Research Article

Effects of Xuefu Zhuyu Decoction on Cell Migration and Ocular Tumor Invasion in Drosophila

Sitong Wang,1 Fanwu Wu,1 Bin Ye,1 Shiping Zhang,2 Xingjun Wang,3 Qian Xu,4 Guowang Li,1 Menglong Zhang,1 Shuai Wang,1 Yongsen Jia1,1 Chunhua Jiang1,1 Xiaojin La,1 Hong Chang1,1 Zixue Zhao,1 Peng Li,1 Ji-an Li1,1 and Chenxi Wu1

1Tangshan Key Laboratory of Traditional Chinese Medicine Pharmacology, College of Traditional Chinese Medicine, North China University of Science and Technology, 21 Bohai Road, Tangshan 063210, China
2School of Life Science and Technology, ShanghaiTech University, 393 Middle Huaxia Road, Shanghai 201210, China
3Department of Neuroscience, Scripps Research Institute, 130 Scripps Way Jupiter, Florida 33458, USA
4College of Integrative Medicine, Fujian University of Traditional Chinese Medicine, Fuzhou 350122, China

Correspondence should be addressed to Chenxi Wu; chenxi.wu@ncst.edu.cn

Received 28 November 2019; Revised 15 February 2020; Accepted 3 April 2020; Published 25 April 2020

Guest Editor: Md. Atiar Rahman

Copyright © 2020 Sitong Wang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Xuefu Zhuyu Decoction (XFZYD), a Traditional Chinese Medicine (TCM) decoction mainly for treating blood stasis syndrome, has been widely investigated and applied in clinic and in laboratory. XFZYD contains 11 herbs and has been identified to promoting blood circulation to remove blood stasis for cardiovascular disease. Meanwhile, blood stasis is directly related to malignant tumor according to TCM basic theory. However, the effects of XFZYD on tumor metastasis and the underlying mechanisms are still largely unknown.

Here, we employed well-established Drosophila cell migration and tumor invasion models to explore whether XFZYD has the anticancer activity on tumor metastasis in vivo. Our work has demonstrated that XFZYD could suppress cell migration and tumor invasion at the moderate concentrations. In addition, XFZYD altered the expression of MMP1, β-integrin, and E-cadherin to impede cell migration. Moreover, XFZYD inhibited ocular tumor invasion presumably by reducing the activity of Notch signaling. Together, these evidences reveal a positive role of XFZYD in suppressing cell migration and tumor metastasis, providing the potential drug targets and key clues for cancer clinical treatment strategies.

1. Introduction

With the increased incidence, cancer has become a worldwide disease and represents one of serious health-care issues for human race [1, 2]. More than 90% fatalities of human cancers are due to the ability of tumor metastasis at late stage, or form discontinuous secondary tumor foci at distant locations, instead of the localized primary tumor growth [3, 4]. Tumor invasion development is an extensive complex progress with multiple steps. Firstly, with the metastatic feature of decrease in adhesive force and increase in superficial charges, cancer cells could separate from the initial tumor. Through releasing numerous proteolytic enzymes and other factors, they possess the abilities of penetrating basement membrane (BM) and destroying cellular matrix, thus infiltrating into peripheral tissue [5]. After cancer enters the vascular and lymphatic vessels, these tumor cells survive and spread across the body through the circulatory system. Finally, they pierce through the vessel wall and settle at a secondary location to expand the metastatic focus [6–8]. A large number of genes and signaling pathways participate in this biological behavior, providing potential drug targets and
valuable therapeutic strategies for clinical cancer treatments [9–12]. Due to the adverse effects and high cost, synthetic chemotherapeutic anticancer drugs have not been as successful as expected in preventing and fighting against cancers in the clinic. Hence, the need to develop effective, safe, and affordable anticancer drugs from alternative and complementary herbal resources is becoming more and more urgent.

Traditional Chinese Medicine (TCM), with ancient application history in China or other regions of Asia, has gained considerable importance in defending against cancer during the last two decades [13–15]. Blood stasis, characterized by blood flow retardation and blood stagnation [16–18], is an essential factor for tumor metastasis during tumorigenesis in the TCM basic theory system [19, 20]. Usually, researchers utilize the hemorheology indexes to evaluate the patients with blood stasis syndrome or the established blood stasis animal models rather than pathological methods [21, 22]. Gao et al. checked the nail fold microcirculation in tumor patients and found that microcirculatory disturbance makes blood flow slow down, whose erythrocyte aggregation, exudation, and hemorrhage in turn promote the generation, development, and metastasis of tumor [23]. And, the association between blood stasis and cancer has received increasing attention in the studies related to TCM and integrative Chinese and Western medicine [24–26]. Thus, promoting blood circulation to remove blood stasis has become one of the major treatment principles for cancer in TCM. While the blood-activating stasis-resolving drugs (BAHRDs) have been well documented in antitumor therapy, their roles in cell migration and tumor metastasis remain controversial. Several reports have indicated that BAHRDs could inhibit tumor cell proliferation, invasion, and metastasis and reduce adverse reactions of radiotherapy and chemotherapy [27–29]. Conversely, other studies suggested that BAHRDs can improve local microcirculation and provide a richer blood supply for tumor growth, thereby promoting tumor metastasis [30]. A possible explanation for the discrepancy is that this category of drugs has multiple active ingredients, which are mainly categorized into the following: flavonoids, terpenoids, alkaloids, and fatty oil [31]. Different compounds and various combinations may trigger different gene expression profiles and signaling pathways, thus producing diverse even opposite effects. Additionally, most work was done in vitro or in cultured cancer cell lines that could not adequately recreate the in vivo condition.

