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UCP2 promotes proliferation and chemoresistance through regulating the NF- κ B/ β -catenin axis and mitochondrial ROS in gallbladder cancer

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Abstract

Uncoupling protein 2 (UCP2) is a mitochondrial anion carrier which plays a key role in energy homeostasis. UCP2 is deregulated in several human cancers and has been suggested to regulate cancer metabolism. However, the role of UCP2 in gallbladder cancer has not been defined.

Using clinical samples, we found highly expressed UCP2 in gallbladder cancer tissues, and higher expression levels of UCP2 correlated with worse clinical characteristics. To study whether UCP2 promotes gallbladder cancer growth, UCP2 stable knockdown cells were generated, and cell proliferation was suppressed in these knockdown cells. Further studies demonstrated that glycolysis was inhibited and IKK β , as well as the downstream signaling molecules NF- κ B/FAK/ β -catenin, were downregulated in UCP2 knockdown cells. More importantly, gallbladder cancer cells became sensitive to gemcitabine treatments when UCP2 was inhibited. UCP2 knockdown suppressed the activation of the NF- κ B/ β -catenin axis and promoted the increases in mitochondrial ROS in gallbladder cancer cells exposed to gemcitabine treatments. The UCP2 inhibitor genipin suppressed xenograft tumor growth and sensitized grafted tumors to gemcitabine treatments. These results suggest targeting UCP2 as a novel therapeutic strategy for the treatment of gallbladder cancer.

Graphical Abstract

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Authors Contributions: JY, LS, WL, BL, and YZ conducted the experiments; JY, LS, and YZ analyzed the data; JY and YZ wrote the manuscript.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

Keywords

UCP2; gallbladder cancer; glycolysis; chemotherapy resistance; β -catenin; NF- κ B; ROS

1. Introduction

As the most frequently encountered malignancy of the biliary tract, gallbladder cancer (GBC) has various incidence in different parts of the world. In certain ethnic populations, such as Hispanic, Indian, and East Asian, gallbladder cancer has a relatively high incidence, from 3.9 to 8.6/100,000^[1,2]. The relative uncommonness of gallbladder cancer causes a poor understanding of the disease^[1]. Because the majority of cases are diagnosed in advanced stages, gallbladder cancer has a dismal overall survival even in the present day: the overall 5-year survival rate for unresectable gallbladder cancer is less than 5%^[3]. Therefore, identifying novel molecular mechanisms associated with tumorigenesis and chemoresistance may provide a potential therapeutic approach for the treatment of this aggressive type of cancer.

Uncoupling protein 2 (UCP2), a member of the mitochondrial uncoupling protein family, is ubiquitously expressed in vertebrate cells. The physiological function of uncoupling proteins is to decrease the proton gradient between mitochondrial inner and outer membrane, known as “proton leak reactions”, resulting in decreases in electrochemical potential (ψ_m)^[4]. UCP2 is aberrantly expressed in several cancers including colon, skin, lung, prostate, pancreatic, and breast cancer^[5–8]. Highly expressed UCP2 in cancer cells has been regarded as an important regulator of metabolic plasticity: shifting from oxidative phosphorylation to glycolysis^[9,10]. Moreover, because of the activity of reducing electron transport chain-derived reactive oxygen species (ROS), UCP2 is also considered as a member of the mitochondrial antioxidant system^[11,12].

In this study, the expression levels of UCP2 in human gallbladder cancer tissues were detected and the association between UCP2 expression and disease progression was evaluated. Both a UCP2 inhibitor and UCP2 stable knockdown gallbladder cancer cells were used to study whether UCP2 contributes to gallbladder cancer progression and to understand the underlying mechanism.

2. Materials and methods

2.1. Cell culture

Gallbladder cancer (GBC) cell line G-415 was purchased from the RIKENBioResource Center (Ibaraki, Japan). GBC-SD gallbladder cancer cell line was obtained from the Chinese Academy of Sciences Shanghai Branch Cell Bank (Shanghai, China). Mycoplasma

contamination was routinely monitored. G-415 and GBC-SD cells were cultured in RPMI-1640 medium (VWR Corporate, Radnor, PA, USA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin.

