Fall 9-29-2014

Withaferin a alone and in combination with Cisplatin suppresses growth and metastasis of ovarian cancer by targeting putative cancer stem cells.

Sham S. Kakar
University of Louisville

Mariusz Z. Ratajczak
University of Louisville

Karen S. Powell
University of Louisville

Mana Moghadamfalahi
University of Louisville

Donald M. Miller
University of Louisville

See next page for additional authors

Authors
Sham S. Kakar, Mariusz Z. Ratajczak, Karen S. Powell, Mana Moghadamfalahi, Donald M. Miller, Surinder K. Batra, and Sanjay K. Singh

OPEN ACCESS Freely available online

PLOS ONE

Withaferin A Alone and in Combination with Cisplatin Suppresses Growth and Metastasis of Ovarian Cancer by Targeting Putative Cancer Stem Cells

Sham S. Kakar[1,2]*, Mariusz Z. Ratajczak[2,3], Karen S. Powell[4], Mana Moghadamfalahi[5], Donald M. Miller[2,3], Surinder K. Batra[6], Sanjay K. Singh[1,2]

1 Department of Physiology and Biophysics, University of Louisville, Louisville, Kentucky, United States of America, **2** James Graham Brown Cancer Center, University of Louisville, Louisville, Kentucky, United States of America, **3** Department of Medicine, University of Louisville, Louisville, Kentucky, United States of America, **4** Research Resources Center, University of Louisville, Louisville, Kentucky, United States of America, **5** Department of Pathology, University of Louisville, Louisville, Kentucky, United States of America, **6** Department of Biochemistry and Molecular Biology, Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska, United States of America

Abstract

Currently, the treatment for ovarian cancer entails cytoreductive surgery followed by chemotherapy, mainly, carboplatin combined with paclitaxel. Although this regimen is initially effective in a high percentage of cases, unfortunately within few months of initial treatment, tumor relapse occurs because of platinum-resistance. This is attributed to chemo-resistance of cancer stem cells (CSCs). Herein we show for the first time that withaferin A (WFA), a bioactive compound isolated from the plant Withania somnifera, when used alone or in combination with cisplatin (CIS) targets putative CSCs. Treatment of nude mice bearing orthotopic ovarian tumors generated by injecting human ovarian epithelial cancer cell line (A2780) with WFA and cisplatin (WFA) alone or in combination resulted in a 70 to 80% reduction in tumor growth and complete inhibition of metastasis to other organs compared to untreated controls. Histochemical and Western blot analysis of the tumors revealed that inclusion of WFA (2 mg/kg) resulted in a highly significant elimination of cells expressing CSC markers - CD44, CD24, CD34, CD117 and Oct4 and downregulation of Notch1, Hes1 and Hey1 genes. In contrast treatment of mice with CIS alone (6 mg/kg) had opposite effect on those cells. Increase in cells expressing CSC markers and Notch1 signaling pathway in tumors exposed to CIS may explain recurrence of cancer in patients treated with carboplatin and paclitaxel. Since, WFA alone or in combination with CIS eliminates putative CSCs, we conclude that WFA in combination with CIS may present more efficacious therapy for ovarian cancer.

Citation: Kakar SS, Ratajczak MZ, Powell KS, Moghadamfalahi M, Miller DM, et al. (2014) Withaferin A Alone and in Combination with Cisplatin Suppresses Growth and Metastasis of Ovarian Cancer by Targeting Putative Cancer Stem Cells. PLoS ONE 9(9): e107596. doi:10.1371/journal.pone.0107596

Editor: Deodutta Roy, Florida International University, United States of America

Received July 16, 2014; **Accepted** August 5, 2014; **Published** September 29, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. I confirm that all data underlying the findings in my study are freely available in the manuscript.

Funding: NIH/NCI CA124630, James Graham Brown Cancer Center Research Funds, School of Medicine Basic Research program Research Grant. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Co-author Surinder Batra is a PLOS ONE Editorial Board member. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

* Email: sskaka01@louisville.edu

Introduction

Epithelial ovarian cancer (EOC) remains the leading cause of death in women among gynecologic cancers and is the 5th highest cause of cancer-related deaths in women in the United States [1,2]. The majority of ovarian cancers are diagnosed at advanced stage due to the mainly non-specific symptoms. Currently, the treatment for ovarian cancer entails cytoreductive surgery followed by chemotherapy, employing mainly platinum/taxane combination [3]. Although this regimen is initially effective in a high percentage of cases (70 to 80%), unfortunately 70% of women develop recurrent cancer within few months of initial treatment as a result of platinum-resistance [4,5]. In addition, cisplatin (CIS) is associated with multiple severe side effects such as nausea, vomiting, myelosuppression, hepatotoxicity, neurotoxicity, nephrotoxicity and ototoxicity [4,6–9]. Therefore, need for new treatment options that target cancer cells and in particular putative cancer stem cells is mandatory either at first-line setting or even more at the first- and second-line management of recurrent ovarian cancer.

In our previous studies [10], we showed for a first time that withaferin A (WFA), a bioactive compound isolated from the plant Withania somnifera, when used alone or in combination with CIS had a time- and dose-dependent synergistic effect on inhibition of cell proliferation and induction of cell death, thus reducing required dosage of cisplatin. We also showed that while WFA achieves its antitumor effect through generation of ROS leading to DNA damage, CIS achieves its effects though direct binding to DNA causing the formation of DNA adducts. Combination treatment also resulted in a significant enhancement of reactive oxygen species (ROS) production and DNA damage.

WFA has been a part of Indian traditional medicine for centuries. It is available in US over-the-counter as a dietary

supplement and is known to treat various disorders due to its anti-inflammatory [11,12], anti-bacterial [13], and cardio protective properties [14]. In recent years, WFA has been suggested as a potential anti-cancer compound shown to prevent tumor growth, angiogenesis, and metastasis [15,16] in various types of cancer [17–26]. Mechanisms by which WFA attains its anticancer activity include inactivation of Akt and NF-κB [27] to achieve apoptosis, decrease in pro-survival protein Bcl-2 [28], G2/M cell cycle arrest [29,30], generation of reactive oxygen species (ROS) [31,32], induction of Par-4 [17], activation of caspase 3 and 9 activities, DNA damage [10], inhibition of HSP90 [20], regulation of FOXO3a and Bim [15] inhibition of Notch-1 [33] and down regulation of expression of HPV E6 and E7 oncoproteins [19].

Development of drug resistance and recurrence of ovarian cancer has been a major clinical problem. A number of mechanisms that induce drug resistance have been proposed. Over the last several years, there has been increasing evidence that "cancer stem cells (CSCs)", are the most important trigger of tumor progression, chemo-resistance and relapse after initial treatment [34,35]. First evidence for the existence of cancer stem cells came in the year 1997, with the identification of leukemia stem cells [36,37]. In the year 2003, Al-Hajj et al. [38] experimentally demonstrated the hierarchical stem cell origin in breast cancer. However, until recently the existence of putative cancer stem cells within solid tumors had remained controversial [39]. In recent studies using murine models for brain, skin and intestinal tumors, three independent groups have provided convincing evidence for the existence of CSCs in tumors and their role in tumor expansion [40–42]. Accordingly, CSCs within tumor mass undergo self-renewal and give rise to heterogeneous cancer lineages that comprise tumor tissue. CSCs purified accordingly to some surface markers are able to form tumors when injected into nude mice [36,43,44]. Since, ovarian cancer is very heterogeneous; different cell surface markers have been reported for putative ovarian CSCs. Most commonly reported include CD24, CD34, CD44, CD133, CD117, ALDH1, Oct4, MyD88 and EpCAM [45–53]. Since, CSCs are considered to be major players responsible for developing drug resistance and hence leading to cancer recurrence [52,53], targeting CSCs and inhibiting their self-renewal will lead to reduction of cancer growth [33].

