Oleanolic Acid Inhibits Epithelial–Mesenchymal Transition of Hepatocellular Carcinoma by Promoting iNOS Dimerization

Hongzhi Wang1,2, Weilong Zhong1,2, Jianmin Zhao3, Heng Zhang1,2, Qiang Zhang1,2, Yuan Liang1, Shuang Chen1, Huijuan Liu1, Shumin Zong1, Yixuan Tian1,2, Honggang Zhou1,2, Tao Sun1,2, Yanrong Liu1,4, and Cheng Yang1,2

Abstract

Oleanolic acid exhibits extensive pharmacologic activities and takes significant antitumor effects. Its pharmacologic mechanism, however, still remained to be further clarified. In this study, we demonstrated that oleanolic acid attenuated the migration and invasion abilities, resulting in the suppression of the epithelial–mesenchymal transition (EMT) process in liver cancer cells, and inhibited the tumor growth of the peritoneal lymphocytes–bearing mice. We further proved that inducible nitric oxide synthetase (iNOS) may be the potential target of oleanolic acid. We confirmed that oleanolic acid could promote the dimerization of iNOS, activating it, and subsequently increasing the production of nitric oxide. Further experiments indicated that oleanolic acid promoted the nitrilation of specific proteins and consequently suppressed their EMT-related biological functions. Furthermore, it has been confirmed that oleanolic acid enhanced the antitumor effects of regorafenib in liver cancer treatment. These results deepened our understanding of the pharmacologic mechanism of the antitumor effect oleanolic acid, and the importance of nitric oxide synthetase as a therapeutic target for liver cancer treatment.

Introduction

The 5-year survival rates of patients with liver cancer, which is highly metastatic, are relatively low (1). Surgery, chemotherapy, and targeted therapy are currently adopted in liver cancer treatment, but these treatments have limited therapeutic efficacy and easily lead to relapse and metastasis (1, 2). Therefore, developing new drugs that can inhibit the metastasis of liver cancer should be developed. Oleanolic acid is a pentacyclic triterpenoid that has long been used as an effective drug in treating hepatitis. Oleanolic acid also exhibits antitumor effects in various cancers, including leukemia (3), pancreatic cancer (4), gallbladder cancer (5), melanoma (6), breast cancer (7), liver cancer (8), lung cancer (9), and osteosarcoma (10), by inducing apoptosis and cell-cycle arrest or through the radiosensitizing effect of radiotherapy. Some potential targets for oleanolic acid have been reported in cancer and other diseases, such as aldo–keto reductase family member 1B10 (AKR1B10), CD81, G-protein coupled bile acid receptor 1, 11b-hydroxysteroid dehydrogenase type 1, protein tyrosine phosphatase 1B, and cell division cycle 25 phosphatase (11–16). AKR1B10 was suggested as a therapeutic target in colon cancer. Oleanolic acid inhibits cell viability and induces apoptosis by reducing expression level of intercellular adhesion molecule-1 and VEGF in liver cancer (17). Oleanolic acid also has inhibitory effect on hepatocellular carcinoma (HCC) via increase of Bax/Bcl-2 ratio, increased release of cytochrome c, and activation of caspase/PARP (18). However, the direct target of oleanolic acid in liver cancer is still unclear. Besides, the reported antitumor effects of oleanolic acid, we mainly focused on the cytotoxic effects. Whether oleanolic acid has inhibitory effects on epithelial-to-mesenchymal transition (EMT), the vital process in the initiation of tumor (e.g., liver cancer) metastasis (19–21), is also to be explored. This study reported here that oleanolic acid can significantly inhibit EMT of HCC and that its potential target is inducible nitric oxide synthetase (iNOS). iNOS is one of the three isoforms of nitric oxide synthetase (NOS), and its activity is regulated by dimerization and phosphorylation (22, 23). Overexpression of iNOS negatively correlates with histologic grades and clinical stages, and positively correlates with prognosis in liver cancer (TCGA liver cancer database). Nitric oxide is a simple molecule generated by NOS, and is often involved in the development of diseases including cancers. High nitric oxide concentrations exhibit cytotoxic effects by promoting DNA damage, protein dysfunctions, gene mutations, and
tumor cell death, all of which contribute to tumor inhibition (24, 25). Moreover, high concentrations of nitric oxide can inhibit EMT in several cancers (26–28) and may induce nitration (NT) of specific proteins through reacting with reactive oxygen species (ROS) and subsequently forming peroxynitrite (29).

The results of this study suggest that oleanolic acid promotes the dimerization of iNOS and the subsequent excessive production of nitric oxide, which nitrated specific EMTRelated proteins and influences their biological functions and then eventually suppresses the malignant evolution of liver cancer.

This study provides a comprehensive understanding of the pharmacologic mechanism of oleanolic acid and opens an opportunity for oleanolic acid to be developed as an antitumor drug, to be combined with current antitumor drugs for liver cancer treatment.

