Inhibition of XPO1 with KPT-330 induces autophagy-dependent apoptosis in gallbladder cancer by activating the p53/mTOR pathway

Cheng Zhao1,2†, Zi-yi Yang1,2†, Jian Zhang1,2†, Ou Li1,2, Shi-lei Liu1,2, Chen Cai1,2, Yi-jun Shu1,2, Li-jia Pan1,2*, Wei Gong1,2* and Ping Dong1,2*

Abstract

Background: Gallbladder cancer (GBC) is a highly aggressive malignant cancer in the biliary system with poor prognosis. XPO1 (chromosome region maintenance 1 or CRM1) mediates the nuclear export of several proteins, mainly tumor suppressors. Thus, XPO1 functions as a pro-oncogenic factor. KPT-330 (Selinexor) is a United States Food and Drug Administration approved selective inhibitor of XPO1 that demonstrates good therapeutic effects in hematologic cancers. However, the function of XPO1 and the effect of KPT-330 have not been reported in GBC.

Methods: We analyzed the correlation between XPO1 expression levels by q-PCR and clinical features of GBC patients. Cell proliferation assays were used to analyze the in vitro antitumor effects of XPO1 inhibitor KPT-330. mRNA sequencing was used to explore the underlying mechanisms. Western blot was performed to explore the relationship between apoptosis and autophagy. The in vivo antitumor effect of KPT-330 was investigated in a nude mouse model of gallbladder cancer.

Results: We found that high expression of XPO1 was related to poor prognosis of GBC patients. We observed that XPO1 inhibitor KPT-330 inhibited the proliferation of GBC cells in vitro. Furthermore, XPO1 inhibitor KPT-330 induced apoptosis by reducing the mitochondrial membrane potential and triggering autophagy in NOZ and GBC-SD cells. Indeed, XPO1 inhibitor KPT-330 led to nuclear accumulation of p53 and activated the p53/mTOR pathway to regulate autophagy-dependent apoptosis. Importantly, KPT-330 suppressed tumor growth with no obvious toxic effects in vivo.

Conclusion: XPO1 may be a promising prognostic indicator for GBC, and KPT-330 appears to be a potential drug for treating GBC effectively and safely.

Keywords: Chromosome region maintenance 1, Gallbladder cancer, KPT-330, Apoptosis, Autophagy

Introduction

Gallbladder cancer (GBC) is the most common and aggressive malignant tumor of the human biliary system [1]. Due to the lack of typical clinical manifestations and early diagnostic tools, many patients are diagnosed at an advanced stage [2]. Up to now, the only treatment for GBC has been surgical removal, and even than only 10% of patients at the early stages have access to surgery [3, 4]. Thus, patients with advanced...
GBC have rapid tumor progression, and their prognosis is very poor, with a 5-year survival rate of only 5% [5]. Although many chemotherapies and targeted therapies are now available, the 5-year survival rate has not improved significantly [6]. Therefore, it is extremely urgent to discover more effective molecular targets and therapeutic modalities for the treatment of GBC.

Nucleocytoplasmic transport of macromolecules is an important means for tumor cells to survive and proliferate [7]. Chromosome region maintenance 1 (XPO1, exportin 1, or CRM1) belongs to the karyopherin beta family, and is the predominant receptor mediating nucleocytoplasmic transport [8, 9]. XPO1 is responsible for transporting most of the tumor suppressors and growth regulators; up to 220 proteins bearing the nuclear export signal (a leucine-rich region) are recognized by XPO1 [10]. XPO1 is overexpressed in a variety of tumors, including pancreatic cancer, osteosarcoma, glioma, cervical cancer, and hematological malignancies [8]. Therefore, inhibition of XPO1 has the potential to be an effective anticancer therapeutic approach [11, 12].

Leptomycin B specifically recognizes and blocks XPO1 and inhibits cells growth in different tumor cell lines; unfortunately, its strong toxicity limits its clinical application [13]. This inspired scientists to develop new selective inhibitors of nuclear export (SINEs). KPT-330 (Selinexor), a novel oral SINE, has been approved by the United States Food and Drug Administration for the treatment of refractory multiple myeloma and relapsed/refractory diffuse large B-cell lymphoma. Mechanistically, KPT-330 prevents the nucleocytoplasmic transport activity of XPO1, leading to accumulation of molecules that it transports in the nucleus [14–17]. However, the biological function of XPO1 in GBC, and the therapeutic effect of KPT-330 in GBC, have not yet been evaluated.

In this study, we investigated the biological function of XPO1 in GBC and the anti-cancer effects of the XPO1 inhibitor, KPT-330. Our study is the first to demonstrate the significant association between overexpression of XPO1, and poor clinical characteristics and prognosis in GBC patients. It was found that XPO1 inhibitor KPT-330 inhibited proliferation and colony formation of GBC cells. XPO1 inhibitor KPT-330 also induced mitochondria-dependent apoptosis and autophagy of GBC cells. Furthermore, KPT-330 activated the p53/mTOR pathway to induce autophagy-dependent apoptosis. In a nude mouse model of GBC, KPT-330 inhibited tumor growth without causing obvious toxic effects. Overall, XPO1 is a promising prognostic indicator for GBC, and KPT-330, a specific inhibitor of this transporter, might be a more effective and less cytotoxic chemotherapeutic agent for the treatment of GBC.

### Materials and methods

#### Patients and clinicopathological data

Gallbladder cancer tissues and cholecystitis tissue were obtained from the Department of General Surgery in Xinhua Hospital affiliated with Shanghai Jiao Tong University School of Medicine (Shanghai, China). No patient underwent chemotherapy, radiotherapy, or immunotherapy before surgery. This study received the approval of the Ethics Committee of Xinhua Hospital, and all patients provided informed consent. All specimens were confirmed by pathological diagnosis. The cancer stage of each patient was assessed according to the 8th edition of the American Joint Committee on Cancer Staging Manual.

