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Bitter melon juice exerts its efficacy against pancreatic cancer via targeting both bulk and cancer stem cells

Deepanshi Dhar¹, Gagan Deep^{1,+}, Sushil Kumar¹, Michael F. Wempe^{1,2}, Komal Raina^{1,2}, Chapla Agarwal^{1,2}, and Rajesh Agarwal^{1,2,*}

¹Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, Anschutz Medical Campus, Aurora, CO

²University of Colorado Cancer Center, University of Colorado Denver, Anschutz Medical Campus, Aurora, CO

Abstract

Pancreatic cancer (PanC) is one of the deadliest malignancies worldwide and frontline treatment with gemcitabine becomes eventually ineffective due to increasing PanC resistance, suggesting additional approaches are needed to manage PanC. Recently, we have shown the efficacy of bitter melon juice (BMJ) against PanC cells, including those resistant to gemcitabine. Since cancer stem cells (CSCs) are actively involved in PanC initiation, progression, relapse and drug-resistance, here we assessed BMJ ability in targeting pancreatic cancer-associated cancer stem cells (PanC-CSCs). We found BMJ efficacy against CD44⁺/CD24⁺/EpCAM^{high} enriched PanC-CSCs in spheroid assays; BMJ also increased the sensitivity of gemcitabine-resistant PanC-CSCs. Exogenous addition of BMJ to PanC-CSC generated spheroids (not pre-exposed to BMJ) also significantly reduced spheroid number and size. Mechanistically, BMJ effects were associated with a decrease in the expression of genes and proteins involved in PanC-CSC renewal and proliferation. Specifically, immunofluorescence staining showed that BMJ decreases protein expression/nuclear localization of CSC-associated transcription factors SOX2, OCT4 and NANOG, and CSC marker CD44. Immunohistochemical analysis of MiaPaCa2 xenografts from BMJ treated animals also showed a significant decrease in the levels of CSC-associated transcription factors. Together, these results show BMJ potential in targeting PanC-CSC pool and associated regulatory pathways, suggesting the need for further investigation of its efficacy against PanC growth and progression including gemcitabine-resistant PanC.

Keywords

pancreatic cancer; cancer stem cells; bitter melon juice; natural/dietary agents; chemo-resistance

INTRODUCTION

Over the past few decades, pancreatic cancer (PanC) has steadily emerged as a serious threat to mankind, being the fourth leading cause of cancer-related deaths within the United States,

*Correspondence: Rajesh Agarwal; rajesh.agarwal@ucdenver.edu.

⁺Present address: Wake Forest Baptist Comprehensive Cancer Center, Winston-Salem, NC

accounting for 3% of all cancers [1]. It is a highly aggressive disease linked to a poor prognosis and resulting in a dismal 5-year survival rate of <5%, owing to its rapid and symptomless progression to advanced stages. Key survival statistics for the year 2018 reveal ~55,440 new incidences and ~44,330 PanC associated fatalities within the United States [2]. Majority of PanC linked symptoms appear in under 3 years prior to metastasis including heartburn, bloating, abdominal pain, altered bowel schemes, etc., accompanied by extreme fatigue and weight loss [3]. A variety of risk factors are known to be associated with PanC development namely smoking, age, onset of diabetes, chronic pancreatitis, excessive alcohol consumption and high fat diet [4]. Surgical resection is the only available current curative treatment option for PanC patients; however, not entirely reliable with the imminent risk of tumor relapse. Apart from surgery, as majority of the clinical cases present themselves as metastasized or unresectable forms of PanC, the frontline chemotherapeutic agent currently used in clinic is gemcitabine, a nucleoside analogue. Gemcitabine is extensively applied in PanC therapy alone or in combination with other approved cytotoxic agents (5-fluorouracil, cisplatin, oxaliplatin, *etc.*) and biological agents (erlotinib, bevacizumab, *etc.*) [5]. However, there has been a constant increase in resistance to gemcitabine therapy in PanC patients and the survival outcome is recurrently poor [6]. PanC usually arises as noninvasive precursor lesion namely pancreatic intraepithelial neoplasia (PanIN) and gradually evolves to the advanced metastatic form of pancreatic ductal adenocarcinoma (PDAC). PDACs are the most commonly occurring form of PanC (90%), with mutated *KRAS* expressed in 95% of cases. Other frequently mutated genes present in 50% of PanC types are *p16/CDKN2A*, *TP53* and *SMAD4* [7].

Other than genetic alterations, in recent years, cancer stem cells (CSCs) have been identified to play a major role in cancer progression and recurrence in pancreatic as well as other cancers [8,9]. PanC-CSCs were first identified in 2007 by Li *et al.*, in established human PanC xenografts of NOD/SCID mice [8]. CSCs are the cells comprising a very small part (~1%) of the entire tumor mass, with the ability to self-renew and give rise to phenotypically and functionally heterogeneous cancer cell lineages found within the tumor itself [10]. While they share the core regulatory pathways with normal stem cells, CSCs are known to undergo reprogramming and transformation, resulting in their multi-lineage differentiation and self-renewal potential [11]. Owing to their stem cell like properties, although a small population, CSCs are known to mediate initiation, progression, metastasis, relapse and drug resistance in variety of cancers including PanC [12]. It has been frequently reported that CSCs are responsible in inducing chemo-resistance and lead to accelerated progression of PanC to its more aggressive and invasive forms [13]. The idea therefore remains to target the PanC-CSC pool using novel agents with elevated anticancer and therapeutic potential and negligible toxicity to the surrounding normal tissue.