Materials and Methods

Cell culture

HCC cell line HepG2 was cultured in DMEM supplemented with 10% FBS (HyClone) and 1% penicillin–streptomycin (Beitoyime). Peritoneal lymphocytes (PLC-PRF-5) and 7721 were maintained in RPMI1640 supplemented with 10% FBS and 1% penicillin–streptomycin. The three cancer cell lines were maintained at 37°C under a humidified atmosphere of 5% CO2 in an incubator, trypsinized, and passaged every 2 days. The cells were purchased from KeyGen Biotech and the company provided complete cell identification. The cells were periodically authenticated by biomarker detection of hepatocellular carcinoma, morphologic inspection, growth curve analysis, and Mycoplasma testing, and were not used beyond 10 passages.

Target prediction of oleanolic acid

Target prediction was performed by the similarity ensemble approach (SEA). SEA predicted target proteins based on the set-wise chemical similarity among their ligands. Using the similar type of oleanolic acid structure, SEA rapidly searches large compound databases and builds cross-target similarity maps.

Detection of intracellular nitric oxide content and NOS activity

Cells were seeded in 96-well plates and were incubated with a series of oleanolic acid concentrations (purchased from Aladdin, diluted in DMSO to prepare a 5 mmol/L stock solution, and stored at 4°C) 20 hours after cell seeding for approximately 36 hours. Intracellular nitric oxide content and NOS activity were detected with 3-amino,4-aminomethyl-2,7'-difuorescein diacetate, a type of fluorescent probe that reacts with nitric oxide, and NOS Assay Kit (both from Beiyotime), respectively, in accordance with the manufacturer's procedures.

Transfection of siRNAs

Negative control siRNA (siRNA-Ctrl) and siRNA against INOS (siRNA iNOS) were purchased from Santa Cruz Biotechnology. The siRNAs/Lipofectamine 2000 (Invitrogen) complex was prepared at a 1:3 weight ratio by incubation in OptiMEM at room temperature for 20 minutes in accordance with the manufacturer's instructions. After complex formation with Lipofectamine 2000, 25 mmol/L siRNA was transfected into liver cancer cells. At 48 hours after transformation, cells were incubated with oleanolic acid for subsequent testing.

Migration and invasion assays

For migration assays, cells were seeded in a 24-well plate at a density of 2 × 105 cells/well. After 24 hours, a wound was made in the center of the well, and oleanolic acid (50 μmol/L), oleanolic acid (50 μmol/L)/C-PTIO (20 μmol/L), regorafenib (1.5 μmol/L), or oleanolic acid (50 μmol/L)/regorafenib (1.5 μmol/L) was added and incubated for 48 hours. For the oleanolic acid (50 μmol/L)/siRNA iNOS group, after transfection of siRNA iNOS for 48 hours, cells were incubated with or without oleanolic acid for another 48 hours. Subsequently, pictures were taken at different intervals.

Invasion assays were performed with a 24-well plate and 8-μm Matrigel invasion chambers. The chambers were placed in the well of the plate, and 50 μL of Matrigel:medium (Collaborative Biomedical) (1:1) was added to the chambers. The plate and chambers were maintained at 37°C for approximately 30 minutes for the solidification of Matrigel. After 30 minutes, 200 μL of cell suspension without 10% FBS containing approximately 8 × 104 cells was added into the top chamber, and 500 μL of medium with 10% FBS acting as a chemo-attratant was added to the bottom chamber, followed by incubation at 37°C. The cells were treated similarly to those in the migration assays, except that the incubation time was 24 hours. After 24 hours, the chambers were removed, and the medium together with the Matrigel inside it was abandoned. After washing three times with 1 × PBS, the cells transferred through the filter membrane at the bottom of the chambers were fixed in 4% paraformaldehyde (PFA; precooled at 4°C) and stained with crystal violet for 20 minutes at room temperature. Finally, photographs were taken with a microscope (Nikon).

Scanning electron microscopy

PLC cells were seeded on a climbing film and treated and/or transfected with oleanolic acid (50 μmol/L), oleanolic acid (50 μmol/L)/C-PTIO (20 μmol/L), or oleanolic acid (50 μmol/L)/siRNA-iNOS. After 24 hours, the cells were fixed with 4% PFA (precooled at 4°C), dehydrated in acetone/isooamyl acetate (1:1), dried with a gradient concentration of acetonitrile, and then coated with gold. Subsequently, the cell images were captured using a scanning electron microscope (LEO 1530 VP).

