European Journal of Integrative Medicine 25 (2019) 6–12

Contents lists available at ScienceDirect

European Journal of Integrative Medicine

journal homepage: www.elsevier.com/locate/eujim

Research paper

Hydroxysafflor yellow A sensitizes ovarian cancer cells to chemotherapeutic agent by decreasing WSB1 expression

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ARTICLE INFO

Keywords:
Hydroxysafflor yellow A
Ovarian neoplasms
Antineoplastic agents
Microarray analysis

ABSTRACT

Introduction: *Carthamus tinctorius* L. (safflower) is a traditional Chinese medicine, the active ingredient of which is hydroxysafflor yellow A (HSYA).[2] HSYA has been shown to have the potential to inhibit tumor growth. However, the molecular mechanisms whereby HSYA exerts its antitumor functions remain largely unclear. In this study, we investigated the antitumor mechanisms of HSYA in ovarian cancer cell line Skov3.

Methods: The cell proliferation assay was conducted using a Cell Proliferation Assay kit. The cell viability assay was performed using the CellTiter-Blue Cell Viability kit. Microarray was conducted to identify the global gene expression change of ovarian cancer cells caused by HSYA treatment. Small interfering RNA (SiRNA)[3] transfection was conducted to knock down WD repeat and SOCS box-containing protein 1 (WSB1)[4]. WSB1 expression was detected by quantitative reverse transcription-quantitative polymerase chain reaction (qRT-PCR)[5]. The protein expression of extracellular signal-related kinase (Erk)[6]1/2 and phosphorylation-Erk1/2 was detected by western blot.

Results: HSYA inhibited Skov3 cell proliferation in a dose-dependent manner ($P < 0.05$). When cells were cultured with HSYA and doxorubicin, cell viability was further reduced ($P < 0.05$). HSYA could decrease the expression of WSB1. Through knocking down of WSB1, ovarian cancer cell proliferation was inhibited and further reduced by treating with doxorubicin ($P < 0.05$), the expression of Erk1/2 and Erk phosphorylation were downregulated.

Conclusion: HSYA may inhibit ovarian cancer cell line Skov3 proliferation and sensitize Skov3 cells to chemotherapeutic agents through the reduction of WSB1 expression.

Abbreviations: HSYA, hydroxysafflor yellow A; SiRNA, small interfering RNA; WSB1, WD repeat and SOCS box-containing protein 1; qRT-PCR, quantitative reverse transcription-quantitative polymerase chain reaction; Erk, extracellular signal-related kinase; SY, safflor yellow; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; PPAR, peroxisome proliferator-activated receptor; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MMP, metalloproteinase; MAPK, mitogen-activated protein kinase; PPP2R3C, protein phosphatase 2, regulatory subunit B", gamma; INPP4B, inositol poly-phosphate 4-phosphatase type II; PNMA1, paraneoplastic antigen MA1

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[2] HSYA: hydroxysafflor yellow A
[3] SiRNA: Small interfering RNA
[4] WSB1: WD repeat and SOCS box-containing protein 1
[5] qRT-PCR: quantitative reverse transcription-quantitative polymerase chain reaction
[6] Erk: extracellular signal-related kinase

https://doi.org/10.1016/j.eujim.2018.11.007
Received 4 June 2018; Received in revised form 23 November 2018; Accepted 23 November 2018

1. Introduction

Ovarian cancer is the fifth leading cause of cancer-related mortality and is the most lethal gynecologic cancer among women [1]. Epithelial ovarian cancer comprises the most frequent form of ovarian cancer and has the highest mortality rate [2]. Ovarian cancer is typically treated with surgery and/or chemotherapy. Chemotherapy is an important option for patients with ovarian cancer, particularly in instances where the spread of cancer cells precludes surgical debulking [3]. The majority of patients respond to chemotherapy initially. However, many advanced patients suffer high rate recurrence [4]. Chemoresistance is defined by recurrence of the disease less than six months after the first treatment and most mortality induced by ovarian cancer is caused by recurrence and metastasis [5]. Given the limited progression to improve the outcomes of patients with ovarian cancer, further investigations are required to discover novel agents that may inhibit primary ovarian cancer progression or reduce its recurrence.

