

## Gentiopicroside suppresses cell proliferation, migration, and invasion *via* inhibition of the HGF/PI3K/Akt pathway in clear cell renal cell carcinoma

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Accepted October 11, 2026

Published online October 15, 2026

### ABSTRACT

Clear cell renal cell carcinoma (ccRCC) is the most common malignant renal tumour; however, effective therapeutic drugs are still limited. Gentiopicroside, a naturally occurring iridoid glycoside, has demonstrated antitumour activity, but its effects on ccRCC remain unclear. This study aimed to evaluate the role of gentiopicroside in ccRCC and elucidate its underlying mechanisms. Cell viability and proliferation were measured using the CCK-8 assay, while wound healing and Transwell assays evaluated cell migration and invasion, respectively. Bioinformatic analysis of high-throughput gene expression data was performed to predict the mechanisms underlying the anti-ccRCC effects of gentiopicroside. qPCR and Western blotting were used to determine mRNA and protein levels, respectively. The results demonstrated that gentiopicroside dose-dependently suppressed proliferation, migration, and invasion of ccRCC cells. High-throughput bioinformatic analysis suggested that the PI3K/Akt pathway and HGF may mediate the regulatory effects of gentiopicroside in ccRCC. Gentiopicroside inhibited the PI3K/Akt pathway, and activation of this pathway reversed its suppressive effects on malignant phenotypes of ccRCC cells. Furthermore, gentiopicroside downregulated HGF expression, whereas HGF overexpression counteracted gentiopicroside-induced inhibition of the PI3K/Akt pathway and malignant phenotypes. In conclusion, gentiopicroside inhibits ccRCC cell proliferation, migration, and invasion *via* the HGF/PI3K/Akt pathway. These findings not only propose a novel candidate drug for ccRCC treatment but also advance our understanding of the bioactivity and mechanisms of gentiopicroside.

**Keywords:** gentiopicroside, clear cell renal cell carcinoma, cell phenotypes, HGF, PI3K/Akt signalling pathway

### INTRODUCTION

Clear cell renal cell carcinoma (ccRCC) is the most common subtype of kidney cancer, accounting for approximately 75 % of all renal malignancies (1). The five-year relative survival rate for patients with distant-stage renal cancer is 18 % (2). Currently, surgical

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resection remains the primary treatment for ccRCC; however, its efficacy is limited by metastasis and recurrence (3). Additionally, ccRCC is resistant to conventional chemotherapy and radiation therapy, and the therapeutic outcomes of immunotherapy and targeted therapy are suboptimal (4, 5). Therefore, the development of novel therapeutic agents for ccRCC is of considerable clinical importance.

Natural products have long been utilized in cancer therapy, and numerous chemotherapeutic agents have been derived from natural sources. Gentiopicroside ( $C_{16}H_{20}O_9$ ; Fig. 1a), a natural iridoid glycoside derived from *Gentiana* species, exhibits anti-inflammatory and antioxidant properties (6). Its antitumour effects have gradually attracted increasing research interest (7–9). However, the effects of gentiopicroside on ccRCC remain unclear.

The PI3K/Akt pathway is a classical signalling pathway that plays a critical role in cancer progression, including tumour cell proliferation, migration, and invasion (10). Previous studies have shown that gentiopicroside exerts antitumour effects by inhibiting this pathway (8, 11); however, this regulatory mechanism has not been reported in ccRCC. HGF, a pleiotropic cytokine, plays a vital role in promoting malignant cancer phenotypes (12). It mediates biological regulation by activating the PI3K/Akt pathway (13–15). To date, the relationship between gentiopicroside and HGF remains unclear.

In this study, we assessed the potential effects of gentiopicroside on ccRCC, evaluated its therapeutic impact, predicted the underlying mechanisms, and examined the roles of the PI3K/Akt pathway and HGF in gentiopicroside-mediated regulation of ccRCC.

## EXPERIMENTAL

**Drugs and antibodies.** – Gentiopicroside (purity 99.83 %, cat. no. HY-N0494) was purchased from MedChemExpress (USA). Recombinant human IGF-1 protein (cat. no. K20057; Shanghai Yuanye Bio-Technology Co., Ltd., China), a known agonist of the PI3K/Akt pathway (16), was used at a concentration of 100 ng mL<sup>-1</sup>. Drugs were diluted in culture medium. Antibodies used for Western blotting included anti-HGF (1:1000; cat. no. 26881-1-AP; Proteintech Group, Inc., USA), anti- $\beta$ -actin (1:10000; cat. no. 60008-1-Ig; Proteintech Group, Inc.), anti-phospho-Akt (p-Akt; 1:500; cat. no. AF0016; Affinity Biosciences, USA), and anti-total Akt (t-Akt; 1:500; cat. no. AF6261; Affinity Biosciences). Secondary antibodies, goat anti-mouse (1:10000; cat. no. SA00001-1) and goat anti-rabbit (1:10000; cat. no. SA00001-2), were obtained from Proteintech Group, Inc.

**Cell culture and experimental grouping.** – The ccRCC cell line Caki-1 (serial no. SCSP-5064) and the normal renal cell line HK-2 (serial no. SCSP-511) were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, China. Caki-1 cells were cultured in McCoy's 5A medium supplemented with 10 % FBS and 1 % penicillin-streptomycin, unless otherwise specified. HK-2 cells were cultured in DMEM/F12 supplemented with 10 % FBS and 1 % penicillin-streptomycin. All cells were maintained at 37 °C in a 5 % CO<sub>2</sub> incubator. Culture reagents were purchased from Thermo Fisher Scientific, Inc. (USA).

