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Original Research

Ovarian cancer cells with CD133$^+$ phenotype is more resistant against Ngai Bun *Boesenbergia pandurata* extract than original ovarian cancer cells

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ABSTRACT

Introduction: Ovarian cancer is one of the most common cancers in women. Due to the difficulty in early detection and treatment of ovarian cancer, many research studies and clinical trials have been developed to discover more efficient therapies. Besides Western medicine, traditional medicine has gained increased interest as a research field with potential to lead to the production of marketable therapeutic products. With the diversity of tropical plants in Asia, traditional medicine has been very popular and has served as a traditional therapy for generations. The Ngai bun (*Boesenbergia pandurata*) root is used not only as a food spice but also in ethnomedicine. This study aimed to compare the anti-tumor activity of *Boesenbergia pandurata* root extract against ovarian cancer cells and CD133$^+$ovarian cancer cells that were enriched from the original ovarian cancer cells. **Methods**: Crude extract of *Boesenbergia pandurata* roots were prepared in two kinds of solvents (methanol and chloroform). The ovarian cancer cells OVP-10 were used in this study. The population of CD133$^+$ ovarian cancer cells (CD133$^+$OVP-10) were sorted from the OVP-10 cancer cells. Both OVP-10 cells and CD133$^+$OVP-10 cells were treated with these crude extracts. Adipose-derived stem cells (ADSCs) were used as control normal cells for all assays. The anti-tumor activity of extracts were evaluated based on the IC_{50} values. **Results**: Based on the IC_{50} index, the chloroform extract had an anti-tumor activity higher than that of methanol extract, on both OVP-10 and CD133$^+$OPV-10 cells (IC_{50} of methanol and chloroform extracts were 330.1 $\pm$ 16.9 μg/mL and 246.5 $\pm$ 21.2 μg/mL, respectively, for OVP-10 cells; IC_{50} of methanol and chloroform extracts were 411.8 $\pm$ 83.7 μg/mL and 307 $\pm$ 9.2 μg/mL respectively, for CD133$^+$OVP-10 cells). The results also showed that CD133$^+$OVP-10 cells were more resistant to chloroform extract than were OVP-10 cells (307 $\pm$ 9.2 μg/mL *vs.* 246.5 $\pm$ 21.2 μg/mL, respectively, for CD133$^+$OVP-10 *vs.* OVP-10 cells, $p < 0.05$). **Conclusion**: The chloroform extract of *Boesenbergia pandurata* roots displayed strong anti-tumor activity against ovarian cancer cells OVP-10 and CD133$^+$OVP-10; the latter cells were found to be more resistant than the original ovarian cancer cells.

Key words: CD133+ cancer stem cells, ovarian cancer stem cells, ovarian cancer, Boesenbergia pandurata

INTRODUCTION

In 2019, the American Cancer Society estimated ovarian cancer as the leading cause of death in gynecological disease, ranking 5th of the 10 leading cancer types in women [1]. Ovarian cancer is a rare disease, in which early detection is difficult and surgical strategies are the first step in treatment for this cancer [2]. However, the extent of surgery depends on how far the ovarian cancer has spread; chemotherapy must be used in the next steps to eradicate any residual cancer cells still present in the body after surgery. The goal of chemotherapy is to destroy the cancer by inhibiting the proliferation of cancer cells. Chemotherapy is a potential treatment for prolonging the cancer patient's life. There are many kinds of anti-cancer drugs from natural sources, such as plants (*e.g.* vincristine, irinotecan, and camptothecins) and microorganisms (*e.g.* doxorubicin, mitomycin, and bleomycin) [3]. Doxorubicin (DOX) is the most popular anti-cancer drug, which is currently widely used for treatment of many kinds of human cancers, both solid and hematological [4]. DOX was shown to induce resistance in 3D spheroids, at a rate higher than that exhibited in standard 2D cell culture [5]. Additionally, tirapazamine (TPZ; 3-amino-1,2,4-benzotriazine 1,4 dioxide) is a new class of cytotoxic drugs with a focus on treating hypoxic mammalian cells [6]. When cultured in 2D, the toxicity of DOX with target cells is more sensitive than TPZ. Cell viability of cells with the testing drug was identified by using the Alamar Blue assay. By incorporating a fluorometric/colorimetric growth indicator based on detection of metabolic assay, the

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Alamar blue assay can quantitatively measure the proliferation of various human and animal cells. Many new drugs and therapies have been under development and are being tested in ongoing clinical trials, with the aim of finding the most effective therapy of ovarian cancer.

