Garcinol downregulates Notch1 signaling via modulating miR-200c and suppresses oncogenic properties of PANC-1 cancer stem-like cells

Chi-Cheng Huang1,2,3,*, Chien-Min Lin4,*, Yan-Jiun Huang5,6, Wei-Li6,7, Lei-Li-Ting8, Chia-Chun Kuo8, Cheyu Hsu8, Chiou JF8, Alexander TH Wu6,† and Wei-Hwa Lee9,†

1 Department of Surgery, Cathay General Hospital SiJhih, New Taipei City, Taiwan
2 School of Medicine, Fu-Jen Catholic University, New Taipei City, Taiwan
3 School of Medicine, Taipei Medical University, Taipei City, Taiwan
4 Department of Neurosurgery, Taipei Medical University-Shuang Ho Hospital, Taipei, Taiwan
5 Department of Surgery, Taipei Medical University Hospital, Taipei, Taiwan
6 The Ph.D. program for translational medicine, Taipei Medical University and Academia Sinica, Taiwan.
7 Department of Neurosurgery, Taipei Medical University-Wan Fang Hospital, Taipei, Taiwan.
8 Cancer Center and Department of Radiation, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
9 Department of Pathology, Taipei Medical University-Shuang Ho Hospital, Taipei, Taiwan
* These authors contributed equally.
† Corresponding authors:
Alexander TH Wu, The Ph.D. program for translational medicine, Taipei Medical University and Academia Sinica, Taiwan. Phone: 886-2-2736-1661 ext. 7502 E-mail: chaw1211@tmu.edu.tw
Wei-Hwa Lee, Department of Pathology, Taipei Medical University-Shuang Ho Hospital, Taipei, Taiwan. Phone: 886-2-2490088 ext. 8881. FAX: 886-2-2248-0900. E-mail: whlpath97616@s.tmu.edu.tw
Synopsis
Pancreatic cancer represents one of the most aggressive types of malignancy due to its high resistance towards most clinically available treatments. The presence of pancreatic cancer stem-like cells (CSCs) have been attributed to the intrinsically high resistance and highly metastatic potential of this disease. Here, we identified and isolated pancreatic cancer stem-like cells using side-population (SP) method from human pancreatic cancer cell line, PANC-1. We then compared the SP and non-SP PANC-1 cells genetically. PANC-1 SP cells exhibited cancer stem-like cell properties including enhanced self-renewal ability, increased metastatic potential and resistance towards gemcitabine treatment. These cancer stem-like phenotypes were supported by their enhanced expression of ABCG2, Oct4 and CD44. A traditional plant-derived anti-oxidant, garcinol, have been implicated for its anti-cancer properties. Here, we found that garcinol treatment to PANC-1 SP cells significantly suppressed the stem-like properties of PANC-1 SP cells and metastatic potential by down-regulating the expression of Mcl-1, EZH2, ABCG2, Gli-1 and Notch1. More importantly, garcinol treatment led to the up-regulation of several tumor suppressor miRNAs and miR-200c increased by garcinol treatment was found to target and down-regulate Notch1. Thus, PANC-1 SP cells may serve as a model for studying drug-resistant pancreatic cancer stem-like cells and garcinol has the potential as an antagonist against pancreatic CSCs.

Keywords: Drug resistance, Garcinol, Notch1, Pancreatic cancer stem-like cells, Side-population cells,
Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies with worst prognosis. Annually, 37,000 patients are diagnosed with pancreatic cancer, and accounting for 34,000 deaths, which is the fourth leading cause of cancer-related deaths in the United States[1]. Over the past 2 decades, numerous efforts have been made in the treatment strategies including chemotherapy, radiation therapy, gene therapy, and immunotherapy to improve the survival of patients diagnosed with pancreatic cancer; however, the outcome has been very disappointing. Because of the absence of specific early symptoms and the lack of early detection methods, pancreatic cancer is usually diagnosed at an advanced incurable stage[2]. Thus, the median overall survival is approximately 5 to 6 months after conventional therapies, resulting in less than 5 % overall 5-year survival rate[2]. This disappointing survival outcome is, in part, due to late diagnosis as well as intrinsic (de novo) and extrinsic (acquired) therapeutic resistance of pancreatic cancer to conventional therapeutics.