Xuefu Zhuyu Decoction (XFZYD), a traditional Chinese herbal formula, has been widely investigated and applied in the clinic mainly for treating blood stasis syndrome. It contains 11 herbs (Supplementary Table 1 showing the drug composition and medicinal part, as well as the amount of each herb in one dose XFZYD) [32] and is originated from the well-known medical book Yi Lin Gai Cuo (Corrections of Errors Among Physicians) which was written by Wang Qingren in Qing Dynasty [33]. As a representative of blood-activating stasis-resolving recipe, XFZYD is mainly used to treat metabolic syndrome and cardiac-cerebral vascular diseases, including hyperlipidemia, hypertension, coronary heart disease, traumatic brain injury, and ischemic stroke [34–40]. It has been reported that XFZYD displays a significant effect on the pretreatment of the myocardium in sepsis rats by inhibiting myocardial cell apoptosis and antioxidation [41]. In addition, researchers found that XFZYD could protect against retinal ischemic cell apoptosis and antioxidation by downregulating HIF-1α and VEGF via a synergistic inhibition of RBP2 and PKM2 [42]. According to the Chinese medical theory, blood stasis is one of main leading causes for malignant tumor formation. With the efficacy in promoting circulation to remove blood stasis, we considered that XFZYD could be potentially used to develop an effective treatment for malignant tumors. However, the effects of XFZYD on tumor metastasis and the underlying mechanisms remain poorly understood.

With less genome redundancy and more genetic tools, Drosophila melanogaster serves as an elegant in vivo model for the study of tumor metastasis [43–45]. Many genes and signal transduction pathways pivotal for cancer progression were first identified in flies and subsequently verified in mammals. Moreover, several powerful tumor invasion models have been well established in Drosophila melanogaster. For instance, the loss of C-terminal SRC kinase (csk) or cell polarity genes, like scribbled (scrib), disc large (dlg), or lethal giant larvae (lg), in the anterior/posterior (A/P) compartment boundary region triggers invasive cell migration phenotype [10, 46, 47]. Drosophila is also becoming a potential model for studying ocular tumors [48]. Ferres-Marcos et al. found that coupled with overexpression of Delta (the ligand for Notch signal), downregulation of the expression of two epigenetic genes pipsqueak (psp) and longitudinal lacking (loa) induces the formation of Drosophila eye metastatic tumors during development, which is termed eyeful [49]. By using these important in vivo models, the purpose of our study was to analyze the effects of XFZYD extract on cell migration and ocular tumor invasion in Drosophila and to provide the potential molecular mechanisms for XFZYD in clinical treatment on malignant tumors.

2. Materials and Methods

2.1. Traditional Chinese Herbs. XFZYD is composed of Bupleurum chinense, Paeonia lactiflora, Cyathula officinalis, Ligusticum chuanxiong, Angelica sinensis, Prunus persica, Glycyrrhiza uralensis, Carthamus tinctorius, Platycodon grandiflorum, Rehmannia glutinosa, and Citrus aurantium. The 11 Chinese herbs in XFZYD were purchased from Beijing Tongrentang Tangshan Chain Store Drug Store Co., Ltd. Their batch numbers and manufacturers are listed in the Supplementary Table 2. All these materials (Supplementary Figure 1a-k) were all authenticated by Prof. Chunyu Tian in the College of Traditional Chinese Medicine, North China University of Science and Technology [50].

2.2. Preparation of Xuefu Zhuyu Decoction. The method of preparing traditional prescription is water extraction. Firstly, one dose of XFZYD (Supplementary Table 1 showing the amount of each herb in one dose) was weighed and soaked overnight [32]. After being soaked for 12 h, the herbs were extracted in boiled double distilled water (ddH2O) (100 g herbs to 1000 mL ddH2O) at 60°C for 3 hours. The extract was centrifuged at 4200 × g for 30 min at 25°C, followed by...
vacuum filtration with a 0.45 μm filter. The XFZYD extract was stored at -80°C and then freeze dried with a lyophilizer and then reconstituted in ddH₂O in order to obtain a final stock concentration of 0.78 g/mL (Supplementary Figure 1I). XFZYD extract was added directly to regular food from a 0.7800 g/ml aqueous stock to a final concentration of 0.0100, 0.0125, 0.0200, 0.0500, and 0.1000 g/mL. For control food, only water was used.

2.3. Quality Control of Xuefu Zhuyu Decoction. To control the quality of XFZYD, a high-performance liquid chromatography (HPLC) method was performed to establish the fingerprint spectrum. The analyses were performed with Shimadzu LC-20A. The chromatographic column was Agilent Eclipse XDB-C18 (4.6 × 250 mm, 5 μm). The mobile phase flow rate was 1 ml/min, and column temperature was maintained at 25 ± 1°C. The elution gradient was 0-8 min (C: 19%, D: 81%), 8-35 min (C: 19%-50%, D: 81%-50%), 35-36 min (C: 50%-100%, D: 50%-0%), and 36-40 min (C: 100%-19%, D: 0%-81%). Naringin was eluted with a gradient system consisting of methanol (A) and 0.05 M KH₂PO₄ solution (B) (A : B = 40:60). The mobile phase for ammonium glycyrrhizinate is acetonitrile (C) and 0.05% phosphoric acid solution (D). Its elution was performed with Shimadzu LC-20A. The chromatographic column was Agilent Eclipse XDB-C18 (4.6 × 250 mm, 5μm). The paeoniflorin, ammonium glycyrrhizinate, and naringin were dissolved in methanol as control detected by the HPLC method. Paeoniflorin was stored at -80°C and then reconstituted in ddH₂O in order to obtain a final concentration of 0.0100, 0.0200, 0.0500, and 0.1000 g/mL (Supplementary Figure 1I).

2.4. Drosophila Strains and Genetics. Flies were kept on a cornmeal and agar medium at 25°C with a 12 h light-dark cycle incubator according to standard protocols unless indicated. Drosophila stocks used include the following: w¹¹¹⁸ (#3605) and Oregon*RC (#0005) were obtained from Bloomington Drosophila stock center (BDSC); UAS-scrib-IR (#27424) was obtained from Vienna Drosophila RNAi center (VDRC); ptc-GAL4 UAS-GFP (ptc-GFP) was previously described [51, 52]. eye/full/Cyo is a kind gift of Professor Lei Xue from Tongji University, which has been established by the homologous chromosome recombination (HCR) of ey-GAL4 (#8220, BDSC), UAS-Delta (#5614, BDSC), and GS88A8 (previously described [49]) fly strains via the balancer fly stock Sco/Cyo (#2555, BDSC). The crossing scheme for establishing the eye/full model is shown in Supplementary Figure 2. For all fly crossing experiments, healthy unmated male and female parents were randomly assigned to different groups.

