Inhibition of the Hedgehog pathway induces autophagy in pancreatic ductal adenocarcinoma cells

YONGHUA XU1*, YONG AN2*, XUEHAO WANG3, WENZHANG ZHA1 and XIANGCHENG LI3

1Department of General Surgery, Fourth Affiliated Hospital of Nantong University, The First People's Hospital of Yancheng, Yancheng, Jiangsu; 2Hepatobiliary Surgery, The First People's Hospital of Changzhou, Changzhou, Jiangsu; 3Liver Transplantation Center, First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, P.R. China

Received September 16, 2013; Accepted November 4, 2013

DOI: 10.3892/or.2013.2881

Abstract. The HH signaling pathway is a ‘core’ signal transduction pathway in pancreatic cancer that promotes the tumorigenesis of pancreatic cancers via enhancing cell proliferation, increasing invasion and metastasis and protecting against apoptosis. In the present study, we found that HH signaling regulates autophagy in pancreatic cancer cells. Activation of HH signaling inhibits autophagy, while inhibition of the HH pathway induces autophagy. Although the role of autophagy in cell survival and apoptosis may depend on tumor type and the microenvironment, our data clearly demonstrated that GANT61-induced autophagy contributed to reduced viability and increased apoptosis in pancreatic cancer cells both in vivo and in vitro, and these effects were reversed by the autophagy inhibitor, 3-MA. We propose that HH signaling by regulating autophagy plays an important role in determining the cellular response to HH-targeted therapy in pancreatic cancer and further investigation of the interaction between autophagy and HH signaling is particularly important.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal of all human malignancies (1). Great improvements have been made in surgical and chemotherapeutic approaches during the past decades; however, the survival rate of patients with PDAC has not been significantly prolonged. Therefore, an alternative therapy for PDAC is urgently required.

The Hedgehog (HH) signaling pathway is an essential pathway during embryonic development (2). Sonic Hedgehog (Shh) is a secreted mammalian ortholog of the Hedgehog family and plays multiple roles during embryonic development (3). Activation of the Shh pathway triggers a series of intracellular events through the GLI transcriptional effectors Gli-1, Gli-2 and Gli-3 (4,5).

The HH pathway is reactivated in tumor development, and plays an important role in the tumorigenesis and maintenance of tumors (6-8), including pancreatic cancer (9). Evidence has shown that the HH signaling pathway is a ‘core’ signal transduction pathway in pancreatic cancer and is abnormally expressed in almost all pancreatic cancer cells (10). Accumulating data suggest that the HH pathway promotes the tumorigenesis of pancreatic cancers by enhancing cell proliferation (11,12), increasing invasion and metastasis (13,14) and protecting against apoptosis (15,16).

In a phase I trial testing vismodegib (GDC-0449), an inhibitor of Smoothened (SMO), beneficial responses were observed in patients with advanced basal cell carcinoma (BCC) and medulloblastoma. However, only one in eight patients with pancreatic carcinoma experienced a stable disease as the best response (17). The ineffectiveness of a pathway inhibitor for pancreatic cancer can probably be explained by the fact that there are numerous crosstalks between the HH signaling pathway and other signaling pathways in pancreatic cancer (18-20). Feedback from these crosstalk pathways may reduce the therapeutic effectiveness of the HH pathway inhibitor. Therefore, further investigation should be focused on the mechanism of interaction between HH signaling and these crosstalk pathways.

Autophagy was initially reported 50 years ago by Ashford and Porter (21), and it has recently gained considerable attention. Autophagy is a lysosome-mediated protein and organelle degradation process that is characterized by the formation of double-membrane vesicles, referred to as autophagosomes (22,23). Autophagy is involved in several pathophysiological processes and contributes to numerous diseases, particularly to cancer (24,25). However, the function of autophagy in cancer has yet to be fully clarified, as it acts both as a tumor suppressor and as a tumor promoter (26).