2.2. Establishment of UCP2 stable knockdown (KD) clones

G-415 cells were infected with pooled UCP2 shRNA lentivirus (Applied Biological Materials, Vancouver, Canada). The target sequences include 5'-CGGTTACAGATCCAAGGAGAA-3', 5'-GGCCTGTATGATTCTGTCA-3', 5'-GCACCGTCAATGCCTACAA-3' and 5'-CGTGGTCAAGACGAGATACATGAACTCTG-3'. Seventy-two hours post infection, the cells underwent puromycin (1 µg/ml, Invitrogen, Carlsbad, CA, USA) selection for 2 weeks. The resistant colonies were selected and amplified, and Western blot analysis was performed to identify the UCP2 KD clones. The clones infected with the scrambled siRNA lentivirus were similarly selected and used as the control.

2.3. Clinical tissue samples

Tissue samples were obtained from 70 patients at the Shaoxing People's Hospital from January 2013 to February 2018. Informed consent was obtained from the patients and the tissue acquisition protocol was approved by the Ethics Committee of Shaoxing People's Hospital. There were 30 samples from gallbladder cancer patients and 40 samples from patients undergoing cholecystectomy because of gallbladder stones or benign gallbladder polyps. Among the gallbladder cancer patients, 13 patients underwent chemotherapy while 17 patients did not. The clinical characteristics of these patients including lymph node invasion, TNM staging, differentiation grade, histological type, and survival time were collected. Fresh tissues were frozen in liquid nitrogen and used for RNA and protein extraction.

2.4. RNA extraction and polymerase chain reaction

Total RNAs were isolated from tissues using TRIzol (Invitrogen) and reverse transcribed into cDNAs using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. After SYBR Green PCR Master Mix was added, a quantitative real-time PCR was performed using the ABI 7500 Real-time PCR system (Applied Biosystems). The designed PCR primers were as followings: UCP2 forward primer, 5'-TGGTCGGAGATACCAAAGCACC-3'; UCP2 reverse primer, 5'-GCTCAGCACAGTTGACAATGGC-3'; β -actin forward primer, 5'-AGAAGGAGATCACTGCCCTGGCACC-3'; β -actin reverse primer, 5'-CCTGCTTGCTGATCCACATCTGCTG-3'. β -actin was used as an endogenous control. The experiments were repeated three times. Relative expression levels were determined using the 2^{-C_t} methods [13].

2.5. Western blot analysis

Primary antibodies against β -actin, FAK (Focal adhesion kinase), p⁻³⁹⁷Tyr-FAK, IKK α (I-kappaB kinase α), IKK β (I-kappaB kinase β), β -catenin, total-p53, p21, and MnSOD were

purchased from Santa Cruz Biotechnology (Santa Cruz, Dallas, TX, USA). Primary antibodies against UCP2, NF- κ B p65, p-⁵³⁶Ser-NF- κ B p65, S6 ribosomal protein and cleaved caspase 3, were purchased from Cell Signaling Technology (Boston, MA, USA). Primary antibodies against wild type p53 were purchased from MilliporeSigma (Burlington, MA, USA). On a 10% SDS-PAGE gel, 20 μ g total protein was electrophoresed, transferred onto a polyvinylidene fluoride membranes, blocked, incubated with a primary antibody and then with a horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Immunoreactive bands were visualized using a chemiluminescence solution (Genesee Scientific, El Cajon, CA, USA). Experiments were repeated three times. β -actin was employed as an endogenous control. The pixel densities of the protein bands were quantified using ImageJ.

2.6. Cell proliferation assays

Two thousand viable GBC cells were seeded in 96-well plates for cell proliferation assay. Cell proliferation assays were performed using the IncuCyte live-cell system (Essen BioScience, Ann Arbor, MI, USA). Cells were imaged every 4 h and the proliferation rates based on confluency were determined using the IncuCyte software. Eight replicate wells from each cell were analyzed.

2.7. Detection of mitochondrial membrane potential (ψ_m)

Ten thousand GBC cells were seeded in 96-well plates and incubated overnight. Cells were incubated with growth medium containing 2 μ g/ml of JC-1 (5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide, Thermo Fisher, Waltham, MA, USA) for 30 min. The dye was removed and cells were washed with PBS. Fluorescence intensity was measured immediately by fluorescence spectrometry (For JC-1 green, Ex=485 nm, Em=525 nm; for JC-1 red, Ex=535 nm, Em=590 nm). Six replicate wells from each cell were analyzed.