#### Immunohistochemistry (IHC) and scoring

Immunohistochemistry (IHC) staining was conducted using the Servicebio Technology (Wuhan, China). Immunohistochemistry was conducted according to a standard protocol. The IHC score was calculated according to the staining extent and intensity as follows: Negative (1): <10% immunoreactive cells; Weak (2): 10%-49% immunoreactive cells; Moderate (3): 50%-74% immunoreactive cells; Strong (4): ≥75% immunoreactive cells. The staining color was scored as no staining (0), light yellow (1), brownish yellow (2) and brown (3). The final score was calculated by multiplying the two scores. Involved antibodies were given in Table 1.

#### Cell culture and reagents

The human GBC cell lines (NOZ and GBC-SD) were obtained from the cell bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM medium (Gibco) and were supplemented 10% fetal bovine serum (Gibco), at 37 °C in a humidified chamber containing 5% CO2. KPT-330, rapamycin (treatment concentration of 0.4 μM and processing time of 6 h), chloroquine (treatment concentration of 0.2 μM and processing time of 6 h), Z-VAD-FMK (treatment concentration of 0.1 μM and processing time of 6 h), and MHY1485 (treatment concentration of 0.5 μM and processing time of 6 h) were obtained from Selleck Chemicals (Houston).

#### Table 1 Information of antibody

| Antibody | Catalogue | Dilution | Company                  |
|----------|-----------|----------|--------------------------|
| XPO1     | 46,249    | 1:2000   | Cell Signaling Technology, USA |
| PCNA     | 131105    | 1:2000   | Cell Signaling Technology, USA |
| Ki67     | 91295     | 1:2000   | Cell Signaling Technology, USA |
**TdT-mediated dUTP nick-end labeling assay**

One Step TUNEL Apoptosis Assay Kit (Beyotime) was used to conduct TdT-mediated dUTP nick-end labeling (TUNEL) assays according to the manufacturer’s instructions. The cells were examined under a fluorescence microscope (Leica).

**Flow cytometry**

Flow cytometry was used to conduct the apoptosis, cell cycle assays and mitochondrial membrane potential assay. NOZ and GBC-SD cells were treated with KPT-330 for 48 h in the respective IC50 values. Annexin V-FITC kit (BD Biosciences, CatLog: 556,547), Cell Cycle Analysis Kit (Beyotime) and mitochondrial membrane potential assay kit with JC-1(Beyotime) were used according to the manufacturer’s protocol. In the end, the samples were analyzed using flow cytometry (CytoFLEX, Beckman Coulter).

**Cell transfection**

The siRNAs were synthesized by Genomeditech (PRC). The siRNAs used were listed in Additional file 1: Table S1. RFect reagent (Baidai) was used for transfection according to the manufacturer’s protocol. In brief, cells were seeded in 6-well plates at about 30% density. siRNA was diluted to 50 nM with 50 uL of Opti-MEM (Gibco) and 5 uL of RFect (Baidai) was diluted with 50 uL of Opti-MEM (Gibco). Then transfection reagent dilution and siRNA dilution were mixed and left for 20 min at room temperature. The mixture was then added to the cell culture dish containing 1 mL of Opti-MEM (Gibco) and replaced with fresh medium after 6 h. After 48 h, cells were collected for further experiments.

**Quantitative real-time PCR**

Total RNAs were extracted from tissue samples, NOZ and GBC-SD cells by using Trizol reagent (Invitrogen). cDNA was generated using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa) according to the manufacturer’s instructions. The primers used for amplification were listed in Additional file 1: Table S2. Target gene expression level was detected using SYBR Green method and the StepOne-Plus Real-time PCR System (Applied Biosystems).

**Western blot analysis**

Firstly, proteins were isolated with RIPA Lysis buffer (Beyotime) and quantified using BCA assay (Beyotime). Next, proteins were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore). Then, 5% skim milk was used to block the blots for 2 h at room temperature. A series of primary antibodies were added to the appropriate position of the PVDF membranes and incubated overnight at 4 °C. Finally, all blots reacted with the suitable HRP-conjugated secondary antibody (Beyotime) and the immunoreactive bands were detected by chemiluminescence and visualized using a Gel Doc 2000 (Bio-Rad). The list of antibodies is given in Table 2.

**Ad-mCherry-GFP-LC3B transfection**

Cells were seeded in 96-well plates and then transfected with Ad-mCherry-GFP-LC3B adenovirus (Beyotime) at an MOI of 4 for 6 h, following treatment with KPT-330 for 48 h in the respective IC50 values. Then, images were taken with a fluorescence microscope (Leica).

Viruses used in infection were calculated according to Eq.

\[
\text{Virus(µL)} = \frac{\text{MOI} \times \text{Cell number}}{\text{Virus titer (PFU mL)}} \times 1000
\]

**Table 2 Information of antibody**

| Antibody       | Catalogue | Dilution | Company                             |
|----------------|-----------|----------|-------------------------------------|
| GAPDH          | 5174      | 1:1000   | Cell Signaling Technology, USA      |
| XPO1           | 46,249    | 1:1000   | Cell Signaling Technology, USA      |
| p53            | 2527      | 1:1000   | Cell Signaling Technology, USA      |
| LC3-I/II       | 12,741    | 1:1000   | Cell Signaling Technology, USA      |
| Bcl-2          | 4223      | 1:1000   | Cell Signaling Technology, USA      |
| BAX            | 5023      | 1:1000   | Cell Signaling Technology, USA      |
| PARP           | 9532      | 1:1000   | Cell Signaling Technology, USA      |
| Cleaved-PARP   | 5625      | 1:1000   | Cell Signaling Technology, USA      |
| Caspase9       | 9504      | 1:1000   | Cell Signaling Technology, USA      |
| Cleaved-Caspase9 | 9509    | 1:1000   | Cell Signaling Technology, USA      |
| Caspase3       | 9662      | 1:1000   | Cell Signaling Technology, USA      |
| Cleaved-Caspase3 | 9664   | 1:1000   | Cell Signaling Technology, USA      |
| p-mTOR (Ser2448) | 5536   | 1:1000   | Cell Signaling Technology, USA      |
| mTOR           | 2983      | 1:1000   | Cell Signaling Technology, USA      |
| p21            | 2947      | 1:1000   | Cell Signaling Technology, USA      |
| p27            | 3686      | 1:1000   | Cell Signaling Technology, USA      |
| H3             | 60,932    | 1:1000   | Cell Signaling Technology, USA      |
Cell proliferation assay and IC50 assay