Recently, besides modern medicine, ethnomedicine has also become a potential direction for the development of new drugs. Ethnomedicine is a study of the traditional medicine based on the use of plant compounds. Ngai bun (*Boesenbergia pandurata*) is a ginger species belonging to the *Zingiberaceae* family; it is cultivated in tropical countries such as Southeast Asia, India, Sri Lanka and South China. This species has many local names, such as "ngai bun" in Vietnam[7], "temu kunci" in Indonesia[8], krachai (or krachai-dang) in Thailand, and Chinese key (or fingerroot) in English[9]. This plant is mainly used as a spice and contains several prenylated chalcones and flavonoids with many biological activities, including antifungal, antibacterial, anti-inflammatory and anticancer functions[7,8].

The cancer stem cell (CSC) theory proposes that in the cancer cell population, there exists a small population of stem cells which have the capability of self-renewal and tumor-initiation. Recent research studies had revealed that CSCs in ovarian cancer are not only responsible for primary tumor growth and metastasis but also for chemotherapy resistance[10]. In studies on ovarian cancer stem cells, CD133 is one of most popular cell surface markers which has been used for the isolation of CSCs from various type of cancers, such as breast, colon, ovarian and prostate cancer[10,11].

In this research study, we aimed to test the toxicity of Ngai bun (*Boesenbergia pandurata*) extract in different solvents on ovarian cancer OVP-10 cells and $CD133^+$ sorted OVP-10 cells ($CD133^+$OVP-10 cells) to assess the therapeutic potential of this plant for ovarian cancer treatment.

MATERIALS AND METHODS

Cell culture

Human ovarian cancer OVP-10 cells were grown in DMEM/F12 with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO). The cells were maintained in cell culture flasks (25-cm^2 and 75-cm^2) in an incubator set at 37^0C and 5% CO_2. Cells were cultured and passaged two to three times before processing in the bioassays.

$CD133^+$ cell sorting

OVP-10 cells were labelled with CD133 magnetic beads and sorted by magnetic-activated cell sorting (MACS), per the instructions of the Human CD133 MicroBead kit (kit and MACS sorting multi-stand were obtained from Miltenyi Biotec Inc., Auburn, CA). The $CD133^+$ cancer cells were expanded to obtain enough cells for the bioassays.

Drugs

The standard drugs used in this study were doxorubicin (DOX) and tirapazamine (TPZ); both were purchased from Sigma-Aldrich. Ngai bun extract was isolated from fresh root, following a previously published protocol, and dissolved in the different solvents[7].

Alamar Blue assay

The cell viability of ovarian cancer cells was tested by Alamar Blue assay. Cells were plated in a 96-well plate at a density of 2 x 10^4 cells/well. After plating for 24 h, cells were treated with the drugs at six differing concentrations (2000, 1000, 500, 250, 125, 62, and 0 μg/mL) for 48 h. The culture medium was then removed and the wells were replaced with fresh media. As a negative control, fresh media was also added to empty wells. All wells were added with 10 μl of the Alamar Blue solution and then re-incubated at 37^0C, 5% CO_2 for 4 h. Data was collected by using a DTX880 system (Beckman-Coulter, Brea, CA), and fluorescence was monitored at 530-560 nm excitation wavelength and 590 nm emission wavelength.

Statistical analysis

Each experiment was repeated three times. The IC_{50} and significant differences between mean values were calculated by using GraphPad Prism 7.0 (GraphPad Inc., La Jolla, CA), with p-value < 0.05 set as statistical significance.