2.8. 3D spheroid growth

96-well round-bottom plates (ultra-low attachment, Corning, NY, USA) were used for seeding cells. Five thousand viable GBC cells were seeded in 90 μ l medium containing 5% Matrigel (BD Bioscience, San Jose, CA, USA). After centrifuging at 700 rpm for 5 min, plates were incubated overnight to form spheroids. The next day, 10- μ l treatments (genipin and/or gemcitabine) were added to the culture. The spheroids were imaged every 48 h and the volumes were calculated with the following formula: volume = $\frac{4}{3} \pi b^2 c$ (b=semi-major axis, c=semi-minor axis). Six replicate wells from each cell were analyzed.

2.9. Chemosemitivity assays

For the chemosensitivity assays, cisplatin and gemcitabine (Cayman Chemical, Ann Arbor, MI, USA) were used as the chemo drugs to evaluate the effect of genipin (a specific UCP2 inhibitor [14], purchased from Cayman Chemical) on chemosensitivity. GBC Cells were seeded into 96-well plates (four thousand cells/well) and allowed to attach overnight. Cells were treated with various concentrations of chemicals and 50 μ M genipin concomitantly in at least six replicate wells. After incubated for 48 h, viable cells were determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide, Sigma, St. Louis,

MO, USA) assay. One-tenth volume of MTT diluted in serum-free medium was added to each well, and the plates were further incubated at 37°C for 4 h. Formazan crystals were dissolved in DMSO. The absorbance at 595 nm was measured using a spectrometer (Bio-Rad, Philadelphia, PA, USA).

2.10. Lactate assay

Intracellular levels of lactate were measured using the Lactate Assay Kit from Biovision (Milpitas, CA, USA) according to the manufacturer's instructions. Lactate levels were normalized to the amount of proteins extracted from cells. Experiments were repeated three times.

2.11. Flow cytometry for cell cycle analysis

1×10^6 GBC cells were seeded into 60-mm dishes and harvested after 24 h of culture. After washing with PBS, cells were fixed with 70% ice-cold ethanol at 4°C overnight. After washing, cells were stained with propidium iodide (PI, the final concentration at 50 μ g/ml, Fisher Scientific) and 10% RNase A (Fisher Scientific) in PBS for 30 min at 37°C. A total of 1×10^4 events were analyzed per assay by FACSscan analysis using the LSRII SORP (Becton-Dickinson, Franklin, NJ, USA). Experiments were repeated three times.

2.12. Nude mice xenografts

Eight-week-old male athymic nude mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All animals were kept in sterile laminar flow cabinets under appropriate pathogen-free conditions. All procedures were approved by the Animal Care and Use Committee of Zhejiang University and conformed to the Care and Use of Laboratory Animals guide published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). 2×10^6 GBC cells in log-phase growth were suspended in 0.1 ml PBS and subcutaneously injected into the dorsal flank of nude mice. One week later, two experiments were performed to study the effect on tumor growth inhibition of genipin by itself or in combination with gemcitabine. In the genipin alone study, 12 mice were randomly assigned as either Vehicle or Gen (genipin) group ($n=6$ per group). The mice in the Gen group were injected intraperitoneally with genipin (60 mg/kg). The injections were performed three times per week for three weeks. For the chemosensitivity study, 12 mice were randomly assigned as either GEM (gemcitabine) or GEM+Gen group ($n=6$ per group). In the GEM group, mice were injected intraperitoneally with gemcitabine (4 mg/kg). In the GEM+Gen group, mice were injected intraperitoneally with gemcitabine (4 mg/kg) and genipin (60 mg/kg). These injections were also performed three times per week for three weeks. Mice were euthanized and tumor tissues were harvested and weighed.

2.13. Detection of mitochondrial ROS (mtROS)

MitoSOXTM Red probe (Invitrogen) was used to detect mitochondrial ROS. Flow cytometry assays were performed as previously described [15, 16]. Briefly, 1×10^6 GBC cells were suspended in 0.5 ml RPMI-1640 medium containing 2.5 μ M MitoSOX in a 37 °C water bath, protected from light, for 40 min. After washed with PBS, the red fluorescence was

detected by FAC Scan using the LSRII SORP (Becton-Dickinson). Experiments were repeated three times.

