Figure 2C). Comprehensive results suggested that GSG2 knockdown promote CCA cell apoptosis and arrest cell cycle in G2 phase.

**Knockdown of GSG2 inhibits CCA cell migration in vitro**

Migration capacity of CCA cells with or without GSG2 knockdown was identified by wound-healing assay. The results displayed that the migration rate of HCCC-9810 cells in shGSG2 group in 8 h was decreased by 57% compared with shCtrl group (P < 0.001). Meanwhile, the migration rate of QBC939 cells at 48 h was decreased by 83% (P < 0.001) (Figure 2D). Additionally, the expression of EMT biomarkers was detected by Western Blot analysis and the results were shown in the Figure 2E. The protein level of E-cadherin was upregulated in shGSG2 group compared with shCtrl group in HCCC-9810 and QBC939 cells; contrarily, protein expression of N-cadherin, Vimentin were downregulated. Obviously, knockdown of GSG2 inhibited CCA cell migration by suppressing EMT.

**Exploration of downstream molecular mechanism of GSG2 in CCA**

The expression of related proteins in the Human Apoptosis signaling pathway was detected after knocking down the expression of GSG2 in QBC939 cells. Results showed that the protein expression levels of BIM, Caspase3, HSP60, p21, p53 were significantly upregulated, while the protein expression of IGFBP-2, Survivin and TNF-β was obviously downregulated (P < 0.05) (Figure 3A). Otherwise, downstream molecular mechanism of GSG2 in CCA cell was elicited through Western Blot (Figure 3B). The results showed that the protein expression of p-Akt, CCND1 and PIK3CA was downregulated in the experimental group compared with the control group; while MAPK9 proteins expression was upregulated, and there was no significant alteration in Akt. In a word, GSG2 was involved in progression of CCA by regulating apoptosis-related factors and downstream signaling.

**Knockdown of GSG2 in CCA cells impaired tumor growth in vivo**

HUCCT1 cells infected with shGSG2 or shCtrl were subcutaneously injected into nude mice to establish xenograft model. Importantly, average volume of tumor in shGSG2 group was significantly reduced by 33.85 ± 10.92 mm³ compared with the shCtrl group (P <0.01) (Figure 4A). In particular, the average tumor weight of mice inoculated with shGSG2 cells was significantly lighter than that of the shCtrl group (P <0.01) (Figure 4B). Additionally, in vivo imaging indicated that bioluminescence expression was apparently weaker in shGSG2 than that in shCtrl (P < 0.01), also indicating the lower tumor burden in shGSG2 group (Figure 4C). Moreover, Ki67 staining displayed that the proliferative activity of tumors in shGSG2 group was significantly lower than that in shCtrl group (P < 0.01) (Figure 4D). In a word, knockdown of GSG2 impaired tumorigenicity in vivo, which was in accordance with the aforementioned in vitro results.

Table 1. Expression patterns in cholangiocarcinoma cancer tissues and para-carcinoma tissues revealed in immunohistochemistry analysis.
### Table 2. Relationship between GSG2 expression and tumor characteristics in patients with cholangiocarcinoma cancer

<table>
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</thead>
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<td></td>
<td></td>
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</tr>
<tr>
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### Table 3. Relationship between GSG2 expression and tumor characteristics in patients with cholangiocarcinoma cancer
Discussion

The physiological function of GSG2 has not been well illustrated, and the underlying mechanism of how GSG2 correlates with tumor progression is far from clear. In our study, we demonstrated for the first time that GSG2 promoted the development of CCA. Through *in vitro* and *in vivo* experiments, we investigated the role of GSG2 in CCA progression. It was found that GSG2 knockdown significantly suppressed cell proliferation and tumor growth. Conversely, CCA cell apoptosis was obviously promoted upon GSG2 knockdown, which may be resulted from the regulation of apoptosis-related proteins such as BIM, Caspase3, HSP60, p21, p53, IGFBP-2, Survivin and TNF-β. Moreover, the results of wound-healing assay revealed that GSG2 may regulate cell migration through influencing EMT related proteins. Moreover, we estimated that GSG2 was involved in CCA progression via Akt signaling.

Unlimited growth, aggressive, reduced apoptosis and cycle disorders are markers of cancer and play an important role in the development of cancer [22]. A lot of studies termed that the epithelial-to-mesenchymal transition (EMT) promotes invasion and metastasis in various types of tumor [23]. This process involved the downregulation of epithelial-specific marker E-cadherin and upregulation of mesenchymal markers including Vimentin, and N-cadherin [24]. In our study, knockdown of GSG2 inhibited CCA cell migration by inducing EMT, which including E-cadherin upregulation and N-cadherin, Vimentin downregulation.