Taken together, it is clear that several efforts are being made to control, manage, and treat PanC; however, conventional chemotherapeutic drugs have drug-resistance and often fail to effectively target and eliminate CSCs, thereby resulting in tumor relapse and metastasis. This feature is further emphasized in PanC particularly; mainly credited to its high intrinsic resistance resulting from relatively higher percentage and enrichment of CSCs following chemotherapeutic regimes [14]. Clearly, better approaches as well as agents are needed to target the mechanisms and/or pathways driving both PanC and PanC-CSCs, with minimal

associated toxicity to healthy cells. In this regard, in recent years, our focus is predominantly on the efficacy of Bitter melon juice (BMJ) in PanC therapy and preventive intervention. BMJ is isolated from Bitter melon (*Momordica charantia*, Family: Cucurbitaceae), a vegetable widely consumed in Asia, Africa and parts of South America. In recent years, bitter melon has shown efficacy against a variety of diseases and medical conditions including inflammation, hypoglycemia and various cancers, such as head and neck, ovarian, lung, leukemia, bladder, hepatocellular, breast, and prostate [15,16]. Regarding PanC, our published studies have shown promising anticancer activity of BMJ against PanC cells in both culture and nude mice xenografts, *via* Adenosine monophosphate-activated protein kinase (AMPK) modulation, together with its efficacy in gemcitabine-resistant PanC cells [17,18]. Based on these significant findings, here we assessed BMJ efficacy in targeting PanC-CSCs and associated mechanisms.

MATERIALS AND METHODS

Cell lines and reagents

Human pancreatic adenocarcinoma cells MiaPaCa2, PANC1 and AsPC1, were purchased from ATCC (Manassas, VA, USA). MiaPaCa2 and PANC1 cells were grown under standard culture conditions (37°C, 95% humidified air and 5% CO₂) with 10% FBS, 1% Penicillin-Streptomycin, and additional 2.5% horse serum for MiaPaCa2, in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, from ATCC. AsPC1 cells were grown in RPMI 1640 (1×) with 10% FBS, 1% Penicillin-Streptomycin and essential amino acids. DMEM/F12 (1:1) 1× media supplemented with B27 (50×) and N2 (100×) from life technologies with 1% Penicillin-Streptomycin was used for spheroid generation assays. EGF and FGF were from Invitrogen (Grand Island, NY, USA). HPLC grade gemcitabine hydrochloride was purchased from Sigma. Antibody for PDX1 was from Abcam (Cambridge, MA, USA). SOX2 and NANOG antibodies were from Cell signaling (Beverly, CA), and OCT4 and CD44 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

BMJ preparation

Chinese variety of commercially available bitter melon was used for our study. The fruits were washed with water and air dried. Once the water was completely drained, the melons were cut open, deseeded and the remaining fruit was juiced using a household juicer. The juice was then subjected to centrifugation at 3000g for 30 mins. The supernatant was collected, sterile filtered and aliquoted for storage at -80°C, while the pellet was discarded. The liquid supernatant was employed for all *in vitro* studies; for the *in vivo* studies, we employed the lyophilized BMJ powder stored at 4°C as detailed earlier [17,18].

FACS sorting

PANC1 cells were trypsin digested and stained with CD44-FITC, CD24-APC, and EpCAM-PE antibodies from BD Pharmingen and then subjected to cell sorting by FACS using Flow Cytometry Shared Resources of the University of Colorado Cancer Center. Isolated CD44⁺/CD24⁺/EpCAM⁺ triple positive and CD44⁻/CD24⁻/EpCAM⁻ triple negative populations were subjected to limiting dilution assays for sphere formation. Sorted triple positive cells

and unsorted cells were seeded at varying densities (200 cells/well to 10,000 cells/well) in 6-well ultra-low attachment plates in DMEM/F12 (1:1) 1× media supplemented with B27 (50×) and N2 (100×) and 1% Penicillin-Streptomycin, and observed for their sphere forming ability over a course of 11 days. Consequently, the effect of BMJ (0.5%–2%, v/v) was examined on sorted triple positive cells versus unsorted PANC1 cells.

Gemcitabine exposure

PANC1 and AsPC1 cells were trypsin digested and seeded at 2500 cells/well density, in 6-well ultra-low attachment plates in DMEM/F12 (1:1) 1× media supplemented with B27 (50×) and N2 (100×) and 1% Penicillin-Streptomycin. At 6 hours post seeding, cells were treated with 2.5 and 5.0 μM gemcitabine. At 24 hours after gemcitabine treatments, cultures were treated with BMJ (0.5%–2%, v/v) without washing out any residual gemcitabine and sphere formation was followed for 11 days. EGF (20ng/ml) and FGF (10ng/ml) were added at every 72 hours.