2.14. Superoxide dismutase (SOD) activity assay

The SOD activity was measured using the Superoxide Dismutase Assay Kit (Cayman Chemical). After exposed to gemcitabine (10 $\mu\text{g/ml}$) for 24 h, whole-cell lysate was prepared by sonication in PBS (containing proteinase inhibitors). This experiment was performed according to the manufacturer's protocol. The final SOD activity was normalized by protein concentration. Experiments were repeated three times.

2.15. Statistical analysis

Data were presented as means $\pm$ SD. Statistical significance between two groups was determined using the Student's t-test. One-way ANOVA followed by the Tukey–Kramer adjustment was used to examine differences among multiple groups. The χ^2 test was used to show differences in categorical variables. $p < 0.05$ was considered to be significant. All statistical analyses were conducted using SPSS 13.0.

3. Results

3.1. UCP2 expression was upregulated in gallbladder cancer

UCP2 mRNA levels in GBC tissues were determined by real-time PCR analysis. Gallbladder stones (GBS) and gallbladder polyps (GBP), potential risk factors for GBC, are more common and often result in cholecystectomy [17]. The normal epitheliums from resected gallbladder due to GBS or GBP were employed as the control. As shown in Fig. 1A, the mRNA levels of UCP2 were higher in tumor samples, compared to normal epithelium samples from GBS and GBP. Similarly, the protein levels of UCP2 were also higher in tumor tissues, compared to the tumor-adjacent normal tissues from the same patient (Fig. 1B).

Patients enrolled in this study were classified according to the mRNA levels of UCP2 and clinical characteristics were compared. Interestingly, we found that TNM staging and grade of differentiation were positively whereas prognosis was negatively associated with UCP2 expression levels (Table 1). These results suggest that the upregulation of UCP2 may be common in GBC, which affects disease progression and treatment outcomes.

3.2. Inhibition of UCP2 suppressed proliferation of GBC cells in vitro and in vivo

Genipin has been identified as an inhibitor of UCP2 and a potential drug candidate for cancer [14]. To determine whether genipin could inhibit the uncoupling activity of UCP2, mitochondrial membrane potential (ψ_m) was examined in GBC cells. The results showed that mitochondrial membrane potential was significantly increased after genipin treatments (Fig. 2A). The decreased intracellular levels of lactate suggested that genipin inhibited the metabolic activity of UCP2 (promoting glycolysis) (Fig. 2B).

Given that UCP2 is upregulated and its levels are negatively associated with prognosis, whether inhibition of UCP2 by genipin suppresses GBC cell proliferation was examined. As

shown in Fig. 2C, all three concentrations of genipin, (25 μ M, 50 μ M, and 100 μ M) significantly inhibited the growth of G-415 and GBC-SD cells. Next, 3D spheroid growth of G-415 cells was used to mimic the ability of tumor formation *in vitro*. The results showed that G-415 cells formed smaller spheroids after treated with a moderate (50 μ M) or a high concentration (100 μ M) of genipin, comparing to the vehicle-treated group (Fig. 2D).

Finally, xenograft tumor studies were performed using GBC-SD cells. Genipin administered by peritoneal injection significantly reduced the tumor weight, compared to the vehicle-treated group (Fig. 2E).

3.3. UCP2 knockdown inhibited cell proliferation and suppressed the NF- κ B/FAK/ β -catenin signaling in gallbladder cancer cells

UCP2 knockdown (KD) clones from G-415 cells were established for mechanistic studies. Proliferation assays were performed to confirm the consistent effect between UCP2 KD and UCP2 inhibition by genipin. The results showed that UCP2 KD G-415 cells grew significantly slower compared to the control cells (Fig. 3A).

Given the uncoupling and metabolic reprogramming activities of UCP2, mitochondrial membrane potential and cellular lactate levels were detected in UCP2 KD cells. As shown in Fig. 3B & 3C, mitochondrial membrane potential was significantly higher whereas the lactate level was remarkably lower in UCP2 KD cells compared to the control cells. These results suggest that aerobic glycolysis is inhibited due to increased membrane potential caused by UCP2 knockdown.

