Solanine Attenuated Hepatocarcinoma Migration and Invasion Induced by Acetylcholine

Liang-Tzung Lin[1,2], Chen-Yen Choong[3],
and Chen-Jei Tai[4,5] (iD)

Abstract

Aim: Evidence has provided an explanation of the correlation between the nervous system and the tumor microenvironment. Neurotransmitters may be involved in different aspects of cancer progression. The glycoalkaloid solanine has been reported to suppress neural signaling pathways and exists in numerous plants, including *Solanum nigrum*, which have been demonstrated to inhibit cancer cell proliferation. **Methods:** We evaluated the potentials of solanine on inhibiting acetylcholine-induced cell proliferation and migration in hepatocellular carcinoma cells. **Results:** The results indicated that solanine markedly attenuated cell proliferation and migration via inhibiting epithelial-mesenchymal transition and matrix metalloproteinases in acetylcholine-treated Hep G2 cells. In addition, exosomes derived from acetylcholine-treated Hep G2 cells were isolated, and solanine showed inhibiting effects of extrahepatic metastasis on blocking cell proliferation in exosome-treated A549 lung carcinoma cells through regulating microRNA-21 expression. **Conclusion:** Solanine has strong potential for application in integrative cancer therapy.

Keywords

solanine, acetylcholine, migration, hepatocarcinoma, exosome

Introduction

Several lines of evidence have linked the nervous system (including neurotransmitters) to the tumor microenvironment, thereby regulating proliferation, tumor growth, metastasis, and progression; in addition, migration of carcinoma cells along nerves into nerves is termed "perineural invasion."[1,2] In preclinical studies, nerve ablation resulted in a profound impact on tumorigenesis progression and delayed tumor growth and metastasis.[1,2] Several compounds including nicotine, norepinephrine, neuropeptide Y, glutamate, serotonin, and acetylcholine were reported to upregulate cell proliferation, invasion, and angiogenesis.[1,3,4] The neurotransmitter acetylcholine acts as an autocrine growth factor for human cancer, and acetylcholine signaling has been found to relate to cancer risk.[5,6] The role of acetylcholine is involved in angiogenesis, growth promotion, and tumor-protecting effects in tumor cells.[7,8] These reports found that acetylcholine-signaling inhibitors may be used in cancer therapy.

Solanine is a glycoalkaloid that has been found in various plants such as potato, tomato, and *Solanum nigrum*.[9-11] In our recent studies, we found that the *Solanum nigrum* could inhibit cell proliferation in cancer cell lines, including breast cancer cells,[9] hepatocellular carcinoma cells (HCCs),[10] ovarian carcinoma cells,[11] colorectal carcinoma cells,[12] and endometrial carcinoma cells.[13] However, the mechanism of solanine obtained from *S nigrum* potentially regulating

[1]Department of Microbiology and Immunology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[2]Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan
[3]Division of Hematology and Oncology, Department of Internal Medicine, Taipei Medicine University Hospital, Taipei 11042, Taiwan
[4]Department of Traditional Chinese Medicine, Taipei Medical University Hospital, Taipei, Taiwan
[5]Department of Obstetrics and Gynecology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan

Corresponding Author:
Chen-Jei Tai, Department of Traditional Chinese Medicine, Department of Internal Medicine, Taipei Medical University Hospital, 252 Wu-Xing Street, Xin-Yi District, Taipei 110.
Email: chenjtai@tmu.edu.tw

neuronal pathways for anticancer is unclear, even though solanine has been reported to significantly suppress neural signaling pathway.[14] The aim of this study is to evaluate the potential of solanine inhibiting the proliferation, migration, and epithelial-mesenchymal transition (EMT) in HCCs induced by acetylcholine.

Materials and Methods

Materials

Crystal violet, propidium iodide, acetylcholine, solanine, sodium dodecyl sulfate (SDS), Triton X-100, trypsin, and trypan blue were purchased from Sigma Chemical Co (St. Louis, MO). Fetal bovine serum was purchased from Life Technologies (Auckland, New Zealand). Dimethyl sulfoxide was purchased from Wako Pure Chemical Industries (Saitama, Japan). Vimentin, E-cadherin, and GAPDH antibodies were purchased from Santa Cruz (Santa Cruz, CA).

Cell Culture and Treatment

The human HCC line Hep G2 was grown in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) containing 2 mM L-glutamine and 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum (Gibco BRL) and 2% penicillin-streptomycin (10 000 U/mL penicillin and 10 mg/mL streptomycin). The cells were cultured in a humidified incubator at 37°C under 5% CO_2. The Hep G2 cells were treated by acetylcholine with or without solanine for various concentrations and times. And the cell viability and proliferation were evaluated.