The experimental groups ( $n = 3$ ) were designed as follows. For tolerance validation, Caki-1 and HK-2 cells were treated with increasing concentrations of gentiopicroside. For malignant phenotype assessment, groups included a control group (no treatment) and gentiopicroside-treated groups (30, 60, or 120  $\mu$ mol L<sup>-1</sup>). To evaluate the role of the PI3K/

Akt pathway, the groups were as follows: a control group (no treatment), a gentiopicroside group ( $120 \mu\text{mol L}^{-1}$ ), and a gentiopicroside + IGF-1 group ( $120 \mu\text{mol L}^{-1}$  gentiopicroside +  $100 \text{ ng mL}^{-1}$  IGF-1). For Western blot analysis of Akt phosphorylation, cells were co-treated with gentiopicroside and IGF-1 for 24 h before sample collection. In addition, Western blot analyses of Akt phosphorylation and HGF expression were independently repeated under serum-reduced (1 % FBS) conditions, with all other procedures identical. To investigate the role of HGF, groups included a control group (transfected with control plasmids), a gentiopicroside group (control plasmids +  $120 \mu\text{mol L}^{-1}$  gentiopicroside), and a gentiopicroside + HGF-OE group ( $120 \mu\text{mol L}^{-1}$  gentiopicroside + HGF overexpression plasmids).

**CCK-8 assay.** – Cell viability and proliferation were assessed using the CCK-8 assay. For viability testing, Caki-1 ( $1 \times 10^5$  cells/well) and HK-2 ( $1.3 \times 10^5$  cells/well) cells were seeded into 96-well plates. The cells were incubated overnight (approximately 16–18 hours) to allow complete attachment. After cell attachment, the medium was changed according to experimental requirements. Following 24 h of treatment,  $10 \mu\text{L}$  of CCK-8 reagent (cat. no. 40203ES60; Yeasen Biotechnology Co., Ltd., China) was added to each well. After 2 h of incubation at  $37^\circ\text{C}$ , absorbance (OD value) was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.). Cell viability (%) was calculated as follows:  $(\text{OD value of gentiopicroside treated-group} - \text{OD value of blank}) / (\text{OD value of control group} - \text{OD value of blank}) \times 100$ .

For proliferation assays, ccRCC cells (3,000 cells/well) were seeded into 96-well plates, and OD values were measured at 24, 48, and 72 h post-treatment. The detection methods were identical to those used for viability testing. Results were expressed as OD values.

**Wound-healing assay.** – Cell migration was evaluated using a wound-healing assay. Caki-1 cells ( $2.5 \times 10^6$  cells/well) were seeded into 6-well plates. After attachment, a scratch was created using a  $200\text{-}\mu\text{L}$  pipette tip. After scratching, the cells were cultured in medium containing 1 % FBS to minimize proliferation. Images were captured at 0 and 48 h (40 $\times$  magnification) from four randomly selected fields per well. The wound area was quantified using ImageJ software (version 1.51; National Institutes of Health, USA), and migration rate (%) was calculated as  $(\text{wound area at 0 h} - \text{wound area at 48 h}) / (\text{wound area at 0 h}) \times 100$ .

**Transwell assay.** – Cell invasion was assessed using 8- $\mu\text{m}$  pore Transwell plates (cat. no. 14241; Labgic Technology Co., Ltd., China) pre-coated with Matrigel (cat. no. 356234; BD Biosciences, USA). Cells ( $1.5 \times 10^5$ /well) were seeded in serum-free medium in the upper chamber, while the lower chamber contained medium supplemented with 10 % FBS. After 24 h of incubation, non-invading cells were removed. Since the cells were seeded in serum-free medium in the upper chamber and the assay duration was limited to 24 h, cell proliferation during the assay was negligible; the number of invaded cells therefore primarily reflects invasive capacity rather than proliferative differences among groups. Invaded cells were fixed with 4 % paraformaldehyde (cat. no. P1110; Beijing Solarbio Science & Technology Co., Ltd., China) for 20 min at room temperature (RT) and then stained with 1 % Crystal Violet Ammonium Oxalate Solution (cat. no. G1062; Solarbio Science & Technology Co., Ltd.) for 15 min at RT. Images were captured from four randomly selected fields per well (200 $\times$  magnification), and cell numbers were quantified using the ImageJ software. Results were expressed as the number of invaded cells.

**Bioinformatic analysis of high-throughput gene expression data.** – Gene expression data (control: GSM2286398 and GSM2286399; gentiopicroside-treated: GSM2286361 and

GSM2286360) were obtained from the GEO database (GSE85871) (17). Differentially expressed genes (DEGs) ( $|\log_2FC| \geq 2$ ,  $p < 0.05$ ) were identified using GEO2R ([www.ncbi.nlm.nih.gov/geo/geo2r](http://www.ncbi.nlm.nih.gov/geo/geo2r)). ccRCC-related genes were retrieved from GeneCards (<https://www.genecards.org/>) and filtered using a relevance score threshold ( $> 50$ ) (18). The results were visualized as volcano plots using the WeiShengXin online tools. The intersection of DEGs and ccRCC-related genes was visualized using a Venn diagram using the WeiShengXin online tools (<http://www.bioinformatics.com.cn>) (19). KEGG pathway enrichment analysis was performed using the DAVID database (<https://davidbioinformatics.nih.gov/tools.jsp>) (20). Results were ranked by  $p$  value, and the top 10 terms were displayed in a bubble plot (WeiShengXin online tools). The gentiopicroside-DEGs-PI3K/Akt pathway network was constructed using Cytoscape software. A heatmap displaying the expression trends and ccRCC associations of target genes was generated using GraphPad Prism software (version 8.0; Dotmatics).

