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Research

Flavonoid GL-V9 suppresses development of human hepatocellular cancer cells by inhibiting Wnt/β-Cantenin signaling pathway

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Abstract
Distant metastasis and post-operative recurrence of tumours are the main causes of death in patients with hepatocellular carcinoma (HCC). In recent years, flavonoids have been found to achieve effective anticancer effects by inhibiting cancer cell proliferation and inducing apoptosis, inhibiting cancer cell invasion and metastasis and neovascularization. GL-V9 is a newly synthesized flavonoid that has been demonstrated anticancer effects in a variety of tumors, but its anticancer effects in HCC and its related mechanisms are still unclear. In this study, we investigated the anti-proliferative, anti-invasive and anti-migratory activities of GL-V9 in HCC cells by MTT method cell proliferation assay, plate cloning assay, transwell invasion assay and cell scratching assay. Based on the results, we found that GL-V9 inhibits the EMT process through a pathway that inhibits Wnt/β-Cantenin pathway signaling, thereby reducing the proliferation, migration and invasion ability of HCC cells. Therefore, GL-V9 may be a novel potential therapeutic agent to inhibit HCC cell metastasis.

Keywords GL-V9 · Hepatocellular carcinoma cells · Wnt/β-Cantenin signaling pathway · EMT

1 Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer with high aggressiveness and mortality, and is the second most deadly malignancy in China, after lung cancer. The 5-year survival rate is only 14%, according to relevant statistics [1]. At present, surgical resection is still the most important treatment for hepatocellular carcinoma, but high bloodstream metastatic spreading ability and tumour recurrence are one of the main causes of death for hepatocellular carcinoma patients [2], so there is an urgent need to find an effective drug to inhibit tumour cell proliferation and metastasis to improve the overall survival rate of hepatocellular carcinoma patients.GL-V9 (5-hydroxy-8-methoxy-2-phenyl-7-

(4-(pyrrolidin-1-yl) butoxy) 4H- chromen-4-one) is a newly synthesized natural flavonoid derivative that has been reported to have effective anti-cancer effects in breast cancer, squamous cell carcinoma of the skin, gastric cancer, etc.

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[3–5]. However, whether GL-V9 can affect the metastasis of HCC remains unclear. In this study, we examined the effect of GL-V9 on the proliferation, invasion and migration of HCC cells in vitro, and further investigated the pathways and potential mechanisms involved in producing this effect. Therefore, GL-V9 is expected to be a novel potential therapeutic agent for the clinical treatment of HCC.

2 Materials and methods

2.1 Reagent processing

GL-V9 ($C_{24}H_{27}NO_5$, MW: 409.47, purity > 99%) was kindly provided by Dr. Bin Di (China Pharmaceutical University, China) and dissolved in dimethyl sulfoxide (DMSO) to prepare a master solution and stored at -20℃ as a stock solution. For cell experiments, GL-V9 was dissolved in sterilized dimethyl sulfoxide (DMSO) and diluted with cell culture medium to different final concentrations of 10, 20 and 40 μmol/L, respectively. The final concentration of dimethyl sulfoxide did not exceed 0.1% (v/v) throughout the study.

2.2 Cell culture

A kind of human hepatocellular carcinoma cell line (SMMC-7721) was cultured in RPMI1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) at 37 ℃ in a 5% CO2 cell culture incubator. The cells were digested with 0.25% trypsin when they had grown adductively to 80% abundance, and the digestion was terminated when an increase in cell gap and retraction of tentacles were observed under an inverted microscope, and the cells were divided and cultured, and the experiment was started when they entered logarithmic growth.

2.3 Cell proliferation assay

We determined the effect of GL-V9 on the proliferation of HCC cells by MTT assay. SMMC-7721 cells of logarithmic growth phase were taken, and the cells were inoculated in 96-well cell culture plates at a density of 5×10^3 cells/well, 100μL, and cultured for 24 h; 100 μL of RPMI-1640 medium was added to the control group, and 100 μL of RPMI-1640 medium containing different concentrations of GL-V9 was added to each other group, and 5 replicate wells were set up for each group. After 12 h incubation, 20 μL of MTT solution (5 mg/ml) was added to each well. Each group of cell culture plate was incubated for 4 h at 37 ℃ in a humidified environment with 5% CO_2, the clear solution was discarded, and 100 μL of dimethyl sulfoxide was added to each well. After gently shaking and mixing to fully dissolve the MTT reduction product, the absorbance at 570 nm was measured by enzyme marker to calculate the growth rate of cells. The growth rate (%) was calculated using the following formula: Growth rate (%) = OD value of experimental group/value of control group × 100%. The inhibition rate (%) was calculated using the following formula: Inhibition ratio (%) = [(control OD—experimental OD) / (control OD—blank OD)] × 100%. IC50 value is the concentration at which 50% inhibition of cell viability was achieved; OD value is the average absorbance of parallel experiments.