Cell Viability

The effects of solanine and acetylcholine on proliferation of Hep G2 cells were measured using a crystal violet staining assay. Cells were seeded on 24-well plates (3×10^4 cells/well) and treated with various solanine or acetylcholine concentrations for 24 hours. The medium was then removed, washed with phosphate-buffered saline (PBS), stained with 2 g/L crystal violet in phosphate-buffered formalin for 20 minutes, and washed with water. The crystal violet bound to the cells was dissolved in 20 g/L SDS solution and its absorbance was measured at 600 nm.[15]

Western Blot

Cells were rinsed with ice-cold PBS and lysed by radio immunoprecipitation assay lysis buffer with protease and phosphatase inhibitors for 20 minutes on ice. Then the cells were centrifuged at 12 000 $\times$ g for 10 minutes at 4°C. Protein extracts (20 μg) were resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 200 V, 45 minutes). The protein bands were electro-transferred to nitrocellulose membranes (80 V, 120 minutes). Membranes were then treated with a 5% enhanced chemiluminescence blocking agent (GE Healthcare Bio-Sciences, Piscataway, NJ) in saline buffer (T-Tris-buffered saline) containing 0.1% Tween-20, 10 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ at a pH of 7.4 for 1 hour, and then incubated with the primary antibody overnight at 4°C. Subsequently, membranes were washed 3 times in T-Tris-buffered saline and bound antibodies were detected using appropriate horseradish peroxidase-conjugated secondary antibodies, followed by analysis in an enhanced chemiluminescence plus Western blotting detection system (GE Healthcare Bio-Science).

Assay for Cell Migration

Cells were cultured and the assay was carried out when the cell density reached 70% to 80% confluence. First, a wound was made using a sterile tip. The wells were gently washed 3 times with PBS. The cells were subsequently cultured in serum-free Dulbecco's modified Eagle's medium. After treatment with acetylcholine or solanine, the distance between the cells was observed by microscope.

Exosome Isolation

Cells were removed from 4-day cell culture supernatant by centrifugation at 500 $\times$ g for 10 minutes to remove any cell contamination. To remove any possible apoptotic bodies and large cell debris, the supernatants were then spun at 12 000 g for 20 minutes. Finally, exosomes were collected by spinning at 100 000 g for 70 minutes. Exosomes were washed in 20 mL PBS and pelleted again by ultracentrifugation (Beckman 70Ti rotor). Exosome preparations were verified by electron microscopy. Exosome size and particle number were analyzed using the nanoparticle characterization system (NanoSight, Malvern Instruments, Malvern, England). The final exosome pellet was suspended in PBS and protein concentration was measured by BCA (Pierce, Thermo Fisher Scientific, Waltham, MA).

Real-Time Polymerase Chain Reaction

Total RNA was obtained using the Trizol reagent (Gibco BRL Life Technologies Inc, Gaithersburg, MD), according to the manufacturer's instructions. Primers were synthesized by MD-Bio Inc. (Taipei, Taiwan). The gene expression level was determined through relative quantitative real-time polymerase chain reaction (CFX Cycler System, Bio-Rad Laboratories Inc, Hercules, CA). The U6 gene was used as an internal control and each reaction was performed in triplicate. The primer sequences were as follows: microRNA-21 (miR-21), forward 5′-GCGGCGTAGCTTATCAGACTGA-3′, reverse 5′-GTGCAGGGTCCGAGGT-3′; and U6, forward 5′-CTC GCTTCGGCAGCACA-3′, and reverse 5′-AACGCTTC AC GAATTTGCGT-3′.[16]
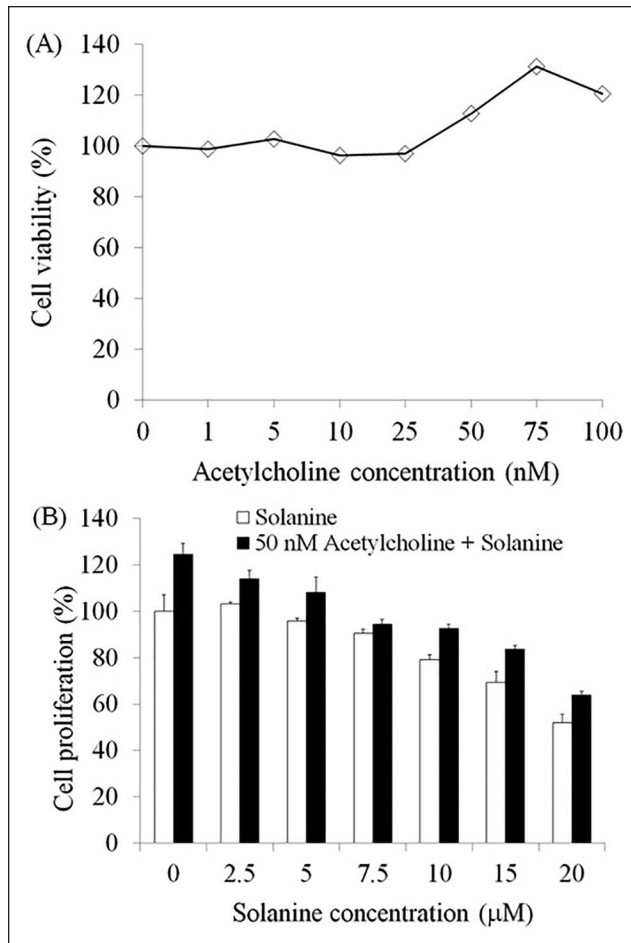
Figure 1. (A) The promotion of proliferation in Hep G2 cells treated by acetylcholine after 48 hours. (B) The effects of solanine limited proliferation in Hep G2 cells induced by acetylcholine. Cell proliferation was evaluated by MTT assay. Data are shown as mean ± standard deviation (n = 3).