**Western blotting.** – Western blotting was performed to detect target protein levels. Total protein was extracted from cell samples using RIPA lysis buffer (cat. no. 9806S; CST Biological Reagents Co., Ltd., China). Protein concentration was determined using a BCA Protein Quantification Kit (cat. no. 20200ES76; Yeasen). Protein samples were separated on a 10 % SDS-PAGE gel (cat. no. 20325ES62; Yeasen), and then electro-transferred onto PVDF membranes. After blocking with TBST (cat. no. T1081; Solarbio) containing 5 % BSA (cat. no. 36105ES60; Yeasen) for 1 h at RT, the membranes were incubated with primary antibodies overnight at 4 °C. Following incubation with the corresponding secondary antibodies for 1 h at RT, proteins were detected using an ECL kit (cat. no. 180-501; Tanon Science & Technology Co., Ltd., China). Band intensities were quantified using the ImageJ software.  $\beta$ -actin served as the loading control. The p-Akt/t-Akt ratio was calculated to assess PI3K/Akt pathway activity.

**Real-time quantitative PCR (qPCR).** – qPCR was conducted to detect mRNA levels. Total RNA from each cell sample was extracted using TRIzol<sup>®</sup> reagent (Thermo Fisher Scientific). cDNA was synthesized *via* reverse transcription using the 1st Strand cDNA Synthesis SuperMix Kit (cat. no. 11141ES; Yeasen). Subsequently, qPCR was performed using the qPCR SYBR Green Master Mix (cat. no. 11201ES; Yeasen) under the following conditions: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. Target gene expression was normalized to  $\beta$ -actin gene (ACTB) and analysed using the  $2^{-\Delta\Delta C_q}$  method (21). The primers used for qPCR are listed in Table I.

**Transfection.** – An HGF-overexpression plasmid was constructed by cloning the human HGF gene (NM\_000601.6) into pcDNA3.1/His B (cat. no. V385-20; Thermo Fisher Scientific). The plasmid was transfected into ccRCC cells using Lipofectamine<sup>®</sup> 3000 transfection reagent (cat. no. L3000075; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The empty vector served as a control. After transfection for 6 h at 37 °C, the culture medium was replaced with fresh medium, and the cells were cultured for an additional 24 h.

### Statistical analysis

All quantitative data are presented as the mean  $\pm$  standard deviation (SD). Statistical analyses were performed, and the corresponding bar charts were generated using GraphPad Prism software (USA). Normality was assessed using the Shapiro-Wilk test.

Table I. Sequences of primers used for qPCR.

|        | Forward (5'→3')        | Reverse (5'→3')        |
|--------|------------------------|------------------------|
| HGF    | GGTAAAGGACGCAGCTACAA   | AGCTGTGTTTCGTGTGGTATC  |
| FGFR2  | GGATAACAACACGCCTCTCTT  | CTTGCCAGTGTCAGCTTAT    |
| ANGPT2 | AGAATGCAGTACAGAACAGAC  | CTTGGGCTTCCACATCAGTTA  |
| COL4A3 | GATGGATTGCCAGGTTTGAAAG | TTTGACTGTGTCGGGTGAAG   |
| IL4    | CACGGACACAAGTGCGATA    | AGGCAGCAAAGATGTCTGTTA  |
| FGF2   | AGCGGCTGTACTGCAAAAACGG | CCTTTGATAGACAACTCCTCTC |
| ACTB   | CTCCATCCTGGCCTCGCTGT   | GCTGTCACCTTCACCGTTCC   |

Multigroup comparisons were performed using one-way ANOVA followed by Dunnett's post hoc test or Sidak's post hoc test for pairwise comparisons, whereas two-group comparisons were performed using Student's unpaired *t*-test. *p*-value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

*Effects of gentiopicroside on proliferation, migration, and invasion of ccRCC cells*

To evaluate the effects of gentiopicroside on ccRCC, cellular phenotype experiments were performed. Cell viability assays were performed to determine appropriate concentrations for subsequent experiments. As shown in Fig. 1a, the  $IC_{50}$  of gentiopicroside in Caki-1 cells was 145.3  $\mu\text{mol L}^{-1}$ . Cytotoxicity screening confirmed that concentrations  $\leq 120 \mu\text{mol L}^{-1}$  were non-toxic to HK-2 cells (Fig. 1b). Accordingly, 30, 60, and 120  $\mu\text{mol L}^{-1}$  were selected for further analysis. CCK-8 assay results (Fig. 1c) showed that OD values in the gentiopicroside-treated groups were significantly lower than those in the control group at 24, 48, and 72 h, with values decreasing as concentrations increased. Wound-healing assays (Fig. 1d) revealed significantly inhibited migration rates after 48 h of treatment, with stronger inhibition at higher concentrations. Transwell assays (Fig. 1e) demonstrated significantly fewer invaded cells in the treatment groups after 24 h, with the 120  $\mu\text{mol L}^{-1}$  group showing the lowest number. These results demonstrate that gentiopicroside inhibits ccRCC cell proliferation, migration, and invasion in a dose-dependent manner. Based on these findings, 120  $\mu\text{mol L}^{-1}$  was selected for subsequent experiments.