2.4 Plate cloning experiment

SMMC-7721 cells at logarithmic growth stage were divided into four groups (GL-V9 10, 20, 40 μmol/L group and control group), and each group of cells was cultured in a wide-mouth flask, digested with 0.25% trypsin, washed with RPMI-1640 medium to terminate the digestion, then RPMI-1640 medium containing 10% fetal bovine serum was used to prepare 1×10^5 mL/1 single cell suspension. The cells were removed, digested again with 0.25% trypsin, sedimented by centrifugation and collected. Resuspend and dilute to the appropriate multiple. Each group was supplemented with RPMI-1640 medium to 10 mL, with three groups adding 100μL of GL-V9 at different concentrations, and incubated for 2 weeks at 37 °C in a 5% CO2 cell incubator. Cultures were terminated when macroscopic cell clones appeared. The excess culture medium was aspirated, washed twice with PBS buffer, fixed with 5 ml of methanol for 15 min and the fixative was discarded. Add hematoxylin staining solution for 20 min rinse with tap water and place in air to dry. The formula for clone formation rate (%) was calculated as follows: Clone formation rate (%) = (number of clones/number of cells inoculated) × 100%.

2.5 Cell invasion assay

Cell invasion assays were performed on the SMMC-7721 cell line using the transwell assay. The cells of each group were configured into 1×10^5 single cell suspension per mL using RPMI-1640 medium containing 10% FBS. 1.5 mL of suspension was inoculated into the upper chamber of 6 Well Matrigel-Transwell chambers and incubated in a 37℃, 5% CO2 cell culture chamber for 24 h. After the cells had grown against the wall, the upper layer of culture medium was aspirated.The experimental group added 100 μL of RPMI-1640 medium containing 20 μM of GL-V9 solution, the control group added 100 μL of RPMI-1640 medium, and the lower chamber added 2.5 mL of RPMI-1640 medium with 10% FBS, and the cells were allowed to migrate freely at 37 ℃ and 5% CO_2 for 48 h. The upper culture medium was aspirated and the cells were fixed with 1% paraformaldehyde. The cells in the lower chamber were fixed and the Matrigel matrix on the upper surface of the 6 Well Matrigel-Transwell chambers and the non-invasive cells on its surface were swabbed off, then stained with 0.1% crystalline violet and the number of cells on the lower surface of the filter membrane (number of membrane penetrating cells) was counted in five randomly selected areas (×200) under an inverted microscope, and the experiment was performed in three times and the average was taken as the final result.

2.6 Wound healing assay

The groups of cells were configured into a single cell suspension of 1×10^5 mL/L using RPMI-1640 medium containing 10% FBS, inoculated into 6-well plates at a density of 2 mL/L/well(cell suspension) and incubated in a 37 ℃, 5% CO2 cell culture chamber for 24 h to allow the cells to grow against the wall. When the cells had grown to 80–90% confluence, excess culture fluid was aspirated and the tip of a 10 μL sterile pipette gun was used to A horizontal line was lightly scored inside the 6-well plate to form a linear wound. The plates were then washed three times with PBS buffer to remove cell debris and the width of the horizontal line was recorded. 1 mL of 20 μM GL-V9 solution was added to the experimental group and 1 mL of RPMI-1640 medium was added to the control group.Culture was continued for 2 weeks at 37℃ in a 5% CO2 cell incubator. During this time the wound edges were photographed and images were captured using an image analysis frame grabber and analysed using image analysis software. Migration was also monitored using an inverted microscope, where the difference between the width of each group of cell scratches and the initial width, i.e. the distance travelled by the cells, was measured under an inverted microscope. The unfilled scratch area was quantified by measuring the distance between the advancing edges of the cells in five randomly selected microscopic fields (×200) at each time point, and the cell migration rate (%) was calculated: cell migration rate (%) = (distance travelled by the cells / initial scratch width) × 100%. The experiment was carried out in three sessions and the average was taken as the final result.