The IKK/NF- κ B pathway plays an important role in the process of aerobic glycolysis [18–21]. Given suppressed glycolysis in UCP2 KD cells, the expression levels of IKK α / β were examined. The results showed that both IKK α and IKK β were downregulated in UCP2 KD cells (Fig. 3D). Moreover, decreased levels of phospho-NF- κ B p65 was also detected in UCP2 KD cells, whereas the total p65 levels remained unaltered (Fig. 3D). NF- κ B is a well-characterized transcription factor that activates many targets including the PTK2 gene, which encodes FAK [22–24]. Not only the total protein levels of FAK but also the phosphorylated form were downregulated in UCP2 KD cells (Fig. 3D), β -catenin is a classical oncogene in various cancers and FAK plays a key role in regulating the expression of β -catenin [25, 26]. We also observed that β -catenin was downregulated in UCP2 KD cells (Fig. 3D).

Given the potential influence of UCP2 on metabolism, the expression levels of S6 ribosomal protein were examined to detect whether there was an overall trend of decreased protein translation in UCP2 knockdown cells. The results shown in Fig. 3D demonstrated that this was not the case.

As a well-known transcription factor, β -catenin regulates various oncogenic genes including cyclin D1, an important regulator of cell cycle and a marker for proliferation [27]. Indeed, cyclin D1 was downregulated in UCP2 KD cells (Fig. 3D). Cell cycle analysis was next performed. We found that more gallbladder cancer cells accumulated at the G0/G1 phase in UCP2 KD cells (Fig. 3E).

3.4. UCP2 inhibition sensitized gallbladder cancer cells to chemotherapy in vitro and in vivo

Chemoresistance is an important factor for the poor prognosis of gallbladder cancer. We found that UCP2 was upregulated in G-415 cells after treated with gemcitabine (Fig. 4A). Therefore, we tested the possibility of using genipin as an adjuvant in combination with chemotherapy in treating gallbladder cancer. Compared to gemcitabine or cisplatin alone, combination treatments induced more cell death in GBC cells including both G-415 and GBC-SD (Fig. 4B).

The effect of UCP2 inhibition on chemosensitivity of GBC was also studied in xenograft tumors of GBC-SD cells. Mice were administered intraperitoneally with both gemcitabine and genipin (GEM+Gen group), or gemcitabine alone (GEM group) one week after cells were seeded. As shown in Fig. 4C, the xenograft tumors in the combination group were significantly smaller than those in the gemcitabine alone group.

3.5. NF- κ B/ β -catenin signaling and mitochondrial ROS were involved in UCP2-contributed chemoresistance

To study the underlying mechanism linking the aberrant expression of UCP2 and chemoresistance, the sensitivity of UCP2 KD cells to chemo drugs was detected. The results from both the MTT assay and 3D culture showed that UCP2 KD cells were more sensitive to both gemcitabine and cisplatin treatments compared to the control cells (Fig. 5A and 5B). In consistent, more cleaved Caspase-3 proteins were detected in UCP2 KD cells after gemcitabine treatments (Fig. 5C).

Both NF- κ B and β -catenin were upregulated after gemcitabine treatments in GBC cells, coinciding with the expression levels of UCP2 (Fig. 5C). However, these increases were not observed in UCP2 KD cells after treated with gemcitabine (Fig. 5C), which suggests that the NF- κ B/ β -catenin axis may at least partially, mediate UCP2's drug resistance activity.

Previous studies have demonstrated that UCP2 could regulate the levels of mitochondrial ROS [28, 29]. In GBC cells, MnSOD, an important scavenger in the mitochondrion's oxidant scavenging systems [30], was upregulated after gemcitabine treatments (Fig. 4A). This suggests that oxidative stress in mitochondria may play an important role during chemotherapy of GBC cells. Using the flow cytometry assay, we found that mtROS levels were much higher in UCP2 KD cells after gemcitabine treatments, compared to the control cells (Fig. 5D). In consistent, MnSOD was only upregulated in the control cells, not in UCP2 KD cells (Fig. 5C). SOD activities were also measured in these cells after gemcitabine treatments. The levels of SOD activity in UCP2 KD cells were significantly lower than those in the control cells (Fig. 5E). These results indicate that NF κ B/ β -catenin signaling and mitochondrial ROS are involved in UCP2-mediated chemoresistance.

