Embelin enhances the sensitivity of renal cancer cells to axitinib by inhibiting HIF signaling pathway

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Abstract

Background: Renal cell carcinoma (RCC) is a common malignant tumor of urinary system with high recurrence rate and easy metastasis. Current clinical drugs for renal cell carcinoma include immunotherapy and targeted drugs. Axitinib is a clinical targeted drug for the treatment of renal cell carcinoma, which has some shortcomings such as unstable efficacy and easy drug resistance. The aim of this study was to determine whether embelin can enhance the sensitivity of renal cancer cells to axitinib and explore its regulatory pathways.

Results: Embelin enhanced the sensitivity of renal cancer cells to axitinib in the following aspects: enhancing the inhibition of cell proliferation by axitinib, and the induction of cell apoptosis. HIF was a potential pathway of Embelin action. After IOX2 regulated the HIF-1 α pathway, the enhancing effect of embelin on axitinib was weakened. Moreover, after PT2977 regulated the HIF-2 α pathway, the enhancing effect of embelin on axitinib was also weakened.

Conclusions: Embelin enhanced the sensitivity of both A498 and 786-O renal cancer cells to axitinib by inhibiting the HIF pathway.

Background

Renal cell carcinoma (RCC), which originates from the renal parenchyma urinary tubule epithelia, is one of the three most common malignant tumors in the urinary system. The incidence rate of renal cell carcinoma is approximately 3% of all adult malignant tumors, second only to bladder cancer.[1] Results of epidemiological studies show that the incidence of renal cell carcinoma varies in different countries or regions around the world. In general, the incidence rate in developed countries is higher than that in developing countries, urban areas are higher than rural areas. There are more males than females, with a ratio of about 2:1, and the high incidence age is between 50-70 years old.[1] The pathogenesis of renal cell carcinoma is still unclear, but studies have suggested that it is related to diet, occupation, living environment, smoking, and genetic mutations.[2,3] For early-stage RCC, partial nephrectomy or radical nephrectomy may be performed, but for later-stage patients with metastatic disease, drug therapy is the best option.[4] Drug therapy is mainly divided into immunotherapy and targeted drug therapy. Immunotherapy drugs have a synergistic effect with radiotherapy on early-stage patients and can activate immune cells and kill tumor cells in patients after surgery. However, immunotherapy has low effectiveness in patients with advanced renal cell carcinoma. Multi-target small molecule antitumor drugs can interact with specific targets of tumor cells, and ultimately achieve the purpose of blocking the growth and proliferation of tumor cells through specific means. Targeted drugs play an important role in prolonging the survival of patients and improving survival therapy.

Axitinib is a multi-target anticancer agent that blocks vascular endothelial growth factor receptor 1/2/3, platelet-derived growth factor receptor α /p, and stem cell receptor factor c-Kit.[5] It has been approved for the treatment of advanced renal cell carcinoma and has shown good efficacy in multiple tumor models.

[6] Studies have reported that axitinib has a lower incidence of adverse reactions than other drugs, but there have been reports of fatal adverse reactions. Therefore, looking for drugs to enhance the efficacy of axitinib is an effective way to reduce the dosage of axitinib and reduce adverse reactions.

Embelin is a quinone compound extracted from the plant *Embelia ribes*, which is a common Indian herb used to treat a variety of diseases.[7] Embelin (Embelic acid), a potent, non-peptidic XIAP inhibitor, can inhibit the anti-apoptotic effect of XIAP and is a promising anti-cancer drug. The LD50 of Embelin is as high as 2000 mg/kg, and it has no fatal or adverse reactions in rats and mice, indicating its high safety. [8] In addition, the median lethal dose of Embelin is 44 mg/kg when administered intraperitoneally. Oral doses of embelin ranged from 10 mg to 3 g/kg in rats and mice, showing a wide safety range and no toxic effects. Subcutaneous injections of 10 mg/kg of embelin for 10 consecutive weeks showed no cardiac effects. The toxicity of embelin in the liver, kidney, and bone marrow also indicates that embelin is highly safe.