2.7 Western blotting assay

Western blotting was carried out as previously described [6]. The total protein in HCC cells was extracted using Radio immunoprecipitation assay (RIPA) buffer(Beyotime, China). The protein samples were analysed by 12% SDS polyacrylamidegel and then eletretransferred to polyvinylidene fluoride membranes.To block the membranes, 5% non-fat dry milk in Tris-buffered saline was used.Then, membranes were incubated using the respective primary antibodies overnight at 4 °C. Finally, the appropriate HRP-conjugated secondary antibodies were applied and signals were detected by enhanced chemiluminescence following the manufacturer's instructions.

2.8 Statistical analyses

Analyses were carried out using GraphPad Prism 8 and SPSS 22. Associations between two groups were analyzed by student *t* tests. All data are presented as the mean ± SEM and $p < 0.05$ was defined as statistically significant.

3 Results

3.1 GL-V9 inhibits the proliferation of HCC cells

We first investigated the effect of GL-V9 on the proliferation and viability of HCC cells. As measured by the MTT assay, we found that continuous duration of GL-V9 treatment showed a time-dependent inhibition of the proliferation and viability

of the HCC cell line. There was little difference in the proliferation of HCC cells treated with GL-V9 and those without GL-V9 treatment after 24 h. However, the change in proliferation and viability of the two groups of cells increased significantly after GL-V9 treatment for 72 h. The OD value of SMMC-7221 cells treated with GL-V9 was (0.26 ± 0.01), whereas the OD value of control SMMC-7221 cells had an OD of (0.37 ± 0.01)%. After 5 days of the experiment, the OD of HCC cell lines treated with GL-V9 was (0.39 ± 0.02)%, while the OD of HCC cell lines without GL-V9 treatment was (0.55 ± 0.02)% (Fig. 1A). This shows that the proliferation level of HCC cell lines under GL-V9 treatment was significantly inhibited, and the inhibition effect became more pronounced as the treatment time increased. To further confirm the ability of GL-V9 to inhibit the proliferation and survival of HCC cells, a plate cloning assay was performed. Analysis of the data showed that the cloning rate of SMMC-7221 cells treated with GL-V9 was (9.92 ± 2.18)% and that of SMMC-7221 cells not treated with GL-V9 was (35.58 ± 1.72)%(Fig. 1D). We found that the growth and proliferation ability of the SMMC-7221 cell line treated with GL-V9 was significantly reduced. Thus, our results suggest that GL-V9 significantly inhibited the proliferation and viability of HCC cells.

3.2 GL-V9 inhibits the invasion and migration of HCC cells

Subsequently, we performed transwell invasion assay and cell scoring assay to investigate the effect of GL-V9 on the invasion and migration ability of HCC cells. The data from the transwell invasion assay showed that after 24 h of continuous treatment with GL-V9, the non-GL-V9 treated HCC cells were able to invade freely through the matrix gel with a membrane penetrating cell count of (157.83 ± 6.12), while the number of HCC cells treated with GL-V9 had a significantly lower cell count through the matrix gel with a membrane penetrating cell count of (68.67 ± 5.50) (Fig. 1B). The results indicated that treatment with GL-V9 resulted in a significant reduction in the invasive ability of HCC cells. Furthermore, cell scratching assay showed that GL-V9 treatment significantly reduced the migratory ability of HCC cells. We could find that after 24 h of migration, the degree of wound closure of SMMC-7221 cells treated with 20 μM GL-V9 was (98.17 ± 1.94) % compared to (41.83 ± 6.05) % of SMMC-7221 cells in the control group that were not treated with GL-V9(Fig. 1C).