The cell proliferation and IC50 assay were performed with a cell counting Kit-8 assay (CCK-8) (Yeasen). Approximately 100 cells of NOZ and GBC-SD cells were seeded in 96-well plates. The cell proliferation and IC50 value curves were plotted using absorbance at 450 nm. As for cell proliferation assay, after specific treatment, according to the manufacturer’s instructions, 10 μL CCK-8 solution and 100 μL culture medium were added to each well of the plate. The plate was incubated for 1.5 h in the incubator and the OD450 was measured by the microplate reader. As for IC50 assay, after cells were
treated with KPT-330 for 48 h, 10 μL CCK-8 solution and 100 μL culture medium were added to each well of the plate. The plate was incubated for 1.5 h in the incubator and the OD450 was measured by the microplate reader.

**Colony formation assay**

NOZ and GBC-SD cells were seeded in 6-well plates at a density of 1000 cells per well for 10 days. The cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Only colonies with more than 50 cells were counted.

**mRNA Sequencing and bioinformatics analysis**

mRNA sequencing and bioinformatics analysis of NOZ cells treated with KPT-330 for 48 h in IC50 value were conducted by BGI company (Shenzhen, China). Briefly, after filtering the sequencing data using SOAPnuke (v1.5.2, https://github.com/BGI-flexlab/SOAPnuke), clean reads were obtained in FATSTQ format [18]. Firstly, to calculate the gene expression levels of each sample, we used Bowtie2 (v2.2.5) to align clean reads to the reference gene sequences and then used RSEM (v1.2.8), while differential gene expression was analyzed by using DESeq2(v1.4.5) under the condition of |Log2 FC| ≥ 2, and Q-value ≤ 0.05 [19, 20]. Based on differential gene expression, heatmap (v1.0.8) was used to draw a heatmap (https://cran.r-project.org/web/packages/pheatmap/). Then, to conduct GO analysis (http://www.geneontology.org/) and KEGG analysis (https://www.kegg.jp/), Phyper (https://stat.ethz.ch/R-manual/R-devel/library/stats/html/Hypergeometric.html) was used to calculated P value, which was then corrected for multiple testing by Q-value (https://bioconductor.org/packages/release/bioc/html/qvalue.html) [21].

**Xenograft model**

Male nude mice were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). The in vivo study was approved by the Ethics Committee of Xinhua Hospital. To establish a subcutaneous xenograft model, NOZ cells (2 × 10^6 cells in 100 µL PBS) were injected subcutaneously in two groups of nude mice (6 mice/group). The two groups were treated via oral gavage once every two days: vehicle (0.3% CMC-Na) or KPT-330 (20 mg/kg) dissolved in vehicle (0.3% CMC-Na). Tumor volumes (1/2 × width^2 × length) were measured weekly using a caliper. After 4 weeks of treatment, tumors and tissues were collected for further assays.

**Statistical analysis**

Statistical analyses were performed with Prism 8 (GraphPad Software) and SPSS 24 (SPSS Inc.). Student’s t test was performed between two groups and analysis between multiple groups was conducted by one-way analysis of variance, data are recorded int the form of mean ± SD. P values of < 0.05 were considered statistically significant (p < 0.05: *, p < 0.01: **, p < 0.001: ***). We also use the Pearson’s χ2 test to detect the correlation between XPO1 expression and clinicopathologic data. Also, Kaplan–Meier test for the univariate survival analysis.

**Results**

**Overexpression of XPO1 correlated to poor prognosis of GBC patients**

To clarify the expression of XPO1 in GBC, we first examined its mRNA level in 35 pairs of GBC and adjacent non-cancer tissues by the qRT-PCR. The results showed that XPO1 mRNA expression was significantly increased in GBC tissues compared with that in corresponding non-cancer tissues assessed by Student’s t test (Fig. 1A and B). We also assessed XPO1 protein expression in...
94 tissue samples of GBC and 69 cases of cholecystitis that were established as control by IHC (Fig. 1C). As shown in Fig. 1D, XPO1 was barely detectable in most of cholecystitis specimens compared with cancer samples calculated by Student’s t test. To analyze the correlation between XPO1 expression levels and clinicopathological
Fig. 3  XPO1 inhibitor KPT-330 induced changes in multiple tumor pathways related to the transport function of XPO1. A Heatmap and Go function analysis of differential genes ($|\log_2 FC| \geq 2$ and $Q$-value $\leq 0.05$) of NOZ after KPT-330 treatment for 48 h. B GO cellular component analysis of differential genes. C KEGG pathway analysis of differential genes. D Apoptosis pathway GSEA analysis of differential genes. E p53 pathway GSEA analysis of differential genes.
Fig. 4  XPO1 inhibitor KPT-330 induced GBC cells apoptosis by reducing mitochondrial membrane potential.  

A  Fluorescence images of TUNEL in NOZ and GBC-SD showed increased green fluorescence after KPT-330 treatment relative to the DMSO treatment group. Scale bars represent 100 μm. 

B  Flow cytometry using PI/Annexin V-FITC double staining showed KPT-330 induced apoptosis of NOZ and GBC-SD. 

C and D Expression of BAX, Bcl-2, Cleaved PARP/PARP, Cleaved caspase 9/caspase 9, Cleaved caspase 3/caspase 3 was detected by western blot. 

E  Flow cytometry analysis by using JC-1 staining. The horizontal axis channel “FL1-H” is the same as the FITC channel; the vertical axis channel “FL2-H” is the same as the PI channel. Student’s t test was applied to the statistical analysis in this figure. Data presented as mean±SD (n = 3)
data of GBC patients, we used 57 samples from GBC patients with clinical follow-up data. Based on the fold change, we divided patients into high level of XPO1 (fold change ≥2) and low level of XPO1 (fold change < 2). High level of XPO1 was closely associated with pathologic T stage (P < 0.001), lymph node metastasis (P = 0.047), and TNM stage (P = 0.001) evaluated by Pearson’s χ2 test (Table 3). Furthermore, the overall survival time of GBC patients after surgery with high XPO1 expression was significantly shorter than patients with low XPO1 expression assessed by Kaplan–Meier test (Fig. 1E).