Gentiopicroside has been reported to exert anticancer effects in some cancer types. In ovarian cancer, gentiopicroside inhibits migration and invasion while promoting apoptosis in SKOV3 cells (7). In another study using ovarian cancer cells (OVCAR-3), Tian *et al.* (22) found that gentiopicroside inhibits cell growth and induces apoptosis. In gastric cancer, Chen *et al.* (8) demonstrated that gentiopicroside inhibits cancer progression both *in vitro* and *in vivo*. Furthermore, gentiopicroside inhibits gastric cancer progression by inducing necroptosis (23). Additionally, gentiopicroside induces apoptosis in colorectal cancer cells (HCT116) (9). In this study, we demonstrated that gentiopicroside inhibited ccRCC by suppressing cell proliferation, migration, and invasion. These results indicate

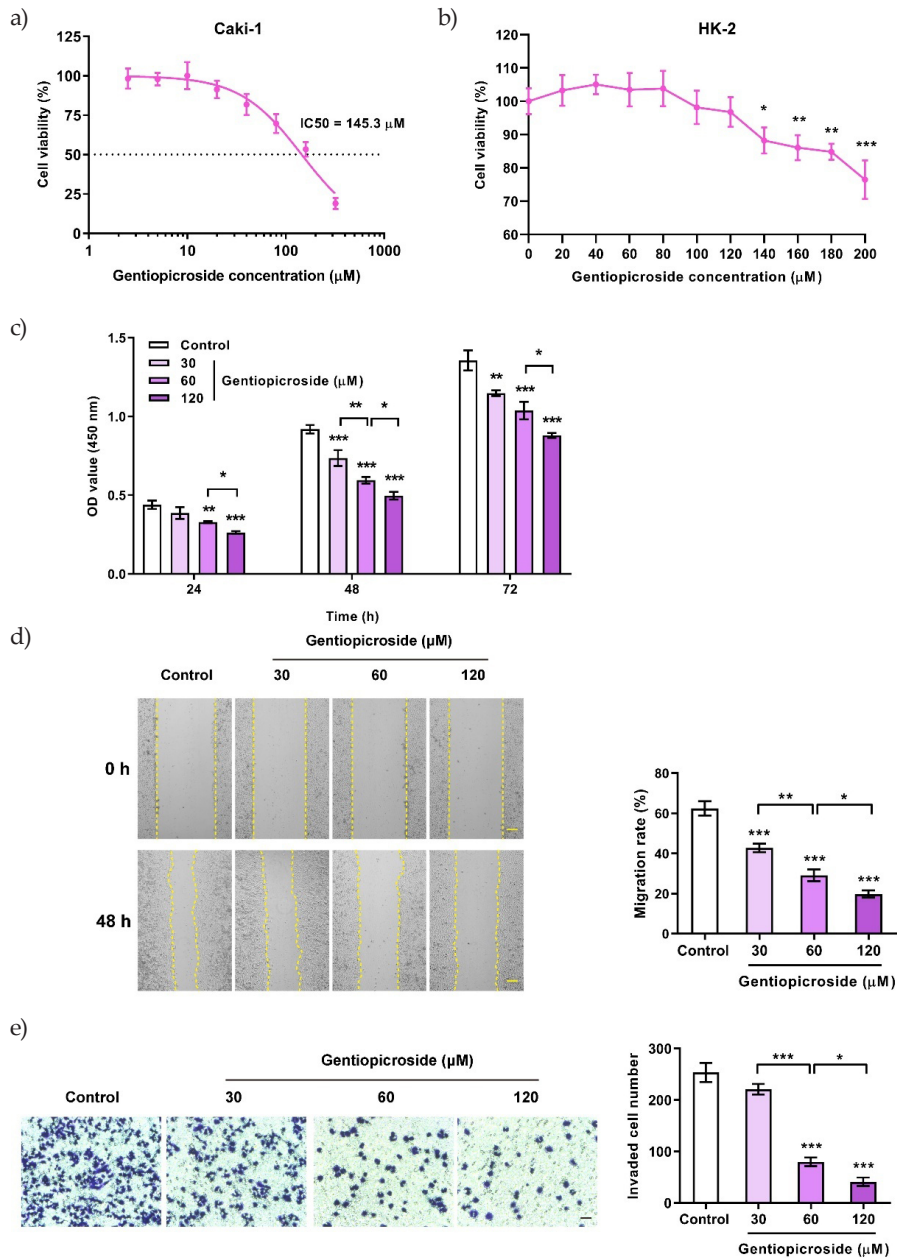


Fig. 1. Effects of gentiopicroside on ccRCC cell proliferation, migration, and invasion: a) IC<sub>50</sub> in Caki-1 cells; b) cytotoxicity in HK-2 cells; c) proliferation; d) migration (scale bar = 200 μm); e) invasion (scale bar = 50 μm). Data are mean ± SD (n = 3). One-way ANOVA with Dunnett's (b) or Sidak's (c–e) post hoc test. \* p < 0.05, \*\* p < 0.01, and \*\*\* p < 0.001 vs. corresponding control.

that gentiopicroside may possess broad anti-cancer bioactivity; however, its effects in other cancers warrant further investigation. Moreover, as only Caki-1 cells were tested, the generalizability of these findings to other ccRCC models remains to be determined. The appropriate drug concentration for *in vivo* application also requires further evaluation in animal studies.

