Lenvatinib Inhibits Intrahepatic Cholangiocarcinoma Growth via Gadd45a-Mediated Cell Cycle Arrest

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Abstract

Background: Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer, and its 5-year survival rate is less than 10%. Fibroblast growth factor receptor (FGFR) changes have been observed in 6%-50% of ICC patients, and patients with FGFR mutations have been shown to have more inert tumour biological activity than patients with wild-type FGFRs. Thus, as a pan-FGFR inhibitor, lenvatinib is supposed to play an anti-tumour role in ICC. However, no relevant experiments have been reported.

Methods: Patients derived xenograft (PDX) model and cell line derived xenograft (CDX) model were both used for the in vivo study. For in vivo work, ICC cell lines were applied to analyse the effect of Lenvatinib on cell proliferation, cell cycle progression, apoptosis, and the molecular mechanism.

Results: In the present study, we found that lenvatinib dramatically hindered in vivo tumor growth in ICC patient-derived xenograft models. In addition, by using in vitro experiments in ICC cell lines, we found that lenvatinib dose- and time-dependently inhibited the proliferation of ICC cells and induced cell cycle arrest in the G₀/G₁ phase. Transcriptional profiling analysis further applied indicated that lenvatinib might inhibit cell proliferation through the induction of cell-cycle arrestment via activating of Gadd45a, it was evidenced by that the knockout of Gadd45a significantly attenuated the cycle arrest induced by lenvatinib, as well as the inhibitory effect of lenvatinib on ICC.

Conclusion: Our work firstly found that lenvatinib exerted excellent antitumor effect on ICC, mainly via inducing Gadd45a mediated cell cycle arrest. Our work provides evidence and a rationale for the future use of lenvatinib in the treatment of ICC.

Introduction

Intrahepatic cholangiocarcinoma (ICC) is a relatively rare and malignant epithelial tumour arising within the liver. In the past decade, although the prognosis of most solid tumours has improved, the incidence and mortality of ICC have been increasing worldwide (1, 2). For ICC patients, surgery remains the best option for long-term survival. However, most patients have early nonspecific symptoms and therefore lose the chance to undergo surgical treatment because ICC has developed into an advanced stage by the time of diagnosis. The standard systemic therapy for ICC is platinum-based chemotherapy combined with gemcitabine, but it provides only a modest survival benefit (3-7). Thus, explorations of new therapeutic targets and drugs are urgently needed to improve the status of ICC.

Fibroblast growth factor receptor (FGFR), a member of the receptor tyrosine kinase family, can be activated by ligand binding, leading to the dimerization and autophosphorylation of the intracellular domains of the receptor and receptor-related ligands (8). Recent studies have shown that FGFRs are overexpressed in most solid tumours, including gastric cancer, pancreatic cancer, oesophageal cancer and liver cancer. Studies have found that the prognosis of patients with FGFR overexpression is usually poor (9-11). In ICC, FGFR changes have been observed in 6%-50% of patients, and these changes were

related to an improvement in survival(12-14). In addition, it has been confirmed that ICC patients with FGFR mutations have more inert tumour biological activity than patients with wild-type FGFRs(15).

As an oral multi-target tyrosine kinase inhibitor (TKI), lenvatinib has been found to exert its antitumour effect by impacting VEGFR1-3, FGFR1-4, PDGFR α , KIT- and RET-related angiogenesis, cell proliferation and apoptosis and was first approved by the Food and Drug Administration (FDA) and European Drug Administration (EMA) in 2015 for the treatment of invasive, locally advanced or metastatic differentiated thyroid cancer and later for the treatment of advanced renal cell carcinoma and unresectable hepatocellular carcinoma(16, 17). Recently, its clinical efficacy in ICC has been increasingly noticed. As a pan-FGFR inhibitor, lenvatinib is expected to be a new opportunity for ICC patients and have a better therapeutic effect by prolonging progression-free survival times and overall survival times(18, 19). However, the efficacy of lenvatinib and its potential molecular mechanisms remain to be fully characterized.