Statistical Analysis

Data were expressed as mean ± standard deviation. Statistical significance was determined using one-way analysis of variance with the general linear model procedure of SPSS Version 17.0 (SPSS Institute Inc, Chicago, IL), followed by one-way analysis of variance with Duncan's test.

Results

The Effects of Acetylcholine on Survival and Proliferation in Hep G2 Cells

The tumor microenvironment, including blood vessels and fibroblasts, regulates tumorigenesis, proliferation, and metastasis during cancer development in humans. Recent studies suggested that nerves are an important component in the cancer microenvironment.[17] Previous reports indicate that sensory neurons have a central role in benign inflammatory disease of the pancreas. Sensory neurons play important roles in the pancreatic ductal adenocarcinoma, and the ablation of sensory neurons could prevent inflammation and prolongation of survival in pancreatic ductal adenocarcinoma mice.[1] In addition, the clinical results suggested that cancer patients taking neural inhibitors have lower recurrence rates and mortality.[18]

Activation of the acetylcholine signaling pathway has been found to protect cell survival against apoptosis and promoting proliferation.[19] We found that 50 nM, 75 nM, or 100 nM of acetylcholine could significantly promote cell proliferation in Hep G2 cells after 48 hours of treatment (Figure 1A). Solanine is a glycoalkaloid and has been found to result in neurological impairments.[20] However, whether this character could be applied in HCCs protection is unclear. We investigated the potential of solanine on suppression of cell survival in acetylcholine-induced Hep G2 cells. Results indicated that solanine markedly attenuated the proliferation of Hep G2 caused by acetylcholine induction, and solanine also displayed anticancer activity in Hep G2 cells without acetylcholine treatment (Figure 1B).

The Inhibition of Solanine on Metastasis in Acetylcholine-Induced Hep G2 Cells

Epithelial-mesenchymal transition is characterized by loosening intercellular junctions and increasing cellular mobility.[21] Several factors including EMT, cancer stem cells, and microRNA (miRNA) are involved in tumor metastasis and recurrence.[19] E-cadherin is a molecule for regulating adhesion, which has been considered as the first stage of cancer cell metastasis.[22] Moreover, E-cadherin, N-cadherin, fibronectin, and vimentin biomarkers have been identified as being involved in EMT.[22,23] We found that the promotion of vimentin and inhibition of E-cadherin were being regulated by acetylcholine (50 nM) treatment in a time-dependent manner in Hep G2 cells as shown in Figure 2A. In contrast, the regulation of acetylcholine on vimentin and E-cadherin in Hep G2 cells was abolished by solanine treatment for 36 hours (Figure 2B). These results suggested that solanine could attenuate acetylcholine resulting in EMT in Hep G2 cells.

