Original Article

Neoalbaconol inhibits cell growth of human cholangiocarcinoma cells by up-regulating PTEN

Guang-Yao Zhou1*, Chen-Wei Pan1*, Ling-Xiang Jin1, Jian-Jian Zheng2, Yong-Xiang Yi3

1Department of Infectious Disease, The Second Affiliated Hospital & Yuying Children’s Hospital of Wenzhou Medical University, Wenzhou 325027, China; 2Wenzhou Key Laboratory of Surgery, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325000, China; 3Department of Hepatobiliary Surgery, The Second Hospital of Nanjing, Affiliated to Southeast University, Nanjing 210003, China. *Equal contributors.

Received September 27, 2015; Accepted December 31, 2015; Epub February 15, 2016; Published February 29, 2016

Abstract: The recently isolated small-molecule neoalbaconol (NA) from Albatrellus confluens has been suggested to possess the ability to inhibit cell growth of many cancer cells. In this study, we investigated the role of NA in the regulation of cell apoptosis in human cholangiocarcinoma cell lines both in vitro and in vivo. Our results indicate that NA could induce cancer cell death via the AKT pathway by targeting phosphorate and tension homolog detected on chromosome 10 (PTEN) and supported the feasibility of NA being a novel chemotherapeutic treatment for human cholangiocarcinoma.

Keywords: Neoalbaconol, cholangiocarcinoma, PTEN

Introduction

Cholangiocarcinoma (CCA) is a lethal malignancy with poor prognosis that makes up 10-25% of all primary liver cancer diagnosed worldwide, and surgical resection represents the mainstay of curative intent treatment [1, 2]. Surgical treatment for hilar cholangiocarcinoma (HC), also known as Klatskin’s tumor, is difficult because of the tumor’s central location in the liver hilum and its intimate relationships with the adjacent liver parenchyma, portal vein, and hepatic arteries [3-5]. The current standard chemotherapy regimens for unresectable HC are platinum-based in combination with gemcitabine. While these regimens have demonstrated small improvements in survival in randomized control clinical trials as well as in small retrospective series, it is still emergent to uncover novel chemotherapeutic agents [3, 5].

Albatrellus confluens, mainly distributed in Southwest China, is a member of the Polyporaceae family. Several compounds with anticancer potential have been isolated from this fungus, of which neoalbaconol (NA) with a drimane-type sesquiterpenoid structure was recently isolated from the fruiting body [6-8]. NA has proven to be efficacious in inhibiting the growth of a broad spectrum of tumor cell lines [7-9], but there is no report on the effects of NA on hilar cholangiocarcinoma. In this study, we found that NA could inhibit cell growth of two human hilar cholangiocarcinoma cell lines both in vitro and in vivo via the AKT pathway by up-regulating phosphatase and tension homolog detected on chromosome 10 (PTEN).

Materials and methods

Cell culture

The human cholangiocarcinoma cell lines QBC939 and FRH0201 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 (Sigma, St. Louis, MO, USA) medium supplemented with 10% fetal bovine serum and penicillin.

Reagents and antibodies

NA was isolated and identified from the mushroom Albatrellus confluens. The primary rabbit antibodies against GAPDH, PTEN, AKT, p-AKT,
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BAD and p-BAD were all purchased from Cell Signaling Technologies (Beverly, MA, USA). The horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from ZSGB-BIO (Beijing, China).

Cell viability analysis

QBC939 and FRH0201 cells were seeded into 96-well plates (4,000 per well) in a final volume of 200 µl. After a 24 h attachment period, cells were incubated with NA at different doses from 0.5-40 µM with incubation for another 24 h or 48 h. The cell viability was measured using a cell counting kit-8 purchased from Dojindo Molecular Technologies (Shanghai, China).

TUNEL assay

QBC939 and FRH0201 cells were incubated with NA at concentrations of 0, 10, 20 and 40 µM for 24 h, and apoptotic DNA fragmentation was detected using a DNA fragmentation imaging kit (F. Hoffman-La Roche Ltd, Basel, Switzerland) following the manufacturer’s instructions. Based on the TUNEL reaction, fluorescence detection of cells with apoptotic DNA strand breaks was performed. To examine total cell numbers, nuclei were labeled simultaneously with 4',6-diamidino-2-phenylindole (DAPI). Merged images of both channels were shown using a fluorescence microscope (Olympus, Tokyo, Japan) at 100× magnification.