2.5. Drosophila Cell Migration Model. To set up the cell migration model, female flies with genotype ptc-GFP were crossed to male flies with genotype UAS-scrib-IR. These fruit flies mated and laid eggs in one tube, which contains normal food. When the offspring reach the 3rd instar larval stage, the larvae with genotype ptc-GFP/UAS-scrib-IR were collected and dissected (Supplementary Figure 1n). After fixation, the migrated cells were observed and recorded, which are marked by green fluorescent protein (GFP) in the wing pouch region through a fluorescence microscope. Simultaneously, the migrating cell number was counted and the migrating distance was measured (Supplementary Figure 3). For the XFZYD-treated groups, the offspring were kept on the XFZYD-added medium. Meanwhile, female flies with genotype ptc-GFP were crossed to male flies of the strain w¹¹¹⁸ on the regular food, and the progeny larvae with genotype ptc-GFP/+ were collected as the control group. For cell migration experiments, all crosses were raised at 25°C for 2 days, then shifted to 29°C for additional 3 days; the 3rd instar larvae were dissected [10].

2.6. Drosophila Ocular Tumor Invasion Model. For ocular tumor invasion assays, female w¹¹¹⁸ flies and male flies with genotype eye/full/Cyo were crossed. The parental flies mated and laid eggs in one tube, which contains normal food. When the offspring reach the adult stage, the flies with genotype eye/full/+ were collected as the model tumor invasion group. Their ocular cells were observed under a stereo microscope, their primary growth and invasion location were recorded (Supplementary Table 3), and the percentage was calculated. For the XFZYD-treated groups, the offspring were kept on the XFZYD-containing medium. Meanwhile, Oregon*RC flies which were raised on the regular fly food were used as the control group.

2.7. Immunohistochemistry. Larval discs were dissected and fixed in 40% formaldehyde for 20 min at room temperature (RT). Cold PBS was added to rinse 3 times. After several washes with 0.3% (v/v) PBST, discs were stained with primary antibodies overnight at 4°C and the following secondary antibodies for 4 h at RT. The primary antibodies used in this study include the following: mouse anti-MMP1 (1:200, Developmental Studies Hybridoma Bank, DSHB, 3A6B4), mouse anti-βPS-integrin (1:100, DSHB, CF.6G11), and rat anti-E-cadherin (1:100, DSHB, DCAD2). The following secondary antibodies were used: goat anti-Mouse-Cyanine3 (Cy3) (1:1000, Life technologies, A10521) and goat anti-Rat-Cy3 (1:1000, Life technologies, A10522). Vectashield mounting media (Vector Laboratories, H-1500) with DAPI (4’,6-diamidino-2-phenylindole) was used for mounting. The fluorophores of DAPI, GFP, and Cy3 were excited and visualized by a fluorescent inverted microscope system (Olympus, IX51).

2.8. qRT-PCR. TRIzol (Invitrogen) was used to isolate total RNA from ten wing imaginal discs dissected from the third instar larvae or thirty adult heads collected from freshly eclosed flies of indicated genotypes, and qRT-PCR was performed as previously described [53] using the following primers:

1. For rp49: sense—5’-TACAGGCCCAAGATCG TGAA-3’; antisense—5’-TCTCCTTGCGCTTCTT GGA-3’

2. For Su(H): sense—5’-CTTGCTGCCGGGTCTCT TAC-3’; antisense—5’-CTCGGCCATGTACTTC TCCA-3’
2.9. Data and Statistics. All data were verified in at least three independent experiments. Results were presented as bar graphs or scatter plots created with GraphPad Prism 8.0. For statistical significance, one-way ANOVA with Bonferroni’s multiple comparison test or chi-squared test was applied. P value less than 0.05 was considered significant and center values as the mean. Error bars indicated standard deviation. P value less than 0.05 was considered significant (ns was not significant; \( P \geq 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001 \).

3. Results

3.1. Xuefu Zhuyu Decoction Preparation and HPLC Analysis. To examine the quality of the Xuefu Zhuyu Decoction (XFZYD), three major compounds were used as quality control standards. When XFZYD high-performance liquid chromatography (HPLC) chromatogram was compared with the standard controls (Supplementary Figure 4a-c), we observed the distinguished peaks for paeoniflorin, ammonium glycyrhrizinate, and naringin in the chemical fingerprints of XFZYD (Figures 1(a)–1(c)). In addition, the content of these three compounds in XFZYD determined by the HPLC method is 0.140%, 0.039%, and 0.220%, respectively. Taken together, these results indicated that the prepared XFZYD consists of the necessary active ingredients and is suitable to carry out the study.

3.2. XFZYD Inhibits Cell Migration in Drosophila Wing Discs. To investigate the XFZYD’s suppressive effect on cell migration in vivo, we employed a well-established cell migration model in Drosophila 3rd instar larval wing imaginal discs [10, 47]. Compared with the control (Figures 2(a), (a’)), depletion of the cell polarity gene scribble (scribble) driven by patched-GAL4 (ptc-GAL4), which was mediated by RNA interference (RNAi), triggered massive cell migration phenotype (Figures 2(b), (b’)). In the ptc>GFPUAS-scrib-IR- (ptc>scrib-IR-) induced cell migration model, the green fluorescent protein- (GFP-) labeled migrating cells were detached from the anterior/posterior (A/P) compartment boundary and moved toward the posterior part of the wing imaginal discs (Figure 2(b’)). To further quantify the cell migration phenotype, we calculated the total number of migrating cells and measured the median or maximum (max) migrating distance in the selected wing pouch region (Supplementary Figure 3) and observed a significant increase in migrating cell number and migrating distance (Figures 2(h)–2(j)).

