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Original Article

Synergistic anti-tumor effects of Danshen injections in combination with chemotherapy and antiangiogenic therapy via alleviating tumor stroma fibrosis state

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Abstract: Cancer-associated hypercoagulation is a negative factor for anticancer treatment. Anticoagulant therapy might reverse treatment bottleneck. The efficacy of Danshen injections, in combination with chemotherapy and anti-VEGF targeted therapy, was evaluated based on previously established colon carcinoma patient-derived xenografts (PDXs). Immunohistochemistry and pharmacological experiments were conducted to explore the mechanisms underlying the synergistic effects of Danshen. Finally, Danshen injections showed significant synergistic effects, in combination with FP3 and eloxatin. According to immunofluorescence and Immunohistochemistry, markers of fibrosis in tumor stroma (such as, TGF-β, PDGF-β, TIMP1, MMP-2 and TNC) were suppressed. In conclusion, Danshen injections showed significant efficacy, in combination with chemotherapy and anti-VEGF therapy, for treatment of metastatic colon carcinoma. Possible mechanisms of improving drug distribution are the alleviation of the tumor stroma fibrosis state.

Keywords: Danshen injections, anti-VEGF therapy, chemotherapy, synergistic effects, anticoagulant

Introduction

Cancer-associated hypercoagulation is a common in malignancy, directly affecting the sensitivity of anticancer drugs and the prognosis of patients with cancer. Chemotherapies are prone to deteriorating cancer patient hypercoagulation [1]. It has been hypothesized that anticoagulant herb extracts, in combination with chemotherapy and anti-VEGF targeted therapy, might alleviate hypercoagulation, resulting in synergistic effects for treatment of solid tumors [2-4].

Danshen (Salvia miltiorrhiza Bunge), an herb, has been widely used in Traditional Chinese Medicine for the treatment of coronary heart diseases, such as myocardial infarction and angina pectoris. Danshen has been recently reported to possess efficacy against human cancer cells, in addition to its function in cardiovascular systems [5-8].

Patient-derived xenografts (PDXs), in which patient tumors are directly engrafted into immunocompromised mice [9], have been increasingly widely used in various types of cancers for translational research [10]. Accumulating evidence has indicated that PDX models recapitulate primary tumor architecture and genetic characteristics, thus becoming a reliable cancer research tool for drug evaluation and personalized medicine applications, superior to traditional cell line xenografts [11]. [Aparicio S, 2015 #21; Chen W, 2016 #89].

A Novel VEGF-trap FP3 (also referred to as KH902 or KH903) is engineered by fusing the 2nd extracellular domain of Flt-1 (VEGF receptor 1) and the 3rd and 4th extracellular domain of KDR (VEGF receptor 2) to the Fc portion of human immunoglobulin G1 [12]. Previous studies have shown that FP3 has antitumor efficacy in PDXs of gastric carcinoma and colorectal cancer, as well as the effect of normalizing vasculature [12].

The aim of this study was to investigate and demonstrate the potential synergistic effects and mechanisms of anticoagulant herb extracts, such as Danshen injections, in combination with chemotherapy, such as capecitabine and anti-VEGF therapy, for treatment of metastatic colon carcinoma. This study used previously established PDX models of colon cancer hepatic metastases via methods of fluorescent imaging, fluorescent immunohistochemistry, and pharmacological experiments.

Materials and methods

Drugs and reagents

FP3 was provided by Kanghong Biotechnology, Inc. ELO (Eloxatin) was purchased from CENEXI-Laboratoires THISSEN S.A. (BN: 14E16). DAN (Danshen injection) was purchased from Shanghai NO.1 Biochemical & Pharmaceutical. CO., LTD. The antibodies against TGF-β, PDGF-β, TIMP-1, MMP-2, and TNC were purchased from Abcam.

Patient and tumor tissues

Colon carcinoma hepatic metastases specimens were obtained from a 54-y-old male patient of the 1st Affiliated Hospital, School of Medicine, Zhejiang University. The study was approved by the Scientific and Ethical Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University. Informed consent was provided by the patient. The tumor was diagnosed as ulcerative moderately differentiated adenocarcinoma.