In our current study, we show for the first time that WFA alone or in combination with CIS if employed to treat mice bearing human orthotopic ovarian tumors not only suppresses tumor growth but targets cells expressing CSC markers as well as inhibits Notch1 and its downstream signaling genes (Hes1 and Hey1) that have been reported to play a crucial role in self-renewal and maintenance of CSCs (33).

Material and Methods

Cell line and cell culture

Ovarian epithelial cancer cell line A2780 was initially obtained from Denise Connolly (Fox Chase Cancer Center) and was maintained in RPMI 1640 medium containing insulin and supplemented with penicillin/streptomycin (100 IU/ml and 100 μg/ml) and 10% fetal bovine serum (FCS) (Hyclone, Atlanta, GA) as described previously [10].

Cell migration Boyden chamber assays

Cell migration in vitro was assayed by determining the ability of cells to migrate through a synthetic basement membrane. The procedure used was as described previously [54]. Briefly, polycarbonate filters (8 μM) were placed in modified Boyden chamber. A2780 cells in log phase were trypsinized and plated in 6 wells plates. After 24 h of plating, cells were treated with WFA and CIS both alone and in combination as described previously [10]. After 24 h of treatment, cells were trypsinized and suspended in serum free medium. A total of 2×10^5 cells were transferred to the top chamber. The medium containing 5% FBS was added to the lower chamber. The cells were incubated at 37°C for 24 h and allowed to migrate through the membrane. Non-migrated cells were removed with a clean cotton swab. Migrated cells on other side of the membrane were stained with crystal violet and counted in three different fields under Olympus microscope. The experiments were repeated for three times. The values represented are the mean ± SEM of three independent experiments.

Generation of orthotopic ovarian tumors in nude mice and treatment with WFA and CIS both alone and in combination

Orthotopic ovarian tumors were generated by injecting ovarian cancer cell line A2780 directly into ovary as described by Nunez Cruz et al. [55]. Briefly, A2780 (1×10^6) cells were directly injected into left ovary of 5 to 6 weeks old nu/nu female mice (Jackson Laboratory) under aseptic conditions and under light anesthesia. After 10 days of post-cell injection, mice were treated with 1) vehicle control (10% DMSO and 90% glyceryl trioctanoate), 2) WFA 2 mg/kg, 3), CIS 6 mg/kg, and 4) WFA 2 mg/kg plus CIS 6 mg/kg. Five animals randomly were included in each group. CIS in saline was injected i.p. once a week, whereas WFA was injected i.p. every other day. After 4 weeks of treatment, animals were sacrificed; tumor and other tissues such as un-injected ovary, lung, kidney, liver, adrenal and heart were collected from each mouse. Tumors were weighted at the time of collection. The tumors and other tissues were divided into two parts, one part was snap frozen, and second part was fixed in 10% buffered formalin. The animals' experiments were approved by the University of Louisville, Institutional Animal Care and Use Committee (IACUC) (protocol # 12063).

Formalin fixed tumor and tissues were processed and embedded in paraffin using standard protocols as described previously [56]. Five μM thick sections of the embedded tumors and tissues were prepared and stained with Hematoxylin and Eosin (H&E). Sections in triplicates were examined under microscope and photographed. Histopatholoigcal analysis of sections was performed by a trained pathologist Dr. Mana Moghadamfalahi.

Immunohistochemistry

Formalin fixed paraffin embedded tissues were deparaffinized in xylene and rehydrated in a decreasing graded series of ethanol as described previously [56]. Sections were heated at 95°C in 10 mM sodium citrate (pH 6.0) for 20 min, cooled to room temperature and then rinsed in PBS. Sections were incubated with 0.3% hydrogen peroxide in methanol for 10 min at room temperature to quench endogenous peroxidase followed by two rinses in PBS (5 min each), and were blocked with normal goat serum using reagents from ABC kit from Vector Laboratories for 60 min at room temperature following the instructions from the supplier. The blocking solution was removed by draining and sections were incubated with specific antibody with appropriate dilution according to instructions from the suppliers at 4°C for overnight in a humidified chamber. The antibodies for CD24 (cat # SAB14202713), CD44 (cat # SAB1405590), CD117 (cat # SAB4300489) and Oct4 (cat # P0873) were obtained from Sigma-Aldrich, and antibody for CD34 (cat # sc-19587) was obtained from Santa Cruz Biotechnology. After rinsing the sections three

times (5 min each) with PBS, sections were incubated with biotinylated anti-rabbit (for polyclonal antibodies) or anti-mouse (for monoclonal antibodies) from the ABC kits (Vector Laboratories) at room temperature for 45 min followed by incubation with streptavidin. After three rinses (5 min each) with PBS, sections were incubated with 3,3′-diaminobenzidine (DAB, Sigma) to develop color. The sections were examined under Nikon Elipse E400 microscope and photographed.

Protein isolation and western blot analysis

A2780 cells were plated into 6 well plates. After 24 h of plating, cells were treated with WFA and CIS both alone and in combination as described previously (10). After 48 h of treatment, cells were lysed in chilled lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 1 mM Na_3VO_4, and 1 mM NaF) supplemented with Complete Mini Protease Inhibitor tablet (Roche Molecular Biochemicals, Indianapolis, IN). To prepare extract from normal and tumor tissues, tissues were suspended in lysis buffer and homogenized on ice using Polytron homogenizer followed by centrifugation at 10,000 rpm for 5 min. The supernatants were collected and protein concentration in each sample was determined using Bradford method (BioRad Laboratories) according to supplier's instructions. Forty μg of protein from each sample was fractionated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes as described previously [56]. Blocking of nonspecific proteins was performed by incubation of the membranes with 5% nonfat dry milk in Tris buffered saline Tween-20 (TBST) for 1 h at room temperature. The membranes were incubated with specific antibody with appropriate dilution as suggested by the suppliers. Antibody for Notch 1 (cat # N6786), Hey1 (cat # SAB1404975) and β-actin (cat # A3854) were obtained from Sigma-Aldrich, and antibody for Hes1 (cat # sc-165996) was obtained from Santa Cruz Biotechnology. The membranes were washed three times (5 min each) with TBST, followed by incubation with horseradish peroxidase conjugated secondary antibody (1:5,000 dilution) in TBST. The membranes were rinsed three times (5 min each) with TBST and the immuno-reactive bands were visualized by enhanced chemiluminescence. Membranes were stripped off for 10 min with methanol containing 3% H_2O_2 and probed with β-actin antibody in order to serve as an internal control.

Statistical analysis

Statistical comparison of data was carried out by the student's t test (for single comparison). Probability of $p<0.05$ determined from the two-sided test was considered significant. The statistical analysis was carried out by using SPSS 10.0 software.