RESULTS

Isolation of human ovarian cancer $CD133^+$OVP-10 cells

Human ovarian cancer OVP-10 cells were cultured and expanded in 75-cm^2 flasks. When cells reached approximately 70% confluency (**Figure 1 B**), cells will be trypsinized and subcultured in DMEM/F12 medium supplemented with 10% FBS. After passing two to three times, OVP-10 cells were subjected to bioassays, and/or sorted for $CD133^+$ cells using

MACS (**Figure 1A**). $CD133^+$OVP-10 cells were labelled with CD133 magnetic beads, isolated and cultured to confluency before treatment with the compounds (**Figure 1C**). The morphology of OVP-10 cells and $CD133^+$OVP-10 cells showed no significant differences; however, in culture, the proliferation time of $CD133^+$OVP-10 cells was slightly longer than that of OVP-10 cells.

Testing an ovarian cell model for drug screening with standard drugs

The cell concentration also affected the *in vitro* bioassay for anti-cancer drug screening[12]. The growth of OVP-10 cells was tested and the model for screening the different extracts was optimized. As seen in **Figure 2A**, OVP-10 cells continued to proliferate after 7 days. The bioassay was performed in 3 days: on the first day, cells were plated in wells and incubated for 24 h; next, cells were treated with compounds for 48 h; on the third day, cells were processed for Alamar blue assay and the IC_{50} indexes were calculated. During these days, OVP-10 cells were still stable and there was little increase in cell number (**Figure 2A**). The OVP-10 cells were treated with standard drugs (doxorubicin or tirapazamine) to confirm that the OVP-10 cell model could be used for screening. Doxorubicin is popular standard drug which used as a control in many studies of drug screening. Specifically, doxorubicin only affected the monolayer cell model but not the three-dimensional (3D) cell culture model. In contrast, tirapazamine was only effective in the 3D cell culture model. As shown in **Figure 2B** and **Figure 2C**, the IC_{50} of doxorubicin in OVP-10 cells (168.9 ± 2.3 nM) and that of $CD133^+$OVP-10 cells (567.7 ± 95.7 nM) were highly different ($p < 0.01$).

Ngai bun extract dissolved in chloroform solvent had a greater effect on both OVP-10 and $CD133^+$OVP-10 cancer cells than methanol solvent

The IC_{50} index of Ngai bun extract in chloroform solvent ($CHCl_3$) had a greater effect on killing ovarian cancer OVP-10 cells when compared with the effect of this extract on adipose-derived stem cells (ADSCs), which were used as the control. The IC_{50} of methanol (MeOH) and chloroform ($CHCl_3$) solvent were 330.1 ± 16.9 μg/mL and 246.5 ± 21.2 μg/mL, respectively. As indicated in**Figure 3A**, the MeOH extract had a different effect on ADSCs and OVP-10 cells, as shown by the IC_{50} index of 497.2 ± 32.4 μg/mL and 330.1 ± 16.9 μg/mL, respectively. However, with $CD133^+$OVP-10 cells, no significant differences were observed between the two cell lines; the IC_{50} of the corresponding cells (ADSCs *vs*. $CD133^+$OVP-10 cells) with MeOH solvent, respectively, were 497.2 ± 32.4 μg/mL and 411.8 ± 83.7 μg/mL (**Figure 3B**) ($p > 0.05$). In **Figure 3C**, with the same cells in a different solvent ($CHCl_3$), the IC_{50} index of OVP-10 cells was 246.5 ± 21.2 μg/mL, which was significantly different from the IC_{50} of 474.6 ± 18.8 μg/mL for ADSCs ($p < 0.05$). The results in**Figure 3D** showed that $CD133^+$OVP-10 cells treated with $CHCl_3$ extract corresponded to an IC_{50} index that was significantly lower than that for ADSC cells (307 ± 9.2 μg/mL *vs*. 474.6 ± 18.8 μg/mL, respectively) ($p < 0.05$).