*Prediction of mechanisms underlying gentiopicroside-induced inhibition*

Bioinformatic analysis of microarray gene expression data was performed to predict the mechanism of gentiopicroside in ccRCC. DEGs were screened based on comparative analysis of gene expression data from MCF7 cells (a human breast cancer cell line) with or without gentiopicroside treatment. It should be noted that no public transcriptomic dataset of gentiopicroside-treated renal or ccRCC cells is currently available. Therefore, GSE85871,

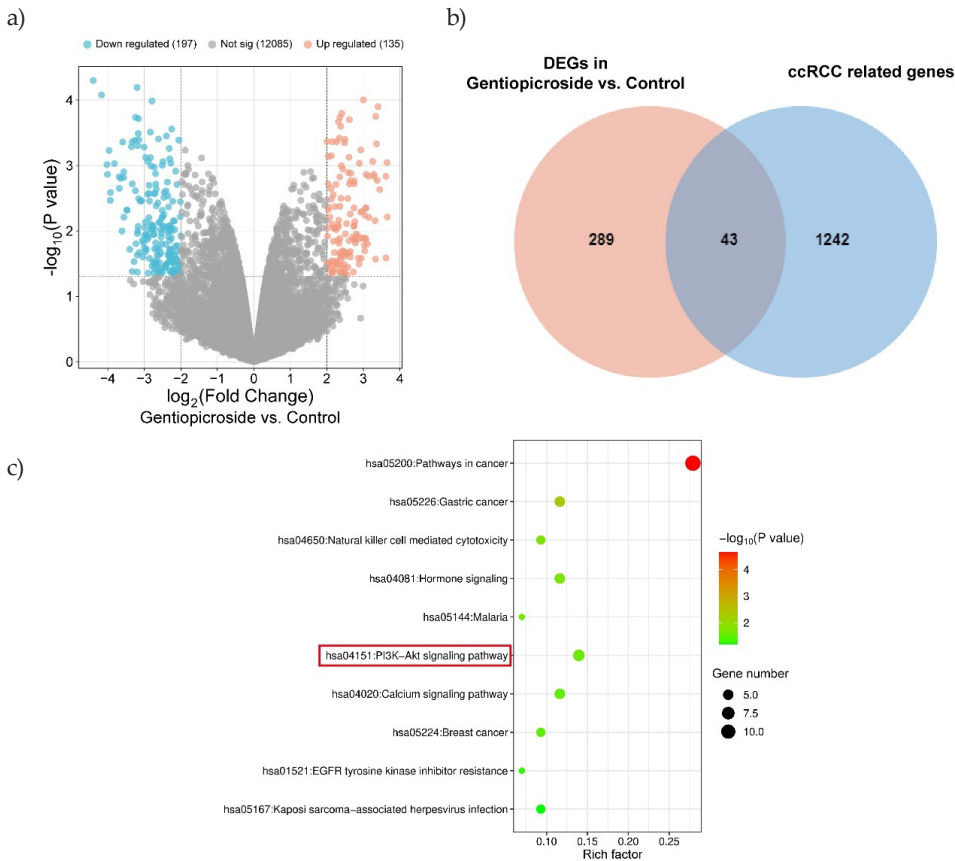


Fig. 2. Bioinformatic prediction of gentiopicroside mechanisms in ccRCC: a) DEG volcano plot; b) Venn diagram of DEGs and ccRCC-related genes; c) KEGG enrichment analysis.

which profiles gentiopicroside-treated MCF7 cells, was used as the closest available dataset, and solely for initial candidate gene screening; all candidate genes were subsequently validated experimentally in Caki-1 ccRCC cells. As shown in Fig. 2a, the gentiopicroside-treated group exhibited 197 downregulated and 135 upregulated genes compared with the control group. The intersection between these DEGs and ccRCC-related genes from GeneCards database yielded 43 genes (Fig. 2b), which was the subset of target genes potentially regulated by gentiopicroside in ccRCC. Further mechanism prediction was performed based on these genes. KEGG enrichment analysis revealed significantly enriched terms, including pathways in cancer, gastric cancer, and natural killer cell mediated cytotoxicity, among others. Among these pathways, the PI3K/Akt signalling pathway, which is associated with ccRCC, was of particular interest (Fig. 2c). These results indicate that gentiopicroside may regulate PI3K/Akt in ccRCC, supporting further validation of the involvement of this pathway.

### *Role of the PI3K/Akt pathway in gentiopicroside-induced phenotype inhibition*

To assess the involvement of the PI3K/Akt pathway, IGF-1, an agonist of this pathway, was used to perform rescue experiments. As shown in Fig. 3a, the p-Akt/t-Akt ratio was significantly lower in the gentiopicroside-treated group than that in the control group, and co-treatment with IGF-1 ( $120 \mu\text{mol L}^{-1}$  gentiopicroside +  $100 \text{ ng mL}^{-1}$  IGF-1) significantly restored this ratio. These results confirmed that gentiopicroside suppressed PI3K/Akt pathway activity, and IGF-1 effectively activated this pathway. Consistent results were obtained when the Western blot analysis was repeated under serum-reduced (1 % FBS) conditions (Fig. 3a). Subsequent phenotypic assays showed that up-regulating the activity of the PI3K/Akt pathway significantly reversed gentiopicroside-induced inhibition of proliferation (Fig. 3b), migration (Fig. 3c), and invasion (Fig. 3d). These findings demonstrate that gentiopicroside inhibits ccRCC cell phenotypes by suppressing PI3K/Akt signalling.

