Gomisin N Exerts Anti-liver Cancer Effects and Regulates PI3K–Akt and mTOR–ULK1 Pathways in Vitro

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INTRODUCTION

Primary liver cancer is a lethal cancer. The phosphatidylinositol 3-kinase (PI3K)–Akt–mammalian target of rapamycin (mTOR) pathway has been implicated in the pathogenesis of liver cancer. Gomisin N (GN), a lignan isolated from the dried fruits of Schisandra chinensis (Turcz.) Baill., has been reported to possess hepatoprotective and anticancer effects. 4,6 Gomisin N (GN) exerts anti-liver cancer effects of GN was determined using MTT (Sigma, U.S.A.) as a 98% purity standard. 8 Gomisin N (GN) was obtained from the growing of Chinese Academy of Sciences (Shanghai, China). HCCCLM3 was obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). HepG2 cell line and the normal liver-derived cell line MIHA were bought from the American Type Culture Collection. All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO, U.S.A.) at 37°C in a humidified incubator with 5% CO₂. GN (Fig. 1A, purity >98% as determined by HPLC) was provided by the Coompo Research Chemicals (Wuhan, Hubei, China).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

HepG2, HCCCLM3 and MIHA cells (4000 cells/well) were seeded in 96-well plates and treated with various concentrations of GN for 48 h. The cytotoxic effects of GN was determined using MTT (Sigma, U.S.A.) assay as reported previously. 8

Cell Apoptosis Analysis

HCCCLM3 cells were cultured in 6-well plates and treated with GN for 48 h. Apoptotic cells were examined using Annexin V-fluorescein isothiocyanate (FITC)/PI double-stain kit (BD Biosciences, Cambridge, MA, U.S.A.) according to the manufacturer’s instruction. 8

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Note

INTRODUCTION

Primary liver cancer is a leading cause of cancer death globally, with an estimation of 782,000 annual deaths. Many molecular alterations, such as over-activation of phosphatidylinositol-3-kinase (PI3K)–Akt and mammalian target of rapamycin (mTOR) pathways, have been identified to promote liver cancer development. 1 mTOR is a canonical target of Akt, but it can also be activated independent of Akt. 2 In addition to promote cell proliferation, mTOR is a master negative regulator of autophagy. 3 Recently, autophagy has gained attention due to its paradoxical roles in the pathophysiology and treatment of various types of cancer, including liver cancer. 5 PI3K–Akt and mTOR pathways have been considered as therapeutic targets for liver cancer. 5 The survival rate of liver cancer patients is still very low because of the advanced stage of presentation, rapid progression of the disease and limited treatment options. Novel targeted therapies are desperately needed.

Gomisin N (GN), a lignan compound isolated from the dried fruits of Schisandra chinensis (Turcz.) Baill., has been reported to possess hepatoprotective and anticancer effects. 4,6 Yim et al. showed that GN exhibits the strongest cytotoxic effects against HepG2 cells, when compared with other studied lignans. 6 However, mechanisms underlying the anti-liver cancer effects of GN remain unknown. In 3T3-L1 preadipocytes, GN was found to inhibit cell proliferation and Akt activation. 7 In the present study, we investigated Akt signaling-related anti-liver cancer mechanisms of GN.

MATERIALS AND METHODS

Cell Culture and Reagents

Human liver cancer cell line HCCCLM3 was obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). HepG2 cell line and the normal liver-derived cell line MIHA were bought from the American Type Culture Collection. All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO, U.S.A.) at 37°C in a humidified incubator with 5% CO₂. GN (Fig. 1A, purity >98% as determined by HPLC) was provided by the Coompo Research Chemicals (Wuhan, Hubei, China).

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Western Blot Analysis  Western blotting was performed as described previously. Primary antibodies including p62, Beclin-1, PI3 Kinase p85, p-P13K p85 (Tyr458)/p55 (Tyr199), Akt, p-Akt (Ser473), Mcl-1, mTOR, p-mTOR (Ser2448), ULK1, p-ULK1 (Ser555) were purchased from Cell Signaling Biotechnology; Antibodies against LC3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were bought from Novus Biologicals and Santa Cruz Biotechnology, respectively. The band density was quantified using Image J software and normalized to that of the control group.

Confocal Microscopy  HepG2 cells grown on coverslips were treated with GN (50 µM) in the presence or absence of chloroquine (CQ) (30 µM) for 24 h and then fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 10 min, blocked in 3% BSA for 30 min. Subsequently, coverslips were incubated with LC3B antibody (1 : 200) at 4°C overnight. After washing with PBS, coverslips were incubated with FITC-conjugated secondary anti-bodies for 1 h in the darkness at room temperature. Coverslips were washed, counterstained with DIPA for 5 min and then visualized under a TCS SP8 spectral confocal microscope (Leica Microsystems, Mannheim, Germany).