Our study is the first to evaluate the anticancer activities of lenvatinib on ICC and its possible molecular mechanisms. This work provides promising evidence and a rationale for clinical therapeutic application of lenvatinib in patients with ICC.

Material And Methods

Tumour xenografts in nude mice

Animal experiments were approved by the Ethics Committee of Animal Experiments of Fudan University and conducted in accordance with “Regulations on the Administration of Laboratory Animal Affairs” (2017 edition).

Patient-derived xenograft (PDX) model: The human tumour tissues used in this study were derived from surgical patients with pathologically confirmed ICC. Ten- to 12-week-old BALB/c-nu mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). All animals were housed under temperature-controlled conditions with proper humidity, lighting (12-h light/12-h dark cycle) and free access to food and water. Fresh tumour specimens with a size of 2*2*2 mm were subcutaneously inoculated into the left flank of each mouse. When the tumours reached a threshold volume, the tumour-bearing mice were sacrificed, and the tumours were dissected and reinoculated into newborn mice according to the above method; these tumours were considered P1. Generally, tissues could be used for subsequent experiments after 2-3 passages, at which point they reached a stable state.

Cell line-derived xenograft (CDX) model: BALB/c-nu mice (6 weeks of age, Shanghai SLAC Laboratory Animal Co., Ltd., China) were housed under specific pathogen-free conditions. A total of 5×10^6 ICC cells suspended in 50 μ L PBS were mixed with 50 μ L Matrigel and implanted subcutaneously into mice. At 14 days after transplantation, the tumour-bearing mice were randomly divided into four groups and treated with 3 mg/kg, 10 mg/kg or 30 mg/kg lenvatinib once daily for 28 days. The same volume of physiological salt was given to the mice in the control group.

In this study, when body weight loss (BWL) was $\geq 20\%$ for any individual mouse, that mouse was given a dosing holiday(s) until its body weight recovered to baseline (BWL, $\leq 10\%$). The length (a) and width (b) of tumour masses were measured by Vernier callipers twice a week, followed by measurement of the mouse body weight. Tumour volumes were calculated as $V = 0.5 \times a \times b^2$. The tumour growth inhibition (TGI) rate was calculated as $TGI (\%) = [1 - (T_i - T_0) / (V_i - V_0)] \times 100$, where T_i and V_i were the average tumour volumes of the experimental group and the control group after the beginning of administration, and T_0 and V_0 were the average tumour volumes of the experimental group and the control group on the first day of administration. Similarly, the body weight (BW) of all tumour-bearing mice was measured twice a week, and the ratio of body weight change (RCBW) after administration (BW_i) was calculated by dividing the daily body weight by that of the same mouse on day 0 (BW_0): $RCBW (\%) = (BW_i - BW_0) / BW_0 \times 100$. The nude mice were sacrificed through cervical dislocation the day after the last administration.

Cells and reagents

The HCCC-9810 and RBE human ICC cell lines were purchased from the cell bank of the Type Culture Collection of the Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). ICC-4389 cells were obtained from 3D Biomedicine Science & Technology Co., Ltd. (Shanghai, China). HCCC-9810 and RBE cells were cultured in RPMI 1640 medium (Biosera, NUAILLE, France). All media were supplemented with 10% FBS (Biosera, NUAILLE, France), and cells were cultured at 37°C in an incubator containing 5% CO₂. Lenvatinib was obtained from Selleck (Houston, TX, USA).

Cell viability assay

Cell viability and cell growth inhibition were determined using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan) according to the manufacturer's instructions. The concentration of lenvatinib that reduced cell viability by 50% was extrapolated from a nonlinear least squares curve that fit the dose-response curves and then used to obtain 50% inhibitory concentration (IC₅₀) values.

Colony formation assay

At an initial density of 1000 cells/well, cells were seeded and cultured in six-well plates. After 2 weeks of cultivation, the cells were stained with 0.1% crystal violet (Sigma, St. Louis, USA) after 4% paraformaldehyde fixation. Colonies containing over 50 cells were counted using a light microscope.