Establishment of PDX model

Surgical tumor tissues were cut into pieces of 2 to 3 mm and transplated s.c. to 4 three-to-four-week-old female BALB/c nude mice (Shanghai Slaccas Laboratory Animal). Tumor volume was calculated with the formula $V = LD \times (SD)^2/2$, where V means the tumor volume and LD and SD are the longest and the shortest tumor diameters, respectively. Tumors were then passed from mouse to mouse. At each generation, tumors were harvested and stored in liquid nitrogen for further experimentation. The use and operation of experimental animals was according to the Principles of Laboratory Animal Care (NIH publication 85-23, revised in 1985). All animal studies were approved by the Institutional Animal Care and Use Committee of Zhejiang University (approval ID: SYXK (ZHE) 2005-0072).

Treatment protocol

Five mice of the 3rd generation PDX models were randomized into each group after tumors grew to a size of 150-200 mm^3. Experimental (control) groups: 1. Danshen injections (NS, normal saline); 2. Danshen injections combined with Eloxatin (Eloxatin); and 3. Danshen injections combined with FP3 and Eloxatin. Dosing was administrated by intravenous injections once per week for FP3 (15 mg/kg, twice per week), by intraperitoneal injections for Eloxatin (3 mg/kg, once per day for 5 days, rest for 5 days, then once per day for 5 days), and by intraperitoneal injections for Danshen (20 mg/kg, twice per week) during the 3 weeks. The mice were weighed and tumor sizes were evaluated twice per week. TGI (relative tumor growth inhibition) was used for evaluation of anti-tumor efficacy. TGI = (1-T/C)%, in which T means relative tumor growth of treated mice and C means relative tumor growth of control mice. Mice tumors were harvested on the 30th day.

Fluorescent immunohistochemistry

Mice with similar tumor sizes were anesthetized with chloral hydrate (5%, 0.2 ml/20 g), injected intramuscularly. The vasculature was perfused with 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) by inserting an 18-gauge cannula into the aorta in the left ventricle. Xenograft tumors were then removed and stored for 2 hours at 4°C. After PBS rinsing, tumor tissues were infiltrated with 30% sucrose overnight and frozen for cryostat sectioning after embedding in OCT. Cryostat sections were fixed in acetone for about 10 minutes. Slides were washed in PBS and dried several times. After blocking nonspecific antibody binding, seven primary antibodies (TGF-β and PDGF-β) were added on the slides overnight at room temperature. The signal was amplified for one hour with fluorescent secondary antibodies. All slides were counterstained with DAPI (Invitrogen). Tissue sections were photographed using an Olympus BX51 Fluorescence Microscope.

Immunohistochemistry

Specimens were fixed in 10% neutral formalin and embedded in paraffin. The tissues were sectioned (5 μm thick) and placed on slides for

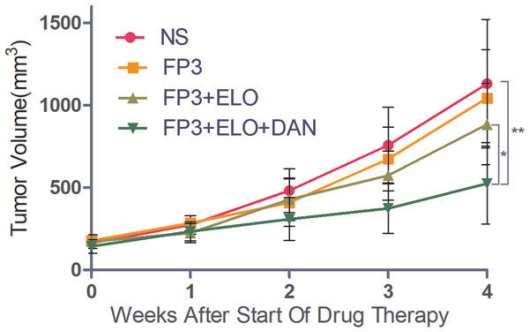

Figure 1. Anti-tumor-growth ability evaluation by endpoint tumor volume. DAN, Danshen injection. ELO, Eloxatin. FP3 in combination with Eloxatin and Danshen injections showed better anti-tumor-growth ability than NS ($p < 0.01$), than that of single FP3 ($p < 0.05$), or than that of FP3 in combination with Eloxatin ($p < 0.05$).

marker analysis. After blocking nonspecific antibody binding, sections were incubated at 4°C with the primary antibodies (TIMP-1, MMP-2, and TNC) overnight. Immunohistochemistry was performed according to Lab Vision streptavidin-biotin peroxidase complex method. Slides were photographed using an Olympus BX60 (Olympus).