Annexin V-FITC/PI staining and flow cytometry analysis

Cell apoptosis was also detected using Annexin V-FITC/PI assays, following the manufacturer’s protocols (Becton-Dickinson Crop, San Jose, CA, USA), and the stained samples were analyzed with a FACSCalibur flow cytometer (Becton-Dickinson).

Western blot analysis

QBC939 and FRH0201 cells were incubated with NA at concentrations of 0, 10, 20 and 30 µM for 24 h and then were harvested and lysed in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na3VO4, leupeptin, and PMSF). Protein concentrations were calculated using a BCA protein assay kit (Thermo Fisher Scientific, USA), and equal amounts of total protein per lane were separated by SDS gel electrophoresis and transferred to nitrocellulose membranes. Non-specific binding of proteins to the membrane was blocked by incubation with TBS-T buffer containing 5% skimmed milk. Membranes were then incubated with the appropriate primary and HRP-conjugated secondary antibodies. GAPDH was used as loading control. Blots were developed with the Phototope HRP Western Blot Detection System (Cell Signaling Technologies).

Animal experiments

Twenty-four 4- to 6-week old male BALB/c nu/nu nude mice (SPF, purchased from Vital River Laboratories, Beijing, China) were divided into three groups: the control group, the NA plus BPV group, and the NA only group. Mice in the NA plus BPV group were intraperitoneally injected with BPV two weeks before tumor inoculation, while mice in the other two groups were injected with PBS as a control. QBC939 cells (1×107) were suspended in 100 µl PBS and subcutaneously injected into the left flank of all the nude mice. NA treatment (100 mg/kg/day) was initiated the second day after tumor transplantation. Four weeks after NA treatment, mice were sacrificed. Tumor size was measured with digital calipers and calculated as V=LS2/2 (where L is the longest diameter and S is the shortest diameter). Tumors were also fixed in 10% neutral formalin and embedded in paraffin for subsequent PTEN immunohistochemical analysis.

Immunohistochemistry detection

Tissue sections (4-6 µm) were mounted and heated at 72°C for 30 min. Sections were deparaffinized with xylene, rehydrated in graded EtOH and rinsed with 0.1 M Tris-HCl (pH 7.6). Non-specific binding was blocked by incubation of the tissue sections with 0.1 M Tris-HCl containing 10% goat serum for 2 h. PTEN expression was then determined by incubating the sections with rabbit anti-PTEN polyclonal antibody at a 1:200 dilution in the above blocking solution in a moist chamber at 4°C overnight. After washes with 0.1 M Tris-HCl, the sections were treated with 1:400 dilutions of biotinylated horse anti-rabbit secondary antibody and incubated at room temperature for 2 h. An additional rinse with 0.1 M Tris-HCl, antibody binding was detected by incubating the tissue sections with HRP-conjugated streptavidin at
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A

B

Figure 1. NA inhibited cell growth of two human cholangiocarcinoma cell lines. QBC939 (A) and FRH0201 (B) were treated 0.5-40 µM of NA, and cell viabilities were analyzed by CCK-8 kit. Values represent the mean ± SD from three independent measurements.

room temperature for 30 min. DAB-H_2O_2 substrate was then added to the slides, which were incubated at room temperature for an additional 4 min. Tissue sections were stained with hematoxylin, dehydrated in graded alcohol, cleared in xylene, and mounted with Permount Mounting Media for visualization by light-microscopy at 400× magnification.

Statistical methods

All statistical analyses were performed using SPSS ver. 13.0 software (SPSS Inc., Chicago, IL, USA). Pearson’s chi-squared (χ^2) test or Fisher’s exact test was used to analyze the differences between groups. Data were presented as the mean ± standard deviation, and the differences between groups were assessed using analysis of variance (ANOVA) or Dunnett’s t-test. A P value of <0.05 was considered to indicate statistical significance.

Results

NA inhibited cell growth

NA inhibited the growth of QBC939 and FRH0201 cells in a dose-dependent manner, while no obvious time-dependent effects were observed (Figure 1). The IC_{50} values were 24.2 µM for QBC939 and 22.8 µM for FRH0201.