Matrix metalloproteinases (MMPs) have been known to promote tumor development and stemness. Nerve growth factor is an essential factor in the normal development of the nervous system, which mediates invasion through MMPs.[25] Neurotrophins also promote invasion by enhancing the production of basement membrane-degradative enzymes,[26] and neurotransmitters upregulate the expression of MMPs in nasopharyngeal cancer cells.[27] However, the effects of acetylcholine on MMPs are unclear. We investigated the regulations of solanine on MMP-2 and MMP-9 activity in acetylcholine-induced Hep G2 cells. Results are shown in Figure 3A and B, wherein we found that solanine significantly attenuated the promotion of MMP-2 and MMP-9 induced by acetylcholine. Moreover, the limitation
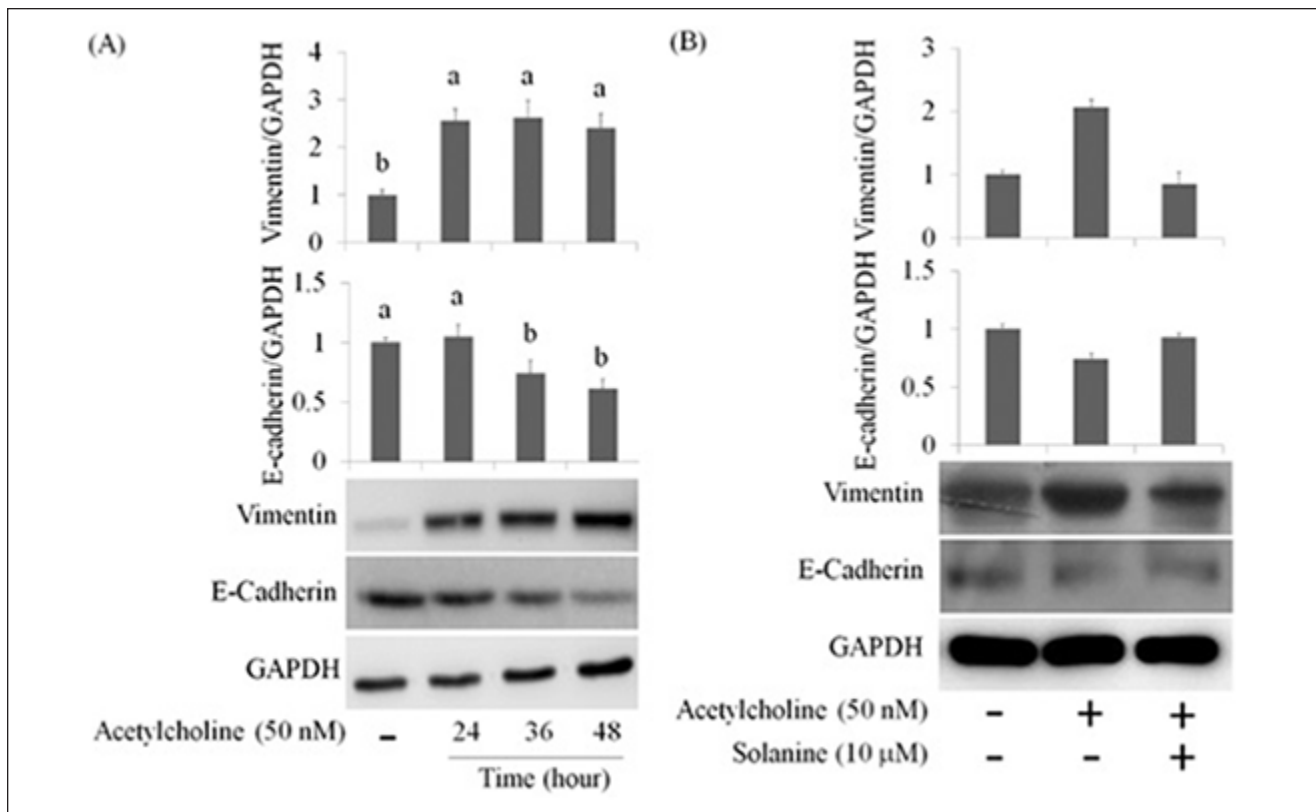
Figure 2. (A) The epithelial-mesenchymal transition (EMT) induction of Hep G2 cells was evaluated after acetylcholine treatment for 24, 36, and 48 hours, respectively. (B) The potential of solanine to attenuate EMT induction in Hep G2 cells by acetylcholine treatment. Data are shown as mean $\pm$ standard deviation (n = 3). Significant difference is shown by different letters ($P < .05$).

of cell migration was observed in acetylcholine-induced Hep G2 cells treated by solanine for 36 hours (Figure 3C). Taking these results together, solanine has potential for suppressing metastasis mediated by inhibitions of EMT, MMP-2/MMP-9, and cell migration.