Results

WFA/CIS combination inhibits cell migration in vitro

Various steps are involved in tumor progression and metastasis including detachment of tumor cells from the primary tumor site, transmigration into lymph- or blood vessels, attachment to endothelium at distant sites of metastasis followed by seeding into new location and subsequent expansion. To examine the effect of WFA and CIS on A2780 cell migration, we treated the A2780 cells with WFA and CIS both alone and in combination for 48 h. As shown in Fig. 1, by employing Boyden chambers we noticed that the treatment of cells with WFA or CIS alone inhibited cell migration in a dose-dependent manner as compared to untreated control cells. While treatment of cells with 20 μM CIS inhibited cell migration, addition of WFA (0.5 μM or 1.5 μM) to CIS resulted in enhanced inhibition of cell migration, suggesting that

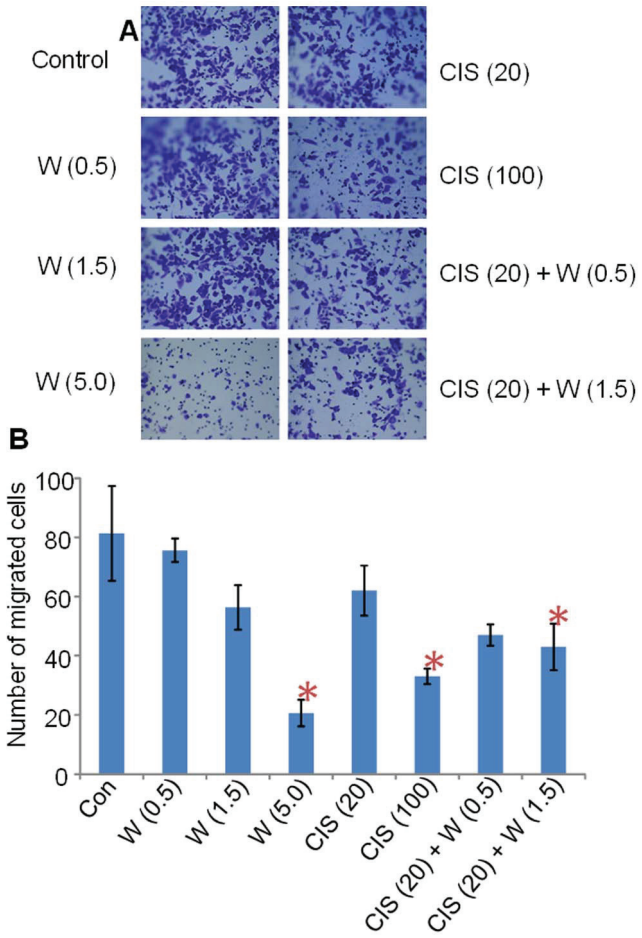

Figure 1. Effect of WFA and CIS both alone and in combination on cell migration. A2780 cells were treated with different concentration of WFA and CIS both alone and in combination for 48 h. The cells were trypsinized and subjected for cell migration using Boyden chamber. Cells were stained with crystal violet and photographed (A). The stained cells were counted under microscope using three different areas; values shown are mean ± SD of three independent experiments. * Represents significant compared to control at $p \leq 0.05$ (B). Con = control, W = WFA. Values shown in parenthesis are μM.
doi:10.1371/journal.pone.0107596.g001

WFA combined with CIS is more effective than each agent employed.

WFA/CIS combination suppresses tumor growth and metastasis in nude mice

In our *in vitro* studies, we showed that treatment of CIS-sensitive cell lines (A2780 and CaOV3) as well as CIS-resistance cell line (A2780/CP70) with WFA and CIS both alone and in combination inhibited cell proliferation in a time- and dose-dependent manner and induced cell apoptosis and DNA damage. Moreover, the combined effect of WFA and CIS was synergistic [10]. To assess the efficacy of WFA/CIS combination on tumor growth and metastasis in vivo, we tested the effect of WFA and CIS both alone and in combination on tumor growth and metastasis in nude mice bearing inoculated orthotopic human ovarian tumors. Murine orthotopic tumors were established by injecting A2780 cells directly into left ovary of 5 to 6 week old nu/nu female mice. Beginning from day 10 after inoculation of tumor cells, animals were treated with WFA and CIS both alone and in

combination as detailed in Materials and Methods section. After 4 weeks of treatment, animals were sacrificed. We noticed that the control mock-treated animals developed highly vascularized and large tumors (Fig. 2). At the same time 4 out of 5 WFA (2 mg/kg) alone-treated animals developed tumors that were significantly smaller in size. Similarly, 3 out of 5 animals treated with CIS (6 mg/kg) developed tumors that were significantly smaller in size as compared to mock-treated controls. Moreover, treatment of animals with WFA (2 mg/kg) in combination with CIS (6 mg/kg) resulted in 70 to 80% reduction in tumor weight compared to untreated control animals (Fig. 2) and out of 5 mice, only three mice developed tumors. No significant differences in tumor weight were observed in mice treated with WFA and CIS alone or in combination (Fig 2).

H&E histo-pathological analysis of un-injected opposite ovaries, livers, and lungs showed metastasis to livers and ovaries in mock-treated animals only. Metastatic cells comprised ~10% of cells in those organs (Fig. 3). In contrast no metastases were observed in WFA and CIS treated groups. These results suggest that combination of low dose of WFA (2 mg/kg) with suboptimal dose of CIS (6 mg/kg) is highly effective in suppressing tumor growth and metastasis of orthotopic ovarian tumors in nude mice. This indicates that it would be possible to reduce therapeutic dose of CIS when combined with WFA in humans to ameliorate side effects associated with high dosage of CIS.

WFA alone or in combination with CIS eliminates putative cancer stem cells in orthotopic ovarian tumors

Chemo-resistance and recurrence of ovarian cancer is a major problem and cause of death. In recent years, a concept of CSCs in solid cancers including ovarian cancers has been proposed [57,58]. CSCs have been reported to be responsible for chemo-resistance, tumor growth and recurrence of cancer after treatment. Putative CSCs have been reported as cancer initiating cells capable to develop tumors when injected into nude mice [57]. To test if WFA when used alone or in combination with CIS targets CSCs, we performed immunohistochemical analysis of the tumors collected from the mock-treated animals and animals treated with WFA and CIS both alone and in combination using the antibodies for markers expressed by putative CSCs including CD44, CD24, CD34, CD117 and Oct4 [56]. As shown in Figs. 4–6, we observed ~10–20% of cells positive for CD44, CD24, CD34, CD117 and Oct4 in tumors collected from untreated animals. However, treatment of animals with WFA (2 mg/kg) resulted in a highly significant reduction in number of those cells. In contrast, treatment of animals with CIS alone at a dose of 6 mg/kg resulted in significant increase in CD44, CD24, CD34, CD117 and Oct 4 positive cells (60%) (Figs. 4–6). More importantly, treatment of animals with WFA in combination with CIS (6 mg/kg) significantly reduced number of cells expressing CSC markers.