Carthamus tinctorius L. (safflower) is a traditional Chinese medicine and has been used to improve blood circulation for a long time [6]. Safflower serves effective roles in inhibition of cell proliferation and the induction of apoptosis in breast cancer [7]. Safflower enhances antitumor activity through promoting the recognition of antigens and facilitating antigen presentation by activating dendritic cells [8]. Safflor yellow (SY) has been identified to be the effective section of safflower, and hydroxysafflor yellow A (HSYA) is the active ingredient of SY [9]. HSYA inhibits proliferation of vascular smooth muscle cells by reducing extracellular signal-related kinase (Erk) 1/2 expression, suggesting its role in inhibiting angiogenesis [10]. HSYA has been suggested to protect cardiomyocytes against apoptosis by upregulating heme oxygenase-1 expression through the phosphoinositide 3-kinase/Akt/Nrf2 signaling pathway [11]. In addition, some studies indicate that HSYA may have tumor inhibitory potential. HSYA inhibits secretion of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR) to suppress liver tumor growth. The underlying mechanism is that HSYA blocks Erk1/2 phosphorylation and decreases the expression of p65 in the nucleus to restrain the activation of NF-kB [12]. Another study has demonstrated that HSYA inhibits the proliferation of human gastric carcinoma BGC-823 cell by promoting the nuclear translocation of peroxisome proliferator-activated receptor (PPAR), induces cell cycle arrest and cell apoptosis [13]. As HSYA has been suggested to have tumor inhibitory functions by several studies, the present study aims to investigate HSYA's impacts on ovarian cancer cell proliferation and sensitizing ovarian cancer cells to chemotherapeutic agents. In addition, the current study aimed to evaluate the effects of a traditional Chinese medicine combined with classical chemotherapeutic agents.

2. Materials and methods

2.1. Cell culture

Skov3 cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all purchased from Gibco; Thermo Fisher Scientific, Inc.). The cells were incubated in 5% CO2 at 37 °C in a humidified incubator.

2.2. Materials and reagents

Triton X-100, Nonidet P40 (NP-40), leupeptin, Phenylmethanesulfonyl fluoride (PMSF) and doxorubicin were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Antibodies against Erk, phosphorylated (p)-Erk and actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). HSYA was purchased from the National Institute for Food and Drug Control (Beijing, China). All other chemicals were of analytical grade.

2.3. Cell proliferation assay

Cells were plated in 96-well plates cultured in high-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were treated with HSYA at 0, 10, 20, 50, 100 and 150 mg/l for 72 h with a medium change every 24 h. The cell proliferation assay was conducted at indicated time points using a Cell Proliferation Assay kit (cat. no. G3580; Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The data was recorded on a SpectraMax M5 microplate reader.

2.4. Cell viability assay

Cells were plated in 96-well plates cultured in high-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were treated with HSYA at indicated concentrations for 48 h with a medium change for every 24 h. Doxorubicin was added at 0, 0.1, 1 and 10 μM to the medium 24 h prior to the assay. A cell viability assay was performed using the CellTiter-Blue Cell Viability kit (cat. no. G8080; Promega Corporation) according to the manufacturer's instructions. The data was collected on a SpectraMax M5 microplate reader.

2.5. Microarray

Shanghai Biotechnology Co., Ltd. (Shanghai, China) processed samples for microarray studies according to the company's standard operating procedures. Briefly, Skov3 cells were treated with HSYA for 48 h. Total RNA was extracted from the cells, and 500 ng total RNA was used for labeling and hybridization, and for running the microarray (Affymetrix, Inc., Santa Clara, CA, USA). The fold change between the control and HSYA groups was ranked.

2.6. Small interfering (si) RNA and transfection

SiRNA and negative controls were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). SiRNA transfection was conducted with Lipofectamine® RNAiMAX Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Cells were harvested after 48 h transfection and subjected to RNA isolation.

2.7. RNA isolation and quantitative reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RNA extraction from cells was performed with an EastepTM Universal RNA Extraction kit (cat. no. LS1030; Promega Corporation) according to the manufacturer's instructions. cDNA synthesis were performed with a Moloney murine leukemia virus system (cat. no. M1705; Promega Corporation) using 1 μg portion of the total RNA. Reverse-transcribed DNA was subjected to PCR. qPCR was conducted with a SYBR Green qPCR kit (cat. no. PR7012; Bioteke Corporation, Beijing, China) according to the manufacturer's instructions. The primers for WSB1 were as follows: Forward 5′-TCTCCTGACTCTTCTATG CTGTGT-3′, and reverse 5′-CATGGTGTATTTATCCATATTCCAAA-3′. The primers were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). qPCR was conducted on AN Abbott m2000 RealTime System (Abbott Laboratories, Chicago, IL, USA). Relative expression was calculated by $2^{-\Delta\Delta CT}$ method.