The Proliferative Effects of Exosomes Obtained From Acetylcholine-Induced Hep G2 Cells on A549 Lung Carcinoma Cells

The proportion of HCC with pulmonary metastases was only 4.56%. But the incidence of lung metastases observed by others was up to 41.6% to 43.6% in the autopsy of HCC.[28] Recently, a study has found that the exosome plays an important role for metastasis.[29] We purified exosomes from Hep G2 cells treated with or without acetylcholine induction or solanine treatment, and the distribution of exosome size is shown in Figure 4A. Results demonstrated that the size distribution of exosome obtained from Hep G2 cells treated by solanine did not show significant differences compared with the blank group.

Furthermore, the promotion of cell proliferation was elevated in A549 lung cells treated with exosomes isolated from

Hep G2 cells after acetylcholine induction compared with that without acetylcholine induction, and these effects were in a dose-dependent and time-dependent manner (Figure 4B and C). These results showed that acetylcholine is a potential factor for accelerating metastasis mediated by exosomes. Therefore, the suppression of solanine on A549 cells treated with acetylcholine-induced Hep G2-derived exosome was evaluated. As shown in Figure 4D, solanine significantly attenuated the proliferation of A549 cells treated by exosome isolated from Hep G2 induced by acetylcholine.

microRNAs are noncoding small RNAs, which have been found to bind to the 3′-untranslated regions of target mRNAs, thereby regulating translation of gene expression.[30] Among various miRNAs, miR-21 is one of the important miRNAs, which has been found to be overexpressed in different types of cancer, and a high level of miR-21 was found in HCC[16]; hence, miR-21 could be used as a potential biomarker for diagnosis and cancer therapy. Inhibition of miR-21 could attenuate proliferation and limit cell cycle thereby suppressing migration and invasion. Our results indicated that the miR-21 level of Hep G2 cells treated by acetylcholine was elevated, but solanine treatment markedly lowered miR-21 level compared with the
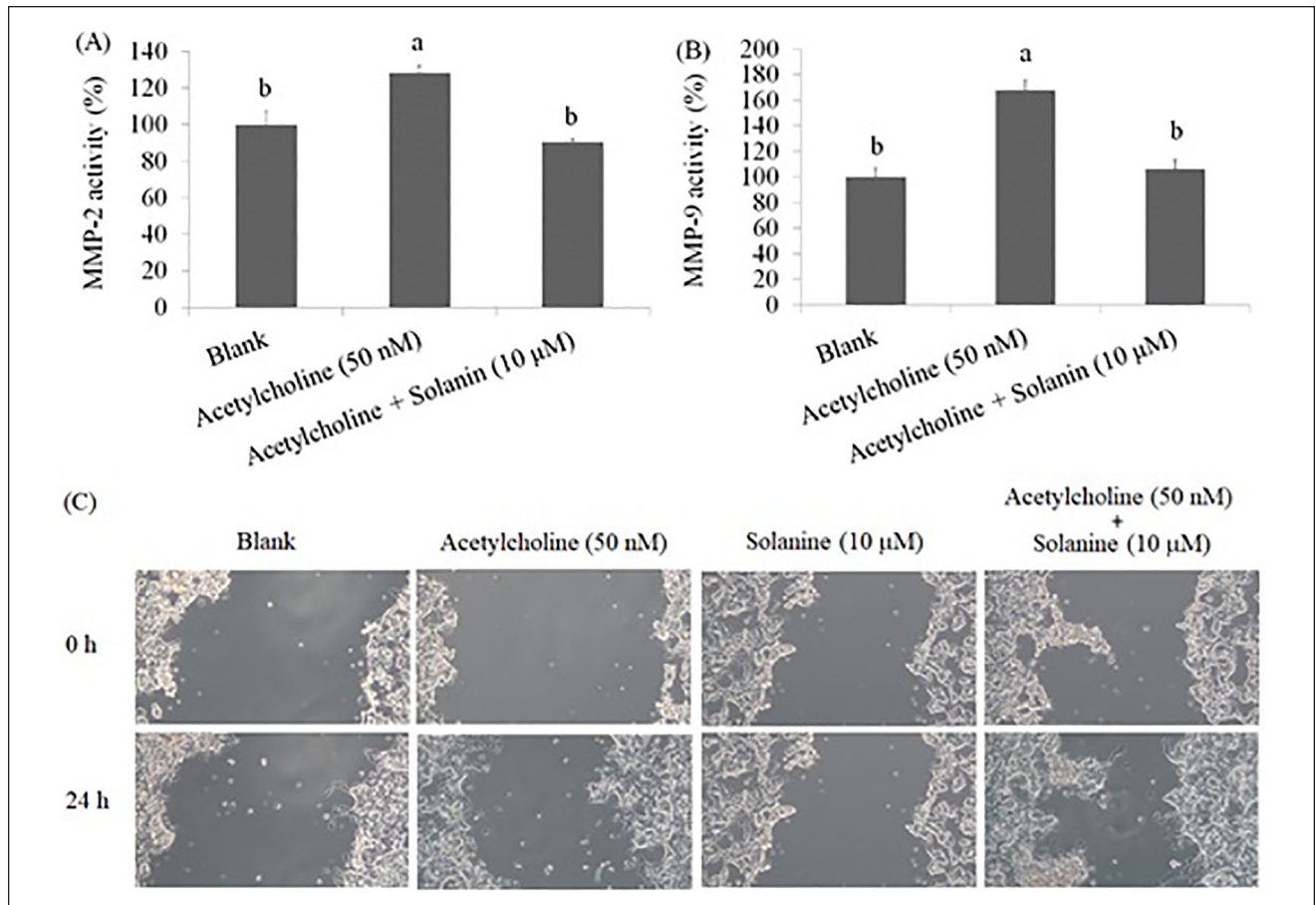
Figure 3. The regulation of solanine on (A) matrix metalloproteinase (MMP)-2 and (B) MMP-9 activity in acetylcholine-treated Hep G2 cells for 36-hour induction by kit assay. Data are shown as mean $\pm$ standard deviation (n = 3). Significant difference is shown by different letters ($P < .05$). (C) Potential of solanine to suppress migration in Hep G2 cells induced by acetylcholine by microscopy after 36-hour treatment.