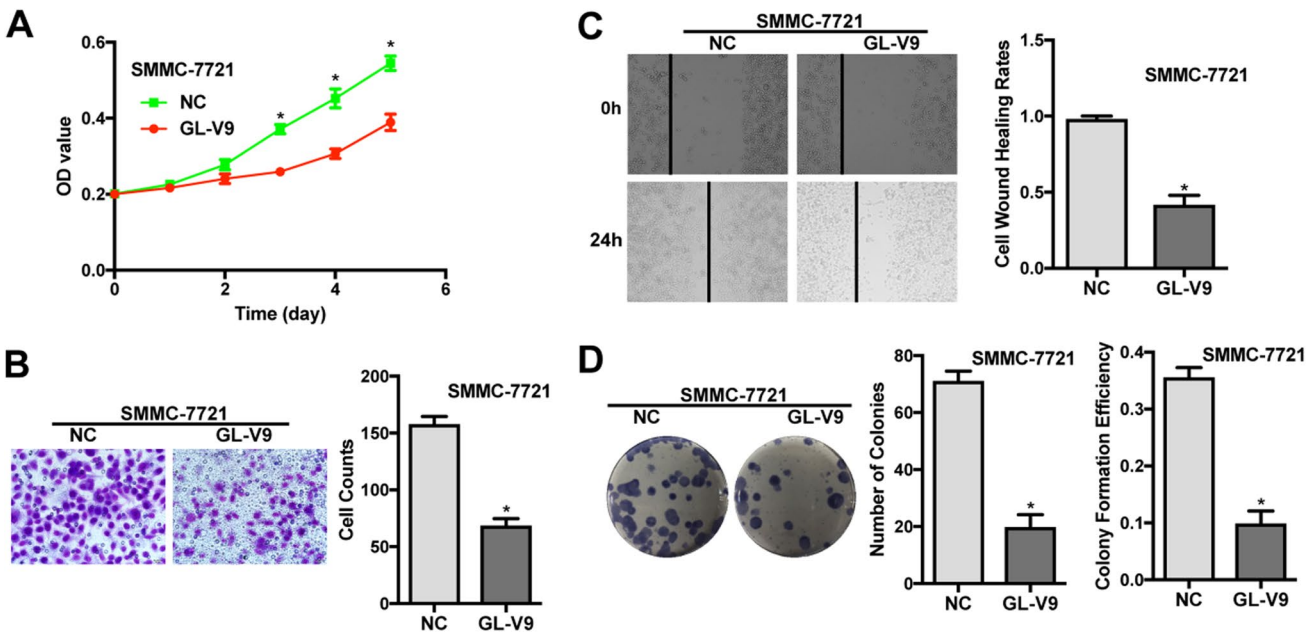

Fig. 1 The inhibitory effect of GL-V9 on the survival ability, invasion ability, migration ability, proliferation ability of HCC cells. **A** The MTT assay is used to detect cell viability after GL-V9 treatment.HCC cells SMMC-7721 were treated with 0–40 μmol/L GL-V9 for 5 day.GL-V9 exhibits cytotoxic effects on HCC cells.Each error bar represents the mean ± SD of six replicate samples. **B** HCC cells SMMC-7721 treated with 20 μM GL-V9 for 24 h demonstrated significantly reduced migration and invasion through extracellular matrix as indicated by the transwell migration assay.Each error bar represents the mean ± SD of three replicate samples. **C** HCC cells SMMC-7721 treated with 20 μM GL-V9 for 24 h demonstrated significantly reduced migratory capacities, as indicated by wound healing assay.Images were taken at 0 and 24 h. Each error bar represents the mean ± SD of three replicate samples. **D** HCC cells SMMC-7721 were treated with 0–40 μmol/L GL-V9 for 2 weeks, and GL-V9 significantly attenuated the proliferation of HCC cells. Each error bar represents the mean ± SD of six replicate samples.$P < 0.05$

3.3 GL-V9 inhibit the Wnt/β-catenin pathway and the EMT process

The Wnt/β-catenin pathway is important in controlling cell proliferation, controlling liver stem cell differentiation and maintaining homeostasis in the mature liver. And it is well known that the EMT process is a key step in the development of metastasis in cancer cells. The EMT process is characterised by a reduction in transmembrane adhesion of E-cadherin and a shift from E-cadherin to N-cadherin. In GL-V9-treated HCC cells, the expression of β-catenin, N-cadherin and Vimentin was significantly reduced. (Fig. 2) These findings suggest that GL-V9 probably inhibits Wnt/β-catenin signaling and the conversion of E-cadherin to N-cadherin in HCC cells.