Accumulating data indicates that malignant tumors harvest a small subset of distinct cell population coined cancer stem cells (CSCs), which are responsible for tumor initiation and propagation. Over the past few years CSCs have been identified in a spectrum of cancer types, including pancreatic cancer [3]. A large number of studies have shown that the resistance towards chemo- and radiation therapies and targeted therapies are, in part, due to the presence of CSCs and their enrichment post therapy. CSCs contain the ability of self-renewal and have the potential to regenerate into all types of differentiated cells within tumor mass and contribute to drive continued tumorigenesis. Thus, the failure to eliminate CSCs is considered to be one of the underlying causes of treatment failure[4]. Thus, the identification of agents which can target and eliminate CSCs may provide the much needed arsenal for combating clinically challenges encountered in patients with pancreatic cancer.

Recently, microRNAs (miRNAs) have been shown to participate in the dynamic regulation of differentiation status in embryonic stem cells (ESCs) as they target transcription factors responsible for maintaining ESC pluripotency. In addition, reports indicate that alterations in the expression of several miRNAs are present in human cancers, suggesting potential roles in pancreatic carcinogenesis. Notably, genes involved in both self-renewal and differentiation of CSCs such as Notch (Notch1), Oct4 (POU5F1), Sox2, Bmi-1 (BMI1) and KLF4 were found to be regulated by miRNAs[5-7]. As CSCs are shown to be responsible for
drug resistance and metastasis and miRNAs have been shown to regulate genes and biological processes involved in CSCs maintenance, we sought to identify the candidate miRNAs which are involved in the generation of pancreatic CSCs for potential therapeutics development.

Garcinol, a polyisoprenylated benzophenone derivative, is found in Guttiferae plants, which are shrubs native to India and South East Asia [8]. Traditionally, garcinol has been used as a food ingredient, garnish and cosmetic constituent, as well as a medicine for treating inflammation and other disorders [9]. Garcinol also showed interesting biological activities in cell culture studies, including induction of apoptosis, suppression of COX-2 and inducible nitric oxide synthase (iNOS) expression, and inhibition of proteasome protease activity [10-12]. In addition, garcinol treatment has been reported to suppress tumorigenesis via modulating the signal transduction pathways that control cell growth, apoptosis, inflammation, angiogenesis and metastasis [12]. Based on these findings, garcinol is proposed to be a candidate agent for targeting and suppressing pancreatic cancer stem cells.

In this study, we identified and isolated pancreatic cancer stem cell-like cells using side-population method. Subsequently we demonstrated that these PANC-1 SP cells exhibited cancer stem-like cell properties, particularly enhanced self-renewal, metastatic and drug resistant abilities. More importantly, we provided evidence that garcinol treatment significantly suppressed self-renewing ability, decreased metastatic potential and increased drug sensitivity in PANC-1 SP cells. Mechanistically, garcinol treatment-mediated effects was through the modulation of miR-200c which targets Notch1 expression.

Materials and Methods

**Cell culture.** The human pancreatic cancer cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained in the culture media recommended by ATCC. Media were supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) (Gibco). Cells were maintained in a humidified incubator at 37°C containing 5% CO2.

**Isolation of pancreatic side-population (SP) cells by Fluorescence-activated cell sorting (FACS).** Side-population PANC-1, PANC-10.05 and AsPC-1 cells were identified and isolated using FACSARia™ III sorter (BD Biosciences, Taiwan). Verapamil (100 µM final
concentration) was used to specifically inhibit ABC pumps, 15 min before Hoechst incubation and was used as control to confirm SP identification. SP cells which express ATP-binding cassette ABCG2 and Hoechst 33342 efflux activity was identified and sorted for further analyses.

**Cell viability assay.** Sulforhodamine B (SRB) dye (Sigma-Aldrich Chemie GmbH, Munich, Germany) was used to test cell viability of PANC-1 SP and non-SP cells treated with gemcitabine was dissolved in DMSO before diluting with growth medium to a final DMSO concentration of <0.05%. PANC-1 cells (SP, non-SP and parental) cells were seeded into 96 well plates at 3000 cells/well. The cells were incubated in the presence of gemcitabine (0, 5, 10, 15, 25, 75 and 100 μg/mL) for 48 h. The cells were harvested and fixed with TCA (50%) to each well to a final TCA concentration of 10% with subsequent incubation for 1 h at 4°C. The plates were then washed and dried. Followed by staining with 100 μl of 0.4% (w/v) SRB prepared in 1% (v/v) acetic acid. 1% acetic acid was used to remove unbound dye. The bound dye was solubilized in 20 mmol/L Tris base (100 μl/well) for 5 minutes and the optical densities were measured and recorded using a microplate reader (Molecular Devices, Sunnyvale, California) at 562 nm.