Statistical analysis

Drug efficacy data are reported as mean ± SD. Calculation and statistics were performed with Excel 2010 (Microsoft, Redmond, WA) and GraphPad Prism 5 (GraphPad Software, San Diego, CA). One-way ANOVA were used to analyze the significance of differences among groups. $P < 0.05$ indicates statistical significance.

Results

Efficacy evaluation of Danshen injections based on PDX model

To test whether Danshen injections have potential synergistic effects, in combination with chemotherapy and anti-VEGF therapy, for treatment of metastatic colon carcinoma, a PDX model of colon cancer hepatic metastases was established. It was based on which efficacy of Danshen injections was evaluated, in terms of single agent, Danshen combined with Eloxatin, and Danshen combined with Eloxatin and FP3, respectively. Since the fourth generation of PDX tumor volume reached 150-200 mm^3, dosing was administrated during the 3 weeks. Mice were killed and tumors were measured on day 30. Relative tumor growth inhibition (TGI) was calculated per the following formula: (1-T/C)%, where T is average relative tumor volume of drug treated group mice and C is average relative tumor volume of control group mice. Danshen injections showed anti-tumor-growth ability, in combination with Eloxatin, more than single Eloxatin. They showed more significant synergistic effects in combination with FP3 and Eloxatin. No significant anti-tumor-growth effects of Danshen were shown as a single agent (**Figure 1**).

Mechanisms underlying synergistic effects of Danshen injection combination therapy

To explore the mechanisms of the synergistic effects of Danshen injections, in combination with chemotherapy and anti-VEGF therapy, changes both in terms of tumor vasculature and stroma were observed via methods of fluorescent imaging, fluorescent immunohistochemistry, and pharmacological experiments.

Evaluating tumor stroma changes as a result of Danshen injection administration, it was found that immunofluorescence expression of PDGF-β (Platelet-Derived Growth Factor Beta) and TGF-β (Transforming Growth Factor Beta) was suppressed (**Figure 2**). Similar results were noted for TIMP1 (Tissue Inhibitor of Metalloproteinase-1), MMP-2 (matrix metalloproteinase-2), and TNC (Tenascin C) by Immunohistochemistry (**Figure 3**), indicating a reduction of tumor stroma fibrosis after Danshen injection combination treatment.

Discussion

The present study established a patient-derived colorectal cancer xenograft model for evaluation of Danshen injections, in combination with chemotherapy and anti-VEGF therapy. Danshen injections showed anti-tumor-growth ability in combination with Eloxatin, more than single Eloxatin. They showed more significant synergistic effects in combination with FP3 and Eloxatin, indicating that Danshen injections might be a novel efficient combination therapy in the treatment of metastatic colon carcinoma (**Figure 1**). No significant anti-tumor-growth effects of Danshen were noted as a single agent, indicating that Danshen injections contribute to tumor treatment as a combination therapy but not as a single agent.

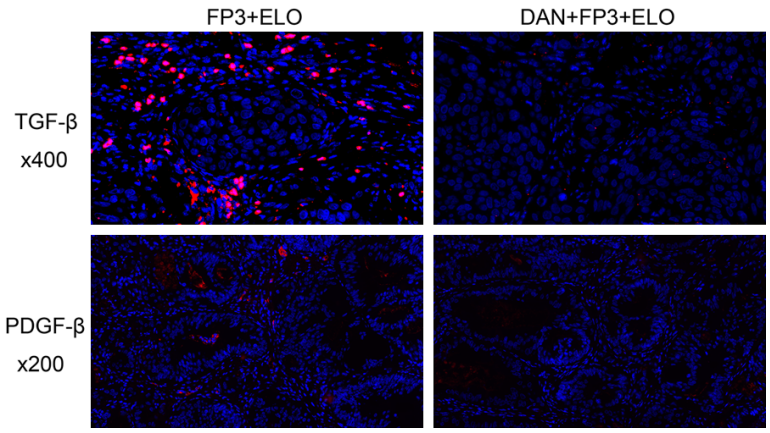

Figure 2. Reduction of tumor stroma fibrosis after Danshen injection treatment. Immunofluorescence expression of stroma fibrosis related biomarkers (such as, PDGF-β, and TGF-β) was suppressed posttreatment.