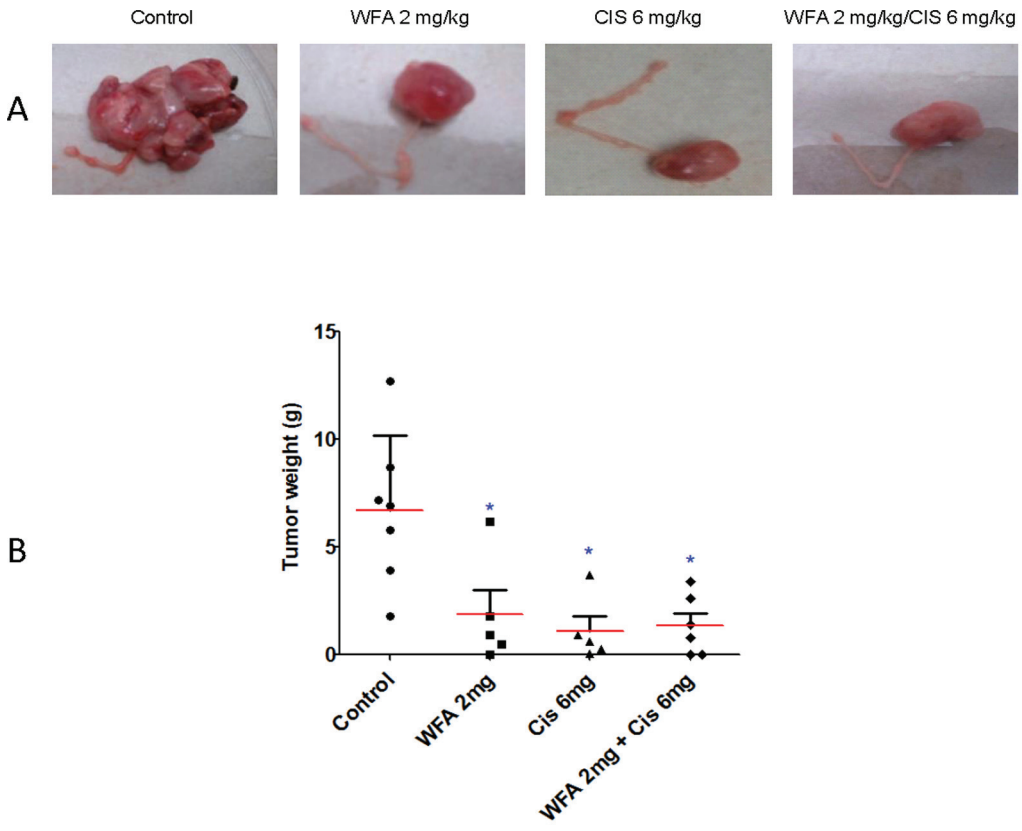

Figure 2. Effect of WFA and CIS treatment on tumor growth. A: 1×10^6 A2780 cells were injected into female mouse ovary. After 10 days of post-injection, mice were treated with WFA and CIS both alone or in combination for four weeks. Mice were sacrificed; tumors were excised out, photographed and weighted. Tumors shown are representative from each group. B: Tumors weight was plotted from each group. Horizontal line represents median weight of each group. Treated group showed significantly lower weight than untreated mice. Results are mean (red line) and ± SD (vertical bar). * Represents significant compared to control at $p \leq 0.05$.
doi:10.1371/journal.pone.0107596.g002

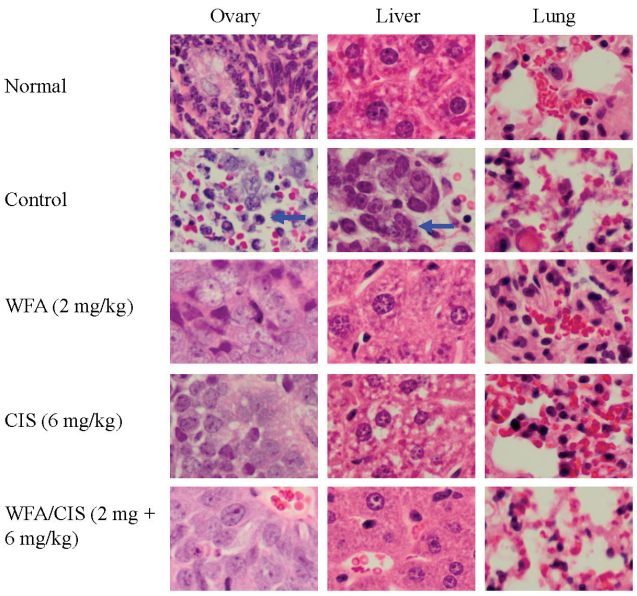

Figure 3. Effect of WFA and CIS both alone and in combination on tumor metastasis. Mice were treated with WFA and CIS as indicated in Figure 2. Tumors and other tissues sections were stained with H&E and examined by a trained pathologist. Metastasis (shown by arrows) was observed in un-injected ovaries and livers and represent approximate 10% of the cells.
doi:10.1371/journal.pone.0107596.g003

WFA alone or in combination with CIS down regulates the expression of CSC-related markers

To confirm our immuno-histochemical analysis of CD44, CD24, CD34, CD117 and Oct4 positive cells in orthotopic tumors, we performed Western blot analysis of the tumor extracts using specific antibody for markers detected by immuno-histochemical staining. As shown in Fig. 7, expression of CD24, CD34, CD44, and Oct4 antigens was significantly down-regulated in tumors collected from animals treated with WFA alone as compared to tumors from mock-treated animals. In contrast a significant increase in expression of CD24, CD34, CD44 and Oct4 was observed in tumor extracts from animals treated with CIS (6 mg/kg) as compared to mock-treated mice or mice treated with WFA (2 mg/kg) alone. Interestingly, treatment of animals with WFA (2 mg/kg) in combination with CIS (6 mg/kg) resulted in a significant elimination of cells expressing CD44, CD24, CD34 and Oct4 antigens.

Increase in number of cells expressing markers of putative CSCs in tumors collected from animals treated with CIS as analyzed by immuno-staining as well as Western blot analysis suggests that treatment by CIS may increase number of cells expressing these markers and may explain development of chemo-resistance and reoccurrence of ovarian cancer in patients treated with CIS or its derivative such as carboplatin in combination with paclitaxel that are commonly used in chemotherapy. In contrast elimination of cells expressing CSC markers in tumors on treatment with WFA alone or in combination with CIS (6 mg/kg) demonstrates that WFA is highly effective in eliminating cells expressing CSC markers.

WFA alone or in combination with CIS inhibits Notch 1 and its downstream signaling genes (Hes1 and Hey1)

Self-renewal, drug resistance and differentiation are key characteristics of CSCs. Sonic Hedgehog (Shh), Notch1, Twist1, Snail and Wnt1 signaling transduction pathways play major roles in the self-renewal of these cells [33,59–68]. WFA has been reported to inhibit Notch-1 and downstream signaling genes (Hes1 and Hey1) [33,68]. Notch 1 signaling pathway is associated with regulation of cell fate at several distinct developmental stages and has been implicated in cancer initiation and progression [63,69,70]. In our present study as shown in Fig. 8, we noticed highly significant inhibition of expression of Notch 1 and its downstream signaling genes Hes1 and Hey1 in tumors collected from mice treated with WFA (2 mg/kg) as compared to tumors from control mock-treated animals. In contrast, animals treated with CIS (6 mg/kg) showed a highly significant increase in levels of Notch1, Hes1 and Hey1 genes. What is important, tumors collected from mice treated with WFA (2 mg/kg) in combination with CIS (6 mg/kg) showed significant decreased levels of Notch1 as well as Hes1 and Hey1 proteins (Fig. 8), suggesting downregulation of Notch1 signaling by WFA alone or in combination with CIS leading to elimination of putative CSCs.