**Verification of stem cell marker in sphere cells.** Immunofluorescence and RT-PCR analyses were used to verify the expression ABCG 2, CD44 and Oct4.

Primer sequences used for RT-PCR are listed:

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCG 2</td>
<td>5’-GGTGGAGGCAAATCTTCGTATTAGA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GAGTGCCCCATCACAACATCATCTT-3’</td>
</tr>
<tr>
<td>CD44</td>
<td>5’-AGAAGGTGTTGGGCGAGAAGAA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-AAATGCACCATTCTCCTGAGA-3’</td>
</tr>
<tr>
<td>Oct4</td>
<td>5’-CCTGAAGCAGAAGAGGTAGCA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-CCGCAGCTTACACATGTCTT-3’</td>
</tr>
</tbody>
</table>

**Isolation of RNA and detection of mRNA expression levels in PANC-1 cells using quantitative real time-PCR.** The total RNA was isolated from the pancreatic cancer stem cells and pancreatic cell lines using TRIlzol (Life Technologies) according to the manufacturer’s instructions. The RNA pellets were then frozen and stored at -80°C until use.

This article is protected by copyright. All rights reserved.
qRT-PCR analysis of microRNAs was performed using TaqMan microRNA assays (Applied Biosystems) using an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems, Foster City, CA). Ten nanograms of total RNA were reverse transcribed by a TaqManH MicroRNA Reverse Transcription (RT) kit from Applied Biosystems. Each RT reaction contained 1x stem loop RT specific primer, 1x reaction buffer, 0.25 μM each of dNTPs, 3.33 U/ml Multiscribe RT enzyme and 0.25 U/ml RNase inhibitor. The reactions were incubated for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C and then held at 4°C. The PCR reaction was performed using a standard TaqManH PCR kit protocol (Applied Biosystems).

**Transfecting cells with pre-miRsTM and anti-miRs.** Up- and down-regulation of miRNAs were performed by transfecting cells with miRNA precursor (Ambion) or anti-miRNA (Ambion), respectively. Fifty nmol of pre-miRTM, anti-miR and Negative control pre anti-miRNA (Ambion), respectively, were transfected into PANC-1 and AsPC-1 cells using lipofectamine 2000 (Invitrogen). 24hs post-transfection, the cells were treated in presence or absence of garcinol and the Total-RNA and Protein were isolated 48 hs post treatment. Experiments were performed in triplicates.

**Western blot analysis.** PANC-1 cells (SP, non-SP, and garcinol-treated samples) after different treatments were harvested for western blot analyses. Briefly, cells were washed with cold PBS and lysed in ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 2 mM EGTA, 10 mM b-glycerophosphate, 150 mM NaCl, 0.5% NP-40, 1 mM phenyl-methyl sulfonyl fluoride, 1 mM NaF, 1 mM DTT, 1% b-mercaptoethanol and 4 mg/ml complete protease inhibitor cocktail (EMD Biosciences). Cell lysates were centrifuged at 15,000 g and protein concentration was determined using the Coomassie Plus protein assay reagent (Pierce, Rockford, IL) and bovine serum albumin as standard. Protein samples (40μg each) were mixed with SDS sample buffer, denatured and separated using 12% SDS-PAGE gels. Gels were subsequently transferred to nitrocellulose membranes and blocked in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% Tween-20, 5% nonfat milk) for 2h. Blots were sequentially incubated with the primary and secondary antibodies, washed in TBS-T. Membranes were developed by enhanced chemiluminescence (ECL-Plus, Amersham Pharmacia Biotech, Piscataway, NJ) and detected using BioSpectrum® Imaging System (Upland, CA).
**Motility assay.** Scratch migration assay was used to study the horizontal movement of cells. A confluent monolayer of cells was established and then a scratch is made through the monolayer, using a standard 1–200 μl pipet tip, which creates a cellular gap (wound), washed twice with PBS, and replaced in media with or without garcinol. The width of the scratch gap is examined microscopically in 4 separate areas each day until the gap is completely filled in the untreated control wells. Three replicates were performed.