2.8. Western blot

Cells were rocked in radioimmunoprecipitation assay buffer overnight at 4 °C, and then centrifuged at 14,000 rpm for 30 min at 4 °C. Protein concentration of cell lysates were standardized with a Protein Concentration Assay kit (cat. no. SK3021; Sangon Biotech Co., Ltd., Shanghai, China) and resolved by sodium dodecyl sulfate

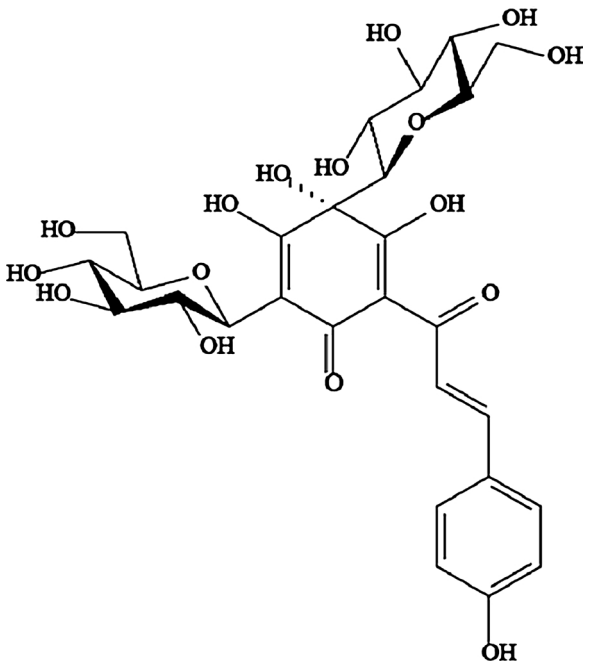

Fig. 1. Molecular structure of hydroxysafflor yellow A.

polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis and immunoblotting were performed as described previously [14].

2.9. Statistical analysis

The experimental data were expressed as mean values ± standard deviation, multi-group comparison were evaluated using One-Way ANOVA test analysis. All statistical analyses were performed by SPSS20.0 statistical software (IBM, Armonk, NY, USA). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Molecular structure of HSYA

The molecular structure of HSYA is shown in Fig. 1. The molecular weight of HSYA is 612. HSYA is a water soluble agent and has been used as one of the oral medicines for a long time in China. Thus, in this study HSYA was directly used to treat ovarian cancer Skov3 cells.

3.2. HSYA decreases ovarian cancer cell proliferation

As HSYA has been previously reported to inhibit tumor growth, the effects of HSYA on ovarian cancer cell Skov3 proliferation were investigated. Skov3 cells were treated with HSYA at different concentrations for 72 h. As shown in Fig. 2, HSYA inhibits Skov3 cell proliferation in a dose-dependent manner. When the concentration of HSYA was increased to 100 mg/l, the highest inhibitory ratio could be observed. The result indicated that HSYA could significantly inhibit ovarian cancer cell proliferation ($P < 0.05$), which is in line with the results from animal studies.

A cell proliferation assay was performed 72 h after treatment with HSYA at various concentrations. The data represent the mean values ± standard deviation of four independent experiments. *$P < 0.05$ vs. 0 mg/l HYSA. **$P < 0.01$ vs. 0 mg/l HYSA. HSYA: hydroxysafflor yellow A; OD: optical density.

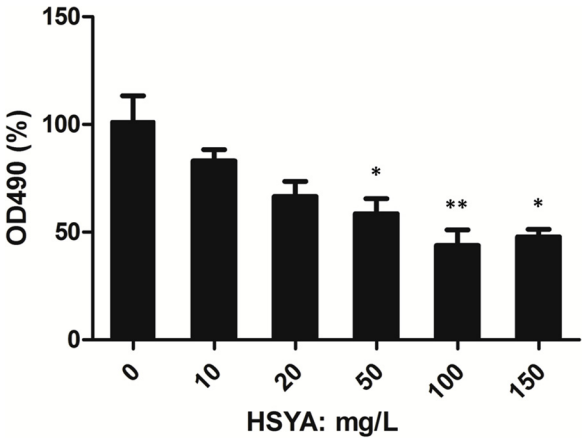

Fig. 2. HSYA inhibits Skov3 cell proliferation.

