Int J Clin Exp Med 2017;10(4):6437-6444
www.ijcem.com /ISSN:1940-5901/IJCEM0045284

Original Article

Synergistic effects and mechanism of Danshen injection in combination with chemotherapy and antiangiogenic therapy for colon cancer

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Received October 27, 2016; Accepted February 1, 2017; Epub April 15, 2017; Published April 30, 2017

Abstract: Cancer-associated hypercoagulation is a negative factor for anticancer treatment. Anticoagulant therapy might reverse the treatment bottleneck. The efficacy of Danshen injection in combination with chemotherapy and anti-VEGF targeted therapy was evaluated based on a previously established colon carcinoma patient-derived xenografts (PDXs). Immunohistochemistry and pharmacological experiments were conducted to explore the mechanism underlying the synergistic effects of Danshen. Finally, Danshen injection shown significant synergistic effect in combination with bevacizumab and Eloxatin. After Danshen injection combination treatment, hypoxia markers CAIX, GLUT1 and VEGF were suppressed, immunofluorescence expressions of fibrosis markers (PDGF-β, TGF-β, TIMP1 and desmin) were suppressed, and tumor vasculatures (visualized by CD31 and α-SMA immunostaining) shown more normalization. In addition, no tumor distant metastases (such as, liver, spleen, kidney, lung and brain) were found. In conclusion, we first reported Danshen injection as a novel efficient combination therapy with chemotherapy and anti-VEGF therapy for treatment of metastatic colon carcinoma through several possible mechanisms of improving drug distribution, such as by improving tumor hypercoagulable state and blood flow, by alleviating the tumor stroma fibrosis state, and by enhancing the tumor vasculature normalization effect of anti-VEGF therapy. In addition, Danshen injection showed no side effect of tumor distant metastases.

Keywords: Colon carcinoma, Danshen injection, anti-VEGF therapy, chemotherapy, synergistic effect, anticoagulant

Introduction

Cancer-associated hypercoagulation is really common in malignancy and directly affects the sensitivity of anticancer drug and the prognosis of patients with cancer, and chemotherapies are prone to deteriorate cancer patients' hypercoagulation [1]. It is hypothesized that anticoagulant herb extracts in combination with chemotherapy and anti-VEGF targeted therapy might alleviate hypercoagulation and result 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 some efficacy against human cancer cells, in addition to its functions 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 in recent years [10]. Accumulating evidences indicate that PDX models recapitulate primary tumor architecture and genetic characteristics, thus become an reliable cancer research tool for drug evalua-

tion and personalized medicine applications, superior to traditional cell line xenografts [11].

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

Materials and methods

Drugs and reagents

BEV (Avastin, bevacizumab) was kindly provided by Department of Chemotherapy, the 1st Affiliated Hospital, College of Medicine, Zhejiang University. 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 antibody against CD31, α-SMA, Desmin, PDGF, TGF-β, TIMP1, GLUT1, CAIX, VEGF 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 signed 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 as formula $V = LD \times (SD)^2/2$, where V means the tumor volume, while LD and SD are the longest and the shortest tumor diameter, respectively. Tumors were then passaged from mice to mice. At each generation, tumors were harvested and stored in liquid nitrogen and used for further experiments. The use and operation of experimental animals were 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 grown to a size of 150-200 mm^3. The experimental (control) groups: 1. Danshen injection (NS, normal saline); 2. Danshen injection combined with Eloxatin (Eloxatin); 3. Danshen injection combined with Bevacizumab and Eloxatin (Bevacizumab and Eloxatin). Then dosing was administrated by intravenous injection once per week for bevacizumab (15 mg/kg, twice per week), by intraperitoneal injection 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 injection for Danshen injection (20 mg/kg, twice per week) during 3 weeks. Mice were weighed and tumor size was 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 size 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. Then, xengraft tumor was removed and stored in fixative for 2 hours at 4°C. After PBS rinse, tumor tissues were infiltrated with 30% sucrose overnight, and frozen for cryostat sectioning after embedded in OCT. Cryostat sections were fixed in acetone for about 10 min. Then slides were washed in PBS and dried for several times. After blocking nonspecific antibody binding, seven primary antibodies (CD31, α-SMA, Desmin, PDGF, TGF-β, TIMP1, GLUT1) was added on the slides overnight at room temperature. The signal was amplified for one hour with fluo-