Flow cytometry

Cells were harvested and washed with PBS, and 7% cold alcohol was added overnight at 4°C. The cells were washed and suspended in PBS and incubated with 100 mg/ml RNase and 40 mg/ml PI for 30 minutes at 4°C. The cell cycle distribution was evaluated on a FACSCalibur flow cytometer (BD Bioscience, San Diego, CA, USA).

Western blot analysis

Upon treatment, cells were washed and lysed on ice in RIPA buffer containing a protease inhibitor cocktail. Protein concentrations were assessed using a bicinchoninic acid kit. Subsequently, protein samples (12µg) were loaded and separated by 10% SDS-PAGE for the detection of various proteins. The proteins were then transferred to a polyvinylidene fluoride membrane, which was then incubated with diluted primary antibodies (Abcam Inc., Cambridge, UK), including rabbit antibodies against Actin (1:5000, Proteintech, 23660-1-AP), CDK1 (1:1000, Cell Signaling Technology, 9116), CDK2 (1:1000, Cell Signaling Technology, 18048), CyclinB1 (1:1000, Proteintech, 55004-1-AP), Cyclin E (1:1000, Proteintech, 11554-1-AP), p21 (1:5000, Abcam Inc., ab109520) and Gadd45a (1:1000, Abcam Inc., ab180768), overnight at 4°C. Then, the blot was washed three times with PBS. Finally, the blot was incubated with a goat anti-rabbit immunoglobulin G (IgG) secondary antibody (HAF008, 1:5000; Novus Biologicals, LLC, Littleton, CO, USA) for 1 h at 37°C. Finally, the proteins were visualized by enhanced chemiluminescence (WBKLS0500; Merck KGaA). Semi-quantification of blots was conducted using ImageJ software (version no. k 1.45; National Institutes of Health).

RNA preparation and quantitative real-time PCR

Total RNA was extracted from cells and tumour samples. Reverse transcription was conducted using a TaKaRa PrimeScript RT reagent kit (TaKaRa, Shanghai, China). The expression of candidate genes was determined using an ABI 7900HT Real-Time PCR system (Applied Biosystems, Inc., USA). The primer used in this study for Gadd45a was 5'-AGAAGAGGAGCATTCAATTCCA-3'.

RNA sequencing

Total RNA was isolated from ICC cells treated with 0µM or 25µM lenvatinib using TRIzol reagent (Invitrogen, CA, USA). Both cell lines were analysed in triplicate.

RNA sequencing was carried out using an Illumina HiSeq 4000 sequencing system. The fragments per kilobase of exon per million mapped reads (FPKM) for each gene were analysed after data pre-processing and collection.

Plasmid and siRNA transfection

Plasmid and siRNA transfection was performed using Lipofectamine 3000 Transfection Reagent (Thermo Fisher, MA, USA) according to the manufacturer's protocol. An empty vector or a nonspecific siRNA was used as the negative control. The sequence of the siRNA specific for Gadd45a used in this study was as follows: siRNA1: 5'-GCCGAAAGGGUAAUCAUA-3'. Samples were digested and used in other experiments 48 h after transfection.

ICC tumour tissues and immunohistochemical (IHC) staining

Human ICC tumour tissues were obtained from patients diagnosed with ICC by histopathological tests at the Fudan University Shanghai Cancer Center (FUSCC, n = 90). All procedures were performed after obtaining approval from the Clinical Research Ethics Committee of the FUSCC, and informed consent

was obtained from each patient prior to the analyses. Two independent pathologists conducted strict pathological diagnosis and postoperative follow-ups. Animal tumour tissue sections were obtained from PDX/CDX model nude mice. Tumour specimens were fixed with paraformaldehyde and then subjected to IHC staining with antibodies against Ki67, Gadd45a, CyclinB1 and Cyclin E using standard procedures. Protein expression levels were calculated by multiplying the positivity (0, <5% of total cells; 1, 5–25%; 2, 25–50%; and 3, >50%) and intensity scores (0, no colouration; 1, pale yellow; 2, yellow; and 3, brown) and were classified as follows: negative (0, –); weakly positive (1–3, +); moderately positive (4–6, ++); and strongly positive (>6, +++).