Next, to test whether there is the inhibitory effect, we prepared feeding media consisting of XFZYD aqueous extract (Supplementary Figure 1m) and raised the ptc>scrib-IR flies from the egg stage to the 3rd instar larval stage. Results showed that the loss-of-scribble-induced increased migrating cell number was notably suppressed by XFZYD extract at the concentration of 0.0125 g/ml or 0.0200 g/ml, but not that of 0.0100 g/ml or 0.0500 g/ml (Figures 2(b)–2(f), (b’)-(f), (h)). For the max or median migrating distance, XFZYD at the concentration of 0.0125 g/ml showed a stronger inhibitory effect than that of 0.0200 g/ml (Figures 2(i) and 2(j)), while the concentrations of 0.0100 g/ml or 0.0500 g/ml did not perform an obvious inhibitory effect. Intriguingly, we found that XFZYD at the concentration of 0.1000 g/ml could strongly aggravate the ptc>scrib-IR-triggered cell migration in cell number, but not in distance (Figures 2(g)–2(j), (g’)). Collectively, XFZYD displayed a dose-independent and biphasic effect on the cell migration in the Drosophila larval stage. And the concentration of 0.0125 g/ml for XFZYD water extracts was chosen, which significantly suppressed cell migration, to further explore the mechanisms.

3.3. XFZYD Alters MMP1, β-Integrin, and E-Cadherin Expression. To further elucidate the underlying mechanisms of XFZYD in inhibiting cell migration, we analyzed several cell migration-related key factors. Matrix metalloproteinase-1 (MMP1), as a member of the MMPs family, has a direct role in enhancing tumor invasion, whose overexpression indicates a poor prognosis [54–56]. Clearly, inhibition of the well-characterized tumor suppressor E-cadherin’s function or expression will lead to cell epithelial-mesenchymal transition, promoting cell migration and metastasis [57]. In addition, as a transmembrane receptor which is composed of α- and β-subunits, integrin adheres to and migrates between cells and is phosphorylated after being subjected to intracellular and extracellular metastasis signals to produce tumor cell migration [58, 59]. In agreement with these previous works, results showed that loss-of-scribble under ptc-GAL4 control along the A/P boundary could trigger strong epithelial-mesenchymal transition (EMT) like phenotype in the wing pouch region, revealed by upregulated expression of MMP1 (Figures 3(a) and 3(b)) and the major β-subunit of integrin (βPS-integrin) (Figures 3(d) and 3(e)) [60, 61] and downregulation of cell adhesion molecule E-cadherin (Figures 4(a) and 4(b)). And the fluorescence intensity analysis results showed that the altered expression of MMP1, βPS-integrin or E-cadherin is nearly located in the ptc>scrib-IR region which was visualized by GFP (Figures 3(b’), 3(e’), and Figure 4(b’’)), indicating that depletion of scribble triggers the increase of MMP1 and integrin, or the decrease of E-cadherin in a cell-autonomous manner. Moreover, the upregulation of MMP1 and β-integrin or the reduction of E-cadherin induced by ptc>scrib-IR was remarkably ameliorated by XFZYD at the concentration of 0.0125 g/ml (Figures 3(c) and 3(f), and Figure 4(c)). Together, these evidences suggest that the strong inhibitory effect of XFZYD on cell migration may be due to altering the expression of MMP1, β-integrin, and E-cadherin.

3.4. Xuefu Zhuyu Decoction Suppresses Ocular Tumor Invasion. Considering cell migration is a central step for metastasis during tumorogenesis, we introduced a Drosophila ocular tumor invasion model, which is named eyeful [49], to further analyze the roles of XFZYD in metastasis. In line with a previous study, we found that exogenous ectopic expression of Delta, which functions as the ligand for the Notch signaling pathway, under the control of ey-GAL4 in Drosophila compound eye, and simultaneous depletion of epigenetic genes psp and lola leads to overproliferation in situ and metastatic tumor formation of Drosophila eye tissue cells.
Figure 1: The HPLC chromatogram of XFZYD. Three major compounds (paeoniflorin, ammonium glycyrrhizinate, and naringin) of XFZYD were identified and compared to the standards by the HPLC method. The tested peaks for paeoniflorin (a, 230 nm), ammonium glycyrrhizinate (b, 237 nm), and naringin (c, 283 nm) are indicated with green, blue, and red arrows, respectively.
Again, 0.0200 g/ml or 0.0500 g/ml XFZYD significantly suppressed the migration rate of the eyeful model (Figure 5(i)), whereas it slightly increased the migration level at the concentration of 0.1000 g/ml (Figure 5(i)). Meanwhile, XFZYD had almost no suppressive effect on Drosophila ocular tumor invasion at the concentration of 0.0100 g/ml or 0.0125 g/ml (Figures 5(i)–5(k)). Given that the Notch signal is upregulated in the eyeful tumor model, it was hypothesized that XFZYD may inhibit tumor invasion through modulating Notch signaling activity. To verify our assumption, a real-time quantitative PCR (qRT-PCR) assay was performed. In accordance with a previous
Figure 3: XFZYD downregulates MMP1 and β-integrin expression. Merged fluorescence micrographs of the 3rd instar larval wing discs stained with anti-MMP1 (a–c) or anti-βPS-integrin antibody (d–f) are shown. The individual channels detecting only GFP (green, a’–f’), only MMP1 (red, a”–c”), and only βPS-integrin signal (red, d”–f”). Nuclei (DNA) were labelled with DAPI (4’,6-diamidino-2-phenylindole, blue). (a””–f””) Graphs of total intensity sum of each fluorophore with respect to the region of interest (indicated by the yellow solid line) as shown in (a–f), respectively. Fluorescence intensities were measured in pixels using Image-Pro Plus 6.0. Scale bar: 50 μm (a–f).
study, we found that the mRNA level of the **Suppressor of Hairless** (**Su(H)**), which acts as the transcription factor in Notch pathway [62], was upregulated in the **eyeful** invasion tumor model (Figure 5(l)). And XFZYD remarkably impeded the **eyeful**-induced elevated transcription level of **Su(H)** at the concentration of 0.0500 g/ml (Figure 5(l)). Hence, we conclude that XFZYD inhibits tumor invasion through downregulating Notch signaling activity.

**4. Discussion**

Cancer has been a worldwide fatal disease that seriously threatens human health. As more than 90% of cancer patients died from tumor migration, suppression of tumor cell migration has emerged as the central target for cancer treatments [3, 4]. *Drosophila melanogaster* is regarded as an excellent model system to study the genetic and molecular mechanisms of tumorigenesis and metastasis [63, 64]. In present study, the *ptc>*scrib-IR-induced cell migration model and *eyeful* ocular tumor invasion model were established and used to investigate the protective effect of XFZYD.