Studies have shown that embelin induces apoptosis of lung cancer cells by activating the p38/JNK pathway.[9] Embelin inhibits the expression of proteins CDC2, CDC25c, and CDK2, thus inhibiting the cell cycle of gastric cancer cells in the S phase and G2/M phase and reducing the activity of gastric cancer cells.[10] Embelin plays a cytotoxic role on pancreatic cancer cells by inhibiting STAT3 and activating the p53 signaling pathway, including inhibiting tumor cell invasion and proliferation and inducing tumor cell apoptosis, thereby slowing the progression of pancreatic cancer.[11] Embelin inhibits the proliferation of breast cancer cells, promotes the apoptosis of breast cancer cells, and inhibits the expression of key oncogenes in breast cancer,[12] suggesting that embelin has a potential therapeutic effect on breast cancer. In addition, embelin can promote apoptosis of prostate cancer cells by inhibiting the Akt signaling pathway.[13] Embelin induces apoptosis of glioma cells through the mitochondrial apoptosis pathway. [14] Studies have shown that embelin inhibits the proliferation and metastasis of breast cancer cells, and can induce the apoptosis of breast cancer cells.[15] Other studies have shown that embelin prevents tumor growth and new angiogenesis during wound healing by depleting the low-respiratory reserve of proliferating ECs without adversely affecting resting ECs.[16]

Based on the above analysis, we inferred that embelin enhanced the sensitivity of renal cancer cells to axitinib. To test this hypothesis, we used two renal cancer cell lines, A498 and 786-O, to identify the effects of embelin and axitinib. Then, cell proliferation and apoptosis were detected to determine the enhanced anticancer effect of embelin at different concentrations on axitinib. Using bioinformatics and network pharmacology, the signaling pathway of embelin action was predicted and initially verified. Through the above experiments, we hope to confirm that embelin can enhance the sensitivity of renal cancer cells to axitinib, and explore its mechanism, to provide theoretical support for finding new ways of drug therapy for renal cancer.

Results

Axitinib and Embelin can inhibit tumor cell viability independently

Axitinib and embelin inhibited tumor cell viability was first identified separately. As shown in Figure 1A, the structure of embelin is significantly different from axitinib, which is already used clinically for the treatment of kidney cancer, and it largely excludes cases where it has the same site of action. Embelin or axitinib were used alone to act on cancer cells. As shown in Figure 1B, as the dosage increased, the inhibitory effect of the drugs on cell activity gradually increased in a dose-dependent manner. There was a certain correlation between the time of drug action and the change of cell activity. The inhibitory effect of the drug on cell activity increased with the time of drug action.

Embelin enhances the anticancer activity of axitinib

Subsequently, we examined the enhancing effect of embelin on the anticancer activity of axitinib. As shown in Figure 2A, axitinib inhibits cell proliferation in two renal cancer cell lines A498 and 786-O. Different concentrations of embelin can enhance the anti-cancer effect of axitinib, and there is a positive correlation between the dose and time of action. Crystal violet staining further confirmed embelin could enhance the tumor inhibition effect of axitinib. As shown in Figure 2B, cells that survived treatment with different drug concentrations were stained with crystal violet. The use of axitinib alone has a certain inhibitory effect on renal cancer cells, but it is not significant. Embelin at 1.25 μ M, 2.5 μ M, and 5 μ M all enhanced the inhibitory effect of axitinib, and the higher the concentration of embelin, the stronger the enhancing effect.

Embelin enhances the apoptosis induced by axitinib in cancer cells

By Annexin V-FITC staining We examined the enhanced effect of embelin on axitinib-induced apoptosis in cancer cells. As shown in Figure 3A, axitinib induced apoptosis in two renal cancer cell lines A498 and 786-O. Different concentrations of embelin can enhance the apoptosis induced by axitinib, and there is a positive correlation with the dose. By western blot, we detected the changes in the expression of apoptosis-related proteins under the action of drugs. As shown in Figure 3B, axitinib upregulated Bax, cleaved caspase-3 and p53 expression and down-regulated Bcl-2 expression in A498 and 786-O renal cancer cell lines. Different concentrations of embelin can enhance the regulation ability of axitinib on apoptosis-related proteins, and there is a positive correlation with the dose.

Embelin may enhance the efficacy of axitinib through the HIF pathway

In order to explore the signaling pathways that may be affected by Embelin, three databases, CTD, Batman, and Stitch, were used. By retrieving Embelin-related pathways in the three databases and intersections with the results obtained from renal cancer retrieval in CTD, three possible pathways were screened out (as shown in Figure 4A). A large number of studies have shown that HIF is highly expressed in a variety of tumor cells[17-19] and plays a key role in renal cell carcinoma.[20] We first used western blot to preliminarily explore the regulation of axitinib and embelin on HIF-1 α , HIF-2 α , PGK-1, VEGF and EPO expression in both A498 and 786-O renal cancer cell lines. As shown in Figure 4B, embelin enhanced axitinib-induced downregulation of HIF-1 α , HIF-2 α , PGK-1, VEGF and EPO in a dose-dependent manner.