**Invasiveness assay.** To quantitate vertical motility, an in vitro transwell invasion assay was used. In this assay, 1x10⁵ cells were plated in the top chamber onto the Matrigel coated membrane (24-well insert; pore size, 8 mm; Corning Costar). Each well was coated with Matrigel (60μg, BD Bioscience). Cells were plated in complete medium and medium supplemented with garcinol was used in the lower chamber. The cells were incubated for 48 hours and cells that did not invade through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were fixed with methanol and stained with crystal violet. The number of cells invading through the membrane was counted under a light microscope (40X, three random fields per well).

**Soft agar colony assay for assessment of tumorigenic potential in vitro.** To examine the anchorage independent growth, PANC-1 cells (SP and non-SP) were suspended (10³ cells/ml) in 2 ml of 0.3% agar with 1% N2 Supplement (Invitrogen), 2% B27 Supplement (Invitrogen), 20 ng/ml human platelet growth factor (Sigma-Aldrich), 100 ng/ml epidermal growth factor (Invitrogen) and 1% antibiotic- antmycotic (Invitrogen) overlaid into six-well plates containing a 0.5% agar base. All samples were plated in triplicate. After 3 weeks, colonies (approximately 0.0.2 mm in diameter) were stained with 0.001% crystal violet blue and counted.

**Immunofluorescence.**

Briefly, PANC-1 cells (with different treatments) were plated in six-well chamber slides (Nunc™, Thermo Fisher Scientific) overnight and fixed in 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 in 0.01 M phosphate-buffered saline (PBS), pH 7.4 containing 0.2% bovine serum albumin, air dried and rehydrated in PBS. Fixed samples were incubated with a rabbit polyclonal antibody against Oct4 and Notch1 (Thermo Scientific, Rockford, IL) and diluted 1:100 in PBS containing 3% normal goat serum for 2 h at room temperature. Negative controls were performed by omitting the primary antibodies.
Anti-mouse IgG fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) were used (1:500 in PBS). The cells were incubated for 1 h at room temperature followed by washes in PBS and mounted in Vectashield mounting medium with 4’, 6-diamidino-2-phenylindole (DAPI) to stain DNA. Images were captured using a Zeiss Axioptot (Carl Zeiss) fluorescence microscope.

**Statistical Analysis.** All experiments were carried out at least three times. Two-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparison test and two-tailed t tests were used to analyze the in vitro and in vivo data. The statistical analysis was performed with the GraphPad Prism software and with the SPSS v. 13.  

**p < 0.05** was defined as statistically significant and is indicated with an asterisk.

**Results**

**PANC-1 side-population (SP) cells exhibited enhanced self-renewal ability and expressed higher stemness markers**

Using flow cytometric technique, we were able to identify a subpopulation (approximately 2%) of PANC-1 cells as side-population (SP) cells or cancer stem-like cells (Fig.1 A). When these cells were under serum-deprived culture conditions, tumor spheres were formed (Fig.1B, i) and increased in size over the period of 21 days (Fig. 1B, ii). More importantly, these PANC-1 tumor spheres showed an elevated stemness makers such as Oct4 (Fig. 1B, iii) and CD44 (Fig. 1B, iv). Using RT-PCR method, we provided additional support that PANC-1 spheres characteristically resemble stem-like cells as reflected by the significantly higher mRNA level in genes such as ABCG2 (drug resistance), Oct4 and CD44 (stemness) as compared to the parental and non-SP cells (Fig.1C).

**PANC-1 SP cells possessed increased tumorigenic properties**

Subsequently, we examined and compared the tumorigenic properties among parental, non-SP and SP PANC-1 cells. In agreement with the increased stemness characteristics, SP PANC-1 showed a significantly increased colony-forming efficiency (CFE), approximately a 9-fold increase as compared to the parental and non-SP counterparts (Fig 2A). In addition, PANC-1 SP cells exhibited an enhanced migratory ability as reflected by wound-scratch assay (Fig. 2B). Using matrigel assay, we also demonstrated that PANC-1 SP cells with an increased invasive potential as compared to parental and non-SP counterparts by nearly 2-fold (Fig. 2C).
**PANC-1 SP cells exhibited chemotherapeutic resistance**

Another important characteristic of cancer stem-like cells contributing to the poor prognosis in pancreatic cancer patients is resistance against chemotherapeutic agents. Here, we performed cell viability assay to compare gemcitabine sensitivity among parental, non-SP and SP PANC-1 cells. We found that parental cells (mixed with non-SP and SP PANC-1 cells) with the intermediate gemcitabine sensitivity with an IC₅₀ value of 25.3 µg/mL and 18.5µg/mL for non-SP cells, respectively. While a significantly higher IC₅₀ value of 125µg/mL was obtained in the PANC-1 SP cells (Fig. 3), supporting our notion that cancer stem-like cells contribute to drug resistance.