Immunofluorescence

Approximately 2 × 105 cells were seeded on the slide inside the well of the 24-well plate. After 20 hours, the cells were treated and/or transfected with oleanolic acid (50 μmol/L), oleanolic acid (50 μmol/L)/C-PTIO (20 μmol/L), or oleanolic acid (50 μmol/L)/siRNA-INOS and incubated at 37°C for 24 hours. The cells were washed three times with 1 × PBS, fixed in 4% PFA (precooled at 4°C) for 20 minutes, and then blocked with 5% BSA containing 0.1% Triton X-100 for 30 minutes at room temperature. The cells were incubated with primary antibodies diluted in 5% BSA. Washing again thrice with 1 × PBS, the cells were incubated with fluorescently conjugated secondary antibodies diluted in 5% BSA for approximately 50 minutes at room temperature at conditions protected from light. After four washes with 1 × PBS, the cells were sealed with the mounting medium (DAPI-containing), and
pictures were taken with a laser scanning confocal A1 microscope (Nikon).

**Western blot assay**

Cells were precultured for 24 hours and then treated and/or transfected with oleic acid (25 μmol/L), oleic acid (50 μmol/L)/C-PTIO (20 μmol/L) or oleic acid (50 μmol/L)/siRNA-iNOS. After 24 hours, the proteins were extracted and whole-cell lysates were centrifuged at 4°C. The protein content was determined with a BCA Protein Assay Kit (Thermo Fisher Scientific). The supernatant, fixed with 5 × loading buffer, was boiled for 10 minutes at 99°C. Equal amounts of proteins (30 μg) were subjected to 10% SDS-PAGE, transferred onto polyvinylidene difluoride (Millipore) membranes, and then blocked with blocking solution (5% nonfat milk dissolved in Tris-buffered saline/Tween 20) for 2 hours on the shaker at room temperature. The proteins were incubated with primary antibodies, including rabbit anti-E-cadherin, iNOS antibodies (Affinity) and mouse anti-vimentin, glyceraldehyde-3-phosphate dehydrogenase antibodies (Affinity), and anti-Nitrotyrosine antibody (Abcam) diluted (1:1,000) in a blocking solution overnight at 4°C. The membranes were then incubated with horseradish peroxidase–conjugated secondary antibody (1:5,000) for 1 hour at room temperature. Finally, the target protein bands were detected with ultrasensitive enhanced chemiluminescence luminescence solution (CLINX Science Instruments) and imaged with a Western Blot Imaging System (CLINX Science Instruments).

**In vivo treatment of PLC-bearing mice**

All mice were purchased from Charles River Laboratories. Six- to 8-week old male BALB/c nu/nu mice were kept in a specific pathogen-free animal care facility in accordance with the institutional guidelines. All animals in this experiment were well taken care of. The 1 × 10^6 cells (suspended in PBS) were subjected to inoculation by subcutaneous injection, and the mice were subsequently randomly divided into four groups (n = 10). Two weeks after inoculation, the mice in the experiment groups were gavaged with gavage with oleic acid (50 mg/kg daily), regorafenib (10 mg/kg daily), or a combination of oleic acid (50 mg/kg daily) and regorafenib (10 mg/kg daily) for another 2 weeks. Meanwhile, the mice in the control group were treated with the same volume of saline for 2 weeks. The tumor volumes (mm^3) determined by the formula 

\[ V = \frac{ab^2}{2} \]

(a = length of tumor, b = width of tumor) were measured every 2 days until the mice were euthanized. All animal experiments were performed under approved protocols of the institutional animal use and care committee of Tianjin International Joint Academy of Biomedicine (Tianjin, China). The metastasis in the lung tissues was counted under microscope after hematoxylin-eosin staining.

**IHC staining**

After the mice were sacrificed, the xenografts were harvested for histologic examination staining. The xenograft tissues were fixed in formalin solution, embedded in paraffin, cut into 4-μm slices, and placed on the slides. Tissues were deparaffinized with xylene and dehydrated in decreasing concentrations of ethanol. Tissue slices of the xenografts were incubated with 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity and then treated with citrate-buffered saline (pH 6.0) for 10 minutes at 95°C to retrieve antigens. After incubation with normal goat serum for 1 hour at room temperature to block unspecific labeling, the tissues were incubated with primary antibodies, including anti-E-cadherin, anti-Vimentin, anti-iNOS, anti-MMP2, anti-MMP9 (all purchased from Affinity), and anti-Nitrotyrosine antibody (obtained from Abcam), in a humidified chamber overnight at 4°C. Diaminobenzidine was utilized for color development and hematoxylin for counterstaining. The expression levels of these proteins were independently evaluated by two investigators. Tumor cells with brown-stained cytoplasm, nuclei, or membranes were considered positive and scored as follows: staining degree: none (0), weak brown (1+), moderate brown (2+), and strong brown (3+). The percentage of stained tumor cells was divided into five classes: 0 for negative cells, 1 for 1%–25%, 2 for 25%–50%, 3 for 50%–75%, and 4 for >75%. Multiplication (staining intensity) of intensity and percentage scores was utilized to determine the result.