As a well-known TCM herbal formula, XFZYD is categorized into the BAHRDs and has been extensively applied for treating metabolic syndrome and cardiac-cerebral vascular diseases [34–40]. Although blood stasis is also a main pathogenic mechanism for tumor metastasis in the TCM theory, the application of XFZYD in treating malignant tumors is largely unknown. Interestingly, our *in vivo* data showed that XFZYD decreased cell migration and invasion at the low dose (the concentration of 0.0125 g/ml for cell migration and that of 0.05 g/ml for ocular tumor invasion) but increased cell migration and invasion at a high dose (the concentration of 0.1000 g/ml for both). Collectively, the effects of XFZYD on cell migration and tumor invasion in *Drosophila* displayed a dose-independent and biphasic manner, whereas, in a septic shock rat model, XFZYD may reduce myocardial damage and a protective role for the heart structure and function in a dose-dependent manner [41]. Besides, we have noticed that the doses of XFZYD that affect cell migration and tumor invasion are different, which may be due to distinct genetic backgrounds of two fly models. Several previous studies showed that multiple cancer-related factors and signal
Figure 5: XFZYD performs a suppressive effect on eyeful ocular tumor invasion. Light micrographs of Drosophila adult eye (a–i) and body (j–r) are shown. (a) and (j) are the wild-type controls, and (b–d) and (k–m) are the representative images of the eyeful model. When the red compound eye cells are observed outside the eye tissue, the ocular tumor is considered to have migrated. According to the number of folds within Drosophila eye, eyeful flies without migrated tumors are divided into three degrees: I (no obvious fold, mild), II (1-2 folds, moderate), and III (≥3 folds, severe) (b–d). According to the invasion location of the ocular tumor, eyeful flies with tumor metastasis are divided into the head (proximal), thorax (middle), and abdomen (distal) subgroups (k–m, the red eye tissue cells are indicated by black arrow). (e–i) and (n–r) are the representative images of the eyeful model when treated with XFZYD at a concentration of 0.0100, 0.0125, 0.0200, 0.0500, or 0.100 g/ml, respectively. (s) A quantification of eyeful tumor invasion percentage. The number of flies in each group with or without migrated tumors was recorded; then the invasion ratio was calculated. The columns from left to right are (1) wild type (Oregon R, 0.00%, n = 65), (2) eyeful (19.57%, n = 92), (3) eyeful+XFZYD 0.0100 g/ml (18.06%, n = 72), (4) eyeful+XFZYD 0.0125 g/ml (18.75%, n = 64), (5) eyeful+XFZYD 0.0200 g/ml (12.07%, n = 116), (6) eyeful+XFZYD 0.0500 g/ml (9.52%, n = 84), and (7) eyeful+XFZYD 0.1000 g/ml (21.88%, n = 96). A chi-squared test was applied. Stacked bar graphs of primary growth (t) and invasion location (u) for eyeful tumor assay are shown (sample size details are shown in Supplementary Table 3). (v) Histogram showing the levels of Su(H) mRNA as measured by qRT-PCR. Error bars represent standard deviation from three independent experiments. One-way ANOVA with Bonferroni’s multiple comparison test was used to compute P values: **P < 0.01 and *P < 0.05. Scale bar: 50 μm (a–i) and 150 μm (j–r).
transduction pathways exhibit varied expression levels and activities in the ptc->scrib-IR cell migration and eyeful tumor models [10, 47–49]. And another reasonable explanation is that the best inhibitory effects of different doses on the two models may be caused by different active ingredients (or different combinations of active ingredients) in XFZYD.

Based on quantifications of daily Drosophila food intake [65], it is estimated that flies raised on medium containing 0.0125 g/ml, 0.05 g/ml, or 0.1000 g/ml XFZYD ingest about 0.4 mg/kg body weight of the drug per day [66], which is, respectively, comparable to the treatment dosage of 26.04 g, 104.2 g, or 208.3 g per day for human patients. In the clinic, the patients with coronary heart disease were treated with XFZYD one dose (78 g) a day [32]. Thus, according to the normal dose, taking XFZYD 26.04 g/day for human (equivalent to 0.0125 g/ml for fly) is far below the usual medication dosage (78 g/day). Above all, the data may provide a key clue for the application of XFZYD in cancer clinical treatment.

Notch signaling is an evolutionarily conserved pathway that regulates many cellular processes, including cell proliferation, survival, apoptosis, invasion, angiogenesis, and stem cell self-renewal [67–69]. Recently, Notch signal has been regarded as a central regulator in the induction of EMT, which is important for migration and metastasis of cancer cells [70–72]. In addition, several studies revealed the molecular mechanisms behind Notch-mediated EMT regulation during non-small-cell lung cancer and breast tumorigenesis [73, 74]. Consistently, in this study, XFZYD showed its anticancer activities through inhibiting EMT-mediated cell migration (Figures 3 and 4) and downregulating Notch signal activity (Figure 5) in Drosophila. Overexpression of Delta (the ligand in fly) is a very powerful strategy to increase Notch signal by releasing the Notch intracellular domain (NICD), which enters the nucleus and regulates the transcriptional activity of Su(H) [75]. Thus, we would like to examine the subcellular distribution of the NICD protein (tagged by GFP/YFP) and the expression of Notch/Su(H) target genes (Enhancer of split (E (spl)), Cut and Wingless) in vivo to directly monitor Notch pathway activity for further investigation [76–79].

In conclusion, our work proved that XFZYD has anticancer activity on cell migration and tumor invasion at the moderate concentrations. Moreover, XFZYD could alter the expression of MMP1, integrin, or E-cadherin to suppress cell migration. Finally, it is demonstrated that XFZYD impedes ocular tumor invasion presumably by inhibiting Notch signaling activity. In sum, a positive function of XFZYD in suppressing cell migration and tumor metastasis has been shown, and the results indicated the potential drug targets and provide key clues for cancer clinical treatment strategies.

Abbreviations

TCM: Traditional Chinese medicine
BM: Basement membrane
BAHRDs: Blood-activating stasis-resolving drugs
XFZYD: Xuefu Zhuyu decoction
HPLC: High-performance liquid chromatography
HCR: Homologous chromosomes recombination
MMP1: Matrix metalloproteinase-1
A/P: Anterior/posterior
RNAi: RNA interference
GFP: Green fluorescent protein
EMT: Epithelial-mesenchymal transition
NICD: Notch intracellular domain.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare no conflict of interest.