The PI3K/Akt pathway has been implicated in gentiopicroside-induced bioactivity. Previous cancer studies have demonstrated that gentiopicroside exerts anti-tumour effects in gastric cancer (8) and retinoblastoma (11) by inhibiting activation of the PI3K/Akt pathway. Similarly, we found that gentiopicroside inhibited ccRCC by suppressing this pathway. Conversely, Xiao *et al.* (24) showed that gentiopicroside improves insulin-resistant diabetes by activating the PI3K/Akt pathway. In non-cancer contexts, gentiopicroside exerts antispasmodic and relaxant effects *via* PI3K/Akt activation (25). These results indicate that gentiopicroside differentially modulates the PI3K/Akt pathway across diseases. Its inhibitory effect on this pathway may therefore be broadly applicable in cancers. Moreover, given that the pathway agonist used in the rescue experiments in this study was IGF-1, which is known to regulate multiple signalling pathways, potential crosstalk among these pathways may require further clarification.

### *HGF expression in gentiopicroside-treated ccRCC cells*

Genes enriched in PI3K/Akt pathway KEGG terms were analysed to explore the downstream mechanisms of gentiopicroside. As shown in Fig. 4a, six DEGs regulated by gentiopicroside in MCF7 cells were significantly enriched in this pathway. These genes were evaluated based on expression change ( $\log_2\text{FC}$  from microarray analysis) and the

strength of their association with ccRCC (GeneCards relevance scores). As shown in Fig. 4b, gentiopicroside up-regulated HGF, FGFR2, ANGPT2, and COL4A3 while down-regulating IL4 and FGF2 in breast cancer cells. FGFR2 showed the strongest correlation with ccRCC, followed by HGF, IL4, COL4A3, and ANGPT2. Initial qPCR validation in ccRCC cells showed no significant differences in FGFR2, COL4A3, or IL4 mRNA expression,

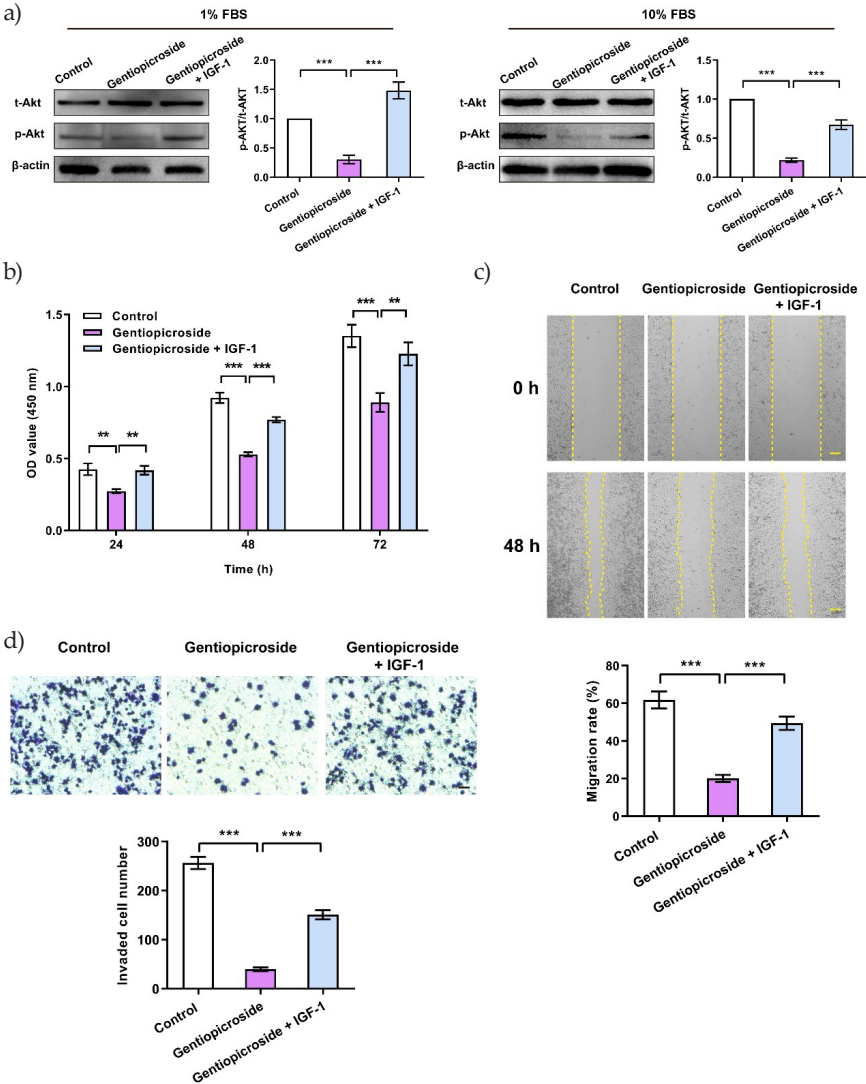


Fig. 3. Role of the PI3K/Akt pathway in gentiopicroside-induced inhibition: a) pathway activation (Western blotting) under standard (10 % FBS) and serum-reduced (1 % FBS) conditions; b) cell proliferation; c) migration (scale bar = 200  $\mu$ m); d) invasion (scale bar = 50  $\mu$ m). Data are mean  $\pm$  SD ( $n = 3$ ). One-way ANOVA with Sidak's post hoc test. \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs. corresponding control.

whereas HGF, ANGPT2, and FGF2 were significantly decreased compared with that in the control cells, with HGF showing the greatest fold change (Fig. 4c). Although HGF was upregulated by gentiopicroside in the MCF7 microarray data (Fig. 4b), it was significantly downregulated in Caki-1 cells. Thus, the bioinformatic screening correctly identified HGF as a candidate target of gentiopicroside but did not predict the direction of its regulation, which is consistent with the cell-type- and context-dependent transcriptional regulation by small-molecule compounds. Based on these results, HGF protein expression was further evaluated using Western blotting, which showed significantly lower levels in the treatment group (Fig. 4d). These findings demonstrate that gentiopicroside inhibits HGF expression in ccRCC cells, suggesting the involvement of HGF in its regulatory effects.