acetylcholine induction group (Figure 5A). Moreover, the miR-21 level in Hep G2-derived exosomes was also lowered by solanine treatment compared with the acetylcholine induction group (Figure 5B).

Discussion

The Solanaceae plant family includes numerous species (such as peppers, aubergines, tomatoes, and varieties of potatoes) that are important for human nutrition. To our knowledge, the aglycone solanidine is glycosylated by solanidine glycosyltransferase enzymes to produce α-solanine and α-chaconine. Solanine is a naturally occurring steroidal glycoalkaloid metabolite produced by *Solanaceae* species, including potatoes (*S tuberosum*), eggplant (*S melongena*), and tomatoes (*S lycopersicum*).[31]

Recent studies suggest that solanine possesses antimicrobial and antitumor activities; hence, the potential of solanine is used in the treatment and management of various cancers (breast and pancreatic cancer cells).[32,33] The proapoptotic effects of solanine relate to cycle arrest[34] and regulation of Bcl-2 level.[35] Moreover, solanine could potentially inhibit AXIN and BMPR2 expressions involved in cell proliferation and cancer metastasis through downregulating the β-catenin pathway.[36] In another study, the impairment of mitochondria in solanine-treated pancreatic cancer cells was found to result in apoptosis.[37] Acetylcholine could bind to nicotinic and muscarinic receptors on lung cancer cells, thereby accelerating their proliferation, migration, and invasion.[38] We found that solanine blocked acetylcholine signal–induced proliferation and migration in Hep G2 cells in current study.