**Microarray assay**

PLC-PRF-5 cells were treated with oleic acid (50 μmol/L) or DMSO for 24 hours. TRIzol Reagent (Invitrogen) was then used to lyse cells and extract the total RNA. The microarray was further performed based on high-throughput gene expression profile. Two times of gene expression difference were selected as the screen standard for further bioinformatics analysis. The detailed microarray expression data can be found in GEO database (GSE120311).

**Molecular docking**

We downloaded the crystal structure of iNOS by using the Protein Data Bank (PDB ID: 1NSI). The iNOS structure was prepared to perform molecular docking. The structure was treated by adding hydrogen, optimizing the H-bond assignment, assigning bond order, and finally minimizing energy to repair and relax protein structure. The protein interface site of the crystal structure was used to define the center site of a docking grid box, and the xz dimension of the docking grid box was 60 × 60 × 60. The Schrodinger software was used to perform molecular docking. The 2D and 3D structures of oleic acid were generated with ChemDraw and LigPrep, respectively, and were minimized with force field OPLS-2005.

**Cell viability assays**

HepG2, SMMC7721, and/or PLC cells (5 × 10^3 cells/mL) were seeded in 96-well culture plates for 20 hours. For combination assays, PLC cells were treated with solvent, oleic acid (50, 65, and 80 μmol/L), regorafenib (1.5 μmol/L), or oleic acid (50, 65, and 80 μmol/L)/regorafenib (1.5 μmol/L), and incubated for 24 hours or 48 hours; for cytotoxicity assay of oleic acid, HepG2, SMMC7721, and PLC cells were treated with solvent and oleic acid (20, 40, 80, 120, 160, 240, and 320 μmol/L), and incubated for 48 hours. Cells were then added with 25 μL of MTT and incubated for 4 hours at 37°C. Subsequently, 125 μL of DMSO was added to dissolve the formazan. Optical density was determined at 570 nm with a microplate reader (Multiskan FC, Thermo Scientific). The combination index (CI) is calculated according to the methods reported previously (30).
Oleanolic Acid Inhibits EMT of Hepatocellular Cancer

Immunoprecipitation assay and in-gel digestion
Cells were cultured and treated similarly to those in Western blot assay. Proteins were extracted with 0.3% NP-40 lysis buffer and quantified by employing the BCA protein assay kit. After centrifugation at 12,000 rpm for 10 minutes, the supernatant containing 2 mg total proteins was incubated with the protein A/G agarose/antibody complex overnight at 4°C. The complexes were previously formed from the incubation of protein A/G agarose and nitro-tyrosine or IgG antibodies (Abcam) for 10 hours at 4°C before harvesting the cells. Then, the protein A/G agarose/antibody/sample complexes and an appropriate quantity of total protein lysate were fixed with 5 × loading buffer and boiled for 10 minutes at 99°C, followed by 10% SDS-PAGE. The SDS-PAGE gel was stained with a Fast Silver Staining Kit (Beyotime Biotec) in accordance with the manufacturer’s instructions and subsequently pictured. The in-gel digestion of proteins in the differential band was then obtained. After ultrasonic extraction and vacuum desiccation, proteins were stored at −20°C until conducting mass spectrometry.

Mass spectrometry
Proteins in differential bands were digested with trypsin for 20 hours at 37°C. The sample was evaporated and resuspended in water/0.1% formic acid and identified by LC/MS-MS (Thermo Fisher Scientific). The experiment was repeated thrice. The Swiss Prot database, Human, which included 20,258 protein sequences, was used to identify tyrosine NT proteins.

Bioinformatics analysis
Cluster and Gene Ontology analyses were performed for the differentially expressed genes. Gene Ontology analysis was used to detect oleanolic acid-induced changes in molecular functions, biological processes, and cellular components. Clinicopathologic and survival analyses were performed by using the HPA (https://www.proteinatlas.org/).

Statistical analysis
All data were expressed as the mean ± SD. After testing for normality and equal variance across the groups, intergroup differences were assessed using Student t tests, ANOVA, and multivariate statistical analysis. All experiments were repeated thrice. P < 0.05 was considered statistically significant.