Cell viability and Spheroid assay

All three PanC cell lines MiaPaCa2, PANC1 and AsPC1 were seeded in 6-well plates (Corning, Inc.) for 24 hours and treated with varying concentrations of BMJ (2–4%, v/v) for 24, 48 and 72 hours. For the control group, media lacking BMJ was added to the wells. The cell viability was determined by trypan blue exclusion assay. The cells stained blue were recorded/counted on the hemocytometer as dead cells. All experiments were done in triplicates.

In the next set of the experiment, MiaPaCa2, PANC1 and AsPC1 cells were cultured in a 2D format/culture conditions (referred to as monolayer here after), and subjected to BMJ treatment (0.5%–2%, v/v) for a course of 9 days (single versus multiple BMJ treatments at every 72 hours). Thereafter, the remaining viable cells on day 9 were trypsin digested and resuspended in stem cell media (DMEM/F12 (1:1) 1× media supplemented with B27 (50×) and N2 (100×) and 1% Penicillin-Streptomycin) and seeded at a density of 2500 cells/well in 6-well ultra-low attachment plates (Costar). During seeding, caution was exerted to specifically seed individual cells and not cell clusters adhering to each other, to minimize the false positives. Additionally, the seeded cells were followed as a function of time to carefully monitor for clumping, if any. The single cell derived spheroid forming ability of these CSCs was monitored over a course of 11 days. Media was replenished and growth factors EGF (20ng/ml) and FGF (10ng/ml) were added at every 72 hours.

In another set of the experiment, BMJ unexposed cells were seeded at a density of 2500 cells/well in 6-well ultra-low attachment plates with DMEM/F12 (1:1) 1× media supplemented with B27 (50×) and N2 (100×) and 1% Penicillin-Streptomycin. At 6 hours post seeding, cells received either a single exposure of BMJ (0.5%–2%, v/v) for 72 hours or repeated exposures of BMJ (0.5%–2%, v/v) at every 72 hours for 9 days. The spheroid generation was monitored for 11 days. Media was replenished, and EGF (20ng/ml) and FGF (10ng/ml) were added at every 72 hours. In a parallel study, cells were allowed to generate spheroids for 4–5 days, which were then treated with exogenous addition of BMJ (2% and 4%, v/v; data not shown for 4%) for 11 days to study the effect of BMJ on mature spheroids.

RT²qPCR array for human stem cell transcription factors

MiaPaCa2 cells were treated with BMJ (2%, v/v) for 72 hours. The cells were collected, washed and RNA isolated (RNeasy Mini Kit, Qiagen). The cDNA conversion was carried out using RT² First Strand Kit (Qiagen). Thermal amplification parameters were: initial denaturation for 5 min at 94°C; 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 54°C and extension for 30 s at 72°C; and final extension for 5 min at 72°C. Quality control was assessed on the nanodrop and ~250ng of the starting material was loaded onto the PCR array plate (Human stem cell transcription factors array PCR, Qiagen). A two-step cycling protocol on ABI 7500 cycler was used involving denaturation for 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The relative quantification of gene expression between the untreated control and BMJ treated cells was conducted by normalization against endogenous *GAPDH* and *β-Actin* using the C_T method of quantification. For data analysis, ABI 7500 and Qiagen software was used to calculate the fold changes.

Immunofluorescence

All three PanC cell lines MiaPaCa2, PANC1 and AsPC1 were grown on coverslips in 6-well culture plates and treated with BMJ (2%, v/v) for 72 hours. Post treatment, cells were harvested and formalin (4% formalin in 1× PBS) fixed. For staining, cells were washed with 0.1% triton X-100 in 1× PBS thrice, 5 min for each wash followed by permeabilization with 0.3% Triton X-100 in 1× PBS for 1–2 hours. Next, cells were blocked with CAS block buffer (Invitrogen, 1:1 in PBS) for an hour at room temperature. Post blocking, primary antibody was added in dilution buffer (1% BSA in PBST) overnight at 4°C in humidified chamber. Following day, cells were washed again with 0.1% triton X-100 in 1× PBS thrice, 5 min for each wash and fluorochrome conjugated secondary antibody was added and incubated in dark with DAPI at RT for 1 hour. Hereon, all steps were carried out in dark. Finally, cells were washed with high salt PBS and subsequent washes with 0.1% triton X-100 in 1× PBS. Lastly, the coverslips were mounted with Prolong® Gold Antifade Reagent/DAPI and allowed to dry. Nikon D-Eclipse C1 confocal microscope (Nikon) was used for imaging and analysis by EZ-C1 Free viewer software. Immunofluorescence for spheres used matrigel as the embedding medium for immobilizing the spheres. Fixing, permeabilization, blocking and antibody incubation was carried out as described in previous publications from our group [19].