3.3. HSYA decreases ovarian cancer cell viability and sensitizes cells to chemotherapeutic agents

As HSYA has been demonstrated to inhibit ovarian cancer cell proliferation, the current study aimed to investigate the influence on cell viability when HSYA was used in combination with doxorubicin, a clinical chemotherapeutic agent for ovarian cancer treatment. Skov3 cells were first treated with HSYA at different concentrations for 24 h. Doxorubicin at different concentrations was added to the medium for another 24 h. As shown in Fig. 3, HSYA decreased Skov3 cell viability in a dose-dependent manner. Furthermore, when cells were cultured together with doxorubicin, cell viability was further reduced. This result indicated that HSYA treatment, in combination with chemotherapeutic agents, may have synergetic effect in ovarian cancer therapy.

Cell viability of Skov3 was evaluated 48 h after treatment with HSYA at various concentrations. Doxorubicin was added at various concentrations 24 h prior to the viability assay. The data represent the mean values ± standard deviation of three independent experiments (*$P < 0.05$). HSYA: hydroxysafflor yellow A.

3.4. HSYA downregulates WSB1 expression

To identify the global gene expression change of ovarian cancer cells caused by HSYA treatment, microarray was conducted in this study. A total of 20 genes were identified as being altered by HSYA treatment, with 9 genes upregulated > 2 fold, and 11 genes downregulated to < 0.5 fold (Fig. 4A). WSB1 was then focused on, as it has been demonstrated to serve roles in the growth, survival and progression of several types of cancer, and three transcripts of WSB1 are downregulated by HSYA [15–17]. The results verified the downregulation of WSB1 expression under HSYA treatment with RT-qPCR. HSYA significantly decreased WSB1 expression at the mRNA level ($P < 0.05$; Fig. 4B). However, the functions of WSB1 in ovarian cancer progression remain largely unknown.

(A) Heat map represents normalized gene expression levels obtained from the microarray data of Skov3 cells with and without HSYA treatment. Three different transcripts of *WSB1* were downregulated by HSYA treatment. (B) Downregulation of *WSB1* expression by HSYA was validated through quantitative reverse transcription-polymerase chain reaction. The data represent the mean values ± standard deviation of three independent experiments. *$P < 0.05$ vs. the control. HSYA: hydroxysafflor yellow A. WSB1: WD repeat and SOCS box-containing protein 1.

3.5. Knockdown of WSB1 decreases ovarian cancer cell proliferation

To investigate the functions of WSB1 in ovarian cancer progression,

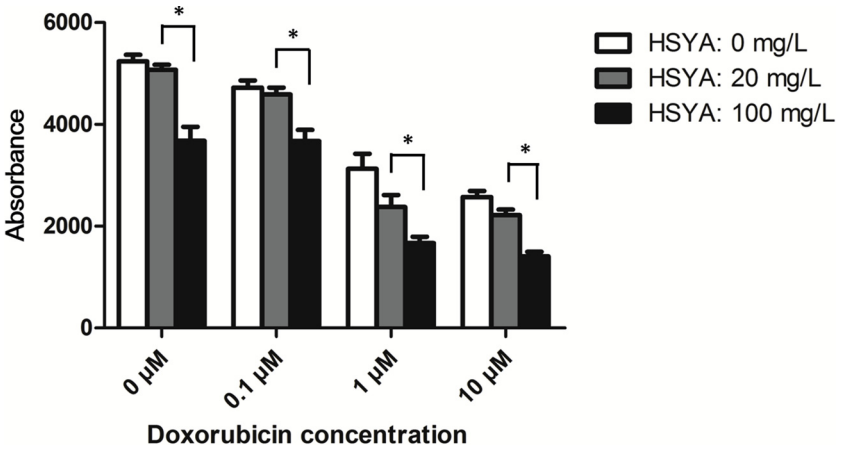

Fig. 3. HSYA decreases cell viability and sensitizes Skov3 cells to doxorubicin.