Results
Oleanolic acid interacts with iNOS and promotes nitric oxide production
The chemical structure of oleanolic acid is shown in Fig. 1A. We summarized the antitumor effects of oleanolic acid on different types of cancers (Fig. 1B) and then identified its potential target by SEA method. Target prediction showed that iNOS was most likely the target of oleanolic acid (Fig. 1C). To confirm this, the relative activity of iNOS and the content of intracellular nitric oxide were examined. Oleanolic acid was used at concentrations of 25 and 50 μmol/L in subsequent studies to exclude the influence of oleanolic acid on cell viability because the survival of HCC is not influenced by these doses (Supplementary Fig. S1). As expected, the relative activity of iNOS and the content of intracellular nitric oxide dramatically increased after oleanolic acid treatment (Fig. 1D and E). Immunofluorescence (IF) was performed to examine the effect of oleanolic acid on iNOS expression. Oleanolic acid treatment significantly increased the fluorescence intensities of iNOS in the PLC, HepG2, and SMC7721 cell lines (Fig. 1F). To further explore the specific regulation mechanism of oleanolic acid on iNOS, molecular docking was then conducted between iNOS (PDB ID: 1NSI) and oleanolic acid, and the docking score implied that oleanolic acid intensively interacts and fits with the interface of the monomer oxidation zone of iNOS (Fig. 1G), suggesting that oleanolic acid good fit iNOS and stabilizes the iNOS dimer. Western blot assays were then performed to further examine the effect of oleanolic acid on the expression levels of monomer and dimerized forms of iNOS. Results indicated that oleanolic acid upregulated the expression of iNOS dimer but did not change that of iNOS monomer (Fig. 1H), suggesting that oleanolic acid can promote dimerization of iNOS, which subsequently increases the production of nitric oxide.

Oleanolic acid reverses mesenchyme phenotype of HCC cells in the nitric oxide–dependent manner
A microarray assay was subsequently performed in PLC to determine the related molecular pathway influenced by oleanolic acid. The double differences were selected as the screening criteria and the differential genes were then analyzed. Results showed that oleanolic acid significantly influenced the gene expression of PLC cells compared with the untreated cells (Fig. 2A). Besides, Gene Ontology analysis results showed the biological process, molecular function, and cellular component were also regulated by oleanolic acid. It was also shown that oleanolic acid promoted protein dimerization and inhibit regulation of cytoskeleton organization (Fig. 2B and C). Results of scanning electron microscopy and migration and invasion assays demonstrated that oleanolic acid treatment significantly inhibited the formation of pseudopodia (Fig. 2D), and the migration (Fig. 2E) and invasion capabilities of HCC cells (Fig. 2F). Moreover, the preceding inhibition effects of oleanolic acid can be recovered by the administration of nitric oxide scavenger (C-PtIO) or transfection of iNOS siRNA (Fig. 2C–E), suggesting that the inhibitory effects of oleanolic acid on the migration and invasion capabilities of HCC are dependent on nitric oxide production.

To further confirm the effects of oleanolic acid on EMT, we determined the changes in the expression levels of the canonical mesenchymal marker vimentin and the epithelial marker E-cadherin by performing IF and Western blot assays. Results revealed that oleanolic acid treatment upregulated the expression levels of E-cadherin and downregulated those of vimentin. However, the treatment of C-PtIO or transfection of iNOS siRNA attenuated the effect of oleanolic acid. The preceding results further support that oleanolic acid inhibits the EMT of HCC, and this effect is dependent on the production of nitric oxide (Fig. 3A and B).

Oleanolic acid promotes the nitrification of specific proteins
Excess nitric oxide may form peroxynitrite by reacting with ROS and subsequently induce nitrification (NT) of a few proteins (31). We then examined the effects of oleanolic acid on protein NT. Immunoprecipitation, Western blot assay, and IF assays with anti-nitrotyrosine (pan) antibody were used to determine the overall NT level of proteins. Results revealed that oleanolic acid treatment promoted the NT modification of specific proteins (Fig. 4A–C). With the differential proteins
Figure 1.
Oleanolic acid (OA) interacts with iNOS and then promotes nitric oxide production. A, Structural formula of oleanolic acid, a pentacyclic triterpenoid. B, Reported antitumor effects of oleanolic acid. C, Result of targeting oleanolic acid prediction indicated that iNOS is its most possible target. D and E, Oleanolic acid treatment increased the relative activity of iNOS and the intracellular nitric oxide (NO) content in HCC cell lines. F, IF assay conducted in HepG2, PLC, and 7721 indicated that iNOS expression levels can be upregulated by oleanolic acid treatment. G, Result of molecular docking between iNOS and oleanolic acid. H, Results of Western blot assay performed in HCC cells showing that oleanolic acid treatment can upregulate the dimer/monomer ratio of iNOS. The results were obtained from three independent experiments, each performed in triplicate. Data are represented as mean ± SEM (*, P < 0.05; **, P < 0.01).
Figure 2.
Oleanolic acid (OA) regulates a series of function and inhibits migration and invasion of HCC cells. A and B, Results of microarray assay performed in PLC cells and Gene Ontology analysis. Oleanolic acid can promote protein dimerization. C, Gene set enrichment analysis showed that oleanolic acid inhibits the cell cytoskeleton. D, Results of scanning electron microscopy assay. Oleanolic acid can reverse the mesenchymal phenotype of PLC cells, whereas treatment of nitric oxide scavenger (C-PTIO) or transfection of siRNA iNOS attenuated the effect of oleanolic acid. E and F, Oleanolic acid treatment can significantly inhibit the migration and invasion capabilities in cancer cells. However, treatment with nitric oxide scavenger (C-PTIO) or transfection of siRNA iNOS can attenuate this inhibitory effect of oleanolic acid. Results were obtained from three independent experiments, each performed in triplicate. Data are represented as mean ± SEM (*, P < 0.05; **, P < 0.01).
obtained from immunoprecipitation, we performed mass spectrometry and obtained 10 nitrotyrosine-containing proteins (Supplementary Table S1). To explore the function of these proteins in cancer aggressiveness, the UniProt database was searched for proteins associated with EMT process (cell adhesion, cytoskeleton, migration, proliferation, and cell stemness) and was then analyzed with the 10 nitrogenated proteins by Venn analysis. Nine of 10 nitrogenated proteins were involved in EMT process (Fig. 4D). We then analyzed the relationship of the expression levels of the nitrogenated proteins with the survival of patients with HCC by TCGA data analysis. Results showed that six proteins were negatively related to the survival of patients with HCC (Fig. 4E). Therefore, we conclude that oleanolic acid exerts an anti-EMT effect in HCC by nitrifying EMT-related proteins, which resulted from the activation of iNOS by oleanolic acid (Fig. 4F).