Immunohistochemistry

MiaPaCa2 tumor xenograft samples from control (water only) and lyophilized BMJ (200mg/kg) exposed groups for 6 weeks from a recently completed study showing strong efficacy of BMJ in inhibiting tumor xenograft growth [17], were processed per our previously published protocol [20]. Tissue samples were stained for transcription factors: SOX2, OCT4, NANOG and PDX1. Brown stained cells were counted positive for each molecule. There were seven animals per group and eight random regions per sample were considered. The final representation of the data is as percent positive cells.

Statistical analysis

All statistical analyses were performed using Sigma Stat software (version 3.5, Jandel Scientific). Quantitative data are presented as mean±SEM. Statistical significance of difference between control and treatment groups was determined through one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. $P < 0.05$ was considered significant. P value of $p = 0.05$ is denoted by *, $p = 0.01$ is denoted by ** and $p = 0.001$ is denoted by ***.

RESULTS

BMJ decreases the number and size of PanC spheroids generated by CD44⁺/CD24⁺/EpCAM^{High} enriched CSCs and unsorted PanC cell populations

CD44⁺/CD24⁺/EpCAM^{High} markers have been validated in a variety of studies for CSC sorting from solid tumors, as the subpopulation of cells expressing the combination of these markers have an increased proliferation potential with poor glandular differentiation correlating to cancer aggressiveness [8]. Importantly, Li *et al.* identified CD44⁺/CD24⁺/EpCAM^{High} side population of cells that accounted for only 0.2 to 0.8% of the total population of PanC cells [8]. However, this population had an enhanced capacity of tumor initiation compared to the non-tumorigenic/bulk tumor population. Here we subjected human PANC1 cell line to FACS assay and CD44⁺/CD24⁺/EpCAM^{High} triple marker was employed for isolating the triple positive population of CSCs (Fig. 1A). The CD44⁺/CD24⁺/EpCAM^{High} enriched (FACS sorted) CSCs were then seeded for limiting dilution assays for spheroid formation, ranging from 200 to 10,000 cells/well (Fig. 1B). Based on these results, appropriate seeding density of 2500 cells/well in a 6-well ultra-low attachment plate was selected for all spheroid assays. The other subpopulation of isolated CD44⁻/CD24⁻/EpCAM⁻ triple negative cells failed to generate any spheroids (data not shown). Addition of BMJ to the CD44⁺/CD24⁺/EpCAM^{High} enriched CSC population showed a pronounced effect; a significant decrease in the generated spheroid number was observed with increasing doses of BMJ (0.5%–2%, v/v), whereby a minimal number of spheroids were formed with 2% BMJ, compared to the untreated control (Fig. 1C). Notably, BMJ treatment dose-dependently decreased the number of spheroids formed by both, unsorted and sorted PANC1 cells (Fig. 1C). BMJ addition targeted and decreased the PanC spheroid number, primarily reflecting its effect on CSC population which in turn is responsible for spheroid generation. A decrease in PanC spheroid size accompanied the decrease in spheroid number, thereby highlighting the potential of BMJ in targeting PanC-CSCs as well as the associated bulk tumor cells, which collectively account for the resulting spheroid mass. Together, these results underline the role of BMJ against CSC-mediated PanC stemness and proliferation. *Note: Given that percentage decrease in spheroids generated by either CSC enriched PanC cells or unsorted PanC population by BMJ was comparable, therefore, in all successive spheroid assays, we employed the unsorted PanC cells to generate spheroids.*

BMJ exposure confers sensitivity to gemcitabine resistant PanC-CSCs

Variety of cell subpopulations from solid tumors resistant to radio- and chemo-therapies and expressing CSC markers have been identified [21]. The most frequently used palliative chemotherapeutic against late stage PanC is gemcitabine. Gemcitabine is effective in PanC

tumor volume reduction and delays the time to proliferation [22]. However, there have been increasing incidences of PanC resistance to gemcitabine treatment [23]. Chemoresistance in PanC might result from extreme desmoplasia, hypovascularization, aberrant activity of membrane transporters/drug efflux pumps and drug metabolizing enzymes and mostly, the CSCs[24]. CSCs have been shown to possess the properties of pro-survival and possible rapid efflux of drugs, thereby conferring chemoresistance [25]. Thus, on the lines of chemoresistance in PanC, we employed BMJ to test its ability in increasing PanC sensitivity to gemcitabine. PANC1 and AsPC1 cell lines resistant to 2.5 μ M (Fig. 2A) and 5 μ M (Fig. 2B) gemcitabine doses were subjected to spheroid assays and exposed to single BMJ treatment (0.5 and 2%). These cells were observed for 11 days in spheroid assays where BMJ was successful in rescuing cells from gemcitabine resistance at both 0.5 and 2% doses; cells from BMJ treated wells were able to form less number of spheroids, where both BMJ doses showed inhibition in sphere formation. Additionally, both 2.5 μ M and 5 μ M gemcitabine resistant cells followed a similar trend in their response to treatment by showing a significant decrease in spheroid formation (number and size) with BMJ exposures. These results indicate the ability of BMJ to reverse CSC-associated gemcitabine resistance in PanC and increasing the sensitivity to the drug, thereby enhancing the response to chemotherapeutics.