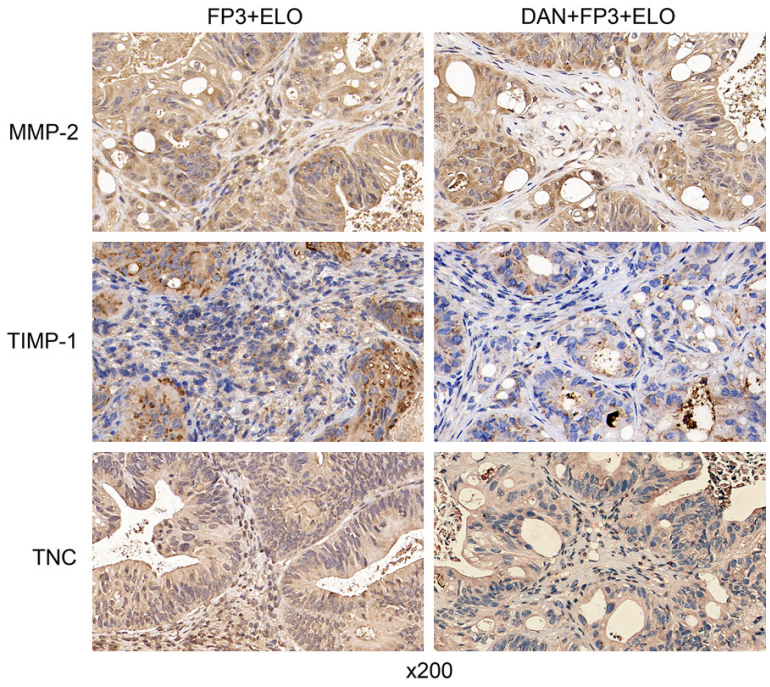

Figure 3. Reduction of tumor stroma fibrosis after Danshen injection treatment. Immunohistochemistry expression of stroma fibrosis related biomarkers (such as, TIMP1, MMP-2, and TNC) was suppressed posttreatment.

Fibrosis (e.g., collagen fibers) in tumor stroma is one of the determinant factors influencing the kinetics of drug distribution within tumors [13-15]. Pro-fibrogenic cytokines, including TGF-β and PDGF-x, are strongly associated with stroma fibrosis [16, 17]. TIMP1, MMP-2, and TNC are also contributory factors in the development of tissue fibrosis [18-20]. Evaluating tumor stroma changes as a result of Danshen injection administration, according to immunofluorescence and Immunohistochemistry, it was found that expression of PDGF-β, TGF-β, TIMP1, MMP-2, and TNC was suppressed. This indicates a reduction of tumor stroma fibrosis after Danshen injection combination treatment (**Figure 3**). Present results indicate that Danshen injections alleviated tumor stroma fibrosis, leading to improvement of drug distribution.

In conclusion, Danshen injections have shown significant efficacy, in combination with chemotherapy and anti-VEGF therapy, for treatment of metastatic colon carcinoma. Possible mechanisms of action include the improvement of drug distribution by alleviating the tumor stroma fibrosis state.

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Disclosure of conflict of interest

None.

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References

[1] Evans CE, Bendahl PO, Belting M, Branco C, Johnson RS. Diverse roles of cell-specific hypoxia-inducible factor 1 in cancer-associated hypercoagulation. Blood 2016; 127: 1355-60.

[2] Lan HR, Lin FQ, Zhou QL, Huang LM, Jin KT. Strategies to improve drug distribution in solid tumor. Int J Clin Exp Med 2016; 2: 658-667.

[3] Stylianopoulos T, Jain RK. Combining two strategies to improve perfusion and drug delivery in solid tumors. Proc Natl Acad Sci U S A 2013; 110: 18632-7.

[4] Khawar IA, Kim JH, Kuh HJ. Improving drug delivery to solid tumors: priming the tumor microenvironment. J Control Release 2015; 201: 78-89.