Oleanolic acid exerts anti-EMT effect in vivo and enhances the antitumor effect of regorafenib
It was reported that oleanolic acid increases the sensitivity of HCC cells to sorafenib via oxidative stress and induces cell death (32). Regorafenib (fluoro-sorafenib), which has a chemical structure closely related to sorafenib, is a potentially stronger inhibitor of angiogenesis than sorafenib and inhibits a range of receptor tyrosine kinase (33). Moreover, regorafenib inhibits the EMT of colorectal and pancreatic carcinoma (34, 35). Considering the inhibitory effect of oleanolic acid on the EMT of HCC, we hypothesized that combining oleanolic acid with regorafenib enhances the antitumor effects of regorafenib.

The effects of oleanolic acid or regorafenib used either alone or combined on cell survival were determined using MTT assays (Fig. 5A). Results showed that the use of oleanolic acid at the dose of 65 or 80 μmol/L enhanced the effects of regorafenib to suppress cell growth of PLC cells. The CI of oleanolic acid and regorafenib is 1.365 in vitro, indicating that the two drugs have a synergistic effect. The results of migration and transwell assays indicated that oleanolic acid sensitizes HCC cells for regorafenib in migration and invasion inhibition (Fig. 5B and C).

The combination effects were then evaluated in the PLC-bearing mouse model. Tumor volumes were significantly smaller in the oleanolic acid- or regorafenib-treated groups than in the control group. Moreover, oleanolic acid enhanced the effect of regorafenib on tumor growth and lung metastasis in the combination group (Fig. 5D and E). The CI of oleanolic acid and regorafenib is 1.18 in vivo, indicating that the two drugs have a synergistic effect (determined when the Q value is greater
Oleanolic acid (OA) nitrified proteins involved in aggressiveness of HCC cells. A and B, Results of immunoprecipitation and Western blot assays performed in the HepG2 cancer cell line. Oleanolic acid can promote NT modification of specific proteins. C, Results of IF assay. Oleanolic acid treatment increased the nitrated proteins in HepG2 and PLC cells. D, Nitrified proteins by oleanolic acid were analyzed with the proteins involved in the cell adhesion, cytoskeleton, migration, proliferation, and cell stemness database search from UniProt (http://www.uniprot.org/) by Venn analysis. The nitrogenated proteins involved in the above EMT-related process are presented. E, The relationship of the proteins obtained by Venn analysis was analyzed with the survival of patients with HCC by TCGA data analysis. The results showed that six proteins were negatively related to the survival of patients with HCC. F, Schematic of this study. Oleanolic acid exhibits anti-EMT effect through iNOS interaction, activation, promotion of nitric oxide production, and enhanced nitration (NT) of EMT-related proteins.
than 1.15; ref. 30). The xenografts were then harvested for IHC staining. The results of IHC implied that oleanolic acid or regorafenib treatment markedly upregulated the expression levels of E-cadherin but downregulated the expression level of vimentin. Furthermore, the expression levels of MMP2 and MMP9, proteases that promote the migration and invasion capabilities of cancer cells, were both attenuated by oleanolic acid or regorafenib. Moreover, oleanolic acid strengthened the effect of regorafenib on E-cadherin, vimentin, MMP2, and MMP9. Furthermore, oleanolic acid treatment increased the levels of iNOS and NT, whereas regorafenib exerted no significant effect on them (Fig. 5F and G). Overall, oleanolic acid plays an anti-EMT role in vivo, thereby further supporting the in vitro experimental results. Oleanolic acid sensitizes HCC cells for regorafenib.