Moreover, apoptosis is a key biological process to prevent uncontrolled cell proliferation and eliminate harmful cells, and anti-apoptotic stimulation is a hallmark of various cancers [25, 26]. Mechanisms of apoptosis and their effector proteins included pro-apoptotic protein, anti-apoptotic Bcl-2 family members, and inhibitor of apoptosis proteins (IAP) [26]. BIM, Caspase3, HSP60, p21 and p53 were all pro-apoptotic proteins, which may contribute to apoptosis induction [27-30]. Caspase3 functions as an executor of apoptosis by activating DNA fragmentation [31]. Alternatively, IGFBP-2 played an important role in cell proliferation, invasion, angiogenesis and apoptosis [32]. Simultaneously, Survivin as an important member of IAP, which were considered to be a regulator of apoptosis related proteins and prevention of apoptosis, and it was strongly expressed in CCA [33-35]. TNF-β also was identified as a key mediator between apoptosis and cancer cell progression [36]. Thus, it was possible that GSG2 knockdown initiate the process of apoptosis through balancing the expression of pro-apoptotic and anti-apoptotic factors.

Previous studies have revealed that PI3K/AKT, CCND1/CDK6 and MAPK pathways play key role in developing CCA [37-40]. For example, Wang *et al.*, clarified that TSPAN1 was involved in CCA progression via PI3K/AKT pathway [41]. Zhang *et al.*, suggested that S100A11 promoted cell proliferation by p38/MAPK signaling pathway in iCCA [42]. This study discovered that GSG2 knockdown contributed to downregulation of P-Akt, CCND1, PIK3CA, and upregulation of MAPK9. Therefore, we elicited that GSG2
exerted effects in CCA cells by modulating protein pathways, such as PI3K/Akt, CCND1/CDK6 and MAPK9.

**Conclusions**

This study found that expression of GSG2 was positively relevant with pathological grade. Importantly, we revealed that GSG2 knockdown inhibited CCA cell progression by regulating cell proliferation, apoptosis, cell cycle, and cell migration. In summary, role and preliminary regulatory mechanisms of GSG2 in CCA were demonstrated. Our findings suggested that GSG2 may be a potential therapeutic target for CCA patients.

**Declarations**

**Ethics approval and consent to participate**

The research was approved by the Ethics Committee of The IRB of Third Xiangya Hospital, Central South University and conducted in accordance with guidelines and protocols for animal care and protection.

**Consent for publication**

Not applicable.

**Availability of data and material**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**

Not applicable.

**Authors’ contributions**

RH designed this program. JZ, JY and CW operated the cell experiments, WN performed animal experiments. ZZ and LM conducted the data collection and analysis. JZ produced the manuscript which was checked and revised by RH. All the authors have confirmed the submission of this manuscript.

**Acknowledgements**

Not applicable.

**References**


Figures
Figure 1

GSG2 is highly expressed in CCA and the construction of GSG2 knockdown cell model. (A) Expression levels of GSG2 in CCA tumor tissues and adjacent normal skin tissues were detected by IHC staining. (B) GSG2 expression in CCA cells was detected by qRT-PCR. (C) qPCR was used to screen knockdown efficiency of GSG2 in shGSG2 (RNAi-00177), shGSG2 (RNAi-10055), and shGSG2 (RNAi-00176). (D) Transfection efficiencies for HCCC-9810 and QBC939 cells were evaluated by expression of green fluorescent protein 72 h post-infection. (E, F) The specificity and validity of the lentivirus-mediated shRNA knockdown of GSG2 expression was verified by qPCR (E) and Western Blot analysis (F). The data were presented as the mean ± SD (n = 3). *P<0.05, **P<0.01, ***P<0.001.
**Figure 2**

Knockdown of GSG2 inhibits cell proliferation and migration, promotes apoptosis in CCA cells. (A) Cell proliferation of HCCC-9810 and QBC939 cells with or without knockdown of GSG2 was evaluated by MTT assay. Flow Cytometry analysis based on Annexin V-APC staining was utilized to detect cell apoptotic ratio (B) and cell cycle distribution (C) for HCCC-9810 and QBC939 cells. (D) Cell migration of HCCC-9810 and QBC939 cells with or without knockdown of GSG2 was evaluated by wound healing assay. (E) EMT marker proteins of HCCC-9810 and QBC939 cells with or without knockdown of GSG2 were detected by Western Blot. The data were presented as mean ± SD (n = 3), *P<0.05, **P<0.01, ***P<0.001.

**Figure 3**

Exploration of downstream molecular mechanism of GSG2 in CCA cells (A, B) Human apoptosis antibody array analysis was performed in QBC939 cells with or without GSG2 knockdown. The difference (A) and mergence (B) were shown separately in figures. (C) Densitometry analysis was performed and the gray values of differentially expressed proteins were shown. (D) The expression of downstream protein pathway was observed by Western Blot in QBC939 cells with or without GSG2 knockdown. The data were presented as mean ± SD (n = 3), *P<0.05, **P<0.01, ***P<0.001.
Figure 4

Knockdown of GSG2 inhibits tumor growth in mice xenograft models. (A) The volume of tumors in shCtrl group and shGSG2 group was measured after post-injection. (B) The images and average weight of tumors in shCtrl group and shGSG2 group. (C) The imaging and total bioluminescent intensity of tumors in shCtrl group and shGSG2 group. (D) The staining images and expression levels of Ki67 in tumor tissues in shCtrl group and shGSG2 group. The data were presented as mean ± SD (n = 3), *P<0.05, **P<0.01, ***P<0.001.