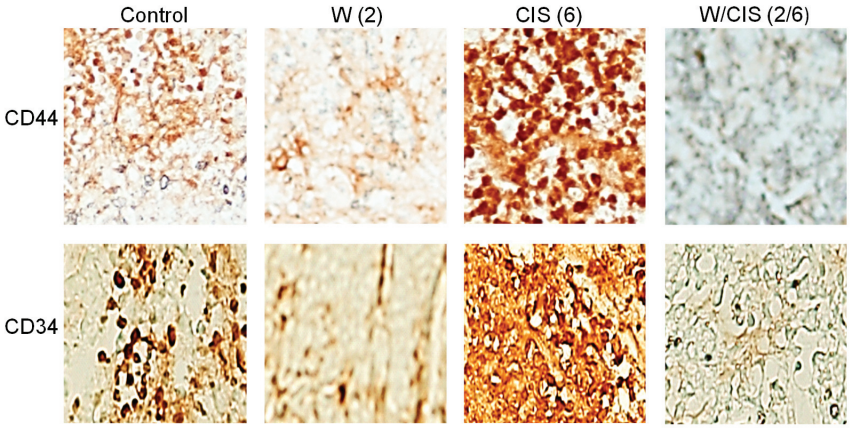

Figure 4. Immunohistochemical analysis of CD44 and CD34 positive cells in tumors collected from mock treated mice (control) and mice treated with WFA and CIS both alone and in combination. The data shown is representative of two independent experiments. W = WFA. Values shown in parenthesis are mg/kg.
doi:10.1371/journal.pone.0107596.g004

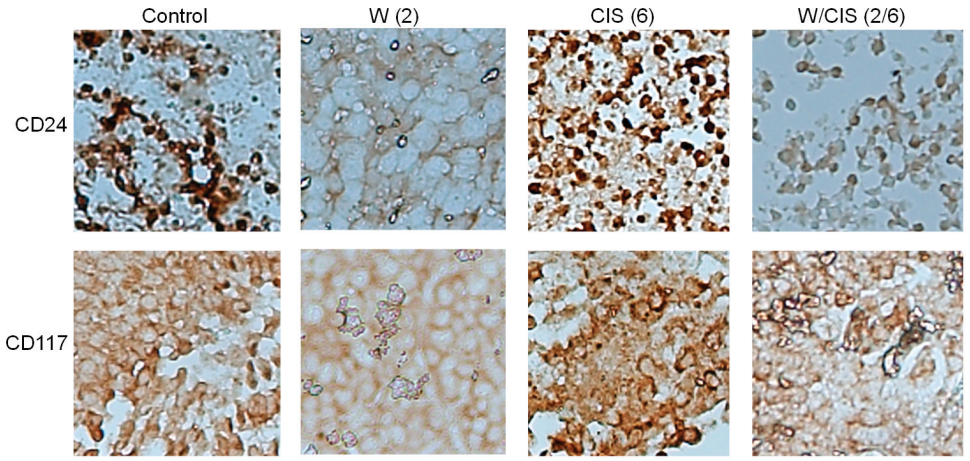

Figure 5. Immunohistochemical analysis of CD24 and CD117 positive cells in tumors collected from mock treated mice (control) and mice treated with WFA and CIS both alone and in combination. The data shown is representative of two independent experiments. W = WFA. Values shown in parenthesis are mg/kg.
doi:10.1371/journal.pone.0107596.g005

Discussion

The most common first line chemotherapy used for ovarian cancer after cytoreductive surgery is carboplatin in combination with paclitaxel. Initial response rate to this combination is very high (70 to 80%), however within 6 to 20 months after initial treatment tumor relapse and patients become resistance to CIS [71]. Resistance to CIS has been associated with number of mechanisms such as increase in glutathione and metallothionein levels, decrease in drug uptake, increase in DNA repair mechanisms (due to enhanced expression of excision repair genes) and tolerance of the formation of platinum-DNA adducts [72]. Change in status of p53 has also been reported to play important role in sensitivity of CIS [73,74].

In recent years, several investigators have reported a presence of small population of CSCs in tumor tissues to be responsible for induction of chemo-resistance and recurrence of cancer [75–77]. The convincing evidence for the role of CSCs in ovarian cancer was provided by Bapat et al. [45] who showed the presence of CSCs at single cell level in the ascites of an ovarian cancer patient, that could sequentially propagate tumor over several generations. Consistent with this, many other investigators reported the presence of CSCs in ovarian cancer cell lines, patients' ovarian tumors and tumor associated-ascites [57,76,77]. As a follow up of these observations CSCs have been isolated based on the presence of some extracellular markers. Most common makers used for ovarian CSCs include CD44, CD24, CD34, CD117 and CD133. CSCs also express ALDH1, Oct4, Myd88 and EpCAM [47,51,57,60,78,79]. An increase in number of CSCs in ovarian tumors correlates with a poor prognosis, including shorter overall and disease free survival [80–82]. Development of chemo-resistance of ovarian cancer could be explained by enrichment for CSCs [77,83–85]. In a recent study, Abubaker et al. [53] demonstrated using two ovarian cancer cell lines (epithelial OVCA433 and mesenchymal HEY) enrichment for a population of cells with high expression of CSC markers at the protein as well as mRNA levels after treatment with CIS, paclitaxel and the combination of both. In addition, these investigators showed

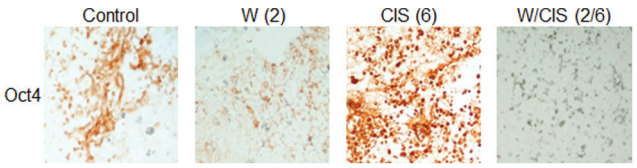

Figure 6. Immunohistochemical analysis of Oct4 positive cells in tumors collected from mock treated mice (control) and mice treated with WFA and CIS both alone and in combination. The data shown is representative of two independent experiments. W = WFA. Values shown in parenthesis are mg/kg.
doi:10.1371/journal.pone.0107596.g006

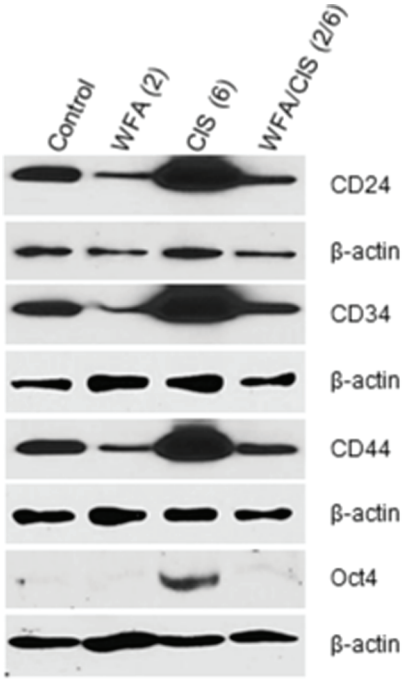

Figure 7. Western lot analysis of CD24, CD34, CD44, and Oct4 proteins from tumors collected from mock treated mice and mice treated with WFA and CIS both alone and in combination. Beta-actin was used as an internal control. The data shown is representative of two independent experiments. Con = control, W = WFA. Values shown in parenthesis are mg/kg.
doi:10.1371/journal.pone.0107596.g007

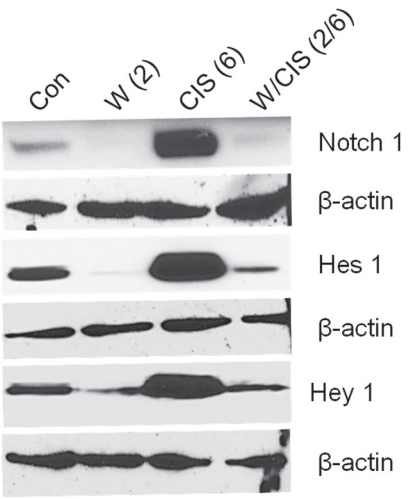

Figure 8. Western blot analysis of Notch1, Hes1 and Hey 1 proteins from tumors collected from mock treated mice and mice treated with WFA and CIS both alone and in combination. Beta-actin was used as an internal control. The data shown is representative of two independent experiments. Con = control, W = WFA. Values shown in parenthesis are mg/kg.
doi:10.1371/journal.pone.0107596.g008

increase in tumorigenic properties of ovarian cancer cells in response to chemotherapy drugs. In the present study, we show somehow in agreement with those studies [53] that the number of CSCs increases in animals bearing orthotopic ovarian tumors treated with CIS at 6 mg/kg. This increase in CSCs population in ovarian tumors of mice with CIS may explain the development of chemo-resistance and reoccurrence of ovarian cancer in patients treated with CIS or its derivative carboplatin employed in combination with paclitaxel.