Figure 5.
Oleanolic acid (OA) exhibits anti-EMT effect in vivo and enhances the antitumor effect of regorafenib. A, Oleanolic acid at the dose of 65 or 80 μmol/L enhanced the effects of regorafenib to suppress cell growth of PLC cells. B and C, Oleanolic acid enhanced the effects of regorafenib in inhibiting migration and invasion of HCC. D, Tumor volumes of PLC-bearing BALB/c-null mice. Oleanolic acid or regorafenib treatment significantly inhibited the tumor growth. Moreover, oleanolic acid can enhance the antitumor effect of regorafenib. E, Oleanolic acid or regorafenib treatment significantly inhibited the lung metastasis. Moreover, oleanolic acid can enhance the inhibition effect of regorafenib. F, Results of IHC assays. The expression levels of E-cadherin were significantly upregulated, whereas those of vimentin, MMP2, and MMP9 were downregulated by oleanolic acid or regorafenib treatment, and oleanolic acid enhanced the effects of regorafenib. The expression levels of iNOS and NT were upregulated by oleanolic acid but not by regorafenib. G, Staining indexes of IHC assays. Data are represented as mean ± SEM (**; P < 0.01; ***; P < 0.001).
High iNOS expression plays a positive role in the prognosis of patients with HCC

The preceding results demonstrate that oleanolic acid plays an anti-EMT effect in HCC by interaction with and activation of iNOS by oleanolic acid. To further determine the role of iNOS in the progression of patients with HCC, data from the Human Protein Atlas (HPA) and The Cancer Genome Atlas (TCGA) liver cancer database were analyzed. The HPA data show that the iNOS expression levels in liver cancer were higher than those in normal liver tissues (Fig. 6A), suggesting that iNOS can be used as a specific target for HCC treatment. The Kaplan–Meier analysis of iNOS in HCC was performed with the TCGA liver cancer database to further determine the role of iNOS in the prognosis of patients with HCC. Results indicated that high iNOS expression was

**Figure 6.** High expression levels of iNOS improve the prognosis of patients with HCC. **A,** iNOS expression levels were significantly upregulated in patients with HCC. **B,** High expression levels of iNOS positively related to the survival probability of patients with HCC. **C,** New primary tumor occurred when significant downregulation of iNOS occurred. **D** and **E,** The expression levels of iNOS in grades 1–3 and stages I–III patients with HCC significantly increased compared with those in normal people. In grade 4 and stage IV patients with HCC, the iNOS expression levels were apparently downregulated compared with those in grades 1–3 and stages I–III patients with HCC (*, P < 0.05; **, P < 0.01).
associated with a high survival probability of patients with HCC, whereas low iNOS expression predicted a relatively low survival probability (Fig. 6B). The relationship between iNOS expression and liver cancer recurrence was then explored. Although iNOS expression was not dramatically related to locoregional, intrahepatic, and extrahepatic recurrence, new primary tumors occurred as iNOS expression significantly decreased (Fig. 6C). In addition, relationships between iNOS expression and stages and grades of patients with HCC were examined using the TCGA liver cancer database. Consistent with the HPA data shown in Fig. 6A, the iNOS expression levels in the patients with HCC (grades I–IV) were significantly higher than those in the normal people (Fig. 6D and E). However, the iNOS expression levels in patients with grade 4 and stage IV HCC were significantly lower compared with those in patients with grades 1–3 and stages I–III HCC. Therefore, increased iNOS expression may predict improved prognosis of patients with HCC.

**Discussion**

Oleanolic acid is an important member of pentacyclic triterpenoids that widely exist in food, medicinal herbs, and other plants. Oleanolic acid (36) is relatively nontoxic and has been used in cosmetics, health products, and drugs for prevention and treatment of hepatitis. It has been demonstrated that oleanolic acid has antitumor activity. Oleanolic acid inhibited the cellular metabolism by targeting AKR1B10 in colon cancer (11). Moreover, oleanolic acid displays antitumor effect in various cancers by inducing apoptosis and cell-cycle arrest via increase of Bax/Bcl-2 ratio, increased release of cytochrome c, and activation of caspase/PARP (18). However, the direct target of oleanolic acid on HCC is not clear.