Authors’ Contributions

Sitong Wang, Fanwu Wu, and Chenxi Wu conceived the project. Sitong Wang, Fanwu Wu, Bin Ye, Shiping Zhang, Xingjun Wang, Guowang Li, Menglong Zhang, Shuai Wang, Zixue Zhao, and Peng Li performed the experiments. Fanwu Wu, Chunhua Jiang, Xiaojin La, Yongsen Jia, and Hong Chang supervised the study and gave advice. Sitong Wang, Fanwu Wu, Qian Xu, and Chenxi Wu analyzed the data and wrote the manuscript. All authors gave the final approval for publication. Sitong Wang and Fanwu Wu contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31701244), the Natural Science Fund of Hebei Province of China (C2018209119 and C2020209032), the Scientific and Technological Research Project of Higher Education of Hebei Province (BJ20190940), and Doctoral Scientific Research Foundation of North China University of Science and Technology (BS2017063) to Chenxi Wu, Graduate Student Innovation Fund of North China University of Science and Technology (2019S36) to Sitong Wang, and Basal Research Fund for the Hebei Provincial Universities (JXC2019001) to Bin Ye. We thank Dr. Lei Xue, Bloomington and VDRC Fly Centers, for fly stocks, and members of the Wu Lab for the discussion and critical comments.

Supplementary Materials

Supplemental Information includes three tables and four figures. Supplementary Table 1 The formulation of XFZYD (one dose). Supplementary Table 2 Batch numbers and manufacturers of XFZYD. Supplementary Figure 1 Composition of Xuefu Zhuyu Decoction. Supplementary Figure 2 A flow chart for establishing the eyeful model. Supplementary Figure 3 Measurement of migrating cell distance. Supplementary Figure 4 The HPLC chromatogram of standard controls. Supplementary Table 3 Ocular tumor’s primary growth and invasion location in adult Drosophila. (Supplementary Materials)
References

[1] L. Gabriele, M. Buoncervello, B. Ascione, M. Bellenghi, P. Matarrese, and A. Care, "The gender perspective in cancer research and therapy: Novel insights and on-going hypotheses," Annali Dell’Istituto Superiore Di Sanita, vol. 52, no. 2, pp. 213–222, 2016.

[2] R. L. Siegel, K. D. Miller, and A. Jemal, "Cancer statistics, 2017," CA: A cancer journal for clinicians, vol. 67, no. 1, pp. 7–30, 2014.

[3] Z. Cai and Q. Liu, "Understanding the global cancer statistics 2018: Implications for cancer control," Science China Life Sciences, 2019.

[4] K. W. Hunter, N. P. Crawford, and J. Alsarraj, "Mechanisms of metastasis," Breast Cancer Research, vol. 10, no. 51, Supplement 1, p. 52, 2008.

[5] B. Breznik, H. Motaln, and T. Lah Turnsek, "Proteases and cytokines as mediators of interactions between cancer and stromal cells in tumours," Biological Chemistry, vol. 398, no. 7, pp. 709–719, 2017.

[6] S. Valastyan and R. A. Weinberg, "Tumor metastasis: Molecular insights and evolving paradigms," Cell, vol. 147, no. 2, pp. 275–292, 2011.

[7] L. Wan, K. Pantel, and Y. Kang, "Tumor metastasis: Moving new biological insights into the clinic," Nature Medicine, vol. 19, no. 11, pp. 1450–1464, 2013.

[8] N. Maishi and K. Hida, "Tumor endothelial cells accelerate tumor metastasis," Cancer Science, vol. 108, no. 10, pp. 1921–1926, 2017.

[9] S. U. Gerlach, T. Eichenlaub, and H. Herranz, "Yorkie and JNK control tumorigenesis in drosophila cells with cytokinesis failure," Cell Reports, vol. 23, no. 5, pp. 1491–1503, 2018.

[10] S. Zhang, X. Guo, H. Wu et al., "Wingless modulates activator protein-1-mediated tumor invasion," Oncogene, vol. 38, no. 20, pp. 3871–3885, 2019.

[11] X. Ma, Y. Chen, S. Zhang et al., "Rho1-Wnd signaling regulates loss-of-cell polarity-induced cell invasion in Drosophila," Oncogene, vol. 35, no. 7, pp. 846–855, 2016.

[12] J. N. Anastas and R. T. Moon, "WNT signalling pathways as therapeutic targets in cancer," Nature Reviews Cancer, vol. 13, no. 1, pp. 11–26, 2006.

[13] S. Yan, Y. Yue, J. Wang et al., "Banxia Xiexin decoction, a traditional Chinese medicine, alleviates colon cancer in nude mice," Annals of Translational Medicine, vol. 7, no. 16, p. 375, 2019.

[14] X. Tian, P. Wang, N. Zhan, Y. Y. Ren, L. Ming, and P. Y. Li, "Advances in studies on antitumor effective components of Chinese herbal medicines," Special Wild Economic Animal and Plant Research, vol. 32, no. 3, pp. 73–76, 2010.

[15] N.-N. Zhou, J. Tang, W.-D. Chen, G. K. Feng, B. F. Xie, and Z. C. Liu, "Houttuynia acuminata, an active constituent of Chinese herbal medicine, inhibits phosphorylation of HER2/neu receptor tyrosine kinase and the tumor growth of HER2/neu-over-expressing cancer cells," Life Sciences, vol. 90, no. 19–20, pp. 770–775, 2012.

[16] N. Ran, Z. Pang, X. Guan et al., "Therapeutic effect and mechanism study of Rhodiola wallichiana var. cholaein injection to acute blood stasis using metabolomics based on UPLC-Q/TOF-MS," Evidence-Based Complementary and Alternative Medicine, vol. 2019, Article ID 1514845, 20 pages, 2019.

[17] L. He, M. Fang, L. Chen et al., “Transcriptome analysis of blood stasis syndrome in subjects with hypertension,” Journal of Traditional Chinese Medicine, vol. 36, no. 2, pp. 173–180, 2016.