Statistical analysis

SPSS 21.0 software (IBM Corp., Armonk, NY, USA) was employed for data analysis. Experiments were repeated at least three times, and the measurement data are expressed as the mean $\pm$ standard deviation. Comparisons of data obeying a normal distribution or homogeneity of variance in an unpaired design between two groups were performed with an unpaired *t*-test, while comparisons of data for ICC tissues and para-cancerous tissues were conducted with a paired *t*-test. Data were compared among multiple groups by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Repeated-measures ANOVA followed by the Bonferroni post hoc test was utilized to compare data at different time points. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Lenvatinib exhibits effective antitumour activity in ICC patient-derived tumour xenograft (PDX) models

A PDX model is a tumour model constructed by directly transplanting the tumour tissue of a patient into immunodeficient mice (Fig.1a). this approach can not only retain the specific biological characteristics of the primary tumour but also have good consistency with clinical research results. Thus, to preliminarily clarify the antitumour effect of lenvatinib in ICC, 5 PDX models were constructed. According to the model results, lenvatinib could significantly inhibit the tumour growth of ICC PDXs, in which dramatic and significant increases in TGI were observed. However, the sensitivity of PDXs from different patients to lenvatinib varied (Fig.1b). Tumour weights in the control group were also significantly greater than those in the lenvatinib treatment group. Compared with the control group, no significant difference in the relative change rate of body weight was noticed (Fig.1c-g). There were also no significant changes with lenvatinib administration in diarrhea, appetite, or mental state in the PDX mouse model. Taken together, these results indicated that lenvatinib had effective antitumour activity and low drug toxicity on ICC in vivo.

Lenvatinib inhibits ICC cell growth in vitro

After we established that lenvatinib does have an inhibitory effect on ICC in vivo, we investigated the potential antitumour effect of lenvatinib using the ICC cell lines (HCCC-9810 and RBE). Lenvatinib exerted anticancer effects on HCCC-9810 and RBE cells in a concentration-dependent manner, with decreased cell

viability observed at high concentrations. The IC₅₀ values of lenvatinib in HCCC-9810 and RBE cells were 22.63 μ M and 34.09 μ M at 72h, respectively (Fig. 2a). Based on the observed IC₅₀ values, lenvatinib was used at concentrations of 0, 12.5, 25, and 50 μ M in subsequent analyses. CCK-8 assay results showed that the rates of cell proliferation were inhibited after 48h of lenvatinib treatment, and significant dose-dependent inhibitory effects between the groups were detected in both cell lines after 72h (Fig. 2b). Furthermore, the colony formation of the cell lines was significantly suppressed by treatment with increasing concentrations of lenvatinib, and the differences were statistically significant (Fig. 2c; $p < 0.05$).

In addition, to examine whether lenvatinib can inhibit ICC cells *in vivo*, a subcutaneous CDX model was also established. Consistent with the *in vitro* findings, mice treated with lenvatinib exhibited significant inhibition of tumour growth parameters, including tumour weight and tumour volume, compared to control mice. Furthermore, lenvatinib had no significant inhibitory effect on mouse body weight (Fig. 2d). Moreover, staining for the proliferation marker Ki-67 in xenograft tumours collected 1 day after the last administration of lenvatinib was performed, and the Ki-67-positive cells in the lenvatinib-treated groups were reduced in number compared with those in the control groups (Fig. 2e).