WSB1 siRNA were synthesized. Three siRNA sequences were designed to knockdown WSB1 expression in Skov3 cells. As shown in Fig. 5A, siRNA 1′ and siRNA 2′ could significantly reduce WSB1 expression ($P < 0.05$). siRNA 3′ showed no obvious effects on WSB1 expression. A cell proliferation assay was performed following WSB1 knockdown. As shown in Fig. 5B, siRNA 1′ and siRNA 2′ inhibited Skov3 cell proliferation, which indicated that HSYA could inhibit ovarian cancer cell proliferation at least partially by decreasing WSB1 expression.

(A) 48 h after transfection of scramble or WSB1 siRNA, mRNA expression of *WSB1* was determined by quantitative reverse transcription-polymerase chain reaction. The data represent the mean values ± standard deviation of three independent experiments. (B) Cell proliferation assay was performed 72 h after transfection of scramble or *WSB1* siRNAs. The data represent the mean values ± standard deviation of four independent experiments. *$P < 0.05$ vs. scramble siRNA. SiRNA: small interfering RNA. WSB1: WD repeat and SOCS box-containing protein 1.

3.6. Knockdown of WSB1 decreases ovarian cancer cell proliferation and sensitizes cells to chemotherapeutic agents

The effects of WSB1 knockdown on Skov3 cell viability were investigated. As shown in Fig. 6, siRNA 1′ and siRNA 2′ of WSB1 decreased Skov3 cell viability. In accordance with the result in Fig. 3, when Skov3 cells were treated with WSB1 siRNA, the cells were more sensitive to doxorubicin. This result indicated that at least partially the

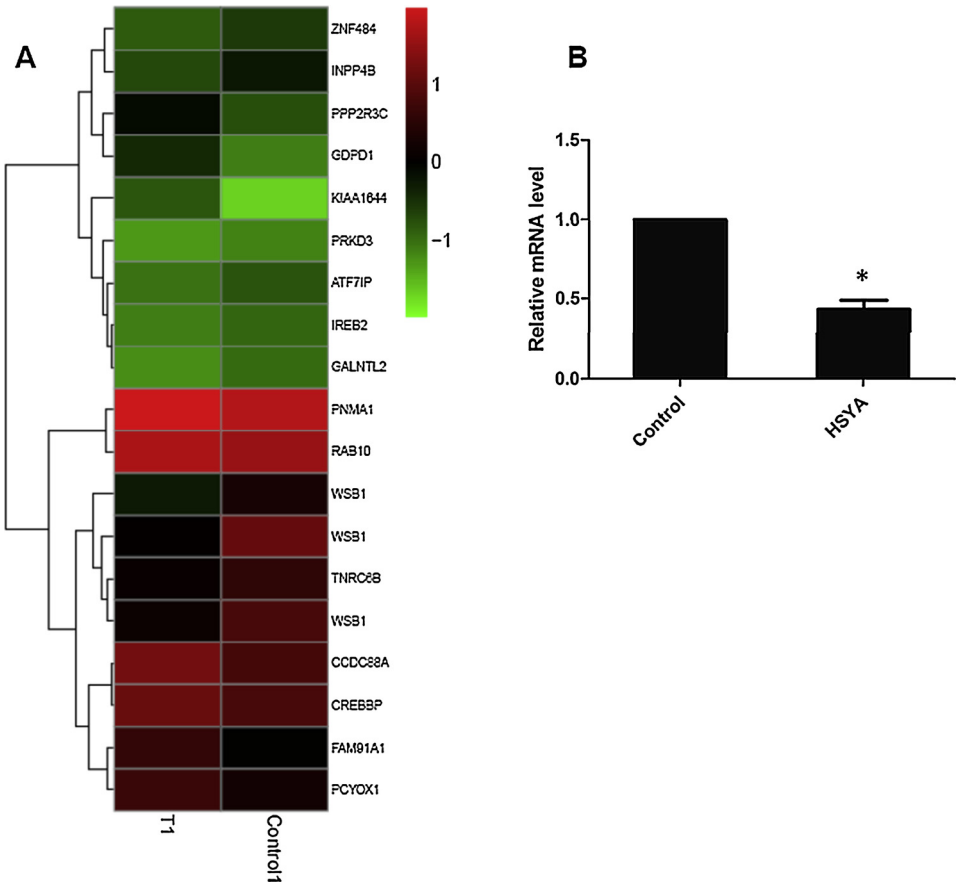

Fig. 4. HSYA decreases WSB1 expression.