Correspondence to: Professor Wenzhang Zha, Department of General Surgery, Fourth Affiliated Hospital of Nantong University, The First People's Hospital of Yancheng, Yancheng, Jiangsu, P.R. China
E-mail: zhawenzhang@sohu.com

Professor Xiangcheng Li, Liver Transplantation Center, First Affiliated Hospital of Nanjing, Medical University, 300 Guanzhou Road, Nanjing, Jiangsu, P.R. China
E-mail: drxcli@njmu.edu.cn

*Contributed equally

Key words: Hedgehog signaling, autophagy, GANT61, pancreatic cancer, apoptosis
During the development of PDAC, autophagy initially suppresses tumor initiation yet supports tumor growth at later stages (27,28). Other studies have reported that pancreatic cancers require autophagy for tumor growth (29) and that autophagy is correlated with poor outcome in PDAC patients (30). Many results suggest that autophagy is a cell death mechanism in response to drugs such as gemcitabine and triptolide. It was confirmed that autophagy is activated by gemcitabine and triptolide in the treatment of pancreatic cancer cells and that activated autophagy plays a role in cancer suppression (31,32). However, it was also reported that activation of autophagy by receptor for advanced glycation end products (RAGE) (33,34), 2-deoxy-D-glucose (2-DG) (35), phosphatidylinositol 3-kinase (PI3K) (36) and Kangai 1 (KA11) (37) promotes tumorigenesis and limits apoptosis in pancreatic cancer cells. Hence, the role and mechanism of autophagy is unclear in pancreatic cancer and requires further investigation.

Although HH signaling is known to inhibit apoptosis, it remains unknown whether HH signaling is able to regulate autophagy in pancreatic cancer cells. The present study describes a novel role of the HH signaling pathway in the regulation of autophagy in PDAC cells. We report that inhibition of the HH pathway induces autophagy and enhances apoptosis in PDAC cells. Our findings suggest that the status of autophagy is a key factor that may influence the cellular response to HH signaling-targeted therapy.

Materials and methods

Cell cultures. Human pancreatic cancer cell line CFPAC-1 was purchased from the Shanghai Cell Bank (Shanghai, China) and propagated in our laboratory by cultivating in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA) at 37°C with 5% CO2, supplemented with 1% penicillin/streptomycin. BALB/c nude mice (4-week-old, female) were purchased from the Model Animal Research Center of Nanjing University, Nanjing, China. Drug treatment included recombinant human N-Shh (R&D Systems, Minneapolis, MN, USA) used at 0.25/0.5 µg/ml; GANT61 (Abcam, Cambridge, MA, USA) at 5/10 µM soluble in DMSO (dimethyl sulfoxide); and 3-MA (3-methyladenine) (Sigma) used at 5 mmol/l.

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) for mRNA quantitation. Total RNA of cells was isolated using TRIzol reagent (Invitrogen). cDNA was synthesized using the PrimeScript RT reagent kit (Takara, Hercules, CA, USA). Quantitative RT-PCR was performed using an ABI 7500 (Applied Biosystems) with FastStart Universal SYBR-Green Master (Roche) (Roche) for mRNA quantitation and with the TaqMan® MicroRNA assay kit (Applied Biosystems). The relative expression was calculated using the ΔCt method (38) normalized to β-actin. Primers for mRNA qPCR were synthesized by Invitrogen (Shanghai, China). The sequences were: Gli-1 sense, 5’-AGCC AAGCACCAATCGGAC-3’ and antisense, 5’-GGTGGTGTC ACATGGGGCTGAC-3’; Gli-2 sense, 5’-GGTGTTGTC AGCCTTGGGAC-3’ and antisense, 5’-ATGCCCAGTCCACGTT

Transmission electron microscopy. For ultrastructural examination, samples (~1 mm3) were fixed with 2% OsO4 and embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and inspected using an electron microscope (JEOL JEM-1010; Jeol, Tokyo, Japan).