The miRNAs are small, noncoding RNAs that inhibit gene expression through mRNA destabilization, translational reduction, and mRNA degradation. The association is clearly exemplified for acetylcholine as a potential target of miRNA-132 (hsa-miR-132), which showed potent apoptotic activity.[39] Various studies have demonstrated that several miRNAs are
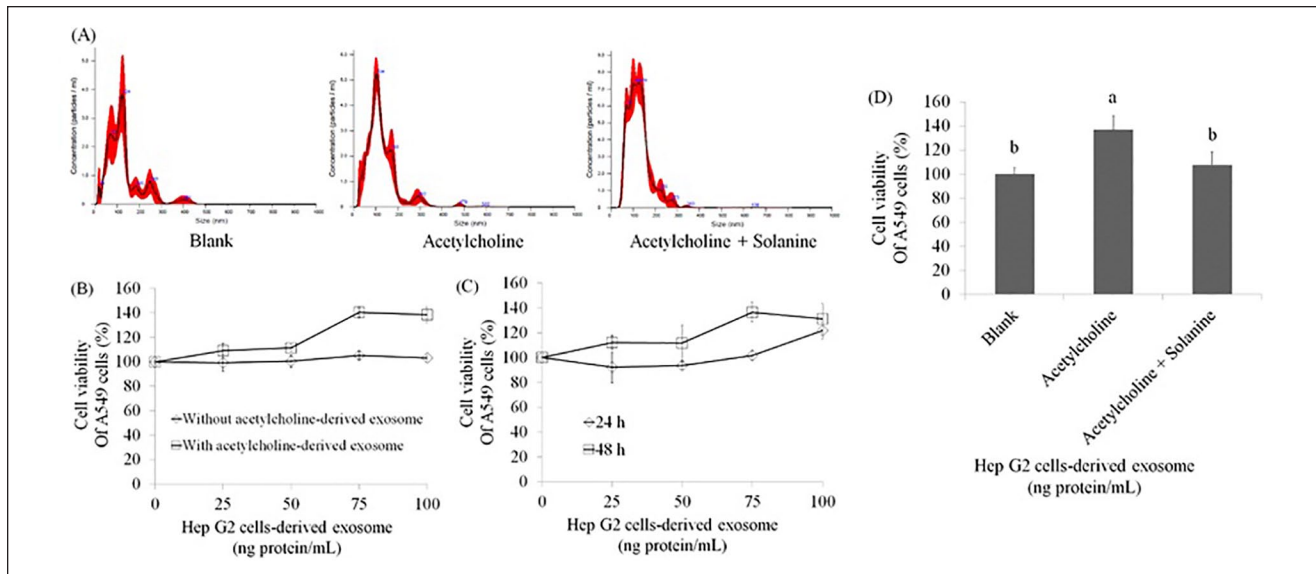
Figure 4. (A) The distribution of exosome size isolated from Hep G2 cells via different treatments and measured by NanoSight assay. (B) The effects of exosomes isolated from Hep G2 cells treated by acetylcholine on proliferation of A549 cells. (C) The proliferation of A549 cells induced by Hep G2-derived exosome in various concentrations after 24-hour or 48-hour treatment. (D) Solanine attenuated the proliferation induction in A549 cells caused by exosomes isolated from acetylcholine-treated Hep G2 cells. Data are shown as mean $\pm$ standard deviation (n = 3). Significant difference is shown by different letters ($P < .05$).
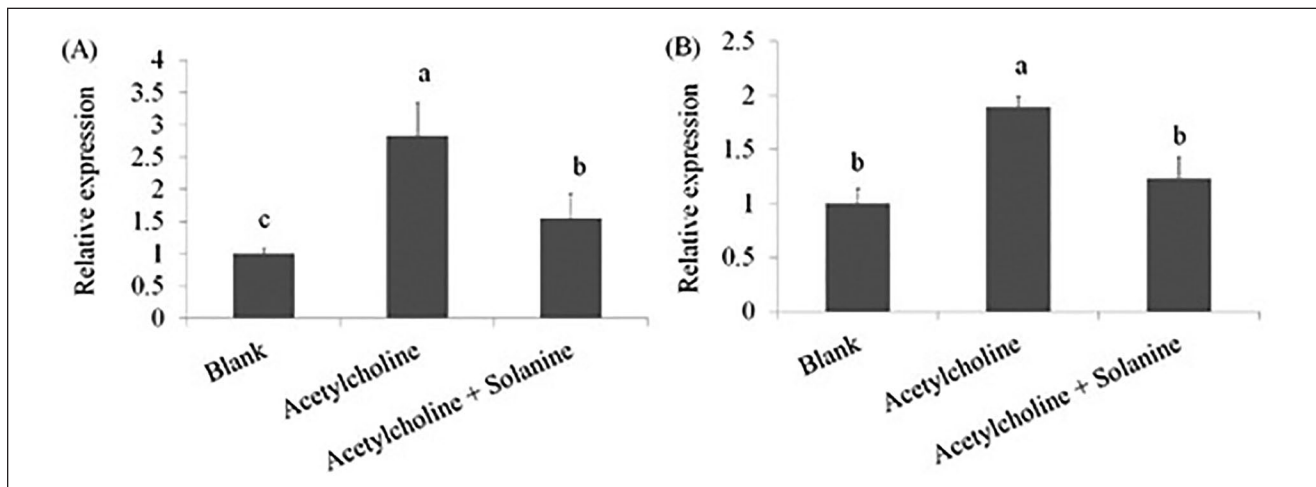

Figure 5. The suppression of solanine on microRNA-21 expression in (A) Hep G2 cells and (B) Hep G2-derived exosome. Data are shown as mean $\pm$ standard deviation (n = 3). Significant difference is shown by different letters ($P < .05$).