Increase in number of CSCs in tumors inoculated in nude mice followed by CIS treatment is result of amplification of CSCs present in human cancer cell line A2780. On other hand growing tumor will attract host-derived normal stem cells that will provide stroma and vasculature for expanding tumor tissue. These cells could provide trophic signals for CSCs, and this is currently investigated in our laboratories.

In the past years a great deal of efforts has been devoted to develop drugs that can kill cancer cells as well as CSCs in order to reduce chemo-resistance and recurrence of cancer after treatment. WFA as reported exhibits an inhibitory effect against several different types of cancer cells. However, its effect on CSCs has not been explored so far. In our previous study [10], we demonstrated that WFA when used alone or in combination with CIS inhibits cell proliferation and induce cell death of both CIS-sensitive (A2780 and CaOV3) as well as CIS-resistant (A2780/CP70) cell lines. In our present follow-up study we show that WFA (2 mg/kg) when used alone or in combination with CIS to treat mice bearing orthotopic ovarian tumor reduced tumor growth by 70 to 80% and prevented metastasis to other organs. In addition, treatment of mice bearing orthotopic ovarian tumors with WFA alone or WFA + CIS eliminated cells that express CSC markers. (CD44, CD24, CD34, CD117 and Oct4). In contrast the number of these cells as mentioned above increased in our hands after treatment by CIS alone. Thus, our results clearly demonstrate that combination of low dose of WFA (2 mg/kg) with suboptimal dose of CIS (6 mg/kg) is highly effective in suppressing the tumor growth and elimination of putative CSCs "expanded" by CIS treatment. Since, therapeutic dose of CIS is 8 mg/kg [19], WFA in combination with CIS has potential to be highly effective and efficacious therapy for ovarian cancer and may ameliorate CIS-therapy related side effects.

Self-renewal, drug resistance and differentiation are key characteristics of CSCs and several developmental pathways such as Sonic Hedgehog (Shh), Notch, Wnt and TGFβ, Twist, and Snail which have been shown to be crucial in these processes [33,59–67,86]. WFA has been reported to inhibit Notch1 and downstream signaling genes (Hes1 and Hey1) [43,77] that have been implicated in cancer initiation and progression [63,69,70].

In our present study, we show for a first time highly significant inhibition of Notch1 and its downstream signaling proteins Hes1 and Hey1 in tumors collected from animals treated with WFA (2 mg/kg) as compared to tumors from mock-treated animals. In contrast, animals treated with CIS (6 mg/kg) alone showed a significant increase in levels of Notch1, Hes1 and Hey1 genes which is consistent with the increase in number of CSCs, suggesting an important role of Notch1 transduction pathway in amplification of those cells. More importantly, treatment of animals with WFA (2 mg/kg) + CIS (6 mg/kg) prevented increase of Notch1, Hes1 and Hey1 expression, suggests that such combined therapy ameliorates unwanted effect of CIS treatment alone and unwanted expansion of CSCs. Thus, treatment of patients that have become resistance to CIS and have developed recurrence cancer could be benefited by WFA treatment alone or in combination with CIS.

Conclusions

The silent observation from this study is that treatment of mice bearing human ovarian tumors with CIS results in an unwanted expansion of cells that express CSC markers, what may lead to CIS resistance and recurrence of ovarian tumor. In contrast, WFA if employed alone or in combination with CIS ameliorates this unwanted effect. The data obtained from our study suggest that WFA alone or in combination with CIS may serve as a safer and more efficacious therapy for both first line and second line options for ovarian cancer.

Acknowledgments

We thank Renu Kakar for expert technical assistance.

Author Contributions

Conceived and designed the experiments: SSK. Performed the experiments: SSK SKS KSP. Analyzed the data: SKS. Wrote the paper: SSK. Contributed reagents/material/planning of experiments: SKB. Edited the manuscript: MZR. Provided input in planning the experiments: DMM. Histopathological analysis of tumors and normal tissues MM.

References

1. Siegel R, Naishadham D, Jemal A (2013) Cancer statistics. CA Cancer J Clin 63: 11–30.
2. Hunn J, Rodriguez GC (2012) Ovarian cancer: etiology, risk factors, and epidemiology. Clin Obstet Gynecol 55: 3–24.
3. National Comprehensive Cancer Network. NCCN Clinical Practice Guidelines in Oncology. Ovarian Cancer. Version 1.2013. Available: HTTP://www.nccn.org/professionals/physician_gls/pdf/ovarian.pdf
4. Piccart MJ, Bertelsen K, Stuart G, Cassidy J, Mangioni C, et al. (2003) Long-term follow-up confirms a survival advantage of the paclitaxel-cisplatin regimen over the cyclophosphamide-cisplatin combination in advanced ovarian cancer. Int J Gynecol Cancer 13 Suppl 2: 144–148.
5. Matsuo K, Lin YG, Roman LD, Sood AK (2010) Overcoming platinum resistance in ovarian carcinoma. Expert Opin Investig Drugs 19: 1339–1354.

6. Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, et al. (2012) Molecular mechanisms of cisplatin resistance. Oncogene 31: 1869–1883.
7. El-Awady SE, Moustafa YM, Abo-Elmatty DM, Radwan A (2011) Cisplatin-induced cardiotoxicity: Mechanisms and cardioprotective strategies. Eur J Pharmacol 650: 335–41.
8. Kintzel PE (2001) Anticancer drug-induced kidney disorders. Drug Saf 24(1): 19–38.
9. Lieberthal W, Triaca V, Levine J (1996) Mechanisms of death induced by cisplatin in proximal tubular epithelial cells: apoptosis vs. necrosis. Am J Physiol 270: F700–708.
10. Kakar SS, Jala VR, Fong MY (2012) Synergistic cytotoxic action of cisplatin and withaferin A on ovarian cancer cell lines. Biochem Biophys Res Commun 423: 819–825.
11. Fugner A (1973) Inhibition of immunologically induced inflammation by the plant steroid withaferin A. Arzneimittelforschung 23: 932–935.
12. Rasool M, Varalakshmi P (2006) Immunomodulatory role of Withania somnifera root powder on experimental induced inflammation: An in vivo and in vitro study. Vascul Pharmacol 44: 406–410.
13. Scartezzini P, Speroni E (2000) Review on some plants of Indian traditional medicine with antioxidant activity. J Ethnopharmacol 71: 23–43.
14. Gupta SK, Mohanty I, Talwar KK, Dinda A, Joshi S, et al. (2004) Cardioprotection from ischemia and reperfusion injury by Withania somnifera: a hemodynamic, biochemical and histopathological assessment. Mol Cell Biochem 260: 39–47.
15. Stan SD, Hahm ER, Warin R, Singh SV (2008) Withaferin A causes FOXO3a- and Bim-dependent apoptosis and inhibits growth of human breast cancer cells in vivo. Cancer Res 68: 7661–7669.
16. Mohan R, Hammers HJ, Bargagna-Mohan P, Zhan XH, Herbstritt CJ, et al. (2004) Withaferin A is a potent inhibitor of angiogenesis. Angiogenesis 7: 115–122.
17. Srinivasan S, Ranga RS, Burikhanov R, Han SS, Chendil D (2007) Par-4-dependent apoptosis by the dietary compound withaferin A in prostate cancer cells. Cancer Res 67: 246–253.
18. Stan SD, Zeng Y, Singh SV (2008) Ayurvedic medicine constituent withaferin a causes G2 and M phase cell cycle arrest in human breast cancer cells. Nutr Cancer 60 Suppl 1: 51–60.
19. Munagala R, Kausar H, Munjal C, Gupta RC (2011) Withaferin A induces p53-dependent apoptosis by repression of HPV oncogenes and upregulation of tumor suppressor proteins in human cervical cancer cells. Carcinogenesis 32: 1697–16705.
20. Yu Y, Hamza A, Zhang T, Gu M, Zou P, et al. (2010) Withaferin A targets heat shock protein 90 in pancreatic cancer cells. Biochem Pharmacol 79: 542–551.
21. Mayola E, Gallerne C, Esposti DD, Martel C, Pervaiz S, et al. (2011) Withaferin A induces apoptosis in human melanoma cells through generation of reactive oxygen species and down-regulation of Bcl-2. Apoptosis 16: 1014–1027.
22. Choi MJ, Park EJ, Min KJ, Park JW, Kwon TK (2011) Endoplasmic reticulum stress mediates withaferin A induced apoptosis in human renal carcinoma cells. Toxicol In Vitro 25: 692–698.
23. Samadi AK, Mukerji R, Shah A, Timmermann BN, Cohen MS (2010) A novel RET inhibitor with potent efficacy against medullary thyroid cancer in vivo. Surgery 148: 1228–1236.
24. Samadi AK, Tong X, Mukerji R, Zhang H, Timmermann BN, et al. (2010) Withaferin A, a cytotoxic steroid from Vassobia breviflora, induces apoptosis in human head and neck squamous cell carcinoma. J Nat Prod 73: 1476–1481.
25. Shah N, Kataria H, Kaul SC, Ishii T, Kaur G, et al. (2009) Effect of the alcoholic extract of Ashwagandha leaves and its components on proliferation, migration, and differentiation of glioblastoma cells: combinational approach for enhanced differentiation. Cancer Sci 100: 1740–1747.
26. Oh JH, Lee TJ, Kim SH, Choi YH, Lee SH, et al. (2008) Induction of apoptosis by withaferin A in human leukemia U937 cells through down-regulation of Akt phosphorylation. Apoptosis 13: 1494–1504.
27. Oh JH, Kwon TK (2009) Withaferin A inhibits tumor necrosis factor-alpha-induced expression of cell adhesion molecules by inactivation of Akt and NF-kappaB in human pulmonary epithelial cells. Int Immunopharmacol 9: 614–619.
28. Mayola E, Gallerne C, Esposti DD, Martel C, Pervaiz S, et al. (2011) Withaferin A induces apoptosis in human melanoma cells through generation of reactive oxygen species and down-regulation of Bcl-2. Apoptosis 16: 1014–27.
29. Roy RV, Suman S, Das TP, Luevano JE, Damodaran C (2013) Withaferin A, a steroidal lactone from Withania somnifera, induces mitotic catastrophe and growth arrest in prostate cancer cells. J Nat Prod 76: 1909–1915.
30. Malik F, Kumar A, Bhushan S, Khan S, Bhatia A, et al. (2007) Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic cell death of human myeloid leukemia HL-60 cells by a dietary compound withaferin A with concomitant protection by N-acetyl cysteine. Apoptosis 12: 2115–2133.
31. Lee TJ, Um HJ, Min do S, Park JW, Choi KS, et al. (2009) Withaferin A sensitizes TRAIL-induced apoptosis through reactive oxygen species-mediated up-regulation of death receptor 5 and downregulation of c-FLIP. Free Radic Biol Med 46: 1639–1649.
32. Hahm ER, Moura MB, Kelley EE, Van Houten B, Shiva S, et al. (2011) Withaferin a-induced apoptosis in human breast cancer cells is mediated by reactive oxygen species. PLoS One 6: e23354.
33. Koduru S, Kumar R, Srinivasan S, Evers MB, Damodaran C (2010) Notch-1 inhibition by Withaferin-A: a therapeutic target against colon carcinogenesis. Mol Cancer Ther 9: 202–210.
34. Hermann PC, Huber SL, Heeschen C (2008) Metastatic cancer stem cells: a new target for anti-cancer therapy? Cell Cycle 7: 188–193.
35. Dean M, Fojo T, Bates S (2005) Tumour stem cells and drug resistance. Nat Rev Cancer 5: 275–284.
36. Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3: 730–737.
37. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, et al. (1994) A cell initiating human acute myeloid leukemia after transplantation into SCID mice. Nature 367: 645–648.
38. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100: 3983–3988.
39. Medema JP (2013) Cancer stem cells: the challenges ahead. Nat Cell Biol 15: 338–344.
40. Chen L, Kasai T, Li Y, Sugii Y, Jin G, et al. (2012) A model of cancer stem cells derived from mouse induced pluripotent stem cells. PLoS One 7: e33544.
41. Driessens G, Beck B, Caauwe A, Simons BD, Blanpain C (2012) Defining the mode of tumour growth by clonal analysis. Nature 488: 527–530.
42. Schepers AG, Snippert HJ, Stange DE, van den Born M, van Es JH, et al. (2012) Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. Science 337(6095): 730–735.
43. Quintana E, Shackleton M, Sabel MS, Fullen DR, Johnson TM, et al. (2008) Efficient tumour formation by single human melanoma cells. Nature 456: 593–598.
44. Dalerba P, Cho RW, Clarke MF (2007) Cancer stem cells: models and concepts. Annu Rev Med 58: 267–284.
45. Bapat SA, Mali AM, Koppikar CB, Kurrey NK (2005) Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res 65: 3025–3029.
46. Kurrey NK, Amit K, Bapat SA (2005) Snail and Slug are major determinants of ovarian cancer invasiveness at the transcription level. Gyne Oncol 97: 155–165.
47. Zhang S, Balch C, Chan MW, Lai HC, Matei D, et al. (2008) Identification and characterization of ovarian cancer-initiating cells from primary human tumors, Cancer Res 68: 4311–4320.
48. Deng S, Yang X, Lassus H, Liang S, Kaur S, et al. (2010) Distinct expression levels and patterns of stem cell marker, aldehyde dehydrogenase isoform 1 (ALDH1), in human epithelial cancers. PLoS ONE 5: e10277.
49. Silva IA, Bai S, McLean K, Yang K, Griffith K, et al. (2011) Aldehyde dehydrogenase and CD133 define angiogenic ovarian cancer stem cells that portend poor patient survival, Cancer Res 71: 3991–4001.
50. Dyall S, Gayther SA, Dafou D (2010) Cancer stem cells and epithelial ovarian cancer. J Oncol 2010: 105269.
51. Alvero AB, Chen R, Fu HH, Montagna M, Schwartz PE, et al. (2009) Molecular phenotyping of human ovarian cancer stem cells unravels the mechanisms for repair and chemoresistance. Cell Cycle 8: 158–166.
52. Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, et al. (2006) Cancer stem cells–perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res 66: 9339–9344.
53. Abubaker K, Latifi A, Luwor R, Nazaretian S, Zhu H, et al. (2013) Short-term single treatment of chemotherapy results in the enrichment of ovarian cancer stem cell like cells leading to an increased tumor burden. Mol Cancer 12: 24.
54. Malik MT, Kakar SS (2006) Regulation of angiogenesis and invasion by human Pituitary tumor transforming gene (PTTG) through increased expression and secretion of matrix metalloproteinase-2 (MMP-2). Mol Cancer 5: 61.
55. Nunez-Cruz S, Connolly DC, Scholler N (2010) An orthotopic model of serous ovarian cancer in immunocompetent mice for in vivo tumor imaging and monitoring of tumor immune responses. J Vis Exp 28.pii: 2146.
56. Fong MY, Jin S, Rane M, Singh RK, Gupta R, et al. (2012) Withaferin A synergizes the therapeutic effect of doxorubicin through ROS-mediated autophagy in ovarian cancer. PLoS One 7: e42265.
57. Tomao F, Papa A, Strudel M, Rossi L, Lo Russo G, et al. (2014) Investigating Molecular Profiles of Ovarian Cancer: An Update on Cancer Stem Cells. J Cancer 5: 301–310.
58. Ramdass B, Duggal R, Minev B, Chowdhary A, Koka P (2013) Functional role of solid tumor stem cells in disease etiology and susceptibility to therapeutic interventions. J Stem Cells 8: 189–231.
59. Ponnusamy MP, Batra SK (2008) Ovarian cancer: emerging concept on cancer stem cells. J Ovarian Res, 1: 4.
60. Alvero AB, Fu HH, Holmberg J, Visintin I, Mor L, et al. (2009) Stem like ovarian cancer cells can serve as tumor vascular progenitors. Stem Cells 7: 2405–2413.
61. Reya T, Clevers H (2005) Wnt signaling in stem cells and cancer. Nature 434: 843
62. Liu S, Dontu G, Mantle ID, Patel S, Ahn NS, et al. (2006) Hedgehogsignaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. Cancer Res 66: 6063–6071.
63. Dontu G, Jackson KW, McNicholas E, Kawamura MJ, Abdallah WM, et al. (2004) Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells, Breast Cancer Res 6: 605–615.