Orthotopic xenograft tumor study. CFPAC-1 cells (2x106) suspended in 100 µl PBS were injected undercapsule of the pancreas in 4-week-old BALB/c nude mice. After 1 week, mice were randomized to three groups; vehicle, GANT61 (50 mg/kg), GANT61 (50 mg/kg) in combination with 3-MA (10 mg/kg) were administered every other day, i.p. for 28 days. At the end of the drug treatment, mice were euthanized and an autopsy was performed to obtain the primary lesions. Tumor volume was determined as V= (L x W2)/2, where L represents

Cell viability assays. Cell viability was determined using an MTT assay. Aliquots of 2x103 cells were plated in 96-well plates with 200 µl culture medium per well accordingly, and the viability was measured at 72 h thereafter using the following procedures. Cells were incubated with 20 µl/well of MTT solution (5 mg/ml; Sigma) at 37°C for 4 h. Then 150 µl of DMSO was substituted for the supernatant, followed by oscillation for 10 min. Absorbance at 490 nm was detected by the Multiskan MK3 microplate reader (Thermo Labsystems, Philadelphia, PA, USA). All results were normalized to corresponding controls and are expressed in terms of percentage.

Analysis of cell apoptosis. Cell apoptosis was assessed by flow cytometry (Becton-Dickinson, San Jose, CA, USA). For cell apoptosis, cells were treated with GANT61 (10 µM), 3-MA (5 mmol/l) and with a combination of GANT61 and 3-MA for 48 h. Cells were then collected, washed, suspended in 100 µl 1X binding buffer and stained with 5 µl fluorescein isothiocyanate (FITC)-Annexin V and 1 µl PI at room temperature for 15 min in the dark. The stained cells were immediately analyzed by flow cytometry.

Western blotting. For western blotting, cells were treated with 400 nM bafilomycin for 2 h before being lysed using RIPA buffer with 1% PMSF on ice. The concentration of total protein was determined using a BCA kit (Nanjing KeyGen Biotech., Co., Ltd., Nanjing, China). Equal amounts of protein (30 µg) were resolved with 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) using a Mini Trans-Blot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were probed with primary antibodies for 12 h at 4°C and then incubated with secondary antibodies for 2 h at room temperature. Primary antibodies used were LC3 (microtubule-associated protein light chain 3) (Novus Biologicals, Littleton, CO, USA) and tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) which was used as an internal control. The secondary antibody was purchased from Beyotime (Santa Cruz Biotechnology). Electrochemiluminescence was performed with a ChemilImager 5500 imaging system (Alpha Innotech Co., San Leandro, CA, USA).

Expression and localization of proteins. Cellular expression and localization of proteins were examined by immunofluorescence staining. Cells were fixed with 4% paraformaldehyde in PBS and blocked with 3% normal goat serum at room temperature for 1 h. The samples were then incubated with primary antibodies at 4°C overnight and following procedures. Cells were incubated with 20 µl/well of 1X binding buffer and stained with 5 µl fluorescein isothiocyanate (FITC)-Annexin V and 1 µl PI at room temperature for 15 min in the dark. The stained cells were immediately analyzed by flow cytometry.
ONCOLOGY REPORTS 31: 707-712, 2014

ONCOLOGY REPORTS 31: 707-712, 2014

709

the length and W the width of the tumor. A portion of each tumor was used for western blot analysis for LC3II.

Statistical analysis. All experiments were repeated in triplicate. All values are expressed as the means ± standard deviation (SD). Statistical significance was determined by a Student's t-test using SPSS 15.0. P-values <0.05 were considered to indicate statistically significant differences.

Results

Effects of N-Shh and GANT61 on the expression of Gli-1 and Gli-2 in CFPAC-1 cells. To determine N-Shh (a recombinant Shh ligand) and GANT61 (a small-molecule inhibitor of Gli-1 and Gli-2) function in CFPAC-1 cells, the cells were treated with N-Shh (0.25/0.5 µg/ml) or GANT61 (5/10 µM) for 12, 24 and 48 h, respectively. The expression levels of Gli-1 and Gli-2 were confirmed by qRT-PCR. The relative levels were normalized to β-actin (P<0.05 compared with the control group, n=3; #P<0.05 compared with Shh-0.25 µg/ml and GANT61-5 µM for Shh-0.5 µg/ml and GANT61-10 µM, respectively, n=3). Each group was analyzed in triplicate, and the data are presented as the average ± SD.

HH signaling regulates autophagy in CFPAC-1 cells. LC3 is widely used to monitor autophagy. The density of the LC3-II band divided by the density of the tubulin band represents the expression level of LC3-II. In our experiment, CFPAC-1 cells were transfected with N-Shh or GANT61 for 48 h. The cells were then treated with bafilomycin (400 nM; Sigma), which significantly inhibits autophagy, for 2 h and used as an autophagy inhibitor.