**XPO1 inhibitor KPT-330 inhibited proliferation and induced G0/G1 cell cycle arrest of GBC cells**

To determine the role of XPO1 in GBC cells, we knocked down XPO1 by siRNAs. And the two different XPO1 siRNAs significantly decreased XPO1 at the protein level (Fig. 2A). The results in Fig. 2B showed that the proliferation of NOZ and GBC-SD cells was significantly inhibited by XPO1-siRNAs evaluated by Student’s t test. This finding tentatively suggested that inhibition of XPO1 could inhibit cell proliferation, then we treated GBC cells with KPT-330, which could specifically inhibit XPO1. The proliferation of NOZ and GBC-SD cells was significantly inhibited by XPO1 inhibitor KPT-330 both time and concentration dependently (Fig. 2C). The KPT-330 IC50 values for NOZ and GBC-SD cells at 48 h were 3.47 and 1.84 μM, respectively (Additional file 1: Fig. S1A); these concentrations and KPT-330 processing time (48 h) were used for subsequent experiments. The western blot results showed that KPT-330 significantly reduced the expression of XPO1 (Additional file 1: Fig. S1B). The colony formation assay showed that KPT-330 significantly reduced the number and size of colonies formed by GBC cells assessed by Student’s t test (Fig. 2D). Similarly, the Edu-488 DNA synthesis assay showed the inhibitory effect of KPT-330 on the proliferation of NOZ and GBC-SD cells (Fig. 2E). Finally, we examined the effect of KPT-330 on the cell cycle in GBC cells by using flow cytometry. The results showed that KPT-330 increased the number of cells in the G0/G1 phase, with a reduced S phase population, compared with the DMSO group (P < 0.05) calculated by Student’s t test (Fig. 2F). These results indicated that XPO1 inhibitor KPT-330 inhibited proliferation of GBC cells and caused G0/G1 cell cycle arrest.

**XPO1 inhibitor KPT-330 induced changes in multiple tumor pathways related to the transport function of XPO1**

To clarify the role of XPO1 in GBC, we extracted total RNA from NOZ cells treated with KPT-330 for 48 h and performed an RNA-seq assay. We set |log2FC| ≥ 2 and the Q-value ≤ 0.05 as inclusive criteria. The results showed that 838 genes were upregulated and 333 were downregulated (Additional file 1: Fig. S1C). According to the Gene Ontology (GO) analysis, the differentially expressed genes were closely associated with “cargo receptor activity”, “binding”, “molecular carrier activity”, and “transporter activity” (Fig. 3A). The GO cellular component showed that the differentially expressed genes were related to “nucleoplasm”, “cytosol”, “nucleus”, and “cytoplasm” (Fig. 3B). Thus, GO analysis results revealed changes were closely related to the role of XPO1 in nucleocytoplasmic transport. The KEGG results suggested important changes in “p53 signaling pathway”, “autophagy”, “mTOR signaling”, and “apoptosis” genes according to the Q-value (Fig. 3C). The GSEA also suggested significant changes in the p53 and apoptosis pathways (Fig. 3D and E). Overall, these bioinformatics analysis results indicated that XPO1 inhibitor KPT-330 could induce important changes related to cancer pathways in GBC cells.

**XPO1 inhibitor KPT-330 induced GBC cells apoptosis by reducing mitochondrial membrane potential**

In the bioinformatics analyses, we found significant changes in apoptosis-related pathways. To verify the existence of apoptosis, we utilized a variety of assays. First, the TUNEL assay confirmed that KPT-330 induced apoptosis in NOZ and GBC cells (Fig. 4A). In addition,
Fig. 5 (See legend on previous page.)
the percentages of cells in both early and late apoptosis were significantly increased, as shown by flow cytometry, in NOZ and GBC-SD cells treated with KPT-330 and stained with PI and Annexin-V-FITC calculated by Student’s t test (Fig. 4B). The expression of proteins associated with apoptosis was evaluated by western blot. The results indicated that the level of cleaved caspase-3 and caspase-9 proteins was increased in GBC cells treated with KPT-330, which activated cleaved PARP, an apoptosis marker (Fig. 4C and D). Further studies assessed the expression of apoptosis-related Bcl-2 family proteins. The results showed that the expression of Bax was remarkably increased, while the expression of Bcl-2 was significantly reduced (Fig. 4C and D).

A decrease in the ΔΨm, which reflects mitochondrial function, is a hallmark event of early apoptosis. According to the previously mentioned cell component of the GO analysis, which revealed changes in mitochondria and the mitochondrial inner membrane, we hypothesized that apoptosis in GBC cells treated with KPT-330 was associated with changes in ΔΨm. To address this hypothesis, NOZ and GBC-SD cells were treated with KPT-330 and stained with JC-1, which selectively enters mitochondria. The results showed that the ΔΨm was markedly diminished assessed by Student’s t test (Fig. 4E).

Together, these results indicated that XPO1 inhibitor KPT-330 induced GBC cells apoptosis through the mitochondria-mediated apoptosis pathway.

**XPO1 inhibitor KPT-330 induced autophagy of GBC cells through the mTOR pathway**

The KEGG pathway analysis indicated that autophagy may exist in NOZ cells treated with KPT-330 (Fig. 3C). To determine whether KPT-330 induced autophagy, GBC cells were treated with KPT-330 for 48 h and their morphology was observed by transmission electron microscopy. GBC cells treated with rapamycin (which activated autophagy) with concentration of 0.4 μM for the same time period were used as a positive control. The results demonstrated KPT-330 and rapamycin treatment could be seen to form a similar autophagosomes and autolysosomes (red arrows point to) compared to control group (Fig. 5A). To assess the extent of autophagosome and autolysosome accumulation separately, we infected NOZ and GBC-SD cells with Ad-mCherry-GFP-LC3B. Green LC3 puncta represent mainly autophagosomes, while red LC3 puncta indicate both autophagosomes and autolysosomes, as mCherry maintains its fluorescence even in the acidic environment of lysosomes, while GFP loses its fluorescence [22]. The numbers of green, red, and yellow (from the merged images) puncta increased greatly in KPT-330 treated cells compared to control, implying an increase in autophagosomes and autolysosomes (Fig. 5B).