[18] H. Goto, “Blood stasis syndrome in Japan and its molecular biological analysis,” Chinese Journal of Integrative Medicine, vol. 20, no. 7, pp. 490–495, 2014.

[19] Y. F. Qian and X. J. Wang, “Effects of blood-activating and stasis-resolving drugs on tumor formation and metastasis,” Journal of Traditional Chinese Medicine, vol. 29, no. 4, pp. 301–310, 2009.

[20] L. M. Liu, Z. Chen, and P. F. Chen, “Thoughts on the treatment of malignant tumor with traditional Chinese medicine for promoting blood circulation and removing blood stasis,” Journal of Traditional Chinese Medicine, vol. 9, pp. 776–779, 2018.

[21] N. Antonova, “Methods in hemorheology and their clinical applications1,” Clinical Hemorheology and Microcirculation, vol. 64, no. 4, pp. 509–515, 2017.

[22] J. X. Zhang, Y. Feng, Y. Zhang, Y. Liu, S. D. Li, and M. H. Yang, “Hemorheology index changes in a rat acute blood stasis model: A systematic review and meta-analysis,” African Journal of Traditional, Complementary and Alternative Medicines, vol. 14, no. 4, pp. 96–107, 2017.

[23] J. Gao, X. Li, and X. J. Yan, "Application of examination of nail fold microcirculation in treating tumor (observation on 119 patients with blood stasis syndrome)," Journal of China-Japan Friendship Hospital, vol. 4, pp. 40–43, 1996.

[24] M. Liu, D. X. Wu, J. Li, N. Hou, Y. L. Zhang, and Y. J. Qiao, “Nature-effect relationship research between pungent and bitter taste of traditional Chinese medicine for promoting blood circulation and removing blood stasis based on nature combination,” Zhongguo Zhong Yao Za Zhi= Zhongguo Zhongyao Zazhi= China Journal of Chinese Materia Medica, vol. 44, no. 2, pp. 218–223, 2019.

[25] F. Peng, H. Zhu, C. W. Meng, Y. R. Ren, O. Dai, and L. Xiong, “New isoflavanes from Spatholobus suberectus and their cytotoxicity against human breast cancer cell lines,” Molecules, vol. 24, no. 18, p. 3218, 2019.

[26] C. C. Pieper, W. A. Willinek, D. Thomas et al., “Incidence and risk factors of early arterial blood flow stasis during first radioembolization of primary and secondary liver malignancy using resin microspheres: An initial single-center analysis,” European Radiology, vol. 26, no. 8, pp. 2779–2789, 2016.

[27] J. Zheng, L.-t. Ma, Q.-y. Ren et al., “Anti-fibrotic effects of salvia miltiorrhiza and ligustrazine injection on LX-2 cells involved with increased N-myc downstream-regulated gene 2 expression,” Chinese Journal of Integrative Medicine, vol. 23, no. 12, pp. 923–928, 2017.

[28] Y. Cao, Q. Tang, L. Luo, Z. Gao, and S. Zhang, “A preliminary study on the antitumor efficacy of caulis spatholobi compound 1802,” Pakistan Journal of Pharmaceutical Sciences, vol. 31, no. 3(Special), pp. 1145–1150, 2018.

[29] X. Chengyong, W. Yuguo, F. Jian, Q. Li, X. Ran, and D. Yongqi, “Effect of optimal combination of Huangqi (radix Astragali Mongolici) and Ezhu (Rhizoma Curcumae Phaeocaulis) on proliferation and apoptosis of A549 lung cancer cells,” Journal of Traditional Chinese Medicine, vol. 38, no. 3, pp. 351–358, 2018.

[30] L. Min and H. Qi, “Effect of different blood-promoting formulas in the tumor-genesis, metastasis and angiogenesis of nude mice bearing human hepatocellular carcinoma xenografts with high metastatic potential,” Chinese Journal of Integrated
C. Gonzalez, "Drosophila melanogaster: A model and a tool to investigate malignancy and identify new therapeutics," *Nature Reviews Cancer*, vol. 13, no. 3, pp. 172–183, 2013.

D. St Johnston, "The art and design of genetic screens: Drosophila melanogaster," *Nature Reviews Genetics*, vol. 3, no. 3, pp. 176–188, 2002.

M. Vidal, D. E. Larson, and R. L. Cagan, "Csk-deficient boundary cells are eliminated from normal drosophila epithelia by exclusion, migration, and apoptosis," *Developmental Cell*, vol. 10, no. 1, pp. 33–44, 2006.

X. Ma, L. Yang, Y. Yang, M. Li, W. Li, and L. Xue, "dUev1a modulates TNF-JNK mediated tumor progression and cell death in Drosophila," *Developmental Biology*, vol. 380, no. 2, pp. 211–221, 2013.

B. Daimark, L. Ekaterina, and C. Neville, "Drosophila as a potential model for ocular tumors," *Ocular Oncology and Pathology*, vol. 1, no. 3, pp. 190–199, 2015.

F. Teng, G. Li, Z. Liu, L. Zhang, and K. Yao, "Xuefuzhuyu decoction alleviates obesity, hepatic steatosis, neuroinflammation, amyloid deposition and cognition impairment in metabolically stressed APPswt/PS1edE9 mice," *Journal of Ethnopharmacology*, vol. 209, pp. 50–61.

S. Q. Tan, X. Geng, J. H. Liu et al., "Xuefuzhuyu decoction inhibition of angiogenesis attenuates liver fibrosis induced by CCl4 in mice," *Journal of Ethnopharmacology*, vol. 153, no. 3, pp. 659–666.

C. W. Yeh, H. K. Liu, L. C. Lin et al., "Xuefu Zhuyu decoction accelerates obesity, hematocrit steatosis, neuroinflammation, amyloid deposition and cognition impairment in metabolically stressed APPswt/PS1edE9 mice," *Journal of Ethnopharmacology*, vol. 209, pp. 50–61.

J. J. Lee, W. H. Hsu, T. L. Yen et al., "Traditional Chinese medicine, Xue-Fu-Zhu-Yu decoction, potentiates tissue plasminogen activator against thromboembolic stroke in rats," *Frontiers in Pharmacology*, vol. 134, no. 3, pp. 824–830, 2011.