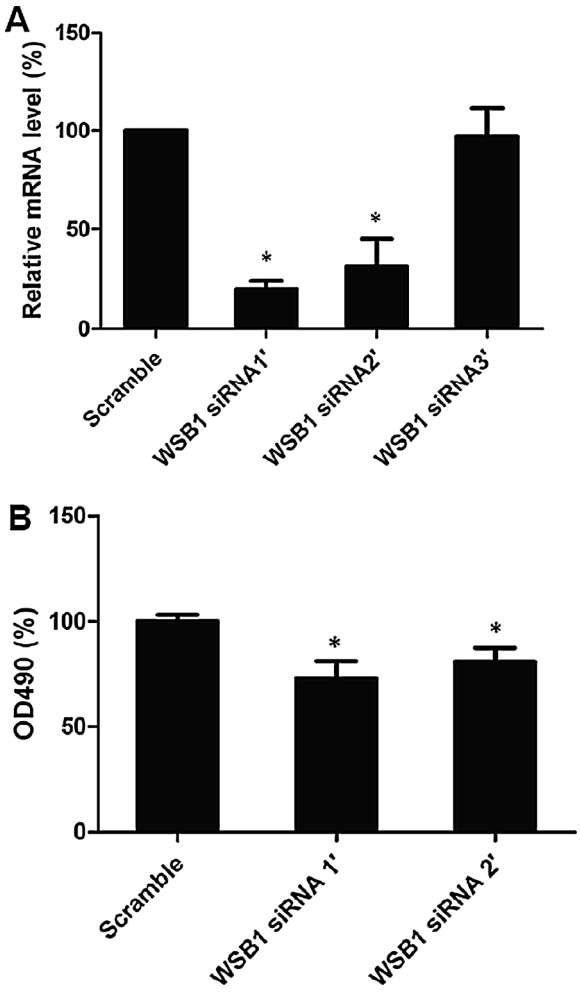

Fig. 5. WSB1 knockdown inhibits Skov3 cell proliferation.

reduction of WSB1 expression could explain the effect of HSYA on ovarian cancer cell viability and the synergetic effects when HSYA was used in combination with doxorubicin.

Cell viability of Skov3 was evaluated 48 h after transfection of scramble or *WSB1* siRNA. Doxorubicin in various concentrations was added 24 h prior to the viability assay. The data represent the mean values ± standard deviation of three independent experiments. *P < 0.05 vs. scramble siRNA. SiRNA: small interfering RNA. WSB1: WD repeat and SOCS box-containing protein 1.

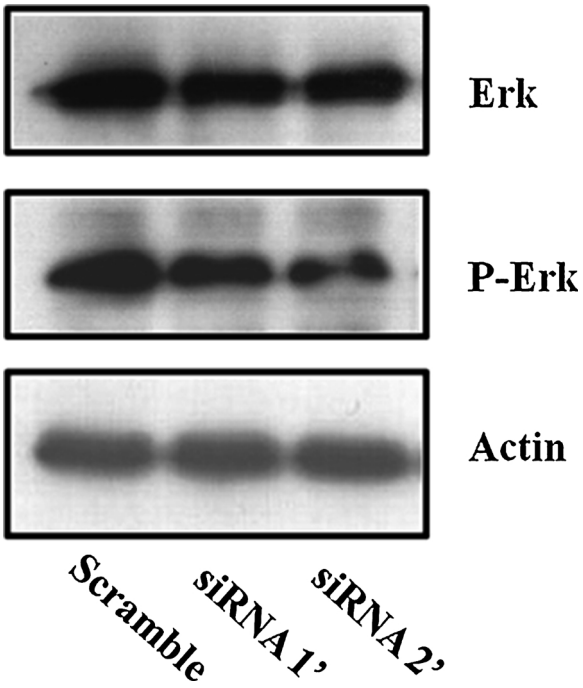

Fig. 7. WSB1 knockdown decreases Erk phosphorylation.

3.7. WSB1 knockdown decreases Erk phosphorylation

The Erk signaling pathway serves important roles in cell proliferation and the development of chemoresistance in a variety of cancers [18–21]. As HSYA was reported to decrease Erk 1/2 expression [10], the present study aimed to determine the influence of WSB1 knockdown on Erk activity. As shown in Fig. 7, WSB1 siRNA decreased Erk phosphorylation. This result suggested that HSYA could at least partially exert its functions to inhibit the Erk signaling pathway activity by downregulating WSB1 expression.