This study showed that iNOS was a potential target of oleanolic acid in HCC by target prediction and subsequent experimental validation. iNOS (37) is one of the three NOS isomers that can produce nitric oxide from l-arginine. In many tumors, iNOS expression is upregulated, and nitric oxide production is elevated (38). However, the role of iNOS and nitric oxide during tumor development is complex and perplexing (39). Although a few studies indicated that nitric oxide may promote cancer formation and progression (40, 41), several studies maintained that iNOS and nitric oxide can exhibit an antitumor and EMT inhibitory effect (27, 42–44). The dual actions of nitric oxide may be due to the microenvironment of the tumor, such as cell types (45), dosage (46), and organs involved (45). For instance, low nitric oxide concentrations may promote tumor progression by resisting apoptosis or inducing angiogenesis, whereas high nitric oxide concentrations may inhibit tumor progression via cytotoxic effects, including protein dysfunctions and possibly anti-EMT effect (25). This phenomenon is true in that nitric oxide donor and gene therapy via iNOS are employed for cancer therapy not only in preclinical experiments but also in phase I/II clinical trials and hopefully in phase III clinical trials (47). Consistent with above research, we also found high iNOS expression plays a positive role in the prognosis of patients with HCC and suggesting that iNOS can be used as a specific target for HCC treatment. Aberrant protein nitration by nitric oxide, produced by dimerization of iNOS, plays a key role in cancer recurrence and development (48).

EMT is crucial in HCC progression and metastasis (49). Therefore, the EMT pathway is of great therapeutic interest in HCC treatment to reduce the morbidity and mortality rates of this disease. This study showed that oleanolic acid exhibited anti-EMT effect by targeting iNOS and eventually nitrifying EMT-related proteins. In specific, oleanolic acid can interact with iNOS and promote its dimerization by activating it and promoting nitric oxide production. The excess nitric oxide then nitrified specific proteins, which are involved in a wide range of tumor biological processes, such as adhesion, cytoskeleton, migration, proliferation, and stem cell production and thus tumor progression can be prevented. This is the first study to report that iNOS was a potential target of oleanolic acid in HCC and oleanolic acid inhibits EMT process by nitrification of EMT-related proteins.

We also proved that oleanolic acid strengthened the inhibitory effect of regorafenib, a potentially stronger inhibitor of HCC growth and invasion than sorafenib in clinical application. Regorafenib reportedly inhibits the EMT of colorectal and pancreatic carcinoma (34, 35). However, regorafenib has drawbacks due to drug resistance and adverse effects when used alone. Combination therapy can successfully overcome the drawbacks of regorafenib (50). This study found that the anti-EMT effects of regorafenib can be further strengthened by combination with oleanolic acid. This study provides new clues as to improve the antitumor effects of regorafenib by combining with natural drugs. Overall, this study demonstrates that oleanolic acid exhibits an anti-EMT effect in HCC by increasing iNOS dimerization, nitric oxide production, and NT of EMT-related proteins. Oleanolic acid may be used in combination with regorafenib as a new therapy to treat liver cancer progression.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: T. Sun, Y. Liu, C. Yang
Development of methodology: T. Sun
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Wang, Q. Zhang, Y. Liang, S. Chen, H. Liu, S. Zong
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Wang, W. Zhong, J. Zhao, H. Zhang, S. Chen, H. Liu, S. Zong, H. Zhou
Writing, review, and/or revision of the manuscript: H. Wang, W. Zhong, Y. Tian, H. Zhou, Y. Liu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Zhao, Y. Tian, H. Zhou, Y. Liu
Study supervision: T. Sun, Y. Liu, C. Yang

**Acknowledgments**

This work was funded by the following: National Biomedical Special Project of International Innovation Park (grant no. 13ZCZDSY2600), Tianjin Natural Science and Technology Fund (grant no. 15CYBC26400), National Natural Science Funds of China (grant no. 81572838, to T. Sun), National Natural Science Funds of China (grant no. 81402973, to Y. Liu), National Natural Science Funds of China (grant no. 81703581; to H. Liu), Tianjin Science and Technology Project (grant no. 15PTGCCX00140), National Science and Technology Major Project (grant no. 2017ZX09306007), and Tianjin Science and Technology Project (grant no. 14ZCZDSY00050).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 3, 2018; revised August 1, 2018; accepted October 3, 2018; published first October 8, 2018.
Oleanolic Acid Inhibits EMT of Hepatocellular Cancer

References


# Molecular Cancer Therapeutics

## Oleanolic Acid Inhibits Epithelial–Mesenchymal Transition of Hepatocellular Carcinoma by Promoting iNOS Dimerization

Hongzhi Wang, Weilong Zhong, Jianmin Zhao, et al.


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-18-0448</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://mct.aacrjournals.org/content/suppl/2018/10/06/1535-7163.MCT-18-0448.DC1">http://mct.aacrjournals.org/content/suppl/2018/10/06/1535-7163.MCT-18-0448.DC1</a></td>
</tr>
</tbody>
</table>

| Cited articles | This article cites 50 articles, 11 of which you can access for free at: http://mct.aacrjournals.org/content/18/1/62.full#ref-list-1 |

| E-mail alerts | Sign up to receive free email-alerts related to this article or journal. |
| Reprints and Subscriptions | To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org. |
| Permissions | To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/18/1/62. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site. |