BMJ affects viability and stemness of human PanC cells

PanC cell lines MiaPaCa2, PANC1 and AsPC1, which are aggressive cancer cell lines [26], were treated with BMJ (2–4%, v/v) and assessed for cell growth and death as well as stemness. The total cell number increased consistently with increasing time points in the controls, but there was a significant decrease in the 2 and 4% BMJ treatment groups (Fig. 3A). Specifically, the total cell number was decreased by 83 and 92% in case of MiaPaCa2, 69 and 97% in case of PANC1, and 80 and 91% in case of AsPC1 cells, following 2 and 4% BMJ treatment for 72 hours, respectively (Fig. 3A). BMJ also strongly induced cell death (with increasing concentration and treatment time) in PANC1 and AsPC1 cells; cell death was induced by BMJ in MiaPaCa2 cells at 24 hours (even though the untreated controls showed higher percentage of dead cells compared to other PanC cells) (Fig. 3B).

Next, we sought to determine the effect of BMJ on PanC-CSCs under different drug exposure conditions. MiaPaCa2, PANC1 and AsPC1 cells in monolayers were treated with single and multiple exposures of BMJ, and then observed for spheroid formation. Cells were treated with BMJ once (Single treatment with 0.5 – 2%, v/v) for 72 hours and seeded for spheroid formation. In parallel, cells were given repeated BMJ treatments (Multiple Treatments with 0.5% - 2%, v/v), every 72 hours until day 9 since study initiation, and the remaining viable cells were seeded for spheroid formation. For the BMJ single treatment assay (Fig. 3C), a dose-dependent decrease in the spheroid number and size per well was observed in all the cell lines with the least number of generated spheroids seen in the 2% BMJ group. The results from BMJ multiple treatments (Fig. 3D) followed similar trends as was seen with single BMJ exposure with regards to reduction in the spheroid number and size over a course of 11 days.

BMJ affects PanC sphere formation with single and multiple exposures as well as pre-formed pancreatic spheres

To study the effect of single treatment of BMJ on PanC spheroid formation (not pre-exposed to BMJ and/or gemcitabine), MiaPaCa2, PANC1 and AsPC1 cells were seeded in stem cell media to generate spheres and, 6 hours post seeding, were treated with BMJ (0.5% - 2%, v/v). Growth factors EGF (20 ng/ml) and FGF (10 ng/ml) were added every 72 hours until day 11. The results show a significant decrease in the sphere number and size in a dose-dependent manner for all the cell lines, with the maximum effect at 2% BMJ, where a 95, 80 and 91% decrease was observed in the number of MiaPaCa2, PANC1 and AsPC1 spheres, respectively (Fig. 4A). Another set of experiments involved repeated treatments of PanC cells with BMJ, where cells were seeded and treated every 72 hours with 0.5%, 1% and 2% BMJ (v/v) until day 11 in the presence of EGF (20ng/ml) and FGF (10ng/ml). Similar to the results seen with single treatment, multiple BMJ treatments also caused a significant reduction in the sphere number and size. By day 11, the 2% BMJ dose caused 91, 88 and 95% reduction in sphere number for MiaPaCa2, PANC1 and AsPC1, respectively (Fig. 4A). In simultaneous studies, spheres were allowed to develop until day 5, following exposure to exogenous dose of 2% BMJ and 4% BMJ (data not shown) for a course of 11 days. As shown in figure (Fig. 4B), 2% BMJ dose for all three PanC cell lines results in a significant decrease in spheroid size and number by day 11 compared to the control groups.

BMJ downregulates mRNA and protein levels of CSC associated transcription factors/regulatory molecules in PanC cells and PanC-CSC spheroids

To test the potency of BMJ in altering the expression of various stem cell transcription factors associated with PanC-CSCs, we employed human stem cell transcription factor RT²qPCR array for determining the changes in levels of ~84 genes linked to stem cells in spheroids generated from PanC cell lines after treatment with BMJ. MiaPaCa2 cells were exposed to 2% BMJ for 72 hours, thereafter harvested and subjected to assessment of various PanC-CSC associated gene expression levels. BMJ treatment exhibited downregulation of a majority of genes as seen in the scatter plot (Fig. 5, top panel). The downregulated genes are displayed as open circles (green). BMJ exposure caused a major change in the expression levels of *SOX2*; it showed the maximum decrease in expression as made evident by the change in fold regulation, followed closely by *OLIG2* and *NR2F2* (Fig. 5, bottom panel). Additional genes contributing to stem cells associated with PanC-CSC that depicted decreased expression in the presence of BMJ, involved molecules such as *GATA6*, *GLI2*, *ISL1*, *JUN*, *MYC*, *NANOG*, *NOTCH2* and *PCNA*. These results highlight the potential of BMJ in targeting especially *SOX2* and *NANOG* levels, which are of utmost importance in their contribution to the CSC pool expansion in PanC [27,28].