64. Giles RH, van Es JH, Clevers H (2003) Caught up in a Wnt storm: wnt signaling in cancer, Biochim Biophys Acta 1653: 1–24.
65. Mimeault M, Batra SK (2012) Novel biomarkers and therapeutic targets for optimizing the therapeutic management of melanomas. World J Clin Oncol 3: 32–42.
66. Wu KJ, Yang MH (2011) Epithelial-mesenchymal transition and cancer stemness: the Twist1-Bmi1connection. Biosci Rep 31: 449–455.
67. Jain P, Alahari SK (2011) Breast cancer stem cells: a new challenge for breast cancer treatment. Front Biosci 16: 1824–1832.
68. Suman S, Das TP, Damodaran C (2013) Silencing NOTCH signaling causes growth arrest in both breast cancer stem cells and breast cancer cells. Br J Cancer, 109: 2587–2596.
69. Shi W, Harris AL (2006) Notch signaling in breast cancer and tumor angiogenesis: cross-talk and therapeutic potentials. J Mammary Gland Biol Neoplasia 11: 41–52.
70. Gangopadhyay S, Nandy A, Hor P, Mukhopadhyay A (2013) Breast cancer stem cells: a novel therapeutic target. Clin Breast Cancer 13: 7–15.
71. Lengyel E (2010) Ovarian cancer development and metastasis. Am J Pathol 177: 1053–1064.
72. Christen RD, Isonishi S, Jones JA, Jekunen AP, Hom DK, et al. (1994) Signaling and drug sensitivity. Cancer Metastasis Rev 13: 175–189.
73. Brown R, Clugston C, Burns P, Edlin A, Vasey P, et al. (1993) Increased accumulation of p53 protein in cisplatin-resistant ovarian cell lines. Int J Cancer 55: 678–684.
74. Perego P, Giarola M, Righetti SC, Supino R, Caserini C, et al. (1996) Association between cisplatin resistance and mutation of p53 gene and reduced bax expression in ovarian carcinoma cell systems. Cancer Res 56: 556–62.
75. Hollier BG, Evans K, Mani SA (2009) The epithelial-to-mesenchymal transition and cancer stem cells: a coalition against cancer therapies. J Mammary Gland Biol Neoplasia 14: 29–43.
76. Ahmed N, Abubaker K, Findlay J, Quinn M (2010) Epithelial mesenchymal transition and cancer stem cell-like phenotypes facilitate chemoresistance in recurrent ovarian cancer. Curr Cancer Drug Targets 10: 268–278.
77. Latifi A, Luwor RB, Bilandzic M, Nazaretian S, Stenvers K, et al. (2012) Isolation and characterization of tumor cells from the ascites of ovarian cancer patients: molecular phenotype of chemoresistant ovarian tumors. PLoS One 7: e46858.
78. Gao MQ, Choi YP, Kang S, Youn JH, Cho NH (2010) CD24+ cells from hierarchically organized ovarian cancer are enriched in cancer stem cells. Oncogene 29: 2672–2680.
79. Landen CN Jr, Goodman B, Katre AA, Steg AD, Nick AM, et al. (2010) Targeting aldehyde dehydrogenase cancer stem cells in ovarian cancer. Mol Cancer Ther 9: 3186–3199.
80. Guo X, Xiong L, Sun T, Peng R, Zou L, et al. (2012) Expression features of SOX9 associate with tumor progression and poor prognosis of hepatocellular carcinoma. Diagn Pathol 7: 44.
81. Surowiak P, Materna V, Maciejczyk A, Kaplenko I, Spaczynski M, et al. (2006) CD46 expression is indicative of shorter revival-free survival for ovarian cancer patients. Anticancer Res 26: 4943–4948.
82. Oh DH, Kim SH, Jung S, Sung YK, Bang SY, et al. (2011) Precuneus hypoperfusion plays an important role in memory impairment of patients with systemic lupus erythematosus. Lupus 20: 855–860.
83. Steg AD, Bevis KS, Katre AA, Ziebarth A, Dobbin ZC, et al. (2012) Stem cell pathways contribute to clinical chemoresistance in ovarian cancer. Clin Cancer Res 18: 869–881.
84. Szotek PP, Pieretti-Vanmarcke R, Masiakos PT, Dinulescu DM, Connolly D, et al. (2006) Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness. Proc Natl Acad Sci U S A 103: 11154–11159.
85. Vathipadiekal V, Saxena D, Mok SC, Hauschka PV, Ozbun L, et al. (2012) Identification of a potential ovarian cancer stem cell gene expression profile from advanced stage papillary serous ovarian cancer. PLoS One 7: e29079.
86. Kwon MJ, Shin YK (2013) Regulation of ovarian cancer stem cells or tumor-initiating cells. Int J Mol Sci 14: 6624–6648.