Lenvatinib could induce cell cycle arrest in ICC

RNA sequencing was performed to reveal the gene expression profiles of cells treated with lenvatinib or the control treatment. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis based on significantly differentially expressed genes revealed a series of enriched signalling pathways, including the cell cycle (Fig. 3a and b). Given that the cell cycle is one of the essential factors for the proliferation of ICC cells, cells were then examined by flow cytometry after incubation with 0, 12.5, 25 or 50 μ M lenvatinib for 72h. Based on our results, dose-dependent arrest in the G₀/G₁ phase of the cell cycle was obviously noticed in HCCC-9810 and RBE cells after treatment with lenvatinib. The G₀/G₁ phase cell percentages were 52.34%, 69.62%, 70.31%, and 77.23% (in RBE cells) and 63.34%, 75.43%, 78.4%, and 82.23% (in HCCC-9810 cells) at 0, 12.5, 25, and 50 μ M lenvatinib, respectively (Fig. 3c). Moreover, these distributions were consistent with the observed levels of important proteins involved in the cell cycle, including CDK1/CyclinB1, CDK2/Cyclin E and p21/Gadd45a (Fig. 3c). IHC staining of tumour tissues indicated that the expression of CyclinB1 and Cyclin E was significantly suppressed in the group treated with lenvatinib (Fig. 3e), which was consistent with the *in vitro* findings. Collectively, our results indicate that lenvatinib can inhibit ICC cell proliferation by inducing cell cycle arrest.

Lenvatinib upregulates Gadd45a *in vitro* and *in vivo*, and Gadd45a expression is positively correlated with ICC prognosis

To further identify the key molecular targets of lenvatinib in ICC, heat map clustering analysis was performed, and the results showed that the lenvatinib and control groups could be obviously distinguished according to differentially expressed genes, with significant Gadd45a upregulation detected in the lenvatinib-treated groups (Fig. 4a-b). To preliminarily investigate the role of Gadd45a in ICC, tissue microarrays (TMAs) containing 90 pairs of patient samples were evaluated. According to the immunohistochemistry score, Gadd45a was expressed at higher levels in tumour tissues than in normal

tissues (Fig.4 c-d). Moreover, after excluding 13 cases with incomplete clinical and follow-up data, an analysis of clinical characteristics of ICC revealed that Gadd45a expression was closely related to tumour differentiation in ICC (Table 1). Furthermore, Kaplan–Meier survival curves revealed an obvious correlation between high Gadd45a expression and a better prognosis in ICC patients ($p= 0.023$; Fig. 4e). According to a Cox regression analysis, Gadd45a expression was an independent prognostic marker of ICC (Supplementary Table).

Gadd45a functions as a target of lenvatinib to induce cell cycle arrest in ICC

Given the crucial findings described above, we hypothesized that activated Gadd45a is involved in the process of ICC inhibition by lenvatinib and serves as a target of lenvatinib. To assess this possibility, HCCC-9810 and RBE cells were transfected with Gadd45a-specific siRNA, and the transfection efficiency was confirmed by western blotting and quantitative RT-PCR (Fig. 5a). As Fig. 5b indicates, Gadd45a silencing obviously weakened the inhibitory effects of lenvatinib on cell cycle progression and cell proliferation in both ICC cell lines, and no significant difference was observed between Gadd45a-knockout ICC cells and scramble siRNA-transfected ICC cells in response to 25 μ M lenvatinib treatment, suggesting that Gadd45a plays a crucial role in cell cycle regulation by ICC. Moreover, the observed levels of CDK1 and CDK2 in Gadd45a-knockout cells following lenvatinib treatment were consistent with the aforementioned results (Fig. 5d), indicating that lenvatinib inhibits ICC cell proliferation via Gadd45a-mediated cell cycle arrest.

Discussion

As an oral multi-target TKI, lenvatinib has been found to be effective against thyroid cancer, hepatocellular carcinoma, and renal cancer(20). Recently, its efficacy in ICC has also been increasingly noticed, but there is no relevant research, and the mechanism remains largely unclear. In this study, we preliminarily confirmed that 1) lenvatinib had excellent antitumour effects in ICC cell lines and ICC PDX models, 2) lenvatinib inhibited ICC cell proliferation through the induction of cell cycle arrest, 3) Gadd45a, a DNA damage-inducing gene, was the key factor in the regulation of the cell cycle by lenvatinib and 4) patients with high expression of Gadd45a had a better prognosis.