[5] Bertotti A, Migliardi G, Galimi F, Sassi F, Torti D, Isella C, Corà D, Di Nicolantonio F, Buscarino M, Petti C, Ribero D, Russolillo N, Muratore A, Massucco P, Pisacane A, Molinaro L, Valtorta E, Sartore-Bianchi A, Risio M, Capussotti L, Gambacorta M, Siena S, Medico E, Sapino A, Marsoni S, Comoglio PM, Bardelli A, Trusolino L. A molecularly annotated platform of patient-derived xenografts ("Xenopatients") identifies her2 as an effective therapeutic target in cetuximab-resistant colorectal cancer. Cancer Discov 2011; 1: 508-23.

[6] Gong Y, Li Y, Abdolmaleky HM, Li L, Zhou JR. Tanshinones inhibit the growth of breast cancer cells through epigenetic modification of aurora a expression and function. PLoS One 2012; 7: e33656.

[7] Nizamutdinova IT, Lee GW, Lee JS, Cho MK, Son KH, Jeon SJ, Kang SS, Kim YS, Lee JH, Seo HG, Chang KC, Kim HJ. Tanshinone I suppresses growth and invasion of human breast cancer cells, MDA-MB-231, through regulation of adhesion molecules. Carcinogenesis 2008; 29: 1885-92.

[8] Lee CY, Sher HF, Chen HW, Liu CC, Chen CH, Lin CS, Yang PC, Tsay HS, Chen JJ. Anticancer effects of tanshinone I in human non-small cell lung cancer. Mol Cancer Ther 2008; 7: 3527-38.

[9] Hidalgo M, Bruckheimer E, Rajeshkumar NV, Garrido-Laguna I, De Oliveira E, Rubio-Viqueira B, Strawn S, Wick MJ, Martell J, Sidransky D. A pilot clinical study of treatment guided by personalized tumorgrafts in patients with advanced cancer. Mol Cancer Ther 2011; 10: 1311-6.

[10] Johnson JI, Decker S, Zaharevitz D, Rubinstein LV, Venditti JM, Schepartz S, Kalyandrug S, Christian M, Arbuck S, Hollingshead M, Sausville EA. Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials. Br J Cancer 2001; 84: 1424-31.

[11] Aparicio S, Hidalgo M, Kung AL. Examining the utility of patientderived xenograft mouse models. Nat Rev Cancer 2015; 15: 311-6.

[12] Jin K, Lan H, Cao F, Xu Z, Han N, Li G, He K, Teng L. Antitumor effect of FP3 in a patient-derived tumor tissue xenograft model of gastric carcinoma through an antiangiogenic mechanism. Oncol Lett 2012; 3: 1052-1058.

[13] Trédan O, Galmarini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. J Natl Cancer Inst 2007; 99: 1441-1454.

[14] Ghajar CM, Bissell MJ. Extracellular matrix control of mammary gland morphogenesis and tumorigenesis: insights from imaging. Histochem Cell Biol 2008; 130: 1105-1118.

[15] Minchinton AI, Tannock IF. Drug penetration in solid tumours. Nat Rev Cancer 2006; 6: 583-592.

[16] Friedman SL. Liver fibrosis -- from bench to bedside. J Hepatol 2003; 38 Suppl 1: S38-53.

[17] Park HJ, Kim HG, Wang JH, Choi MK, Han JM, Lee JS, Son CG. Comparison of TGF-β, PDGF, and CTGF in hepatic fibrosis models using DMN, CCl4, and TAA. Drug Chem Toxicol 2016; 39: 111-8.

[18] Wang K, Lin B, Brems JJ, Gamelli RL. Hepatic apoptosis can modulate liver fibrosis through TIMP1 pathway. Apoptosis 2013; 18: 566-77.

[19] Chapman MA, Zhang J, Banerjee I, Guo LT, Zhang Z, Shelton GD, Ouyang K, Lieber RL, Chen J. Disruption of both nesprin 1 and desmin results in nuclear anchorage defects and fibrosis in skeletal muscle. Hum Mol Genet 2014; 23: 5879-92.

[20] Gandellini P, Andriani F, Merlino G, D'Aiuto F, Roz L, Callari M. Complexity in the tumour microenvironment: cancer associated fibroblast gene expression patterns identify both common and unique features of tumour-stroma crosstalk across cancer types. Semin Cancer Biol 2015; 35: 96-106.