X. Song, J. Wang, P. Wang, N. Tian, M. Yang, and L. Kong, "1H NMR-based metabolomics approach to evaluate the effect of Xue-Fu-Zhu-Yu decoction on hyperlipidemia rats induced by high-fat diet," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 78-79, pp. 202–210, 2013.

T. Tao, T. He, X. Wang, and X. Liu, "Metabolic profiling analysis of patients with coronary heart disease undergoing Xuefu Zhuyu decoction treatment," *Frontiers in Pharmacology*, vol. 10, p. 985, 2019.

P. Wang, X. Xiong, and S. Li, "Efficacy and safety of a traditional Chinese herbal formula Xuefu Zhuyu decoction for hypertension: A systematic review and meta-analysis," *Medicine*, vol. 94, no. 42, p. e1850, 2015.

F. Meng, H. Lai, Z. Luo et al., "Effect of Xuefu Zhuyu decoction pretreatment on myocardium in sepsis rats," *Evidence-Based Complementary and Alternative Medicine*, vol. 2018, Article ID 5936402, 17 pages, 2016.

S. Q. Tan, X. Geng, J. H. Liu et al., "Xue-fu-Zhu-Yu decoction protects rats against retinal ischemia by downregulation of HIF-1α and VEGF via inhibition of RBP2 and PKM2," *BMC Complementary and Alternative Medicine*, vol. 17, no. 1, p. 365, 2017.

G. M. Rubin and E. B. Lewis, "A brief history of Drosophila’s contributions to genome research," *Science*, vol. 287, no. 5461, pp. 2216–2218, 2000.

C. Gonzalez, "Drosophila melanogaster: A model and a tool to investigate malignancy and identify new therapeutics," *Nature Reviews Cancer*, vol. 13, no. 3, pp. 172–183, 2013.

M. L. Brower, M. Wilcox, M. Piovant, R. J. Smith, and L. A. Reger, "Related cell-surface antigens expressed with positional specificity in Drosophila imaginal discs," *Proceedings of the National Academy of Sciences*, vol. 81, no. 23, pp. 7485–7489, 1984.
[61] J. Heino, R. A. Ignotz, M. E. Hemler, C. Crouse, and J. Massague, “Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit,” Journal of Biological Chemistry, vol. 264, no. 1, pp. 380–388, 1989.

[62] N. Perrimon, C. Pitsouli, and B. Z. Shilo, “Signaling mechanisms controlling cell fate and embryonic patterning,” Cold Spring Harbor Perspectives in Biology, vol. 4, no. 8, p. a005975, 2012.

[63] L. F. Willoughby, T. Schlosser, S. A. Manning et al., “An in vivo large-scale chemical screening platform using Drosophila for anti-cancer drug discovery,” Disease Models & Mechanisms, vol. 6, no. 2, pp. 521–529, 2013.

[64] J. C. Pastor-Pareja and T. Xu, “Dissecting social cell biology and tumors using Drosophila genetics,” Annual Review of Genetics, vol. 47, no. 1, pp. 51–74, 2013.

[65] S. A. Deshpande, G. B. Carvalho, A. Amador et al., “Quantifying Drosophila food intake: comparative analysis of current methodology,” Nature Methods, vol. 11, no. 5, pp. 535–540, 2014.

[66] C. Slack, N. Alic, A. Foley, M. Cabecinha, M. P. Hoddinott, and L. Partridge, “The Ras-Erk-ETS-Signaling Pathway Is a Drug Target for Longevity,” Cell, vol. 162, no. 1, pp. 72–83, 2015.

[67] L. Miele and B. Osborne, “Arbiter of differentiation and death: Notch signaling meets apoptosis,” Journal of Cellular Physiology, vol. 181, no. 3, pp. 393–409, 1999.

[68] L. Miele, H. Miao, and B. Nickoloff, “NOTCH signaling as a novel cancer therapeutic target,” Current Cancer Drug Targets, vol. 6, no. 4, pp. 313–323, 2006.

[69] L. Miele, “Notch signaling,” Clinical Cancer Research, vol. 12, no. 4, pp. 1074–1079, 2006.

[70] D. M. Gonzalez and D. Medici, “Signaling mechanisms of the epithelial-mesenchymal transition,” Science Signaling, vol. 7, no. 344, p. re8, 2014.

[71] Z. Wang, Y. Li, D. Kong, and F. H. Sarkar, “The role of Notch signaling pathway in epithelial-mesenchymal transition (EMT) during development and tumor aggressiveness,” Current Drug Targets, vol. 11, no. 6, pp. 745–751, 2010.

[72] I. Espinoza, R. Pochampally, F. Xing, K. Watabe, and L. Miele, “Notch signaling: targeting cancer stem cells and epithelial-to-mesenchymal transition,” OncoTargets and Therapy, vol. 6, pp. 1249–1259, 2013.

[73] X. Yuan, H. Wu, N. Han et al., “Notch signaling and EMT in non-small cell lung cancer: biological significance and therapeutic application,” Journal of Hematology & Oncology, vol. 7, no. 1, p. 87, 2014.

[74] R. Kar, N. K. Jha, S. K. Jha et al., “A “NOTCH” Deeper into the epithelial-to-mesenchymal transition (EMT) program in breast cancer,” Genes, vol. 10, no. 12, 2019.

[75] E. Zacharioudaki and S. J. Bray, “Tools and methods for studying Notch signaling in Drosophila melanogaster,” Methods, vol. 68, no. 1, pp. 173–182, 2014.

[76] C. Slack, A. Foley, M. Cabecinha, M. P. Hoddinott, and L. Partridge, “The Ras-Erk-ETS-Signaling Pathway Is a Drug Target for Longevity,” Cell, vol. 162, no. 1, pp. 72–83, 2015.

[77] W. J. Brook and S. M. Cohen, “Antagonistic interactions between wingless and decapentaplegic responsible for dorsal-ventral pattern in the Drosophila Leg,” Science, vol. 273, no. 5280, pp. 1373–1377, 1996.

[78] A. J. Bardin and F. Schweisguth, “Bearded family members inhibit neuralized-mediated endocytosis and signaling activity of delta in Drosophila,” Developmental Cell, vol. 10, no. 2, pp. 245–255, 2006.