NA induced cell apoptosis

Based on the potency of NA on cell growth inhibition, we chose to use NA at concentrations of 10 µM, 20 µM and 40 µM for both cell lines. TUNEL and Annexin V-FITC/PI co-staining assays were both used to analyze the extent of apoptosis. According to the results shown in Figure 2, NA induced cell apoptosis in a dose-dependent manner. After 24 h treatment, 10 µM, 20 µM and 40 µM of NA induced apoptosis in 8.96%, 71.0% and 94.6% of QBC939 cells, respectively, and 7.32%, 69.1% and 84.2% of FRH0201 cells, respectively (P<0.05).

NA induced up-regulation of PTEN, repression of the AKT pathway and caspase activation

QBC939 and FRH0201 cells were treated with NA at concentrations of 10 µM, 20 µM, and 30 µM for 24 h. Then, cell lysates were prepared, and proteins were detected by Western blotting (Figure 3). Significantly increased PTEN expression was observed in both cell lines after NA treatment in a dose-dependent manner. While the total AKT and BAD protein expression remained nearly unchanged, obvious repressed AKT phosphorylation and upregulated BAD phosphorylation were observed. As postulated, subsequent downstream events of AKT pathway, such as activation of caspase 3 and caspase 9, were also significant.

Effects of NA on cells were reversed by a PTEN inhibitor BPV

To determine whether the inhibition of cell growth and repression of the AKT pathway by NA were induced by upregulation of PTEN, both QBC939 and FRH0201 cell lines were pre-treated with 2.5 µM bisperoxopicolinatox-
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Figure 2. NA induced cell apoptosis of two human cholangiocarcinoma cell lines, QBC939 and FRH0201 cells were collected 24 h after incubation with 10 µM, 20 µM and 40 µM of NA. TUNEL staining (original magnification, ×100) was used to determine the apoptosis of QBC939 (A) and FRH0201 (D); Annexin V-FITC/PI staining levels were analyzed by flow cytometry (B, C for QBC939; E, F for FRH0201). Values represent the mean ± SD from three independent experiments (*P<0.05).
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ovadate (BPV), a PTEN inhibitor (purchased from EMD4 Biosciences, Darmstadt, Germany) for 1 h before NA addition. We found that apoptosis was significantly reduced by Annexin V-FITC/PI staining assay (Figure 4), and the changes in protein expression were obviously recovered by BPV pre-treatment (Figure 5).

In vivo efficacy of NA in the mouse model and immunohistochemical analysis

To explore whether NA could be efficacious in vivo, we subcutaneously inoculated BALB/C nu/nu mice with QBC939 cells. NA treatment (100 mg/kg/day) was performed the day after tumor transplantation. Mice were sacrificed four weeks after NA treatment, and tumors were excised, measured and prepared for subsequent immunohistochemical analysis. The tumor volumes in the NA group were significantly smaller than those of the control group, while the effect of inhibition was recovered by BPV pre-treatment as the tumor volumes in the NA plus BPV group were larger than those of the NA group (Figure 6A and 6B). Furthermore, immunohistochemical staining analysis of PTEN in tumor sections indicated that tumor specimens displayed high levels of PTEN expression in the NA-treated group, and BPV pre-treatment effectively reduced PTEN expression (Figure 6C).

Discussion

CCA, a cancer originating from the epithelial lining of the biliary tree, accounts for approximately 3% of all gastrointestinal tumors and is the second most common primary liver tumor after hepatocellular carcinoma (1,2). It is categorized as intrahepatic (ICC) or extrahepatic (ECC) according to the International Classification of Diseases for Oncology. HC was first described by Altermeier and Klatskin approximately 50 years ago and comprised over 60% of all cholangiocarcinomas, and it has a particularly high prevalence in certain Asian countries, such as
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Figure 4. BPV recovered the apoptotic-inducing effect of NA in both cell lines. QBC939 and FRH0201 cells were pre-treated with 2.5 µM BPV for 1 h prior to the addition of 20 µM NA. Apoptosis levels were detected 24 h after NA incubation using a Annexin V-FITC/PI staining assay followed by flow cytometry analysis (A, C for QBC939, and B, D for FRH0201). Values represent the mean ± SD from three independent measurements (*P<0.05 vs control, #P<0.05 vs 20 µM NA without BPV).