Non-treated cells and the vehicle group (DMSO) were used as controls. Western blot analysis showed that, compared with the non-treated cells, N-Shh reduced the ratio of the LC3-II level/tubulin level (P<0.05; Fig. 2A and B), indicating that the process of autophagy was inhibited. Meanwhile, GANT61 significantly elevated the ratio of the LC3-II level/tubulin level (P<0.05; Fig. 2A and B). These data suggest that activation of HH signaling reduces autophagy, while inhibition of HH signaling induces autophagy. Therefore, HH signaling regulates autophagy in pancreatic cancer CFPAC-1 cells.

Figure 1. HH signaling is active in human pancreatic cancer CFPAC-1 cells. CFPAC-1 cells were treated with N-Shh (0.25/0.5 µg/ml) or GANT61 (5/10 µM) for 12, 24 and 48 h, respectively. mRNA levels of Gli-1 and Gli-2 were confirmed by qRT-PCR. The relative levels were normalized to β-actin (*P<0.05 compared with the control group, n=3; #P<0.05 compared with Shh-0.25 µg/ml and GANT61-5 µM for Shh-0.5 µg/ml and GANT61-10 µM, respectively, n=3). Each group was analyzed in triplicate, and the data are presented as the average ± SD.

Figure 2. HH signaling regulates autophagy. (A) Western blotting for LC3 using lysates from CFPAC-1 cells treated with N-Shh or GANT61. Non-treated cells and vehicle group (DMSO) were used as controls. The ratio of the LC3-II level/tubulin level was used as an indicator of the autophagic level. (B) The ratio of LC3-II/tubulin is presented in the bar chart (*P<0.05 compared with the control group, n=3).
XU et al: INHIBITION OF THE HEDGEHOG PATHWAY INDUCES AUTOPHAGY IN PDAC

GANT61-induced autophagy contributes to reduced viability and increased apoptosis in pancreatic cancer cells. (A) The viability of CFPAC-1 cells treated with GANT61, GANT61 + 3-MA or vehicle over 96 h was measured using MTT assays (*P<0.05; n=3). (B) Cell apoptosis was assessed by flow cytometry. GANT61 treated cells showed a higher apoptosis ratio (28.38±4.12%), whereas the GANT61 + 3-MA group exhibited a lower apoptosis ratio (6.59±0.81%; *P<0.05; n=3). (C) Western blotting for LC3 using lysates from CFPAC-1 cells treated with GANT61 or GANT61 + 3-MA. Non-treated cells and vehicle group (DMSO) were used as controls. The ratio of the LC3-II level/tubulin level was used as an indicator of the autophagic level. (D) The ratio of LC3-II/tubulin is presented in the bar chart (*P<0.05, n=3).

Since both HH signaling and autophagy involve complex crosstalks with many other pathways, we used 3-MA (an inhibitor of autophagy) to inhibit autophagy to confirm whether or not GANT61-induced autophagy is caused by the activation of autophagy. Our data showed that GANT61-induced apoptosis and reduction in cell viability in CFPAC-1 cells were reversed by inhibition of autophagy by 3-MA (P<0.05; Fig. 3A and B). Western blot analysis showed that, compared with the GANT61-treated group, the ratio of the LC3-II level/tubulin level was significantly reduced in the group treated with the combination of GANT61 and 3-MA (P<0.05; Fig. 3C and D), suggesting the GANT61-induced autophagy was inhibited by 3-MA. These results suggest that GANT61-induced autophagy contributes to the viability and apoptosis of CFPAC-1 cell.

**Discussion**

In the present study, we demonstrated that the HH signaling pathway regulates autophagy in pancreatic cancer cells. Our data showed that activation of HH signaling by its ligand, N-Shh, inhibits autophagy, while inhibition of the HH pathway by GANT61 induces autophagy. In addition, GANT61-induced autophagy contributed to reduced viability and increased apoptosis in CFPAC-1 cells and these effects were reversed by the co-treatment with 3-MA, the autophagy inhibitor.