The transition from LC3-I to LC3-II is often considered a marker of autophagy, and can be used to monitor autophagosome formation at an early stage [23]. The LC3-II/LC3-I ratio increased with time in KPT-330 treated NOZ and GBC-SD cells (Fig. 5C and D). Meanwhile, we found that the p-mTOR/mTOR ratio was reduced. To further explore whether inhibition of XPO1 induced mTOR-mediated autophagy, we pre-treated GBC cells with the autophagy inhibitor, chloroquine (concentration of 0.2 μM), or the mTOR inhibitor, rapamycin (concentration of 0.4 μM) for 6 h, then treated cells with or without KPT-330 for 48 h. The results showed that the p-mTOR/mTOR ratio was reduced after treatment with either KPT-330 or rapamycin, and this reduction was enhanced by combining KPT-330 and rapamycin. Furthermore, chloroquine partially abrogated the reduction in the p-mTOR/mTOR ratio by KPT-330 (Fig. 5E). In addition, the cell proliferation results showed that chloroquine significantly prevented the decrease in proliferation caused by KPT-330 analyzed by Student’s t test (Fig. 5F). Overall, these results suggested that the XPO1 inhibitor KPT-330 induced autophagy by regulating the mTOR pathway in GBC cells.
Fig. 6 (See legend on previous page.)
XPO1 inhibitor KPT-330 activated p53/mTOR pathway to induce autophagy-dependent apoptosis

It has been suggested that increased tumor cell apoptosis may be dependent on the presence of autophagy [24, 25]. To determine whether KPT-330 triggered autophagy decreased or increased apoptosis, the extent of apoptosis in NOZ and GBC-SD cells after chloroquine and/or KPT-330 treatment was examined by flow cytometry. The results indicated that the inhibition of autophagy by chloroquine partially abrogated KPT-330-induced apoptosis evaluated by Student’s t test (Additional file 1: Fig. S1D). This result tentatively suggested that inhibition of autophagy may inhibit apoptosis induced by KPT-330. Then we conducted western blot for further analysis. Treatment of these cells with the cell-permeable broad-spectrum caspase inhibitor, Z-VAD-FMK (concentration of 0.1 μM), significantly decreased the levels of cleaved caspase-3 and PARP, whereas the increased LC3-II/LC3-I ratio caused by KPT-330 was not changed (Fig. 6A). Indeed, the rising cleaved forms of caspase-3 and PARP protein expression were effectively prevented by chloroquine in KPT-330 treated cells (Fig. 6B). In addition, the cell proliferation results showed that Z-VAD-FMK significantly prevented the decrease in proliferation caused by KPT-330 calculated by Student’s t test (Additional file 1: Fig. S1E).

The KEGG pathway analysis and GSEA indicated significant changes in the p53 pathway. The western blot analysis of fractionated cellular lysates showed that, p53 level was significantly increased in the nucleus but decreased in the cytoplasm after KPT-330 treatment of NOZ and GBC-SD cells (Fig. 6C). Moreover, immunofluorescence staining of p53 indicated that KPT-330 increased the nuclear accumulation of p53 in both NOZ and GBC-SD cells (Fig. 6D). p53 is involved in regulating cycle-related proteins including P21 and P27 [26]. The nuclear accumulation of p53 could activate its functions [27]. The western blot results confirmed that the expression of P21 and P27 was increased after KPT-330 treatment, which was consistent with the observed G0/G1 cell cycle arrest (Fig. 6E). It has been reported that p53 could affect the mTOR pathway and thereby affect cell autophagy [28–30]. We knocked down p53 by using siRNA (Additional file 1: Fig. S1F). We treated cells with siRNAs for 48 h, then treated cells with KPT-330 for 48 h. This knockdown partially reduced the KPT-330-mediated increase in the phosphorylation level of mTOR, and the LC3-II/LC3-I ratio (Fig. 6F). These results indicated that KPT-330 induced nuclear accumulation of the p53 protein, which then activated the p53/mTOR pathway to regulate cell autophagy. To further confirm that KPT-330 regulation of the p53/mTOR pathway affects autophagy-dependent apoptosis, we performed TUNEL assays and western blot assays. The TUNEL assays indicated that inhibition of autophagy inhibited apoptosis induced by KPT-330 treatment, and inhibition of p53 by p53-siRNA or activation of mTOR by MHY1485 also inhibited apoptosis induced by KPT-330 treatment (Additional file 1: Fig. S2A). The results of western blot showed that inhibition of autophagy could decrease the rising level of cleaved caspase-3 and cleaved PARP protein in KPT-330 treated cells (Fig. 6G). Moreover, the changes of the phosphorylation level of mTOR and LC3-II/LC3-I ratio obtained after knockdown of p53 or activation of mTOR were consistent with the previous results. As shown in Fig. 6H, knockdown of p53 antagonized the anticancer effect of KPT-330 in terms of GBC cell growth assessed by Student’s t test. In summary, XPO1 inhibitor KPT-330 induced the nuclear accumulation of p53 protein and activated p53/mTOR to induce autophagy-dependent apoptosis.

XPO1 inhibitor KPT-330 inhibited growth of GBC in vivo with excellent drug safety

To validate the anticancer effect of KPT-330 in vivo, we injected NOZ cells subcutaneously into the left flank of nude mice to construct a GBC tumor model. One week after injection, the mice were treated with 20 mg/kg KPT-330 for 4 weeks via oral gavage three times per week (Fig. 7A). Compared with vehicle-treated mice, the tumor volume and weight were significantly reduced following KPT-330 treatment calculated by Student’s t test (Fig. 7B-E). IHC staining of tumor tissue showed that expression of the XPO1 protein was dramatically reduced in the KPT-330 treatment group. Similar results were obtained for IHC staining of Ki-67 and PCNA, whose expression is related to the proliferation of GBC cells evaluated by Student’s t test (Fig. 7F and Additional file 1: Fig. S2B). In
Fig. 7 (See legend on previous page.)
terms of safety, no changes were observed in the appearance and morphology of main organs (heart, liver, kidney and lung) of mice treated with KPT-330 (Fig. 7G). In addition, no obvious systemic toxicity was observed in routine blood examinations after KPT-330 treatment (Additional file 1: Table S3). Taken together, these results suggested that the XPO1 inhibitor, KPT-330, inhibited tumor growth in vivo with a good safety profile.