Regulation of the HIF pathway can affect the enhancement of axitinib by embelin

To further confirm that embelin enhances the efficacy of axitinib through the HIF pathway, we used IOX2 (a potent selective inhibitor of HIF-1 α prolyl hydroxylase-2) and PT2977 (a potent selective inhibitor of HIF-2 α). IOX2 or PT2977 was added in combination with axitinib and 5 μ M embelin. As shown in Figure 5A, in both A498 and 786-O renal cancer cell lines, the enhancement of axitinib-induced apoptosis by embelin was partially reversed by IOX2 or PT2977. Western blot results also showed that axitinib or axitinib combined with embelin down-regulated the expression of Bcl-2 in A498 and 786-O cell lines, while IOX2 or PT2977 reversed the down-regulated effect. Axitinib or axitinib combined with embelin up-regulated Bax and cleaved caspase-3 expression, while IOX2 or PT2977 attenuated this up-regulated effect. (Figure 5B).

Discussion

In this study, we demonstrated that embelin enhances the sensitivity of renal cancer cells to axitinib. The results showed that embelin increased the down-regulation effect of axitinib on cell activity, weakened cell proliferation and promoted more apoptosis in two types of renal cancer cells. The role of embelin in the HIF pathway was verified by western blot and pharmacological methods. In conclusion, embelin enhances the sensitivity of renal cancer cells to axitinib by inhibiting HIF signaling pathway.

In solid tumors, tumor cells are mainly regulated by hypoxia inducible factor (HIF) to maintain their adaptation to the hypoxic microenvironment. The HIF family can sense changes in oxygen concentration in the microenvironment and regulate cellular pathways to adapt to hypoxia.[21,22] It has been found that HIF is involved in the direct regulation of more than 800 target genes.[23,24] Several advanced human cancers share HIFs activation as a final common pathway.[25] HIFs are a heterodimeric transcriptional factors composed of an O₂ sensitive α subunits (HIF-1 α , or HIF-2 α or HIF-3 α) and an O₂ insensitive HIF-1 β subunit.[26] HIF-1 α and HIF-2 α are the two most important subtypes of HIFs, and known to be widely expressed in some cancers.[27]

HIF-1 α is the first factor to be identified to regulate the cellular response to hypoxia. HIF-1 α is the functional subunit that plays a major role in oxygen regulation.[28] As a transcription factor, HIF-1 α is involved in the regulation of a variety of downstream target genes related to tumor invasion and metastasis. Up to now, more than 100 target genes that are regulated by HIF-1 α have been identified, which mainly include the following functional categories: tumor angiogenesis,[29-32] glucose metabolism,[33-35] regulation of erythrocyte generation,[36] tumor proliferation,[37-39] apoptosis,[40-42] invasion and metastasis.[43-45] Many studies have shown that HIF-1 α is one of the major regulatory factors of tumorigenic epithelial matrix transformation (EMT).[46-48] Moreover, previous studies have shown that high expression of HIF-1 α is a significant characteristic of renal carcinoma cells.[49,50] Our results confirmed that embelin enhances the efficacy of axitinib by down-regulating HIF-1 α . HIF-2 α has been reported to act as a critical oncogenic protein in RCC.[51] The upregulated HIF-2 α can induce the growth and survival of tumors. For example, excessive HIF-2 α accelerates the invasion and metastasis of

RCC through regulating CXCR4/SDF1a, which causes EMT and increases the generation of RCC-derived cancer stem cells.[52] Our results demonstrated that embelin could enhance the efficacy of axitinib by down-regulating HIF-2α. In addition, we also investigated the effect of embelin on HIF-1α and HIF-2α targets (VEGF, EPO and PGK-1), and found that embelin elevated the efficacy of axitinib via down-regulating the expressions of VEGF, EPO and PGK-1 in both A498 and 786-O cells.

Conclusion

In conclusion, in this study, embelin enhanced the sensitivity of both A498 and 786-O renal cancer cells to axitinib by inhibiting the HIF pathway.