PDX models using patient tumours as the direct tissue source was valid preclinical models for oncology drug development and drug response prediction. studies have found that the biological characteristics of the primary tumour can not only be preserved in a primary transplantation model but also be well preserved in a post-transplantation model. In PDX models of colon cancer, the genetic phenotypes and mutations of primary tumours were consistently and stably expressed in the subculture model. Efficacy experiments conducted with PDX models have as great as 90% consistency with the results of clinical efficacy studies(21, 22). In our study, to determine the value of lenvatinib in ICC treatment, 5 PDX models of ICC were constructed, and the results showed that lenvatinib could significantly inhibit the growth of ICC compared with control treatment, and no significant difference in body weight among groups was

noticed. This result suggested that in clinical practice, the agent is supposed to be safe and effective for ICC patients.

The cell cycle, as one of the important components of tumour cell proliferation, has been proven to play important roles in the occurrence and development of many tumours. Cyclin-dependent protein kinases (CDKs) and cyclin-dependent protein kinase inhibitors (CKIs) are known to be the key regulatory loci of the cell cycle, in which Cyclins can positively regulate CDKs, while CKIs negatively regulate CDKs(23, 24). In our work, dose-dependent arrest in the G0/G1 phase of the cell cycle was obvious in ICC cell lines after treatment with lenvatinib, which was consistent with the observed levels of important proteins involved in the cell cycle, including CDK1/CyclinB1 and CDK2/Cyclin E. Based on transcriptome analysis, we found that Gadd45a was significantly upregulated after the treatment with lenvatinib. As one of the members of the growth arrest and DNA damage gene (Gadd) family, Gadd45a was previously reported to be closely related to the prognosis of tumour patients and was found to be a cell cycle regulator(25-27). On the one hand, activated Gadd45a can directly inhibit the expression of cell cycle-related proteins (CDKs/CKIs) to prevent cell cycle progression; on the other hand, it can inhibit the phosphorylation of Cyclins/CDKs by inducing the expression of p21(28, 29). In our present study, similar results were observed, as increased Gadd45a expression was found to be correlated with better OS in ICC and might serve as a prognostic biomarker for ICC. In addition, loss of Gadd45a expression significantly attenuated the cycle arrest induced by lenvatinib, as well as the inhibitory effect of lenvatinib on ICC, indicating Gadd45a as a key target of lenvatinib for ICC treatment. however, the potential upstream mechanism of its regulation on Gadd45a was not fully elucidated here. As one of the important downstream molecules of p53 in cell cycle regulation, Gadd45a can be regulated in a p53-dependent manner, while the expression of p53 in tumour cells can be significantly inhibited by the PI3K/Akt signalling pathway(30-32). Thus, given recent findings showing that the activation of the PI3K/Akt signalling pathway is closely related to the binding of FGFRs to their ligands(33-35), we speculated that the regulation of Gadd45a by lenvatinib may rely on the upstream PI3K/Akt/p53 signalling pathway, which will be further investigated in our future work.

To sum up, our PDX model-based investigations provided attractive preclinical results. Therefore, prospective clinical trials should be performed to evaluate the efficacy of lenvatinib treatment in patients with ICC. More data should be collected to verify the anti-tumour activity and side effects of lenvatinib before it's clinical application in ICC patients.

Conclusion

The results of our present study indicated that lenvatinib had excellent antitumour activity in ICC cells, it could inhibit ICC proliferation through the induction of Gadd45a mediated cell cycle arrest.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Consent for publication

Not applicable.