*Role of HGF in gentiopicroside-induced regulation*

Rescue experiments were performed by overexpressing HGF to verify its role. ccRCC cells were transfected with HGF-overexpression plasmids, and qPCR analysis confirmed up-regulation of HGF mRNA (Fig. 5a). Consistent with this finding, HGF protein levels

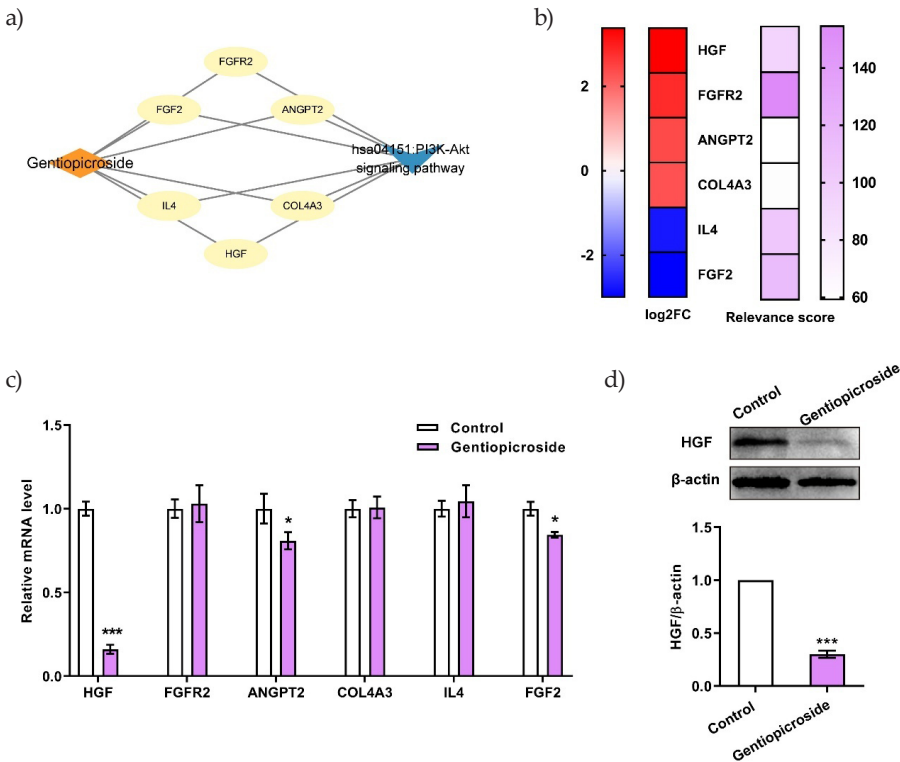


Fig. 4. HGF expression in gentiopicroside-treated ccRCC cells: a) gentiopicroside-DEG-PI3K/Akt pathway network; b) heatmap of PI3K/Akt-enriched DEGs; c) HGF mRNA (qPCR); d) HGF protein (Western blotting). Data are mean  $\pm$  SD ( $n = 3$ ). Student's unpaired  $t$ -test. \* $p < 0.05$  and \*\*\* $p < 0.001$  vs. corresponding control.