4. Discussion

Aerobic glycolysis, also known as the "Warburg effect", is an important acquired ability for malignant cells to adapt to metabolic stress [31]. Gallbladder cancer is an aggressive type of cancer with a dismal prognosis, and recent studies have suggested a potential association

between gallbladder cancer and aerobic glycolysis [32–34]. UCP2 plays an important role in the process of aerobic glycolysis. Aberrant expression of UCP2 accompanied by active glycolysis has been observed in various cancers including colon, skin, and pancreatic cancer [5,10,35]. To our knowledge, this is the first study demonstrating the association between UCP2 expression and gallbladder cancer disease progression. Although the sample size of gallbladder cancer was small ($n=30$), it still provided a meaningful and intriguing result that UCP2 expression is amplified in gallbladder cancers. A large variation in the expression of UCP2 mRNA was observed (Fig. 1A), and it could be due to the fact that inflammation and immune response status among these patients differ, and UCP2 expression is associated with immune response [36,37]. Our study also shows that inhibition of UCP2 reduces the levels of lactate in gallbladder cancer cells, suggesting that highly expressed UCP2 contributes to the metabolic shift to aerobic glycolysis.

Aerobic glycolysis interacts with various metabolism-related signaling molecules in cancer cells. It has been reported that IKK β /NF- κ B can be potentiated by glycolysis-linked protein O-glycosylation [19,20,39]. Our study shows that IKK β is downregulated when glycolysis is inhibited in UCP2 knockdown GBC cells. Subsequently, the phosphorylated NF- κ B-p65, the active form of NF- κ B, is also downregulated in UCP2 knockdown cells. As a NF- κ B and p53 downstream gene [23,24], FAK is overexpressed and promotes tumor progression in various advanced-stage cancers including hepatocellular carcinoma, breast cancer, and ovarian cancer [22,26,40,41]. In our study, both total and phosphorylated FAK are remarkably downregulated in UCP2 knockdown GBC cells. FAK expression can be suppressed by p53 [23,24], and IKK β /NF- κ B can be further activated by glycolysis in p53-deficient cells [19,23]. Besides mitochondrial metabolism, p53 and NF- κ B also regulate cellular metabolism and the Warburg effect [42]. These suggest that p53 mutant or deletion may amplify glycolysis-induced IKK β /NF- κ B/FAK activation in cancer cells. As the most frequently mutated tumor suppressor gene in human cancers, p53 also belongs to one of the most frequent genetic alterations in gallbladder cancer [1,43]. The incidence of p53 mutations in gallbladder cancer can reach 52.4% in the high-incidence areas of this disease, such as Japan and Chile [44]. We detected the status of p53 in G-415 cells used in this study. No band was detected in the cell lysis, using a specific antibody against wild type p53 (unpublished data). Moreover, p21, a canonical p53 target gene [45], was not detected either (unpublished data). A minor band was detected in G-415 cells by a non-specific antibody that recognizes both wild type and mutant p53. These results suggest that G-415 cells may bear mutant p53. GBC-SD, another cell line used in our study, is known to bear mutant p53 [46]. Taken together, our results suggest that UCP2 promotes glycolysis-stimulated IKK β /NF- κ B/FAK activation in gallbladder cancer cells. In future studies, inhibitors to the IKK β /NF- κ B/FAK signaling and glycolysis will be tested for the treatment of gallbladder cancer in an *in vivo* model.

β -catenin in the Wnt/p-catenin pathway is a key oncogenic molecule in many human cancers and it can be potentiated by FAK [25,26,47]. There is one study showing that β -catenin is aberrantly expressed in gallbladder cancer [48]. Our results show that β -catenin is downregulated in UCP2 knockdown GBC cells, potentially as a result of the suppression of the IKK β /NF- κ B/FAK axis. As a direct target of β -catenin, cyclin D1 is downregulated in UCP2 knockdown cells. It is worth noting that the transcriptional regulation of cyclin D1

can also be controlled by NF- κ B [49,50], which is also downregulated in UCP2 knockdown GBC cells. Coincidentally, several studies have demonstrated that overexpression of cyclin D1 in tumor cells suppresses mitochondrial respiration with a consequent shift towards glycolysis [51,52]. Cyclin D1 drives G1–S transition in the cell cycle. More gallbladder cancer cells are present at the G0/G1 phase in UCP2 knockdown cells, which is consistent with the downregulation of cyclin D1 (Fig. 3E). Taken together, our results suggest that inhibition of UCP2 suppresses glycolysis, leading to the inactivation of the IKK β /NF- κ B/FAK/ β -catenin/cyclin D1 axis, and subsequent suppression of cell proliferation.