DISCUSSION

Ovarian cancer is the most serious gynecologic cancer, typically diagnosed at an advanced stage[13,14]. The current standard treatment for ovarian cancer is surgery and chemotherapy. The chemotherapy strategy is faced with many obstacles including cancer metastasis and resistance of tumor with drugs. This has motivated the development of drug discovery to help find novel potentially therapeutic compounds for anti-cancer treatment. In drug screening, cells must be in the proliferation stage and should be stable in the testing with drugs. In this study, the OVP-10 cells continued to proliferate for 7 days and were suitable for our assay. After 24 h of plating, OVP-10 cells showed a low increase in cell number and after 3 days (*i.e.* the day of the Alamar blue assay), cells were still in log phase of proliferation.

Despite the disadvantage of the 2D model and the development of the 3D model, the 2D model is still very popular for drug screening. Evaluation of the 2D model using *in vitro* bioassays, such as MTT or Alamar Blue assay, is necessary to assess the efficiency of the 2D model in anti-cancer drug screening[12]. The cell concentration and drug concentration parameters have a great effect on the success of the *in vitro* bioassays. As shown in this study, the cell concentration for plating in 96-well plates was 1000-2500 cells/well, and 6 parameters of drugs or extract concentrations were at the very least required for calculation of the IC_{50} index[15].

Use of the traditional extract from plants could kill cancer cells with fewer effects on normal cells. In this study, Ngai bun (*Boesenbergia pandurata*) extract was demonstrated to be toxic for OVP-10 cells, when compared with adipose derived stem cells as control. When comparing different *Boesenbergia* species (*B. armeniaca, B. rotunda, or B. pulchella var attenuate*), Jing et al. showed that *Boesenbergia rotunda* extract in