Next, we assessed BMJ effect on protein levels of CSC associated transcription factors/regulatory molecules in PanC cells. MiaPaCa2, PANC1 and AsPC1 PanC cells in monolayers were seeded on coverslips in 6-well plates and treated with BMJ (2%) for 72 hours, then stained for transcription factors, namely SOX2, OCT4, NANOG, and stem cell surface marker CD44. Immunofluorescence imaging results followed by their densitometry analysis clearly depicted a significant decrease in the expression of SOX2, OCT4, NANOG and CD44 in all the cell lines following BMJ treatment, albeit at different levels (Fig. 6).

Together, these results show BMJ activity in targeting CSC associated transcription factors/regulatory molecules in PanC cells at both mRNA and protein levels.

In continuation with our results on PanC cells in monolayers, next we also assessed the effect of BMJ on CSC associated transcription factors/regulatory molecules in PanC-CSC spheroids. PanC cells were exposed to 2% dose of BMJ and followed for spheroid generation for 11 days in stem cell media. Imaging results from Z-stacking of spheroids suggested BMJ to be effective in significantly decreasing the expression of SOX2 and CD44 in all three MiaPaCa2 (Fig. 7A), PANC1 (Fig. 7B) and AsPC1 (Fig. 7C) spheroids. Consistent with our PanC cells in monolayers data (Fig. 6), SOX2 was found to be most significantly downregulated in PanC spheroids as well.

BMJ downregulates the expression of CSC associated transcription factors/regulatory molecules in MiaPaCa2 tumor xenografts

To further establish the significance of our cell culture results in an *in vivo* scenario, paraffin embedded MiaPaCa2 xenograft tissues from control and BMJ-fed nude mice from a recently completed study [17] were processed and stained for transcription factors namely SOX2, OCT4, NANOG and PDX1. Role of transcription factors SOX2, OCT4 and NANOG in CSC driven pancreatic tumorigenesis has been detailed later in the discussion section. PDX1 is a transcription factor crucial for pancreatic development; it is the first transcription factor expressed in developing pancreas and its expression becomes confined to β -cells in adult pancreas. Increased PDX1 levels have been reported in PDACs, mainly in the precursor lesions irrespective of the degree of dysplasia, pointing to its role in PanC progression [29]. Consistent with the finding observed for the *in vitro* studies, there was a significant decrease in the expression of each of the transcription factors (in terms of positively stained cells) examined in the samples from BMJ-treated mice compared to controls (Fig. 8). Quantitative analyses showed a decrease in SOX2 expression by ~80% (Fig. 8A) and that of OCT4, NANOG and PDX1 by ~67% decrease in the samples from BMJ-fed mice compared to controls expression (Fig. 8B-D). Together, these findings suggested that BMJ effectively downregulates the protein expression of the CSC associated transcription factors in both cell culture and animal xenografts, thus signifying its possible efficacy as a novel PanC chemotherapeutic.

DISCUSSION

Lately, the research surrounding CSCs is gaining momentum, mostly due to the preliminary progress achieved in targeting various solid tumors. CSCs possess variable histological characteristics which makes them prone to chemoresistance. Conventional chemotherapeutic agents target and attack the bulk population of cancer cells in a tumor, leaving CSCs unaffected and fully capable of giving rise to the relapsed tumor [30]. This attributes to CSCs being the ideal candidates for developing targeted chemotherapies with recent advances in the assay development [31]. PanC-CSCs were first established in 2007, and since then techniques have been developed to identify them as potential biomarkers of PanC [8].

Phytochemicals have been known to regulate CSC survival by targeting their self-renewal pathways [32]. The advantages of exploiting their chemopreventive and therapeutic efficacy are the minimal associated toxicities to surrounding normal healthy cells and cost effectiveness; particularly with PanC, there have been extensive studies connecting dietary agents to reduced PanC incidences [33,34]. BMJ, a dietary agent extracted from the fruits of *Momordica charantia*, has numerous associated health benefits; antidiabetic, emetic and laxative [35]. Studies using BMJ/BMJ-extracts in animals show lower serum cholesterol levels, hypoglycemia and delayed onset of tumorigenesis, alongside improved glucose tolerance in human subjects without significant alterations to insulin levels with BMJ administration [36–38]. Component analysis of bitter melon characterizes all the important constituents with multiple beneficial effects, mainly cucurbitane-type triterpenoids, triterpene glycoside, phenolic acid, flavonoids, essential oils, fatty acids, saponins and amino acids [35]. The toxicity data is minimal with only evident side effects being diarrhea and abdominal pain that can be relatively easily managed [39]. Variety of bitter melon constituents have actively been screened for their anticancer potential in numerous cancer types [35]. Analysis of bitter melon methanolic extract showed cucurbitane-type triterpenoids to be associated with an elevated anticancer efficacy in a two-stage mouse skin carcinogenesis model [40]. Furthermore, purified fractions of bitter melon methanolic extract containing monogalactosyl diacylglycerol (MGDG, a glycolipid) also displayed DNA polymerase inhibition-mediated growth inhibitory effects in lung, leukemia, colon, cervical and stomach cancer cells; however, no such effects were observed in normal cells [41]. Kuguaoside A; momordicoside I, F₁, and K; and goyaglycoside-b derived from bitter melon ethanolic extract have shown cytotoxicity in breast, colon, laryngeal and medulloblastoma cancer cells [42]. Another vital bitter melon component, cucurbitane-type triterpene 3 β ,7 β -dihydroxy-25-methoxycucurbita-5,23-diene-19-al is reported to exert strong cytotoxic potential against breast cancer cells *via* targeting and activating/altering PPAR γ and -downstream signaling pathways along with inhibition of mTOR-p70S6K signaling mediated by downregulation of AKT and AMPK activation [43]. Our previously published works have also reported BMJ mediated modulation of key pathway molecules such as PI3/Akt and ERK1/2 involvement in PanC associated gemcitabine resistance, and activation of AMPK (master metabolic regulator) leading to nutrient stress and eventually apoptosis in BMJ treated PanC cells and murine tumor xenografts [17,18].