Discussion
XPO1 is a key nuclear export carrier for a variety of tumor suppressor proteins [9]. Maintaining the balance of protein transport between the nucleoplasm is essential for controlling cell survival and death [31]. Thus, altering the nucleoplasmic localization of cargo proteins creates the opportunity to target a variety of unique pathways implicated in carcinogenesis [32]. For example, inhibiting XPO1 diminishes tumor growth and improves the efficacy of cisplatin in ovarian carcinoma [33, 34]. Inhibition of XPO1 also allows BCR-ABL to be trapped in the nucleus and sensitizes leukemic cells to the BCR-ABL inhibitor, imatinib, resulting in a strong decrease in their proliferative potential [35].

An important feature of tumors is the dysregulation of apoptosis. Consequently, apoptosis can be induced with various cytotoxic anticancer agents, making this one of the most effective strategies for the treatment of cancer [36]. Our previous studies and those of other researchers indicated that XPO1 inhibitors exerted its anticancer activities by inducing apoptosis in cancer cells [37–39]. Furthermore, XPO1 inhibitors can trigger p53-mediated caspase-dependent apoptosis in adult T-cell Leukemia [40]. In this study, we found that XPO1 inhibitor KPT-330 induced the nuclear accumulation of p53 protein.

In different cancer cells, autophagy can act as either a tumor suppressor or a tumor promoter, with the specific state depending on the cancer cell environment [41]. Our results showed that autophagy could be detected after KPT-330 treatment. Inhibition of the mTOR pathway has been reported to activate autophagy [42, 43]. Our bioinformatic and western blot analyses verified that KPT-330 could induce mTOR-mediated autophagy. Autophagy is part of a complex network that brings together oversight of metabolism, cell proliferation and apoptosis [44]. Several studies have confirmed that inducing autophagy can promote apoptosis in cancers [45, 46]. Our results confirmed that KPT-330 can induce autophagy-dependent apoptosis. Combined with the fact that tumor cells can undergo apoptosis upon KPT-330 treatment, and that KPT-330 leads to the development of autophagy-dependent apoptosis, we speculated that p53 could regulate autophagy. Based on this conjecture, we knocked down p53 by siRNA and showed that this knockdown reduced the extent of mTOR-mediated autophagy induced by KPT-330. We integrated KPT-330, p53-siRNA, MHY1485 and chloroquine for further experiments and the results confirmed that XPO1 inhibitor KPT-330 activated the p53/mTOR pathway to regulate autophagy-dependent apoptosis.

The in vivo experiment showed that the XPO1 inhibitor, KPT-330, had potent anti-GBC activity. Administration of 20 mg/kg of KPT-330 significantly inhibited the growth of GBC cells in a nude mouse xenograft model without any significant toxic effects. However, some clinical studies with KPT-330 have revealed adverse effects such as decreased appetite, vomiting, and decreased weight, but without significant effects on vital organs [47]. Further studies are needed to investigate in vivo effects of KPT-330 treatment.

In summary, our findings show that overexpression of XPO1 is significantly associated with poor prognosis in patients with GBC. Furthermore, XPO1 inhibitor KPT-330 can activate the p53/mTOR pathway to induce autophagy-dependent apoptosis in GBC cells. Therefore, XPO1 is a potential biomarker for the diagnosis of GBC, and KPT-330 might be a more effective and less cytotoxic chemotherapeutic agent for the treatment of GBC in the future.

Abbreviations
CCK8: Cell Counting Kit 8; GBC: Gallbladder cancer; GSEA: Gene Set Enrichment Analysis; IHC: Immunohistochemistry; KEGG: Kyoto Encyclopedia of Genes and Genomes; mTORL: mammalian target of rapamycin; PARP: Poly(ADP-ribose) polymerase; PCNA: Proliferating cell nuclear antigen; PI: Propidium iodide; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; SINEs: Selective inhibitors of nuclear export; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick-end labeling; ΔΨm: mitochondrial membrane potential; XPO1: Chromosome region maintenance 1; NC: Negative control; TNM: Tumor Node Metastasis; T stage: Tumor stage.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12967-022-03635-w.
mean±SD (n = 3). **Figure S2.** A. Fluorescence images of TUNEL after KPT‑330 or pS3‑siRNA or MHY1485 or chloroquine treatment in NOZ and GBC‑SD. NOZ and GBC‑SD cells were pre‑treated with pS3‑siRNA for 48h or MHY1485 (concentration of 0.5 μM) or chloroquine (concentration of 0.2 μM) for 6h, then were treated with or without KPT‑330 for 48h. Scale bars represent 100 μm. B. Immunohistochemistry score of XPO1, Ki67 and PCNA expression levels of xenograft tumor tissues in KPT‑330 treatment or vehicle group. Student’s t test was applied to the statistical analysis.

Acknowledgements
Sincerely appreciate all the participants involved in this research. Thanks to LCSciences and Mr. Run Qin for your help in the manuscript revision process.

Author contributions
CZ, LP, WG, and PD conceived and designed the research. CZ, ZY and JZ implemented the execution of experiments, data statistics, bioinformatics analysis and manuscript composition. OL, SL, CC and YS revised the manuscript. LP, WG, and PD were responsible for the funding application and the supervision and management of the project. All authors read and approved the final manuscript.

Funding
This study was supported by the National Natural Science Foundation of China (No. 82172628, 81974371, 31701108, 82002503, 82173081), The Shanghai Sailing Program (No.20YF1430200), The Shanghai Scientific and technological innovation plan (22531103600), The national natural science of shanghai (No.20ZR1435200). Hospital Funded Clinical Research, Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

Availability of data and materials
The mRNA‑seq raw data can be found in SRA database: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA827962. All data generated or analyzed during this study are included in this published article and its Additional files.