**Garcinol treatment suppressed cancer stem cell-like properties in PANC-1 SP cells**

After establishing PANC-1 SP cells resemble pancreatic cancer stem-like cells both phenotypically and genetically, we tested a potential anti-cancer phytochemical, garcinol, in our PANC-1 SP cells. First, we found that garcinol treatment dose-dependently suppressed the self-renewing ability of PANC-1 SP cells (Fig. 4A and B). At 10µM, garcinol was able to suppress the formation of PANC-1 tumor spheres at around 10 spheres as compared to approximately 58 spheres in the control group. The morphology of PANC-1 SP tumor spheres also demonstrated that under garcinol treatment, PANC-1 SP cells did not aggregate well and formed a significantly smaller cellular mass (Fig. 4B). Next, we examine several oncogenic and stemness-related markers which have been implicated in pancreatic tumorigenesis in garcinol-treated PANC-1 SP cells. We found that garcinol treatment was able to dose-dependently suppress Mcl-1 (pro-survival factor)[13], EZH2 (a metastatic marker)[14, 15], ABCG2 (drug resistance), Gli-1 and cleaved Notch-1 representatives of Sonic Hedgehog[16, 17] and Notch [18, 19]stemness signaling pathways (Fig. 4C), suggesting garcinol targets multiple signaling pathways in PANC-1 SP cells. Subsequently, we surveyed several anti-oncogenic miRNA molecules namely, miR-29b[20, 21], miR-101[22], miR-181[23], and miR-200c[24] and found that garcinol appeared to effectively induce these anti-oncogenic miRNAs at low concentration (2.5µM).

**Mir-200c suppressed Notch1 and PANC-1 self-renewal ability**

Having established that garcinol treatment led to the increase of several anti-oncogenic miR molecules including miR-200c which has been shown to be an essential key molecule in controlling tumorigenesis in several cancer types including pancreatic cancer[24, 25]. Thus, we selected miR-200c and evaluated its role in PANC-1 cells. First, we used TargetScan
program to predict that miR-200c specifically binds to the 3’UTR of Notch1. It was predicted by the program that miR-200c had a 100% complementary binding to 736-743 nucleotide positions in the 3’UTR of Notch1 gene (Fig. 5A). To verify this prediction, miR-200c mimic and inhibitor molecules were obtained to support our prediction. The addition of miR-200c mimic in PANC-1 cells led to a significant suppressive effect on tumor sphere formation (Fig. 5B). On the contrary, when miR-200c inhibitor was added, the tumor sphere forming ability significantly increased (Fig. 5B). This observation was further supported by the immunofluorescence analysis. When miR-200c mimic was added, the immunofluorescence intensity of Notch1 and Oct4 was significantly lower as compared to the parental counterparts (Fig. 5C). The opposite observation was obtained where both Notch1 and Oct4 immunofluorescence was significantly higher in miR-200c inhibitor-treated PANC-1 tumor spheres as compared to the mimic-treated group (Fig. 5C).

Discussion
Cancer stem-like cells (CSCs) have been identified in a spectrum of cancer types and they are now considered as the culprit of drug resistance, distance metastasis and disease recurrence [4, 16, 26]. Pancreatic cancer also received much attention due to its extremely poor prognosis and difficulty in controlling the disease. Thus far no targeted therapeutic agents in the clinics appear to satisfactorily manage pancreatic cancer. Previous studies have demonstrated that pancreatic cancer stem cells exhibit increased expression of surface markers such as CD44, CD133 and elevated embryonic signaling pathways such as Notch and Oct4 signaling [27]. The isolation and characterization of pancreatic CSCs has recently become a focal research point in the hope of finding key molecular targets for improving the survival opportunity for the patients.