The current study exclusively revolves around BMJ and its anticancer efficacy against PanC exerted through CSC modulation. We utilized a CD44⁺/CD24⁺/EpCAM⁺ triple positive marker for identifying the enriched subset of PanC-CSCs [8]. BMJ potential was evident by PanC growth inhibition observed with lower doses, of upto 2% BMJ, followed by massive cell death inflicted by 4% BMJ over a course of 72 hours. 2% BMJ qualified as the biological dose, hence was selected as the peak concentration for all mechanistic evaluations. The spheroid assays with PanC cells showed a significant decrease in resulting spheroid number and size in BMJ pre-treated PanC cell monolayers and established spheroids, with drug exposures (single and multiple BMJ treatments), highlighting the potential of BMJ to effectively target the CSC pool in solid tumors. Further insight into the PanC-CSC associated transcription factors and regulatory molecules/surface markers by RT²qPCR array of BMJ treated MiaPaCa2 cells displayed altered/decreased fold regulation

for key genes involved in PanC stemness. *SOX2* and *NANOG* are of great significance to this study based on their contribution to the CSC pool in a variety of cancers, particularly PanC. They are specifically responsible for self-renewal, dedifferentiation and impart stemness characteristics by targeting cell cycle regulatory genes, eventually resulting in EMT [27,28,44,45]. Other downregulated genes of interest with BMJ treatment included *NR2F2*; encodes COUP TFII, a downstream molecule in numerous CSC associated PanC progression pathways [46], *GLI2*; downstream molecule of hedgehog pathway and critical to CSC stemness and maintenance in PanC initiation and progression [47], *ISL1*; stage specific marker of pancreatic development/differentiation and highly upregulated in PanC-CSCs [48], *MYC*; overexpressed in PanC and identified as a key determinant of oxidative phosphorylation dependency in PanC-CSCs [25]. Targeting these CSC genes responsible for stemness has been reported to diminish the tumorigenic potential and enhance sensitivity to chemotherapeutic agents by altering core CSC properties [4]. Analysis of protein levels of core molecules involved in PanC-CSC stemness and self-renewal exhibited significantly decreased expressions/fluorescence intensities of SOX2, OCT4, NANOG and CD44 in PanC cell monolayers and spheroids. SOX2, OCT4 and NANOG are embryonic stem cell transcription factors aberrantly expressed in PanC-CSCs, responsible for their self-renewal and pluripotency [28]. CD44 is reported as the most enriched cell surface marker in relapsing PDAC; a suitable therapeutic target in multiple cancers. It is widely accepted on the panel of markers for PanC-CSC isolation [49,50]. Therefore, a reduced expression for these protein markers in PanC monolayers and spheroids correlates to BMJ efficacy in curbing CSC mediated PanC. Overall, BMJ efficiently targets and downregulates these PanC-CSC associated molecules, thereby pointing to inhibition of PanC progression. Lastly, translational relevance of BMJ was confirmed *in vivo* utilizing MiaPaCa2 xenografts where BMJ administration (*via* oral gavage) also caused a significant reduction in PanC-CSC associated transcription factors as examined by immunohistochemical staining of SOX2, OCT4, NANOG and PDX1, thus verifying the results noted from our *in vitro* studies.

In summary, we were able to establish a novel activity of BMJ in targeting both PanC CSC pool as well as bulk tumor population, together with a decrease in the levels of CSC-associated regulatory molecules. The effects observed *in vitro* were further corroborated in mouse xenograft tissue samples showing a decrease in PanC-CSC associated regulatory molecules in BMJ treated samples compared to controls. Notably, these effects of BMJ warrant further studies to establish its efficacy in CSC-driven PanC models and define associated molecular mechanisms and pathways.

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Abbreviations:

PanC	pancreatic cancer
BMJ	bitter melon juice

CSCs	cancer stem cells
IF	immunofluorescence
SOX2	sex determining region Y-box 2
OCT4	octamer binding transcription factor 4
IHC	immunohistochemistry
PanIN	pancreatic intraepithelial neoplasia
PDAC	pancreatic ductal adenocarcinoma
AMPK	adenosine monophosphate activated protein kinase
EpCAM	epithelial cell adhesion molecule
FACS	fluorescence activated cell sorting
EGF	epidermal growth factor
FGF	fibroblast growth factor
PanC-CSCs	pancreatic cancer-associated cancer stem cells
PDX1	pancreas/duodenum homeobox protein 1

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Figure 1. BMJ affects both unsorted and CSC enriched PanC cell spheroids.