Cell lysates from Skov3 cells transfected with scramble or *WSB1* siRNA for 48 h were subjected to immunoblotting with antibodies against Erk, p-Erk and β-actin. SiRNA: small interfering RNA.

4. Discussion

Safflower has been traditionally used to improve blood circulation,

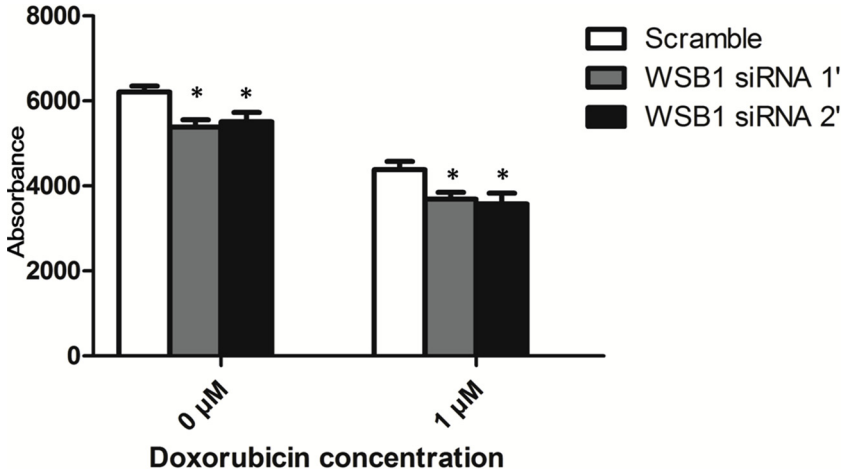

Fig. 6. WSB1 knockdown decreases cell viability and sensitizes Skov3 cells to doxorubicin.

and shows significant antithrombotic effects in mice [22]. However, in recent years, safflower has been demonstrated to possess antitumor functions [7]. Some extracts from the seed of safflower have been reported to inhibit tumor promotion in skin cancer or melanoma [23,24]. HSYA is one of the most effective ingredients of safflower and has been demonstrated to inhibit tumor cells by several studies [12,13]. Chemotherapy is an important therapeutic option for ovarian cancer to date. The majority of patients with ovarian cancer show a response to chemotherapy. However, a significant proportion of advanced patients suffer from a high rate of recurrence [25]. Treatment with some agents in combination with chemotherapeutic agents shows synergetic effects that can result in the cure of ovarian cancer [26]. Doxorubicin is a widely used therapeutic agent for the treatment of various types of cancers. The present study identified that HSYA inhibited ovarian cancer proliferation, and that the proliferation was further suppressed when the cells were treated with HSYA and doxorubicin.

Several studies have demonstrated that WSB1 is important for the development of cancer [27–30]. Metastatic potential was decreased after WSB1 knockdown. WSB1 knockdown could decrease the activity of metalloproteinase (MMP) and vascular endothelial growth factor (VEGF) secretion [31]. Low expression of WSB1 also reduced neuroblastoma cells growth, enhanced its apoptosis rate and increased sensitivity to chemotherapeutic agents [27]. In present study, our result showed that HSYA decreased WSB1 expression, indicating that HSYA may inhibit cell proliferation via blocking WSB1. In addition, a number of other genes were also dysregulated that may contribute to the effects of HSYA to ovarian cancer cells. PPP2R3C was upregulated by HSYA treatment and has been reported to sensitize cells to vincristine and doxorubicin, thus HSYA may sensitize ovarian cancer cells to doxorubicin through the upregulation of PPP2R3C [32]. In addition, INPP4B was downregulated by HSYA. However, it has been demonstrated to possess tumor suppressive functions in melanocytic neoplasms [33]. Furthermore, PNMA1 was reported to promote cell growth in human pancreatic ductal adenocarcinoma, but it was shown to be upregulated in the present study.

Erk is a representative kinase of the mitogen-activated protein kinase (MAPK) family, and a mediator of mitogenic signals. Various types of extracellular stimuli exert effects on cell proliferation, survival and apoptosis through the conserved Ras-Raf-MEK-Erk signaling cascade [19,20]. In response to extracellular signals, the activated Erk translocates to the nucleus and initiates the transcription of targeted genes that promote cell growth and survival. Aberrant regulation of the Erk signaling pathway results in uncontrolled cell proliferation, which has been observed in many types of cancer [34,35]. Acanthopanax trifoliatus (L) Merr was indicated to inhibit prostate cancer cell proliferation by decreasing Erk 1/2 phosphorylation [36]. A number of pharmacological inhibitors have been developed to target this pathway [37]. HSYA has been reported to inhibit vascular smooth muscle cell proliferation by decreasing Erk 1/2 expression [10].