4 Discussion

GL-V9 is a newly synthesized natural flavonoid derived from baicalin, which has been used in the treatment and prevention of hepatocellular carcinoma. In recent years, relevant studies have shown that GL-V9 plays an anti-cancer role in a variety of solid tumours and haematological tumours by promoting apoptosis and inhibiting cell growth and invasion. For example, it inhibits the PI3K/AKT pathway by inhibiting AKT phosphorylation, resulting in a significant reduction in the downstream MMP-2 and MMP-9 activities, thus acting as an anti-breast cancer agent [3]. In skin cancer, GL-V9 is effective against cancer by inducing mitochondrial dysfunction and inhibiting glycolysis to reprogram cellular metabolic processes, by inhibiting AKT-regulated mitochondrial localization of HK2 to induce apoptosis, and by inhibiting the AKT/mTOR pathway to activate autophagy [4]. GL-V9 also increases GRP78, p-PERK, p-eIF2α, ATF 4 and CHOP protein expression, inducing endoplasmic reticulum stress and promoting apoptosis in gastric cancer cells [5]. In T-cell malignancies, it has been shown that GL-V9 activates PTEN to mutate the cell division process, thereby arresting the cell cycle and promoting apoptosis [7].GL-V9 also has a unique beneficial effect in regulating inflammatory responses and drug resistance [8–10]. It inhibits the activation of NLRP3 inflammatory vesicles, which not only has an anti-inflammatory effect, but also activates lysosomal autophagy, effectively treatment of colorectal cancer [8]. In addition to its anti-cancer and anti-inflammatory effects, GL-V9 also inhibits the TGF-β/smad pathway, regulates transcription factors and triggers senescence, thereby reducing liver fibrosis [11, 12].

In the present study, we found that GL-V9 inhibited the EMT process in HCC cells by suppressing Wnt/β-Cantenin pathway signaling, thereby significantly inhibiting the invasive and metastatic ability of HCC cells. MTT assay results showed that GL-V9 inhibited the cell viability of HCC cell line SMMC-7721. The inhibition rate of HCC cell line viability started to be higher than that of untreated HCC cells after 48 h of 20 μM GL-V9 treatment and increased significantly with longer treatment time, with GL-V9 treatment exhibiting a time-dependent inhibition of the proliferation and viability of the HCC cell line. Cell cloning assays likewise provided a strong basis for the inhibition of HCC cell proliferation by GL-V9. The effect of GL-V9 on the metastatic and invasive potential of HCC cells was assessed using the transwell invasion assay and cell scratch assay, and analysis of the experimental data showed that GL-V9 treatment significantly inhibited the invasive, migratory ability of HCC cells. the efficacy of GL-V9 in inhibiting the invasive and migratory ability of skin squamous cell carcinoma cells, breast cancer cells and gastric cancer cells has been previous studies at [3–5], which is consistent with our experimental results. With the above experimental data, GL-V9 is expected to be an effective new drug for the treatment of patients with liver cancer.

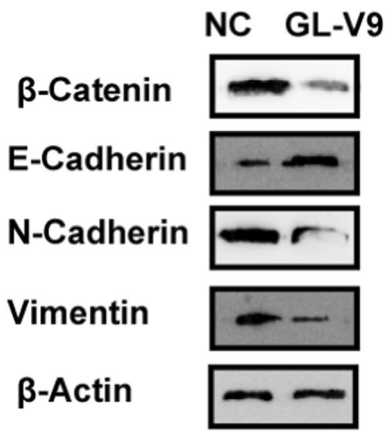

Fig. 2 Effects of GL-V9 on the Wnt/β-Cantenin signaling pathway and EMT. In GL-V9-treated HCC cells, the expression of β-catenin, N-cadherin and Vimentin was significantly reduced, and GL-V9 could inhibit Wnt/β-catenin signaling and the conversion of E-cadherin to N-cadherin in HCC cells

The Wnt/β-catenin pathway is an important regulatory system for normal hepatocytes and is involved in a variety of processes in liver development. It is important in controlling cell proliferation, controlling liver stem cell differentiation and maintaining homeostasis in the mature liver [13]. We found that in HCC cells, Wnt/β-catenin signalling is usually overactive, and β-catenin is heavily concentrated intracellularly and plays a decisive role in the Wnt signalling pathway [14]. Large intracellular accumulation of β-catenin enters the nucleus, thereby activating its mediated downstream target genes, such as CyclinD1 and c-myc, which play a key role in hepatocellular carcinoma and induce changes in tumour pathophysiology such as tumourigenesis, growth and invasion, and cell cycle changes [15, 16]. Therefore,reducing intracytoplasmic β-catenin levels and inhibiting intracytoplasmic β-catenin translocation to the nucleus can effectively inhibit tumorigenesis and progression, and are important molecular targets for anticancer therapeutic research. Through this study, we also found that β-catenin protein was more frequently expressed in HCC cells and that high transcriptional activity of β-catenin was an important factor in the progression, proliferation and colony formation of hepatocellular carcinoma cells [17]. Wei [18] et al. also demonstrated that inhibition of β-catenin/TCF4 transcriptional activity and down-regulation of its target gene expression effectively inhibited the proliferation and induced apoptosis in different hepatocellular carcinoma cell lines. The study also demonstrated that inhibition of β-catenin/TCF4 transcriptional activity and down-regulation of its target gene expression could effectively inhibit the proliferation and induce apoptosis of different liver cancer cell lines.