In this study, we first used side-population (SP) method. This method was developed based on the unique property of stem cells and/or progenitors capable of excluding drugs via enhanced drug transporters such as ABCG2 [1, 28]. PANC-1 cell line was used in this study and we identified approximately 2% of the entire cell population as SP cells with CSC properties. First, PANC-1 SP cells exhibited increased self-renewing ability manifested by the formation of tumor spheres (Fig.1) with increased stemness marker such as Oct4, CD44 and ABCG2. Oct4 has been well established as a key molecule for maintaining undifferentiated status of embryonic stem cells and inducing self-renewing and epithelial-to-mesenchymal transition (EMT) abilities [29-32]. CD44 has been used to established hematopoietic stem cells and CSCs of various types[33, 34], representing another stem cell marker. While the
increased expression of ABCG2 in PANC-1 SP cells reconfirmed the SP method and the drug resistant property of SP or cancer stem cells. Our observations were supported by others using SP method for isolating pancreatic stem cells[12, 35].

Gemcitabine is the standard first line chemotherapeutic agent for patients with advanced pancreatic cancer. However, the overall survival remains poor, and resistance eventually occurs. We found that PANC-1 SP cells exhibited approximately 7-fold higher gemcitabine tolerance as compared to the non-SP counterparts. In addition, PANC-1 SP cells also exhibited enhanced migratory and invasive abilities when comparing to non-SP and parental cells. In agreement, a recently study where a series of gemcitabine resistant pancreatic cancer cell lines which were developed by prolonged gemcitabine exposure, demonstrated up to a 15 fold increase in invasive potential that directly correlates with the level of gemcitabine resistance[36]. These findings suggest a mechanistic relationship between chemoresistance and metastatic potential in pancreatic cancer stem cells; it also implicates that molecular pathways governing the generation and maintenance of SP cells can be exploited for developing therapeutic strategies against refractory and drug-resistant pancreatic cancer.

Phytochemicals have been extensively explored for anti-cancer strategy development. Polyisoprenylated benzophenone, also known as garcinol, isolated from the rind of fruiting bodies of Garcinia indica, has been implicated for its anti-inflammatory and antioxidant properties. Recently, accumulating evidence has demonstrated garcinol also possesses anti-cancer properties. For instance, Garcinol was shown to significantly inhibit proliferation, metastasis, angiogenic processes and induce apoptosis in pancreatic cancer cells[37]. Here we demonstrated that garcinol treatment not only negatively affected tumorigenic properties of PANC-1 cells but also self-renewing ability (one of the key features of cancer stem cells). Mechanistically, garcinol appeared to down-regulate Mcl-1 (pro-survival signal), EZH2 [14, 38] (oncogenic and stemness marker), Gli-1 and Notch-1 (both are key stemness markers). The expression level of several microRNA molecules, namely miR-200c[24], -29b[21, 39], -101[22], and -181[40, 41], which have been suggested to an play important role in a several cancer types, were significantly increased in garcinol-treated PANC-1 SP cells. We found that garcinol treatment was able to increase the aforementioned microRNA expression significantly. Using TargetScan online database, we were able to identify that miR-200c which was induced by garcinol treatment has a high affinity to the 3’UTR of Notch1 gene. In addition, overexpression (mimic) and downregulation (inhibitor) of miR-200c significantly
suppressed and increased the formation of PANC-1 tumor spheres respectively via modulation the level of Notch1 and Oct4 (both essential stemness markers). Our findings implicates that garcinol has a broad range of anti-cancer properties (as mentioned in previous sections); importantly, one of the mechanisms of garcinol-mediated anti-PANC-1 SP appears to be modulating several microRNAs with tumor suppressing ability.

In conclusion, in this study we demonstrated that PANC-1 SP cells with elevated stem and oncogenic expression profiles, resemble stem cells with similar properties including increased colony-forming ability, drug resistance, and enhanced metastatic potential. Importantly, we provided evidence that garcinol, a traditional phytochemical and an anti-oxidant, has the potential as an antagonist for pancreatic cancer stem cells. Garcinol-treated PANC-1 SP cells exhibited a significantly downregulated oncogenic and stem cell properties. Mechanistically, garcinol targets several established tumor suppressor microRNAs, specifically miR-200c which targets and downregulate Notch1 expression. In perspective, garcinol may represent a potential phytochemical for clinical drug development for pancreatic cancer however, further preclinical and clinical experiments are warranted.