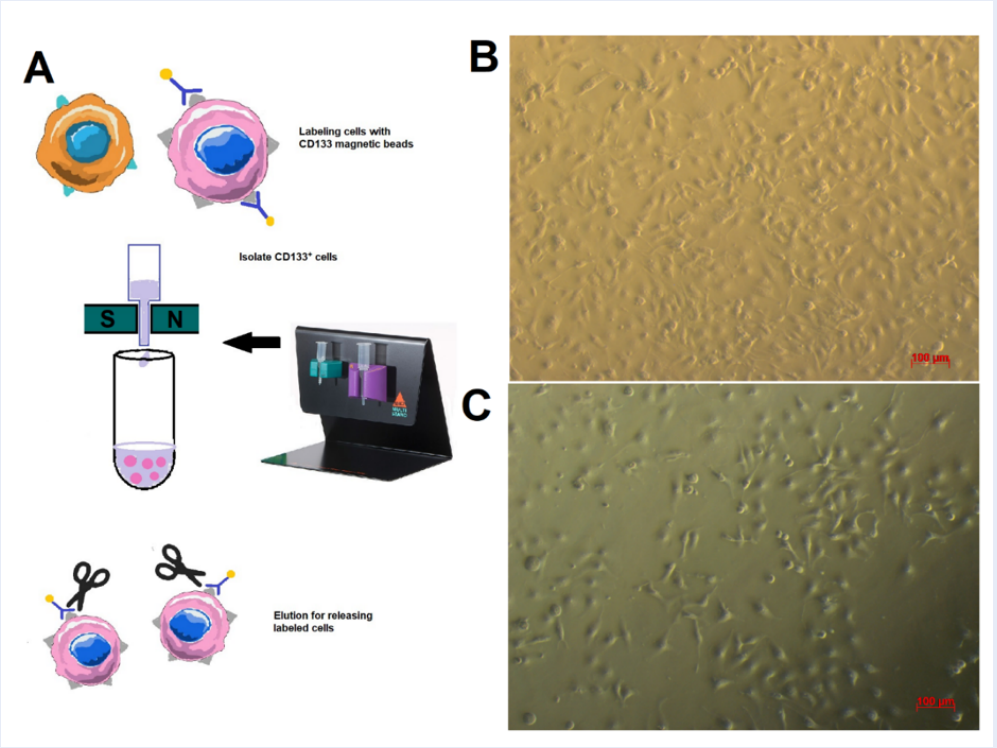

Figure 1: **Isolation and expansion of CD133$^+$OVP-10 cells from OVP-10 ovarian cancer cells**. (**A**) Procedure of CD133$^+$OVP-10 cells isolation by magnetic activated cell sorting. (**B**) Human ovarian cancer OVP-10 cells. (**C**) Human ovarian carcinoma CD133$^+$OVP-10 cells. Pictures were taken at 20 X magnification.

methanol had the strongest inhibitory effects against $CaOV_3$ ovarian cancer and different types of cancers, such as breast cancer MDA-MB231 (IC_{50} 66.5 ± 2.12 μg/mL), MCF7 (IC_{50} 51 μg/mL), cervical cancer HeLa (IC_{50} 66.5 ± 2.12 μg/mL), and colon cancer HT-29 (IC_{50} 52 ± 2.12 μg/mL). *Boesenbergia* genera is potentially potent extract for treatment of ovarian cancer. OVP-10 cells is one of the targets for investigation of drug cytotoxicity against ovarian cancer [16]. Moreover, the synergistic anti-tumor effect would be combined to develop new therapies for ovarian cancer treatment [17]. From different studies, the extract of *Boesenbergia* genera exhibit robust potency that can be utilized as an potential candidates for the development of new anti-cancer drugs. Advances in drug discovery will require identifying and developing new and innovative marketable pharmaceutical products. Future studies from this research should focus further on the discovery of such compounds.

Besides the toxicity towards ovarian cancer OVP-10 cells in a dose-dependent manner, Ngai bun (*Boesenbergia pandurata*) extract in chloroform was demonstrated to inhibit the cell viability of CD133$^+$OVP-10 cells, representing ovarian cancer stem cells. At the IC_{50} of Ngai bun extract which could kill 50% of CD133$^+$OVP-10 cells, that concentration could kill more than 50% of OVP-10 cells but less than 50% of ADSC cells. This shows that the dose of drug used for treatment must be chosen carefully.

In this study, two kind of solvents were chosen to evaluate which was the best solvent for dissolving Ngai bun (*Boesenbergia pandurata*) extract, and still maintain the functions of the extract. As observed, the chloroform-dissolved extract induced better toxicity than the methanol-dissolved extract. Besides the appropriate concentrations, the suitable solvent is also a key factor for determining the success of drug discovery.

CONCLUSION

Overall, the data obtained from this study shows that Ngai bun (*Boesenbergia pandurata*) chloroform-dissolved extract is more toxic on OVP-10 cells than on CD133$^+$OVP-10 cells. The cytotoxicity of the chloroform extract was also higher that of the methanol extract.