Thailand, Korea and China (3). HC is a complex and aggressive disease with a poor prognosis, occurring at the confluence of the right and left hepatic ducts, and it remains controversial whether it should be classified as ICC or ECC or both [10-13]. Though surgical resection is thought to be the best management for HC, complete margin-negative (R0) resection is more curative in early stages of HC than in late stages when the tumor may be close to or invading the major vascular structures surrounding the bile duct, such as the portal vein, hepatic artery, and liver parenchyma. Most commonly, HC invades the portal vein, making
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surgical resection a high-risk procedure [10-12]. With limited and heterogeneous data, Yang et al. [14] suggested in a meta-analysis that patients with advanced biliary tract cancer who are treated with platinum plus gemcitabine may experience better survival outcomes compared with patients who are not treated with this combination of chemotherapy. However, more randomized studies are needed to prove the treatment’s efficacy and to fully understand its capabilities; meanwhile, novel chemotherapeutic agents are demanded [3, 5, 14].

Mutation and genomic loss of PTEN have been identified in many types of human cancer [15-21], and this pathway has been linked to development of cholangiocarcinoma following the discovery that liver-specific deletion of both PTEN and SMAD4 in a mouse model leads to the development of intrahepatic cholangiocellular carcinoma [22, 23]. It is well known that the principal catalytic function of PTEN is to dephosphorylate phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3), which is a potent activator of 3-phosphoinositide-dependent kinase (PDK) and AKT. Following PTEN loss, excessive PtdIns(3,4,5)P3 at the plasma membrane recruits and activates a subset of proteins, including the AKT family and PDK1. Active AKT isoforms drive cell survival, cell proliferation, angiogenesis and cellular metabolism by phosphorylating downstream signaling proteins.

Figure 5. BPV recovered the repression of AKT phosphorylation, the upregulation of BAD phosphorylation, and the activation of caspase 3 and caspase 9 caused by NA. QBC939 and FRH0201 cells were pre-treated with 2.5 µM BPV for 1 h prior to the addition of 20 µM NA. Cell lysates were prepared 24 h after NA incubation, and proteins were detected by Western blotting. Values represent the mean ± SD from three independent measurements (*P<0.05 vs control, #P<0.05 vs 20 µM NA without BPV).
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[18, 19, 24-29]. Energy metabolic reprogramming is another significant feature of most cancers, and a number of small-molecular compounds that target metabolism-related pathways or regulators have been developed and show activity in cancer cells [30-33]. The PTEN-PI3K-AKT-mTOR pathway has a central role in the regulation of glucose metabolism, owing to its position downstream of the insulin receptor (INSR) and IRS adaptor molecules [28]. Deng et al. [8, 9] have suggested in their pioneer studies that NA, a small molecule recently isolated from the fruiting body of Albatrellus confluens, might induce apoptotic and necrotic cell death of various cancer cells via the PDK1-PI3K-AKT signaling pathway and by remodeling cellular energy metabolism. In this study, we explored the biological effects of NA on two human hilar cholangiocarcinoma cell lines, QBC939 and FRH0201. Our results proved that NA could promote cell apoptosis via the PI3K-AKT pathway, which is consistent with previous studies [8, 9]. As Deng et al. [8] proposed that PDK1 is a direct target of NA, we found that NA might trigger PTEN up-regulation as an upstream event by using the PTEN inhibitor BPV, which recovered the repression effect of NA on the AKT pathway in the present study.

The past few years have seen the development and acceptance of the idea that PTEN is pleiotropic in nature and can function as a crucial factor in various processes that are important in cancer development. Therefore, pharmacological manipulation targeting PTEN could undoubtedly serve as a promising treatment.

Acknowledgements

This work was supported by Wenzhou Public Welfare Foundation of Science and Technology Project, Science and Technology Bureau of Wenzhou (Y20150023), Nanjing Medical Science and Technique Development Foundation, Nanjing Department of Health (Grant: QRX11235 and Grant: ZDX12008), Jiangsu Science and Technology Project of Clinical Medicine Foundation, Science and Technology Department of Jiangsu Province (BL2014005).
Disclosure of conflict of interest

None.

Address correspondence to: Yong-Xiang Yi, The Second Hospital of Nanjing, Affiliated to Southeast University, 1-1 Zhongfu Road, Nanjing City, Jiangsu Province, China. Tel: +8618205194323; Fax: +86-025-83626203; E-mail: ian0126@163.com

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