Author Contribution

Xia Yan and Dan Wang contributed equally to this work. Liping Zhuang provided acquisition, analysis, and interpretation of data; Peng Wang helped a lot with the revision of the paper; Zhouyu Ning and Zhiqiang Meng performed study concept and design.

Ethics Statement

All procedures were performed after obtaining approval from the Ethics Committee of the FUSCC, and it was performed in accordance with the Declaration of Helsinki.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Tables

Table1: Demographic and Clinical Characteristics of Patients

Variables	Gadd45a (-) (56)	Gadd45a (+) (31)	p-value
Age (yr)			
≤60	27	19	0.270
>60	29	12	
Gender			
Female	26	13	0.822
Male	30	18	
T stage			
1	12	10	0.337
2	42	21	
3	2	0	
Lymph node status			
Negative	39	22	1.000
Positive	17	9	
TNM stage			
I	12	8	0.889
II	26	14	
III	18	9	
Tumor differentiation			
Well	10	2	0.088
Moderate	39	28	
Poor	7	1	

Figures

Figure 1

Lenvatinib exhibits effective antitumour activity in ICC PDX models. a The flow chart of in vivo experiments; b, Lenvatinib’s response after treatment in five PDX models; c-g, Tumour growth curves and tumour weights box for each PDX case. *P < 0.05; **P < 0.01; ***P < 0.001

Figure 2

Lenvatinib inhibits ICC cell growth in vitro. a HCCC9810 and RBE cells were treated with lenvatinib for 48 and 72 h, Cell viability was measured using CCK8, and IC50 values were calculated; b CCK-8 assay was used to test the effects of Lenvatinib on the viability of ICC cells; c Representative images of the clone formation assay; d, Tumour growth curves and tumour weights for CDX model. E The expression of the proliferation marker Ki-67 was determined in tumour tissue sections from the xenografts using IHC (scale bar, 20 μ m). *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.0001

Figure 3

Lenvatinib could induce cell cycle arrest in ICC. a and b KEGG analysis of differentially expressed genes after treatment with 25 μ M lenvatinib (HCCC-9810); c Cells were treated with lenvatinib at the indicated concentration for 72 h and then stained with PI for flow cytometric analysis. Each experiment was performed in triplicate; d Expression of cell-cycle-related proteins in ICC cell lines after treatment with concentration gradient Lenvatinib for 72 h was detected using western-blot; e The expression of Cyclin B1 and Cyclin E was significantly downregulated, while the expression of Gadd45a was upregulated, based on the immunohistochemistry (IHC) assay after lenvatinib treatment (scale bar, 20 μ m). *P < 0.05; **P < 0.01

Figure 4

Lenvatinib upregulates Gadd45a in vitro and in vivo, and Gadd45a expression is positively correlated with ICC prognosis. a Heatmap showing the clustering of the top 50 differentially expressed genes in ICC cells treated with lenvatinib (25 μ M) and control cells; b Protein-protein interaction network of Gadd45a, based on String; c Representative images of IHC staining for Gadd45a in ICC (scale bar, 20 μ m); d The OS of patients with ICC was assessed using the Kaplan-Meier analysis based on Gadd45a expression (n = 77,

*P = 0.023.); e Gadd45a expression in ICC and adjacent normal tissues, as determined by the IHC score (n= 90 pairs, ****P < 0.0001).

Figure 5

functions as a target of lenvatinib to induce cell cycle arrest in ICC. a Gadd45a silencing in protein and mRNA in ICC cells was confirmed by immunoblotting analysis and quantitative RT-PCR, respectively; b and c The proportion of cells in each phase of the cell cycle and the viability of cells in Gadd45a-knockout and Gadd45a-scramble cell lines following lenvatinib treatment; d Immunoblotting analysis of Gadd45a, CDK1 and CDK2 levels in ICC cells of Gadd45a silencing or scramble following lenvatinib treatment. The grouping of blots cropped from different parts of the same gel or from different gels, fields, or exposures was divided with white space. **P < 0.01, ***P < 0.001; ****P < 0.0001.

Supplementary Files

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