The HH signaling pathway is a ‘core’ signal transduction pathway in pancreatic cancer (10) that promotes the
tumorigenesis of pancreatic cancers via enhancing cell proliferation (11,12), increasing invasion and metastasis (13,14) and protecting against apoptosis (15,16). Therapies targeting HH signaling were believed to be an effective method through which to overcome pancreatic cancer. However, in a phase I trial testing vismodegib (GDC-0449), an inhibitor of Smoothened (SMO), no beneficial responses were observed in pancreatic carcinoma (17). The ineffectiveness of the pathway inhibitor for pancreatic cancer was probably due to the fact there is much crosstalk between the HH signaling pathway and other signaling pathways in pancreatic cancer (18-20). In the present study, we found that HH signaling regulated autophagy in pancreatic cancer cells. Our data revealed that activation of HH signaling by its ligand, N-Shh, inhibited autophagy, while inhibition of the HH pathway by GANT61 induced autophagy. The role of autophagy in pancreatic cancer development and progression is complex. During the development of pancreatic cancer, autophagy initially suppresses tumor initiation yet supports tumor growth at later stages (27,28). Many results suggest that autophagy is a cell death mechanism in response to drugs such as gemcitabine and triptolide. However, it was also reported that activation of autophagy promotes tumorigenesis and limits apoptosis in pancreatic cancer cells. In the present study, we found that GANT61-induced autophagy contributed to reduced viability and increased apoptosis in CFPAC-1 cells both in vivo and in vitro, and these effects were reversed by the blockage of autophagy. Our findings were confirmed by a recent study in which Wang et al (39) found that the HH signaling pathway regulates autophagy in HCC, inhibition of Gli by GANT61 induces autophagy, and blockage of autophagy attenuates GANT61-induced apoptosis. However, in a recent study, we found that miR-101 enhanced apoptosis induced by cisplatin in HCC cells by inhibition of autophagy (40). Although these findings appear contradictory, these inconsistent conclusions confirm that autophagy plays a dual role in tumor initiation and development. The tumor-suppressor or tumor-promoter role depends on the type of cancer and the microenvironment. In addition, inhibition of autophagy by different approaches caused inconsistent effects which may be related to the complex crosstalk between autophagy and many other pathways. Different methods of autophagy inhibition may regulate different crosstalk pathways resulting in a different degree of effectiveness. Thus, further investigation of the mechanism of interaction between autophagy and these crosstalk pathways is particularly important.

In conclusion, the present study showed that HH signaling regulates autophagy in pancreatic cancer cells. Activation of HH signaling inhibits autophagy, while inhibition of the HH pathway induces autophagy. Although the role of autophagy

Figure 4. GANT61 induces autophagy and suppresses pancreatic cancer cell growth in vivo. (A) Orthotopic xenograft model of pancreatic cancer and gross specimens of tumors after receiving treatment with GANT61 or GANT61 + 3-MA. The images are representative of 6 specimens accordingly. (B) Tumor volume of the three groups (P<0.05; n=6). GANT61 treatment significantly inhibited CFPAC-1 tumor growth, and the effectiveness was attenuated by the autophagy inhibitor, 3-MA. (C) Induction of autophagy in GANT61-treated tumors was confirmed by transmission electron microscopy. The white arrows indicate the double membrane-surrounded autophagosomes in the tumor samples. Upper panel, overview images (magnification, x20,000). Scale bars represent 1 µm. Lower panel, enlarged images of cytoplasmic regions of the upper panel indicated within the dashed lines. Scale bars represent 500 nm. (D) The data were quantified by counting the number of autophagosomes per cross-sectioned cell (P<0.05; n=30).
in cell survival and apoptosis may depend on tumor type and microenvironment, our data clearly demonstrated that GANT61-induced autophagy contributes to reduced viability and increased apoptosis in pancreatic cancer cells both \textit{in vivo} and \textit{in vitro}. We propose that HH signaling by regulating autophagy plays an important role in determining the cellular response to HH-targeted therapy in pancreatic cancer and further investigation concerning the interaction between autophagy and HH signaling is particularly important.

Acknowledgements

The present study was supported by grants from the Department of Public Health of Jiangsu Province (no. RC2007056) and the National Natural Science Foundation of China (no. 81170415).

References