In addition to its roles in cell proliferation, Erk activation predicts cell responses to chemotherapeutic agents. Chemoresistance is one of the major obstacles to successful treatment of many types of cancers. The pharmacological or molecular modulation of Erk signaling has been shown to affect the responses of anti-cancer drugs. The effect of doxorubicin on the activation of Emk-Erk predicts its chemotherapeutic response in hepatocellular carcinoma, and the activation of Emk-Erk determines the utility of MEK inhibitor combination treatment [21]. Suppression of Erk activity enhances the sensitivity of cancer cells to doxorubicin. Doxorubicin resistant BEL-7402 (BEL-7402/ADM) cells could be sensitized to doxorubicin by chrysin through suppressing Akt and Erk signaling pathways [21]. Cardiomyocytes could also be protected from doxorubicin-induced apoptosis via Akt and Erk signaling pathways [38]. A number of other studies have shown that doxorubicin caused sustained Erk activation in several types of cancers [39,40]. In addition, other DNA damaging agents caused similar effects on Erk activation [41,42]. In previous studies, sustained activation of Erk was observed to result in cell cycle arrest and apoptosis [39,42]. In the current study, it was observed that HSYA decreased WSB1 expression, and that WSB1 siRNA reduced Erk phosphorylation. Thus, the results of the present study demonstrate the significance of the Mek-Erk signaling pathway in the inhibition of cell proliferation caused by HSYA and it is rational to hypothesize the synergic effects of HSYA and doxorubicin.

For patients with ovarian cancer, chemotherapy is an important option of treatment. However, the response rate has been variable and recurrence is inevitable for many patients. Treatment by chemotherapy with other agents or methods may improve the responses of patients to treatment. For example, combination of paclitaxel and carboplatin enhances the effects on apoptosis induction in the presence of hyperthermia [43]. In addition, the combination of cinobufacini and cisplatin are more efficient in killing osteosarcoma cells [44]. Previously, chemotherapy used together with targeted agents has been demonstrated to increase the clinical responses of patients to treatment [45]. Furthermore, the combination of gefitinib with Erk inhibition restored gefitinib susceptibility [46]. However, few studies have been conducted to investigate the effects of traditional Chinese medicine and chemotherapeutic agents. As HSYA is an effective ingredient of a traditional Chinese medicine, the results of the present study suggest the effectiveness of the combination of Chinese medicines and chemotherapeutic agents in cancer treatment.

Although the present study had some significant results, there are still some limitations. First, the microarray results showed that many genes were upregulated or downregulated by HSYA. These results indicated that HSYA may cause complicated intracellular effects, but for this study we just focused on WSB1. Therefore, more investigations are required to reveal the molecular functions of HSYA. Second, the results of the present study indicated that attenuated Erk activation may be one of the reasons that ovarian cancer cells are more sensitive to doxorubicin. Other signaling pathways may also be altered by WSB1 siRNA or HSYA, which should be explored by further studies. Third, different cell lines may have different responses to HSYA, so further studies on other cell lines are needed.

In conclusion, the results of the present study suggest that HSYA could be used to treat ovarian cancer, as HSYA inhibited ovarian cancer cell proliferation. Intriguingly, HSYA sensitized ovarian cancer cells to chemotherapeutic agents, indicating its synergic effects in treating patients with ovarian cancer together with chemotherapeutic agents. The functions of HSYA in this study could be at least partially explained by decreasing WSB1 expression. More studies are required to further explore the antitumor properties and underlying mechanisms of HSYA.

Conflict of interest

The authors declare that they have no competing conflict of interest.

Funding

This study was supported by the National Natural Science Foundation of China (No. 31500640 and No. 31770849), the Natural Science Foundation of Zhejiang Province (LY15C070002, No. LY16C050001), the fund from Science Technology Department of Zhejiang Province (2015C33131, 2016F10005), the fund from the Science and Technology Bureau of Jiaxing (No. 2015AY23007).

Acknowledgements

Thanks to all the authors for their contribution to this article. Thanks to Dr. Wenbin Ou, for his help with experimental design and data analysis.

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