Statistical Analysis  Data are expressed as mean ± standard deviation (S.D.) of three independent experiments. One-way ANOVA followed by the Dunnett’s multiple comparisons test was performed to determine the significance of differences among multiple groups. All analyses were performed using GraphPad Prism version 7.0 (GraphPad software, San Diego, CA, U.S.A.). Statistical significance was indicated by p < 0.05.

RESULTS AND DISCUSSION  We first evaluated the anti-liver cancer effects of GN on several liver cancer cell lines with different genetic backgrounds. Results in Fig. 1B showed that GN dose-dependently reduced viability of HepG2 and HCCLM3 cells. Although more toxic to human liver cancer cells, GN also reduced the viability of normal liver derived MIHA cells, suggesting that tumor-oriented delivery or structural improvement of GN is warranted. Flow cytometric and Western blot analyses showed that GN triggered apoptosis in liver cancer cells (Figs. 1C, D). These results confirmed that GN reduces viability of, and triggers apoptosis in, liver cancer cells.

Inhibiting PI3K–Akt pathway has been demonstrated to suppress liver tumor in cellular and animal models. Immunoblotting results demonstrated that GN lowered protein levels of p85 PI3K (Tyr458), p-Akt (Ser473) and Mcl-1 (a survival-related molecule) in HepG2 and HCCLM3 cells (Fig. 2A). These results suggest that inhibition of PI3K–Akt signaling is associated with GN-induced apoptosis. Akt is an up-stream positive regulator of mTOR. Unexpectedly, we found that the protein level of p-mTOR (Ser2448) was elevated after GN
Apart from Akt, mTOR is prone to be activated by AMP activated protein kinase (AMPK) and RAS/mitogen-activated protein kinase (MAPK) pathways. Whether the two pathways are involved in GN-mediated mTOR activation needs to be further studied. mTOR has two functionally distinct multiprotein complexes, mTORC1 and mTORC2. Phosphorylation of mTOR at Ser2448 site is regulated by p70S6K which is a robust readout of mTORC1 activity. Our findings indicate that GN inhibits the mTORC1 activity in liver cancer cells. Activating mTORC1 has been shown to repress autophagy through directly inhibiting ULK1 activity. Immunoblotting results demonstrated that GN lowered protein levels of phospho-ULK1 (Ser555) in HepG2 and HCCLM3 cells (Fig. 2B). In addition to ULK1, other mTORC1 downstream molecules, such as TFEB, 4EBP1 and p70S6K, have been reported to participate in multiple cellular processes, including lysosome biogenesis and protein synthesis. Effects of GN on activities of other mTORC1 downstream molecules warrant further investigations.

Immunoblotting also showed that GN lowered LC3-II and Beclin-1 protein levels, while elevated p62 protein level, in HepG2 and HCCLM3 cells (Fig. 3A). LC3-II is an autophagic marker. P62 is an autophagic substrate that is widely used as an autophagy predictor. An increased expression of p62 is associated with the inhibition of autophagic flux. Beclin-1 is a key molecule involved in autophagy initiation. Depletion of Beclin-1 has been shown to block autophagy.

Fig. 2. GN Regulates PI3K/Akt and mTOR/ULK1 Pathways in Liver Cancer Cells

(A) GN inhibits the PI3K/Akt signaling pathway in liver cancer cells. (B) GN regulates the mTOR/ULK1 pathway in liver cancer cells. HepG2 and HCCLM3 cells were separately treated with GN (0, 25, 50, 100 µM) for 24 h, levels of indicated proteins were measured using Western blotting. Representative blots (left) and densitometric analysis (right) are shown. Data are expressed as mean ± S.D. (n = 3). *p < 0.05, **p < 0.01 compared with the control group.
initiation in liver cancer cells. To further determine whether GN inhibits autophagy, CQ, a late stage autophagy inhibitor that inhibits the fusion of autophagosome and lysosomes, was used. CQ has been reported to upregulate the protein level of LC3-II in liver cancer cells.\(^{15}\) We found that CQ enhanced LC3 fluorescence intensity in HepG2 cells; and this effect was attenuated by GN (Fig. 3B). The immunofluorescence analysis results were verified in HCCLM3 cells using Immunoblotting (Fig. 3C), indicating that GN inhibits autophagy in liver cancer cells. In cancer cells, autophagy acts as a pro-survival or pro-death mechanism upon chemotherapeutic treatments. Whether GN-mediated autophagy repression favors or constrains its anti-liver cancer effects should be further investigated.

In summary, our results confirmed that GN has anti-proliferative and apoptotic effects in cell models. Moreover, we found that GN suppresses autophagy. Mechanistically, GN inhibits the PI3K–Akt pathway and regulates the mTOR–ULK1 pathway in liver cancer cells. This is the first study to explore molecular mechanisms underlying the anti-liver cancer effects of GN. This study suggests that GN may serve as a lead compound for developing anti-liver cancer drugs.

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**Conflict of Interest** The authors declare no conflict of interest.

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