Resistance to chemotherapy is tightly associated with dismal prognosis in gallbladder cancer. Aerobic glycolysis can contribute to chemoresistance and targeting glycolysis is being considered as a potential approach to improve chemotherapy [53–55]. Inhibition of UCP2 has also been found to sensitize several cancer cells to chemotherapy [5,56]. Our result shows that in gallbladder cancer, both overall prognosis and prognosis after chemotherapy, are negatively associated with UCP2 expression levels (Table 1), which suggests that highly expressed UCP2 may contribute to chemoresistance in gallbladder cancer. This pro-survival activity of UCP2 may be mediated by the NF- κ B/ β -catenin axis. Both NF- κ B and β -catenin are key transcription factors to regulate downstream targets and help cancer cells survive during chemotherapy [57–60]. Our results show that NF- κ B/ β -catenin is activated upon treatments with chemo drugs in control GBC cells, but not in UCP2 knockdown cells, and these UCP2 knockdown cells are more sensitive to chemotherapy. In future studies, NF- κ B/ β -catenin can be re-expressed in UCP2 knockdown cells to further demonstrate the link between UCP2, NF- κ B/ β -catenin, and chemoresistance. Similarly, inhibitors to β -catenin can also be tested in gallbladder treatments.

A few studies report that UCP2 inhibition triggers ROS-dependent cancer cell death during chemotherapy [5,12,56]. UCP2 location, “Warburg effect” initiation, and ROS generation have an intimate connection with mitochondria. A mutually regulated relationship has been suggested between ROS and the “Warburg effect” [61]. Interestingly, our result demonstrates that MnSOD is upregulated in gallbladder cancer cells exposed to gemcitabine treatments (Fig. 4A), and this increase is diminished in UCP2 knockdown GBC cells under the same treatments, which is accompanied by decreased SOD activities. Concurrently, the levels of mitochondrial ROS are higher in UCP2 knockdown GBC cells (Fig. 5D). These results suggest that MnSOD expression/activity is tight to UCP2 in these GBC cells, although the exact mechanism is unclear. This phenomenon provides another mechanism of chemoresistance for UCP2 highly expressed gallbladder cancer cells.

Our results demonstrate that UCP2 is highly expressed in human gallbladder cancer, which negatively affects diagnosis. Inhibition of UCP2 suppresses cell proliferation and reduces chemoresistance of gallbladder cancer cells *in vivo and in vitro*. As summarized in the schematic figure (Fig. 6), the glycolysis-linked IKK β /NF- κ B/FAK/ β -catenin axis and mitochondrial ROS play the major roles in the whole process. Altogether, targeting UCP2 may serve as a potential therapy for gallbladder cancer.

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Figure 1.

The expression levels of UCP2 were increased in human gallbladder cancer tissues. (A) Real-time PCR was performed to detect the mRNA levels of UCP2 in gallbladder cancer tissue samples. (B) Western blot analysis of UCP2 protein levels in paired gallbladder cancer tissue samples. NGB: normal gallbladder; GBC: gallbladder cancer; N: normal tissues; T: tumor tissues.

Figure 2.

UCP2 inhibition suppressed GBC cell growth *in vitro* and *in vivo*. (A) Detection of mitochondrial membrane potential (ψ_m) in G-415 cells after 24-h genipin treatments. (B) Detection of intracellular lactate levels after genipin treatments. (C) Cell growth curves of G-415 and GBC-SD cells after genipin treatments. (D) Detection of spherical growth of G-415 cells after genipin treatments. (E) Xenograft tumor growth of GBC-SD cells after genipin treatments. Gen: genipin. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus the 0 μM group.

Figure 3.