Acknowledgements: Mr. Ivy Lin’s assistance in PANC-1 SP isolation was greatly appreciated. Mr. Oliver Yang’s assistance in tumor sphere formation was recognized. We are thankful for D. Yeh CT’s generous advice on the manuscript preparation

Funding source: This study is funded to Chi-Cheng Huang and Alexander TH Wu by Cathay General Hospital-Taipei Medical University joint research fund (101CGH-TMU-06).
Reference


invasive phenotype with collateral hypersensitivity to histone deacetylase inhibitors. *Cancer Biol Ther* 8, 0.


Isolation and characterization of pancreatic cancer-stem like cells using side-population methodology. (A) Isolation of cancer stem like cells in PANC-1 cell line. PANC-1 cells were stained with Hoechst 33342 and then analyzed by flow cytometry in the absence or presence of verapamil. SP cells were gated and shown as a percentage. The experiments were performed in triplicate with similar results. (B) Increased stemness genes in PANC-1 tumor spheres generated from SP method. Representative phase contrast photomicrographs of cancer sphere form PANC-1 SP cells cultures in serum free medium supplemented EGF and bFGF. (i) Tumor spheres of PANC-1 SP cells were obtained under serum-deprived conditions demonstrating self-renewal ability. (ii) PANC-1 spheres increased in size over time, imaged on day 21 of culture. (Original magnification, 200x). Representative immunofluorescent images demonstrate that PANC-1 spheres express stem cell markers Oct4 (iii) and CD44 (iv). DAPI nuclear counterstaining (blue), scale bar 50 μm. (C) Expression of cancer stem cell markers of SP and non-SP cells. The elevated expressions of ABCG2, Oct4, and CD44 genes in derived PANC-1 spheres were detected by RT-PCR. Data are presented as the mean of three separate experiments, each performed in triplicate. Bar, SD *P<0.01, compared with parental and non-SP, respectively.
Fig. 2.

SP-isolated PANC-1 cells demonstrate increased malignant phenotypes. (A) Colony formation of PANC-1 side population cells. The clone formation efficiency (CFE) of SP cells was significantly higher than parental and non-SP cells with an equal cell number. The experiments were repeated twice in triplicate. (B) Wound healing (migration) assay demonstrated that SP PANC-1 cells were more mobile as compared to the parental and non-SP counterparts. (C) Matrix gel invasion assay data indicated that SP PANC-1 cells were more invasive than their counterparts by approximately 2-fold. **P<0.01, all experiments were performed in triplicates.
Fig. 3.

Gemcitabine sensitivity in SP and non-SP cells isolated from the PANC-1 cell line. The cells were treated with different doses of gemcitabine for 48 hr. Data are presented as the mean of three separate experiments. **P<0.01
Suppressive effects of garcinol on the tumor sphere formation and stemness expression. (A) Garcinol decreased sphere cells survivals of PANC-1 tumor sphere cells as assessed by SRB assay. Experiments were performed in independent triplicates. *P<0.05; **P<0.01 (as compared to control). (B) Garcinol inhibited the formation of second generation of tumor spheres from PANC-1 cells. (C) Garcinol decreased the expression Mcl-1, EZH2, ABCG2, Gli-1, and cleaved Notch-1. (D) Garcinol increased the miRNA expression of miR-29b, miR-101, miR-181, and miR-200c. **P<0.01 as compared to control. N=3.
MicroRNA 200c targets Notch 1 and Panc-1 self-renewal ability. (A) TargetScan database prediction indicates that Has-miR-200c binds to the 3' UTR of Notch1 transcript. The number indicates the nucleotide position. 

(B) The addition of hsa-miR-200c mimic molecules led to a significant decrease in self-renewal ability in Panc-1 cells while the inhibitor of miR-200c enhanced it. *P<0.05; **P<0.01 (as compared to control). Experiments were performed in triplicates. (C) Immunofluorescence images of Panc-1 spheres. When treated with miR-200c mimic (middle row), the expression of Notch1 was significantly suppressed (green fluorescence) along with Oct4 (red fluorescence); the reverse was observed in Panc-1 spheres treated with miR-200c inhibitor.
本文献由“学霸图书馆-文献云下载”收集自网络，仅供学习交流使用。

学霸图书馆（www.xuebalib.com）是一个“整合众多图书馆数据库资源，提供一站式文献检索和下载服务”的24小时在线不限IP图书馆。

图书馆致力于便利、促进学习与科研，提供最强文献下载服务。

图书馆导航：

图书馆首页    文献云下载    图书馆入口    外文数据库大全    疑难文献辅助工具