Methods

Cell Culture and cell culture reagents

Two Human kidney cell lines, A498 (cat. no. HTB-44, ATCC, Inc, USA) and 786-O (cat. no. HTB-44, CRL-1932, Inc, USA) were maintained in DMEM (cat. no. 11965092, ThermoFisher, Inc, USA) supplemented with 10% FBS (fetal bovine serum, cat. no. 30067334, ThermoFisher, Inc, USA) and 2mM glutamine (cat. no. 25030164, ThermoFisher, Inc, USA). These cells were cultured in DMEM supplemented with 10% FBS, 2mM glutamine, 1% Pen/Strep (cat. no. 15070063, ThermoFisher, Inc, USA). All cells were grown in collagen-coated plastic (50ug/mL) at 37°C in a humidified 5% CO₂ atmosphere.

Cell viability was detected by Cell Counting Kit-8 assay

CCK-8 assay kit (cat. no. C0038, Beyotime, Inc, China) was used to detect cell viability. According to the manufacturer's instructions, logarithmic growth phase A498 or 786-O cells were seeded in 96-well plate at a density of 2000 cells/well. Embelin (cat. no. 178493, Sigma, Inc, USA) or Axitinib (cat. no. PZ0193, Sigma, Inc, USA) of different concentrations were added, and 6 compound holes were set up for each concentration. The cells were cultured at 37 °C for 24 h. Then cells were incubated with 100 μL of medium containing 10% CCK-8 reagent for 2 h. The absorbance values of each well at 450 nm were detected through a spectrometer.

Cell proliferation was detected by MTT assay

MTT Cell Proliferation Assay Kit (cat. no. C0009S, Beyotime, Inc, China) was used to detect cell proliferation following manufacturer's instructions. Cells were seeded into 96-well plates at 2×10^3 per well and incubated under standard culture condition for 72 h. Following Embelin or Embelin treatment, 10 μl of MTT solution was added to each well and the plates were incubated for another 3 h at 37°C, and formazan crystals were dissolved with 100 μl of 0.04 N HCl-isopropanol. After incubated for 4h at 37°C. The absorbance of individual wells was read at 570 nm test wavelength using a microplate reader (Victor Nivo 5s, Perkinelmer, Inc, Finland). Cell proliferation activities were determined by absorbance values.

Cell survival was tested by crystal violet assay

Crystal violet assay was used to detect Cell survival in different concentration of drugs.[53] Briefly, cells were seeded on 48 well cell culture plates at 5×10^3 cells/well and incubated at 37 °C for 24 h. After the addition of drugs, cells were incubated at 37°C for 2 days. At the indicated time points, cells were fixed for 20 min with 4% paraformaldehyde (cat. no. P0099, Beyotime, Inc, China). Subsequently, wells were washed by submersion in a water bath. Then wells were aspirated and 0.5% crystal violet (cat. no. C0121, Beyotime, Inc, China) was added. After 20 min, crystal violet was aspirated and wells were thoroughly washed by submersion in a water bath. Plates were left to dry at 37°C and crystal violet was reconstituted in 10% acetic acid, image of each well was captured and analyzed using ImageJ software.

Apoptosis was assessed by Annexin V-FITC staining

Annexin V-FITC staining kit (cat. no. C1062S, Beyotime, Inc, China) was used to detect cell apoptosis. Following manufacturer's instructions, cells were treated with indicated concentrations of drugs for 48 h. Then cells were collected, washed 3 times with PBS and re-suspended in PBS. Each sample was incubated with 5 µl of Annexin V-FITC and 10 µl propidium iodide staining solution in 195 µl of the binding buffer for 15 min at room temperature in the dark. Then, the samples were analyzed by flow cytometry (ACCURI C6, BD Biosciences, Inc, USA).

Protein expression level was detected by western blot assay

Western blot assay was used to detect protein expression levels. Proteins were extracted using RIPA total protein lysate (cat. no. P0013C, Beyotime, Inc, China) following the manufacturer's instructions. Proteins from each sample were separated by electrophoresis on 10% SDS-PAGE gels and transferred onto PVDF membranes (cat. no. FFP39, Beyotime, Inc, China). After incubation with blocking solution (cat. no. P0252, Beyotime, Inc, China), the PVDF membranes were separately incubated with anti-BCL-2, anti-BAX, anti-cleaved caspase-3, anti-GAPDH, anti-HIF-1 α , anti-HIF-2 α , PGK-1, VEGF, EPO, caspase-3 and p53 (cat.no. ab32124, ab32503, ab32042, ab9485, ab51608, ab243861, ab233135, ab32152, ab226956, ab32351, ab32389, Abcam, Inc, USA,1:1000) at 4°C overnight. After the blots were washed, they were incubated with HRP-conjugated Goat Anti-Rabbit IgG H&L secondary antibody (cat.no. ab6721, Abcam, Inc, USA,1:2000) for 1.5 h and detected by ECL like Western reagent (cat. no. P0018S, Beyotime, Inc, China) with a chemiluminescent imaging system (ChemiDoc MP, Bio-Rad, Inc, USA). For the consideration of saving primary antibodies, the PVDF membranes were cut prior to hybridisation with primary antibodies, thus the images of full length membranes were absence in this manuscript.