UCP2 knockdown suppressed the NF- κ B/FAK/ β -catenin signaling cascade in GBC cells. (A) Cell growth curves of UCP2 knockdown G-415 cells. (B) Detection of mitochondrial membrane potential (ψ_m) in UCP2 knockdown G-415 cells. (C) Detection of intracellular lactate levels in UCP2 knockdown G-415 cells. (D) Detection of the IKK β /NF- κ B/FAK/ β -catenin pathway in UCP2 knockdown G-415 cells. p-NF- κ B p65 was normalized to total p65; whereas other proteins were normalized to β -actin. (E) Cell cycle analysis of UCP2 knockdown G-415 cells. UCP2-KD1: UCP2 knockdown clone 1; UCP2-KD2: UCP2 knockdown clone 2. * P < 0.05; ** P < 0.01; *** P < 0.001 versus the scramble vector-infected cells.

Figure 4.

UCP2 inhibition sensitized gallbladder cancer cells to chemotherapy *in vitro* and *in vivo*. (A) UCP2 expression was upregulated and the NF-κB /β-catenin axis was activated in G-415 cells after exposed to gemcitabine (10 μg/ml). p-NF-κB p65 was normalized to total p65; whereas other proteins were normalized to β-actin. (B) Determination of chemosensitivity of G-415 and GBC-SD cells with or without genipin treatment (50 μM). Eight replicate wells from each cell were analyzed. (C) Inhibition of UCP2 activity by genipin sensitized grafted tumor to gemcitabine treatment *in vivo*. Gen: genipin; GEM: gemcitabine; DDP: cisplatin. * $P < 0.05$ versus the vehicle group. # $P < 0.05$; ## $P < 0.01$, significant differences between the two groups.

Figure 5.

The increases in the NFκB/β-catenin signaling and mitochondrial ROS upon chemo drug treatments were suppressed in UCP2 knockdown G-415 cells. (A) Determination of chemosensitivity of UCP2 knockdown cells. (B) Spheroids growth of UCP2 knockdown G-415 cells after gemcitabine (10 μg/ml) treatment for one week. (C) Detection of the expression levels of UCP2, cleaved Caspase-3, and the NF-κB /β-catenin axis in UCP2 knockdown G-415 cells after gemcitabine treatments. (D) Detection of the levels of mitochondrial ROS in scramble control and UCP2 knockdown G-415 cells with or without gemcitabine treatment. Δ value = (median value *with* gemcitabine) – (median value *without* gemcitabine). (E) Detection of SOD activity in UCP2 knockdown G-415 cells after gemcitabine treatments. UCP2-KD1: UCP2 knockdown clone 1; UCP2-KD2: UCP2 knockdown clone 2; GEM: gemcitabine. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 versus

scramble control cells. # $P < 0.05$, significant difference between the two groups, n.s. = non-significant between the two groups.

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Figure 6.
Schematic representation of how UCP2 promotes growth and chemoresistance in gallbladder cancer cells.

Table 1.

The relationship between UCP2 expression and clinicopathological features of gallbladder cancer.

Variables	N	UCP2		P
		High	Low	
Gender				
Male	9	7	2	0.925
Female	21	16	5	
Age (years)				
60	19	14	5	0.612
<60	11	9	2	
Lymph node invasion				
Present	21	18	3	0.073
Absent	9	5	4	
TNM staging				
I-II	11	6	5	0.029
III-IV	19	17	2	
Serum CEA level (ng/ml)				
>5	8	7	1	0.398
5	22	16	6	
Serum CA19-9 level (U/ml)				
>37	12	10	2	0.481
37	18	13	5	
Serum CA50 level (IU/ml)				
>25	7	7	0	0.096
25	23	16	7	
Serum CA125 level (U/ml)				
>35	7	7	0	0.096
35	23	16	7	
Overall prognosis				
12 month	13	13	0	0.008
>12 month	17	10	7	
Prognosis after chemotherapy				
12 month	4	4	0	0.026
>12 month	9	3	6	
Differentiation				
G1	8	3	5	0.004
G2	9	7	2	
G3	13	13	0	

The expression level of UCP2 in a tumor tissue was higher than the average UCP2 expression level in normal epitheliums of gallbladder, was defined as 'High'; the opposite was defined as 'low'. $P < 0.05$ was considered to be significant and showed in bold. TNM, tumor-node-metastasis classification according to the AJCC/UICC 8th edition; CEA, carcinoembryonic antigen; CA, carbohydrate antigen; G1, well differentiated; G2, moderately-differentiated; G3, poorly-differentiated.