FACS sorting of untreated PANC1 cell line with CD44⁺/CD24⁺/EpCAM⁺ combination of CSC markers (A). The sorted CD44⁺/CD24⁺/EpCAM⁺ was subjected to limiting dilution assay to generate spheroids with cell number ranging from 200–10,000 cells per well for determining the stemness of triple positive population (B). The effect of BMJ was studied by exposing FACS sorted and unsorted cells to varying BMJ concentrations and recording the number of spheroids formed per well (C). $p < 0.05$ is denoted by * and $p < 0.01$ is denoted by ***.

Figure 2. BMJ treatment helps confer sensitivity to gemcitabine resistant PanC cells generated spheroids.

PANC1 and AsPC1 PanC cells surviving 2.5 μ M gemcitabine treatment (**A**), and 5 μ M gemcitabine treatment (**B**), were exposed to 0.5 or 2% BMJ (v/v) treatments in spheroid assay, and the decrease in spheroid number and size was recorded after 11 days. Representative spheroid images are shown. $p < 0.05$ is denoted by * and $p < 0.01$ is denoted by **.

Figure 3. BMJ affects the cell viability and stemness in PanC cell monolayers.

Trypan blue exclusion assay was performed for PanC cell lines MiaPaCa2, PANC1 and AsPC1 with 2–4% BMJ (v/v). The decrease in total cell number (**A**) and increase in percent cell death (**B**), are shown in the top panels for 24, 48 and 72 hours. Also, BMJ effect on PanC stemness was assessed by spheroid assays for PanC cell lines MiaPaCa2, PANC1 and AsPC1 where the cell monolayers were treated with 0.5–2% BMJ (v/v) single (**C**), and multiple treatments (**D**) and observed for 11 days. Representative spheroid images are shown. $p < 0.01$ is denoted by ** and $p < 0.001$ is denoted by ***.

Figure 4. BMJ affects number and size of spheres formed by PanC-CSCs.

PanC cells seeded for sphere formation were exposed to single and multiple treatments of BMJ (0.5–2% BMJ, v/v) and observed for 11 days (**A**). PanC cells were allowed to generate spheroids for 5 days and then treated with 2% BMJ to observe the effects on pre-formed spheroid number and size (**B**). Representative spheroid images are shown. $p < 0.05$ is denoted by *, $p < 0.01$ is denoted by ** and $p < 0.001$ is denoted by ***.

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Figure 5. BMJ alters the mRNA levels of PanC-CSC associated regulatory molecules and transcription factors.

MiaPaCa2 cells were exposed to 2% BMJ for 72 hours, thereafter harvested and subjected to RT²-qPCR array of human stem cell transcription factors for assessment of BMJ effects on gene expression of PanC-CSC associated transcription factors. Top panel depicts scatter plot showing BMJ vs untreated control group (1.5 fold level changes); downregulated genes are displayed as open circles (green). Bottom panel depicts genes displaying the most significant down-regulation with BMJ exposure.

Figure 6. BMJ decreases the protein expression of CSC associated transcription factors and surface markers in PanC cell monolayers.

Immunofluorescence staining of PanC cell monolayers treated with 2% BMJ for 72 hours shows a reduction in SOX2, OCT4, NANOG and CD44 protein expression levels in MiaPaCa2 (A), PANC1 (B), and AsPC1 (C) cells. The results from densitometry by Image J software are also provided and correlate to the data from the images. $p < 0.05$ is denoted by *, $p < 0.01$ is denoted by ** and $p < 0.001$ is denoted by ***.

Figure 7. BMJ decreases the protein expression of CSC associated transcription factor SOX2 and CSC surface marker CD44 in PanC cell generated spheroids.

PanC cells were treated with 2% BMJ (72 hours) and allowed to generate spheroids in stem cell media for 10 days. On day 11, spheroids were harvested and stained by immunofluorescence for SOX2 and CD44 in MiaPaCa2 (A), PANC1 (B), and AsPC1 (C) spheroids. Z-stacking was performed for the spheroids. The results from densitometry by Image J software are also provided and correlate to the data from the images. $p < 0.05$ is denoted by * and $p < 0.01$ is denoted by **.

Figure 8. BMJ targets and downregulates the expression of CSC associated transcription factors in MiaPaCa2 xenografts.

Paraffin embedded MiaPaCa2 xenograft tumor sections from control and BMJ treated groups were subjected to immunohistochemical analysis for determining the *in vivo* effect on the protein levels of PanC-CSC associated transcription factors: SOX2 (A), OCT4 (B), NANOG (C), and PDX1 (D). The percent positive cells were quantitated by counting the brown stained nuclei for each molecule in control versus the BMJ treated groups. $p < 0.05$ is denoted by * and $p < 0.001$ is denoted by ***.