Declarations
Ethics approval and consent to participate
Animal experiments involving nude mice met the requirements and were approved by Xinhua Hospital of Shanghai Jiao Tong University School of Medicine.

Consent for publication
All authors have seen and approved the manuscript and consent publication.

Competing interests
There are no conflicts of interest to disclose.

Author details
1Department of General Surgery, The First Affiliated Hospital of Zhejiang University, Hangzhou, China. 2Department of General Surgery, Xinhua Hospital Affiliated with Shanghai Jiao Tong University School of Medicine, No. 1665 Kongjiang Road, Shanghai 200092, China. 3Department of General Surgery, Xinhua Hospital Affiliated with Shanghai Jiao Tong University School of Medicine, No. 1665 Kongjiang Road, Shanghai 200092, China.

Received: 27 June 2022 Accepted: 11 September 2022
Published online: 30 September 2022

References
1. Kanthan R, Senger J-L, Ahmed S, Kanthan SC. Gallbladder cancer in the 21st Century. J Oncol. 2015;2015:967472.
2. Dutta U. Gallbladder cancer: can newer insights improve the outcome? J Gastroenterol Hepatol. 2012;27:642–53.
3. Li M, Zhang Z, Li X, Ye J, Wu X, Tan Z, et al. Whole‑exome and targeted gene sequencing of gallbladder carcinoma identifies recurrent mutations in the ErbB pathway. Nat Genet. 2014;46:872–6.
4. Zhu AX, Hong TS, Hezel AF, Kooby DA. Current management of gallbladder carcinoma. Oncologist. 2010;15:168–81.
5. Li Y, Yuan R, Ren T, Yang B, Mao H, Liu L, et al. Role of Sciellin in gallbladder cancer proliferation and formation of neutrophil extracellular traps. Cell Death Dis. 2021;12:30.
6. Zeng H, Chen W, Zheng R, Zhang S, Ji JS, Zou X, et al. Changing cancer survival in China during 2003–15: a pooled analysis of 17 population‑based cancer registries. Lancet Glob Health. 2018;6:e555–67.
7. Abeykoon JP, Wu X, Nowakowski KE, Dassari S, Paludo J, Weroha SJ, et al. Salicylates enhance CRM1 inhibitor antitumor activity by induction of S‑phase arrest and impairment of DNA‑damage repair. Blood. 2021;137:513–23.
8. Das A, Wei G, Parikh K, Liu D. Selective inhibitors of nuclear export (SINE) in hematologic malignancies. Exp Hematol Oncol. 2015;4:7.
9. Pan L, Cheng C, Duan P, Chen K, Wu Y, Wu Z. XPO1/CRM1 is a promising prognostic indicator for neuroblastoma and represented a therapeutics target by selective inhibitor verdinexor. J Exp Clin Cancer Res. 2021;40:255.
10. Azizzan NG, Li Y. XPO1‑dependent nuclear export as a target for cancer therapy. J Hematol Oncol. 2020;13:61.
11. Ishizawa J, Koijima K, Hail N Jr, Tabé Y, Andreeff M. Expression, function, and targeting of the nuclear exporter chromosome region maintenance 1 (CRM1) protein. Pharmacol Ther. 2015;153:25–35.
12. Turner JK, Marchion DC, Dawson JL, Emmons MF, Hazelhurst LA, Waishausen P, et al. Human multiple myeloma cells are sensitized to topoisoerase II inhibitors by CRM1 inhibition. Cancer Res. 2009;69:8899–905.
13. Newlands ES, Rustin GJ, Brampton MH. Phase I trial of elacritonib. Br J Cancer. 1996;74:648–9.
14. Liu S, Qiao W, Sun Q, Luo Y. Chromosome Region Maintenance 1 (XPO1/CRM1) as an anticancer target and discovery of its inhibitor. j Med Chem. 2021;64:15534–48.
15. Daelemans D, Aforina E, Nilsson J, Werner G, Kajser L, De Clercq E, et al. A synthetic HIV‑1 Rev inhibitor interfering with the CRM1‑mediated nuclear export. Proc Nat Acad Sci U S A. 2022;119:21484–9.
16. Neegjes J, Vercaigne S, Jacquesm Y, Vanstreels A, Ballegou E, Shachat S, et al. Identifying drug‑target selectivity of small‑molecule CRM1/XPO1 inhibitors by CRISPR/Cas9 genome editing. Chem Biol. 2020;23:107–16.
17. Van Neck T, Panneckecouque C, Vanstreels E, Stevens M, Dehaen W, Daelemans D. Inhibition of the CRM1‑mediated nucleocytoplasmic transport by N‑azolylacrylates: structure‑activity relationship and mechanism of action. Bioorg Med Chem. 2008;16:9487–97.
18. Cock PJ, Fiedler CJ, Goto N, Heuer ML, Rice PM. The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. Nucleic Acids Res. 2010;38:1767–71.
19. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA‑seq data with DESeq2. Genome Biol. 2014;15:50.
20. Langmead B, Salzberg SL. Fast gapped‑read alignment with Bowtie 2. Nat Methods. 2012;9:357–9.
21. Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, et al. KEGG: linking genomes to life and the environment. Nucleic Acids Res. 2008;36:D480‑484.
22. Han J, Pan XY, Yu X, Xiao Y, An Y, Tie L, et al. Curcumin induces autophagy to protect vascular endothelial cell survival from oxidative stress damage. Autophagy. 2012;8:212–5.
23. Zhu Y, He W, Gao X, Mei C, Xu T, et al. Resveratrol overcomes gefitinib resistance by increasing the intracellular gefitinib concentration and triggering apoptosis, autophagy and senescence in PC9/G NSCLC cells. Sci Rep. 2015;5:17730.
24. Wu M-D, Zhang Y-Y, Yi S-Y, Sun B-B, Lan J, Jiang H-M, et al. Acetylshikonin induces autophagy‑dependent apoptosis through the key LKB1‑AMPK and PI3K/Akt‑modulated mTOR signalling pathways in HLR‑60 cells. J Cell Mol Med. 2022;26:1606.
25. Wei X, Tang Z, Wu H, Zuo X, Dong H, Tan L, et al. Biofunctional magnesium‑coated Ti6Al4V scaffolds promote autophagy‑dependent apoptosis in osteosarcoma by activating the AMPK/mTOR/ULK1 signaling pathway. Mater Today Bio. 2021;12:100147.
26. Hume S, Grou CF, Lascaux P, D'Angiolla V, Legrand AJ, Ramadan K, et al. The NUKCS1‑SKP2‑p21/p27 axis controls S phase entry. Nat Commun. 2021;12:6959.
27. Gruffaz M, Yuan H, Meng W, Liu H, Bae S, Kim J-S, et al. CRISPR-Cas9 screening of Kaposi’s sarcoma-associated herpesvirus-transformed cells identifies XPO1 as a vulnerable target of cancer cells. mBio. 2019. https://doi.org/10.1128/mBio.00866-19.