It is well known that local invasion is the primary condition for distant metastasis and that the EMT process is a key step in the development of metastasis in cancer cells [19, 20]. Targeting the EMT process is therefore considered an ideal strategy to prevent cancer metastasis [21]. The EMT process is characterised by a reduction in transmembrane adhesion of E-cadherin and a shift from E-cadherin to N-cadherin [22, 23]. This is accompanied by an increase in the expression of mesenchymal markers such as Vimentin. expression, such as Vimentin, resulting in tumour cells losing their epithelial characteristics and acquiring mesenchymal characteristics to facilitate their detachment from the primary site and their ability to migrate distantly and invasively. In addition to its role in regulating transcriptional activation and target gene transcription, the intracellular β-catenin protein also interacts with E-cadherin protein at the cell membrane and is directly involved in adhesion junctions [24], inhibiting invasive cell migration. E-cadherin needs to be attached to actin via the catenin family (β-catenin/α-catenin) The catenin family (β-catenin/α-catenin) is required to attach to the actin cytoskeleton to maintain stable epithelial cell adhesion. Thus, downregulation of E-cadherin is often closely associated with the transfer of β-catenin from the membrane to the nucleus, and the E-cadherin/catenins complex has been shown to be an important factor involved in the development and progression of HCC [25, 26]. Various studies at home and abroad have shown that the Wnt/β-catenin signaling pathway plays a major role in the EMT process of HCC, therefore targeting the Wnt/β-catenin signaling pathway is one of the measures to effectively inhibit the invasive metastasis of HCC cells. In this study, we found that the expression of β-catenin, N-cadherin and Vimentin was significantly reduced in HCC cell lines treated with GL-V9. GL-V9 treatment not only inhibited the transduction of Wnt/β-catenin signalling pathway in HCC cells, but we also found that E-cadherin to N-cadherin We also found that the expression of E-cadherin to N-cadherin transition and mesenchymal marker Vimentin were also significantly reduced, indicating that GL-V9 treatment could effectively inhibit the EMT process in hepatocellular carcinoma cells.

In conclusion, our study shows that GL-V9 inhibits the proliferation, invasion and metastatic ability of HCC cells in vitro. Upon deeper investigation of its internal molecular mechanism, GL-V9 may inhibit the Wnt/β-catenin signaling pathway in HCC cells, thereby inhibiting the conversion of E-cadherin to N-cadherin and reducing the occurrence of the EMT process in HCC cells, ultimately achieving inhibition of their invasive and metastatic abilities. Combined with previous studies showing its anti-tumour effects, GL-V9 appears to be a promising targeted therapeutic agent for HCC.

5 Limitation

In this research, We found the reducing expression of β-catenin、N-cadherin、Vimentin in HCC cell lines treated with GL-V9, which might mediate the invasive and metastatic abilities in HCC cells. How these mechanisms work needs further verification.Besides, we only focused on the mechanisms of GL-V9 in HCC cell lines in vitro, and further studies are needed to explore in vivo.

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Author contributions Author 1 (First Author) Ye Gu: Conceptualization, Methodology, Software, Investigation, Formal Analysis, Writing-Original Draft Author 2 (Co-first Author) Qiaoxian He: Conceptualization, Investigation, Formal Analysis, Data Curation, Writing-Original Draft Author 3 Lu Xie: Visualization, Investigation Author 4 Fangfang Chen: Resources, Supervision Author 5 Hangbin Jin: Software, Validation Author 6 Lilan Lou: Visualization, Writing-Review & Editing Author 7(Corresponding Author) Xiaofeng Zhang: Conceptualization, Funding Acquisition, Resources, Supervision, Writing-Review & Editing.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.Competing interests The authors declare no competing interests.

Declarations

Competing interests The authors declare no competing interests.

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