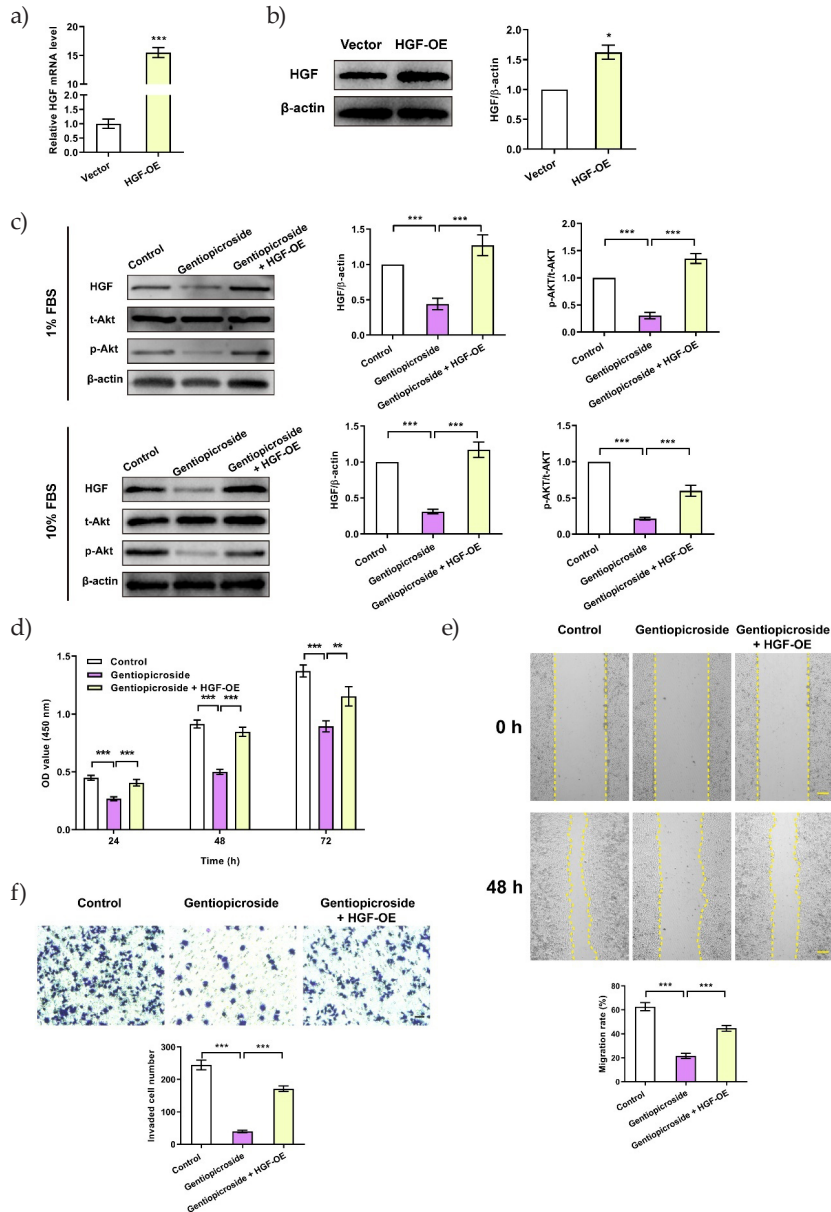


Fig. 5. Role of HGF in gentipicroside-mediated regulation: a) HGF mRNA after transfection (qPCR); b) HGF protein after transfection (Western blotting); c) HGF and PI3K/Akt pathway activation (Western blotting) under standard (10 % FBS) and serum-reduced (1 % FBS) conditions; d) cell proliferation; e) migration (scale bar = 200  $\mu$ m); f) invasion (scale bar = 50  $\mu$ m). Data are mean  $\pm$  SD ( $n = 3$ ). One-way ANOVA with Sidak's post hoc test or Student's unpaired  $t$ -test, as appropriate. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  vs. corresponding control.

were significantly elevated in the HGF-OE group (Fig. 5b), confirming successful HGF overexpression. HGF overexpression significantly reversed the gentiopicroside-induced HGF suppression and restored the p-Akt/t-Akt ratio (Fig. 5c), indicating that gentiopicroside suppresses the PI3K/Akt pathway by inhibiting HGF expression. These effects were also confirmed under serum-reduced (1 % FBS) conditions (Fig. 5c). Phenotypic assays showed that HGF overexpression significantly reversed gentiopicroside-induced inhibition of proliferation (Fig. 5d), migration (Fig. 5e), and invasion (Fig. 5f). Collectively, these findings demonstrate that gentiopicroside-induced suppression of proliferation, migration, and invasion is mediated through the HGF/PI3K/Akt axis.

HGF has been shown to regulate ccRCC and the PI3K/Akt pathway. *In vitro*, HGF stimulation promotes ccRCC cell viability, migration, and invasion (26). The role of the HGF/PI3K/Akt axis has also been reported in other cancers. In testicular germ cell tumours, HGF promotes cell proliferation, migration, and invasion by activating PI3K/Akt (13). In head and neck squamous cell carcinoma, HGF induces chemotaxis and migration through this pathway (14). In breast cancer, HGF promotes cell invasion through activation of the PI3K/Akt pathway (15). In this study, bioinformatic analysis predicted, and experiments confirmed, that gentiopicroside inhibits ccRCC malignant phenotypes *via* the HGF/PI3K/Akt pathway, thereby proposing a novel mechanistic model for its bioactivity. Whether this pathway mediates the anti-tumour effects of gentiopicroside in other cancers requires further investigation. A limitation of this study is that the bioinformatic screening was based on a dataset from MCF7 breast cancer cells rather than ccRCC cells, owing to the lack of a ccRCC-specific gentiopicroside expression dataset. Gene regulation by small-molecule compounds can be highly cell-type specific, as exemplified in this study by the opposite direction of HGF regulation between the MCF7 dataset and Caki-1 cells. Nevertheless, this screening strategy successfully identified HGF as a relevant target, and all key findings were experimentally validated in ccRCC cells. Furthermore, further investigation into the upstream and downstream regulatory mechanisms of the HGF/PI3K/Akt axis is warranted. This includes elucidating the pathways by which gentiopicroside modulates HGF expression, and the subsequent regulation of downstream effectors within the PI3K/Akt cascade. These aspects will constitute the primary focus of our future experimental endeavours, aiming to comprehensively delineate the precise molecular mechanisms underlying the anti-ccRCC effects of gentiopicroside centered on the HGF/PI3K/Akt axis.

## CONCLUSIONS

In conclusion, this study demonstrates that gentiopicroside inhibits ccRCC cell proliferation, migration, and invasion by suppressing the HGF/PI3K/Akt pathway. These findings highlight gentiopicroside as a novel therapeutic candidate for ccRCC and advance our understanding of its mechanisms of bioactivity.

*Availability of data and materials.* – The data generated in this study may be requested from the corresponding author.

*Acknowledgements.* – We thank Editage ([www.editage.com](http://www.editage.com)) for English language editing.

*Conflicts of interest.* – The authors declare no conflict of interest.

*Funding.* – This study was supported by the Natural Science Foundation of Inner Mongolia Autonomous Region of China (Grant Nos. 2026MS0113, 2024QN08078) and the Science and Technology

Program of the Joint Fund of Scientific Research for the Public Hospitals of Inner Mongolia Academy of Medical Sciences (Grant No. 2024GLLH1036).

*Author's contribution.* – Conceptualization, C.B., X.L., and X.W.; methodology, C.B., X.L., and X.W.; analysis, C.B., X.L., and X.W.; investigation, X.W., Z.S., N.Z., and C.Z.; writing, original draft preparation, X.W.; writing, review and editing, C.B. and X.L. All authors have read and agreed to the published version of the manuscript.

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