The target pathway of Embelin was predicted by network pharmacology analysis

To predict the signaling pathways that embelin acts on, three networked pharmacological databases were used for analysis. Embelin was retrieved from CTD Database (Comparative Toxicogenomics Database, <http://ctdbase.org/>), STITCH Database (<http://stitch.embl.de/>) and BATMAN-TCM Database (Bioinformatics Analysis Tool for Molecular mechanism of Traditional Chinese

Medicine, <http://bionet.ncpsb.org.cn/batman-tcm/>) to obtain the corresponding signaling pathways. Subsequently, "Kidney Cancer" was retrieved from the CTD Database and the corresponding signal pathway was obtained. Winn diagram was used to analyze the results of four retrievals and the intersection was selected as the most likely target pathway of embelin.

Statistical analysis

The GraphPad Prism software (version 8.0) was used to perform Statistical analysis and mapping. One-way analysis of variance followed by a Bonferroni post-hoc test was used to analyze differences between groups. Data were considered significantly different when $p < 0.05$. Image analysis was conducted using Image J software (1.53c).

Abbreviations

Renal cell carcinoma (RCC), hypoxia inducible factor (HIF)

Declarations

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Competing interests

The authors declare that they have no conflicts of interest.

Ethics approval and consent to participate

No animal and patients

Consent for publication

Not applicable

Availability of data and material

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

Author contributions

Contributions: Conception and design: Qiong Fang; Collection and assembly of data: Zhiying Li, Ye Xue, Xin Zong and Zuowei Li; Data analysis and interpretation: Wenshuang Ma, Guangmin Xi and XiaoFeng Zhang; Manuscript writing: Qiong Fang; Final approval of manuscript: All authors.

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Figures

Figure 1

Both axitinib and embelin can inhibit tumor cell viability. (A) The chemical structure of embelin and axitinib; (B) CCK-8 assay was used to detect the cell activity under different dosage and treatment time conditions. Measurement data were expressed as mean $\pm$ standard deviation. The cell experiment is repeated three times independently.

Figure 2

Embelin enhanced the sensitivity of cancer cells to axitinib. (A) MTT assay was used to detect the effect of embelin in combination with axitinib at different concentrations on the proliferation of A498 and 786-O cells; (B) Crystal violet staining was used to detect the inhibitory ability of embelin in combination with axitinib at different concentrations on A498 and 786-O cells. * $P < 0.05$ compared with Axitinib group. Measurement data were expressed as mean $\pm$ standard deviation. The cell experiment is repeated three times independently.

Figure 3

Embelin enhanced the apoptosis of cancer cells induced by axitinib. (A) Effects of different concentrations of embelin combined with axitinib on apoptosis in A498 and 786-O cells; (B) Western blot detection of different concentrations of embelin combined with axitinib to regulate apoptosis-related proteins in A498 and 786-O cells. *P < 0.05 compared with Axitinib group. Measurement data were expressed as mean $\pm$ standard deviation. The cell experiment is repeated three times independently.

Figure 4

Embelin might enhance the sensitivity of renal carcinoma cells to axitinib through HIF pathway. (A) Bioinformatics analysis suggested the signaling pathways that embelin may act on; (B) Western blot was used to detect the effect of embelin combined with axitinib at different concentrations on the protein

expression of HIF-1 α , HIF-2 α , PGK-1, VEGF and EPO in A498 and 786-O cells. Measurement data were expressed as mean $\pm$ standard deviation. The cell experiment is repeated three times independently.

Figure 5

Regulation of the HIF pathway affects the sensitivity of renal cancer cells to axitinib. (A) Embelin enhanced axitinib-induced apoptosis in A498 and 786-O cell lines, which was partially reversed by IOX2 or PT2977; (B) Western blot assay showed that IOX2 or PT2977 weakened the regulation of apoptosis related proteins by embelin combined with axitinib. *P < 0.05 compared with Axitinib group, #P < 0.05 compared with Axitinib + 5 group. Measurement data were expressed as mean $\pm$ standard deviation. The cell experiment is repeated three times independently.

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