involved in breast cancer progression and metastasis, including miR-9,[40] miR-10b,[41] and miR-21.[42] Acetylcholine is an ester of acetic acid, which has been known as one of the most important neurotransmitters. This classical neurotransmitter exerts special effects mediated by binding to 2 different classes of receptor, including (1) nicotinic acetylcholine receptor (stimulated by nicotine) and the (2) muscarinic acetylcholine receptors (stimulated by the mushroom poison muscarine).[43] We found that solanine significantly attenuated an increase in miR-21 level caused by acetylcholine induction in Hep G2 cells and Hep G2-derived exosomes. Taken together, solanine was investigated for its inhibitory potential of metastasis in HCC mediated by regulating EMT, exosomes, and miR-21 in this study (Figure 6). It is considered that solanine may be of interest for future experiments in developing integrative cancer therapy.
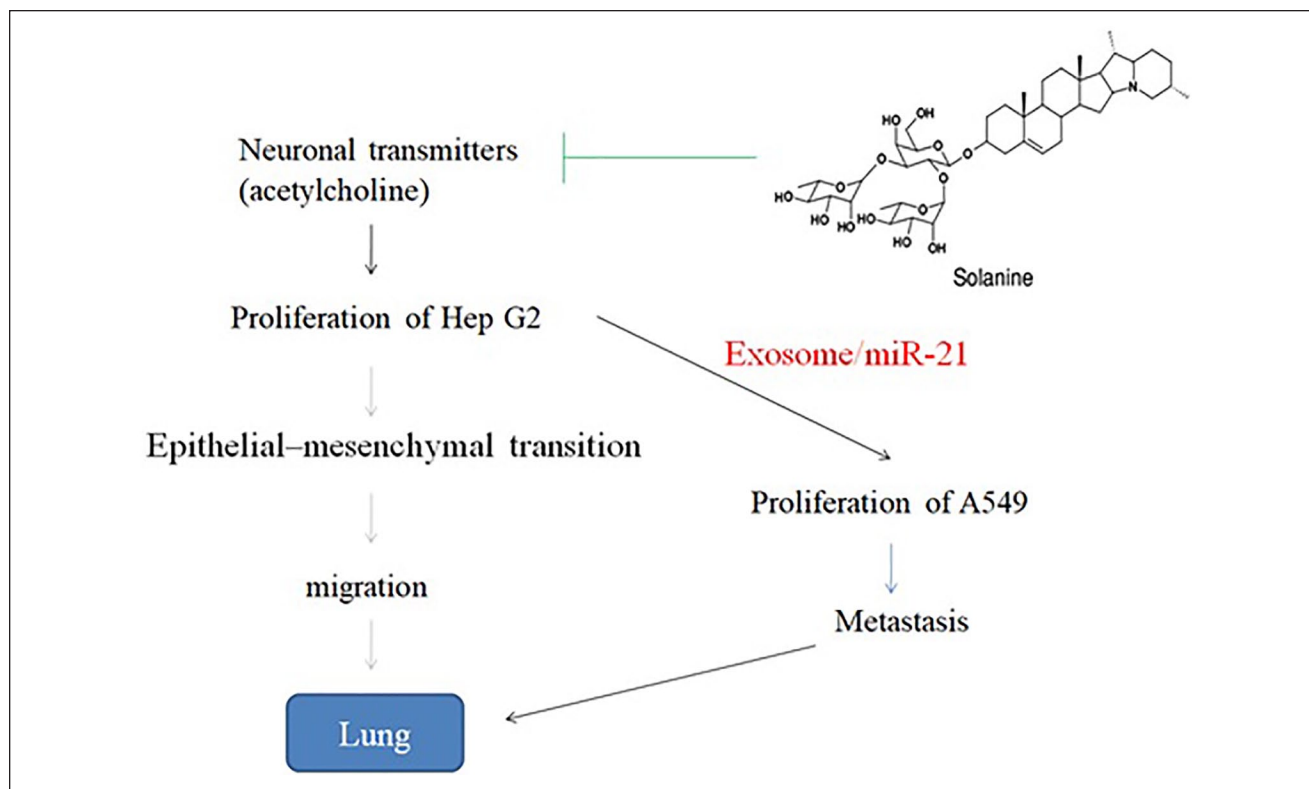
Figure 6. The potential of solanine to suppress migration induced by acetylcholine in Hep G2 cells.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ORCID iD

Chen-Jei Tai [iD] https://orcid.org/0000-0001-7304-6489

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