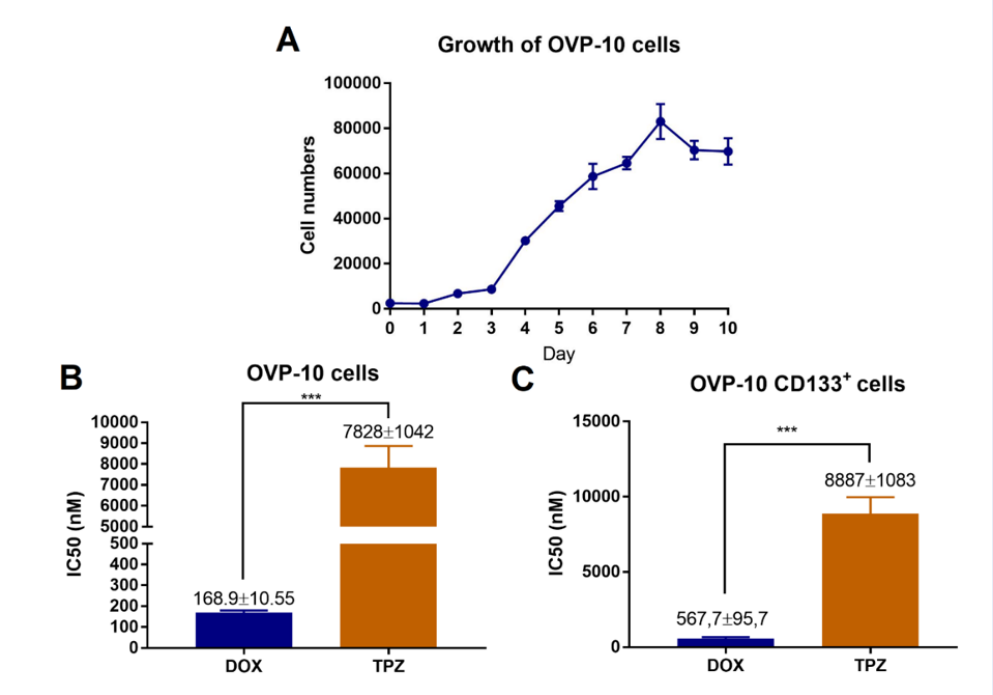

Figure 2: **Model for screening the efficiency of Ngai bun extract**. (**A**) Proliferation of human ovarian cancer OVP-10 cells in 96-well plates over10 days. (**B, C**) Effect of standard drugs (doxorubicin or tirapazamine) on human ovarian cancer OVP-10 cells (**B**) and CD133+ sorted human ovarian cancer (CD133^{+}OVP-10) cells. ***: $p < 0.001$. **Abbreviations**: DOX: dororubicin, TPZ: tirapazamine

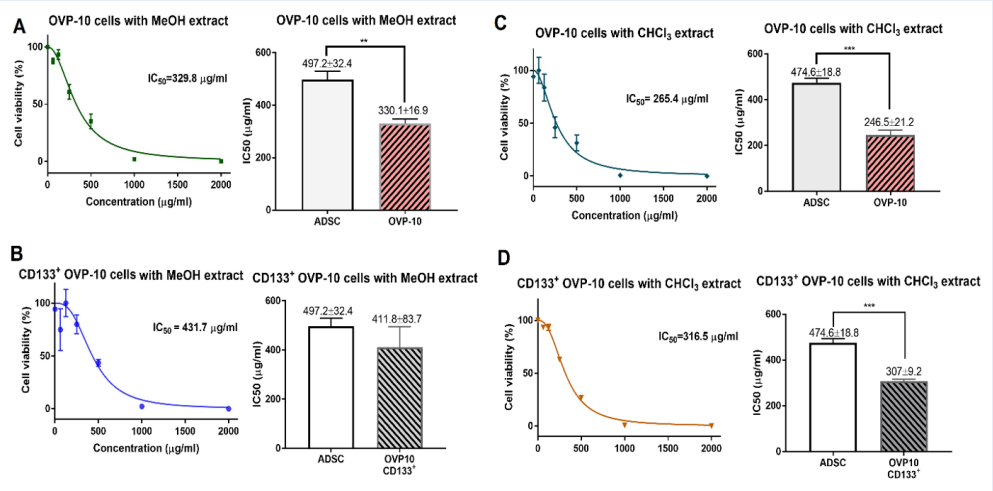

Figure 3: **The IC_{50} of human ovarian cancer OVP-10 cells and CD133^{+}OVP-10 cells in different solutions.** (**A**) methanol (MeOH), (**B**) chloroform (CCl_3). Each experiment was processed three times, and statistical analysis was performed by GraphPad Prism 7.0 with *p<0.05 (** p<0.0021, ***p<0.0002). **Abbreviations**: ADSCs: Adipose derived stem cells.

ABBREVIATIONS

CSC: Cancer stem cell
DOX: Doxorubicin
IC_{50}: the half-maximal inhibitor concentrations
TPZ: Tirapazamine

CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

AUTHORS' CONTRIBUTION

All authors equally contributed in this work and approved the final version of manuscript for submission.

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