28. Cheng B, Lu J-L-T, Meng Z, Liu M, Sun M, et al. 1,3-Dichloro-2-Propanol inhibits autophagy via p53/AMPK/mTOR pathway in HepG2 cells. Food Chem Toxicol. 2018;122:143–50.

29. Zhu J, Ao H, Liu M, Cao K, Ma J. UBE2T promotes autophagy via the p53/AMPK/mTOR signaling pathway in lung adenocarcinoma. J Transl Med. 2021;19:374.

30. Drakos E, Atsaves V, Li J, Leventaki V, Andreeff M, Mederos I, et al. Stabilization and activation of p53 downregulates mTOR signaling through AMPK in mantle cell lymphoma. Leukemia. 2009;23:784–90.

31. Ohno M, Fornerod M, Mattaj I W. Nucleocytoplasmic transport: the last 200 nanometers. Cell. 1998;92:327–36.

32. Balasubramanian SK, Azmi AS, Maciejewski J. Selective inhibition of nuclear export: a promising approach in the shifting treatment paradigms for hematological neoplasms. Leukemia. 2022;36:601.

33. Link W, Fernandez-Marcos PJ. FOXO transcription factors at the interface of metabolism and cancer. Int J Cancer. 2017;141:2379–91.

34. Corno C, Stucchi S, De Cesare M, Carenni N, Stamatatos S, Ciussani E, et al. Foxo-O-1 contributes to the efficacy of the combination of the XPO1 inhibitor selinexor and cisplatin in ovarian carcinoma preclinical models. Biochem Pharmacol. 2018;147:93–103.

35. Aloisi A, Di Gregorio S, Stagno F, Guglielmo P, Mannino F, Sormani MP, et al. BCR-ABL nuclear exportation kills human CML cells: ex vivo study on 35 patients with the combination of imatinib mesylate and leptomycin B. Blood. 2006;107:1591–8.

36. Cui L, Bu W, Song J, Feng L, Xu T, Liu D, et al. Apoptosis induction by alternolactone in breast cancer MDA-MB-231 cells through reactive oxygen species-mediated mitochondrion-dependent pathway. Arch Pharm Res. 2018;41:299–313.

37. Sun H, Lin DC, Cao Q, Guo X, Marjon H, Zhao Z, et al. CRM1 inhibition promotes cytotoxicity in ewing sarcoma cells by repressing EWS-FL1-Dependent IGF-1 signaling. Cancer Res. 2016;76:2687–97.

38. Zheng Y, Gery S, Sun H, Shacham S, Kauffman M, Koeffler HP. KPT-330 inhibitor of XPO1-mediated nuclear export has anti-proliferative activity in hepatocellular carcinoma. Cancer Chemother Pharmacol. 2014;74:487–95.

39. Chen Y, Camacho SC, Silvers TR, Razak AR, Gabrail NY, Gerecitano JF, et al. Inhibition of the nuclear export receptor XPO1 as a therapeutic target for platinum-resistant ovarian cancer. Clin Cancer Res. 2017;23:1552–63.

40. Boorns E, Nogueira TC, Dierckx T, Menezes SM, Jacquemyn M, Tamir S, et al. XPO1 inhibitors represent a novel therapeutic option in Adult T-cell Leukemia, triggering p53-mediated caspase-dependent apoptosis. Blood Cancer J. 2021;11:27.

41. Wang J, Liu G, Li X, Huangfu M, Liu Y, Li X, et al. Curcumol simultaneously induces both apoptosis and autophagy in human nasopharyngeal carcinoma cell lines. PLoS One. 2013;8:57004–17.

42. Zhao J, Zhang T, Chen G, Geng N, Guo Z, Cao S, et al. Non-Structural Protein 3 of Duck Tembusu virus induces autophagy the ERK and PI3K-AKT-mTOR signaling pathways. Front Immunol. 2022;13:746890.

43. Xu P, Zhang X, Cao J, Yang J, Chen Z, Wang W, et al. The novel role of circular RNA ST3GAL6 on blocking gastric cancer malignant behaviours through autophagy regulated by the FOXP2/MET/mTOR axis. Gut. 2022;71:26.

44. Pan Z, Zheng D-D, Wei X-J, Li S-J, Guo H, Yang Q-C. Chitooligosaccharides inhibit tumor progression and induce autophagy through the activation of the p53/mTOR pathway in osteosarcoma. Carbohydr Polym. 2021;258:117596.

45. Liu Z, Wang H, Hu C, Wu C, Wang J, Hu F, et al. Targeting autophagy enhances atezolizumab-induced mitochondria-related apoptosis in osteosarcoma. Cell Death Dis. 2021;12:164.

46. Fu S-C, Lin J-W, Liu J-M, Liu S-H, Fang K-M, Su C-C, et al. Arsenic induces autophagy-dependent apoptosis via Akt inactivation and AMPK activation signaling pathways leading to neuronal cell death. Neurotoxicology. 2021;85:133–44.

47. Lassman AB, Wen PY, van den Bent MJ, Plotkin SR, Walenkamp AME, Green AL, et al. A Phase II study of the efficacy and safety of oral selinexor in recurrent glioblastoma. Clin Cancer Res. 2022;28:452–60.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.