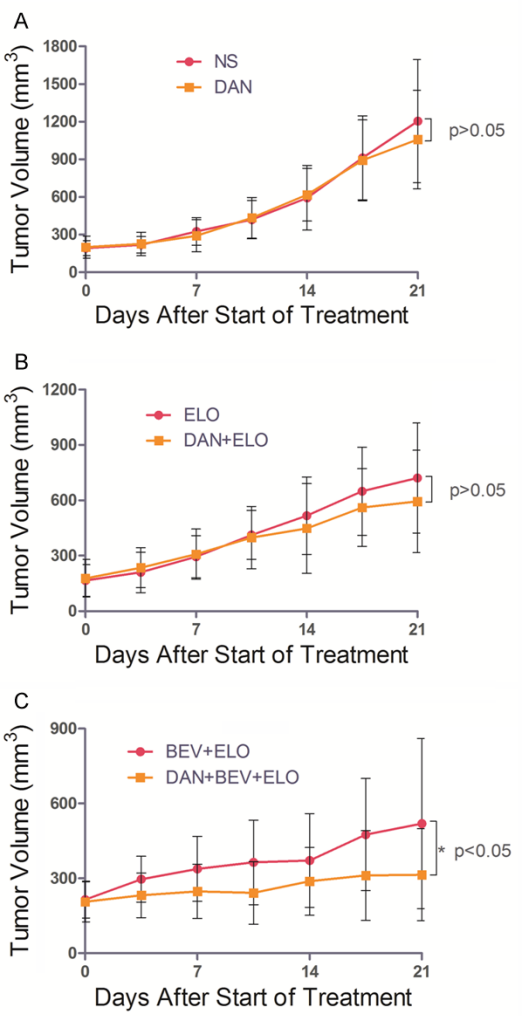

Figure 1. Anti-tumor-growth ability evaluation by endpoint tumor volume. A. Efficacy evaluation of single Danshen injection; B. Combined with Eloxatin, Danshen injection shown better anti-tumor-growth ability than single Eloxatin (without statistical significance); C. Danshen injection shown significant synergistic effect in combination with bevacizumab and Eloxatin.

rescent secondary antibodies. All slides were counterstained with DAPI (Invitrogen). Tissue sections were photographed using a Olympus BX51 Fluorescence Microscope.

Immunohistochemistry

Specimen were fixed in 10% neutral formalin and embedded in paraffin. Then tissues were sectioned (5 μm thick) and placed on slides for marker analysis. After blocking nonspecific antibody binding, sections were incubated at 4°C with the primary antibodies (CAIX, VEGF) 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 were 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. $P < 0.05$ was considered statistically significant.

Results

Efficacy evaluation of Danshen injection based on PDX model

To test whether Danshen injection has 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, based on which efficacy of Danshen injection was evaluated, in terms of single agent, Danshen combined with Eloxatin, and Danshen combined with Eloxatin and bevacizumab, respectively. Since the fourth generation PDX tumors volume reached 150-200 mm^3, dosing was administrated during 3 weeks. Mice were killed, and tumors were measured on day 30. Relative tumor growth inhibition (TGI) was calculated as 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. We found that Danshen injection shown significant synergistic effect in combination with bevacizumab and Eloxatin, while Danshen injection shown better anti-tumor-growth ability in combination with Eloxatin than single Eloxatin but wihout statistical significance. No significant anti-tumor-growth effect of Danshen was shown as a single agent (**Figure 1**).

Mechanism underlying synergistic effects of Danshen injection combination therapy

To order to explore the machanism of the synergistic effects of Danshen injection in combination with chemotherapy and anti-VEGF therapy, changes both in terms of tumor vasculature and stroma were observed via the methods of

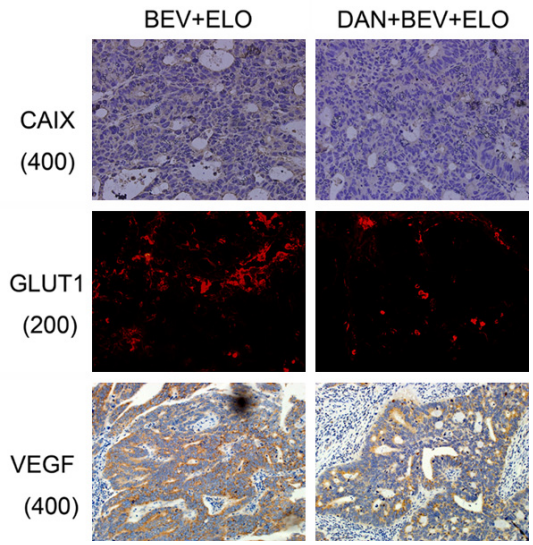

Figure 2. Remission of tumor hypoxia after Danshen injection treatment. Immunohistochemical expression of CAIX (bar = 50 μm), VEGF (bar = 100 μm) and immunofluorescence expression of GLUT1 (bar = 100 μm) and were suppressed posttreatment.

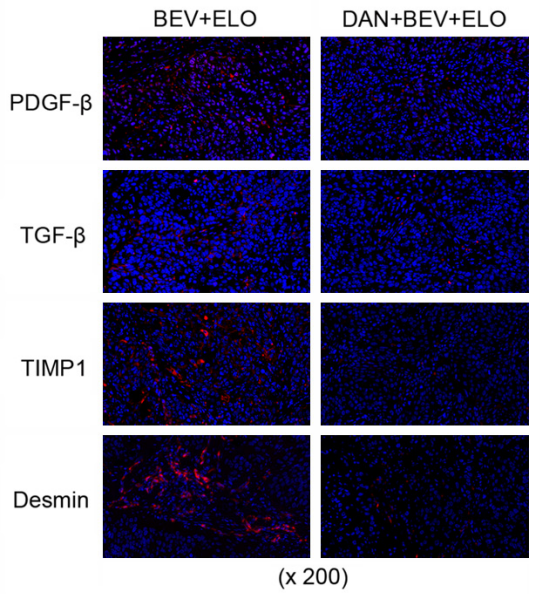

Figure 3. Reduction of tumor stroma fibrosis after Danshen injection treatment. Immunofluorescence expressions of stroma fibrosis related biomarkers (such as, PDGF-β, TGF-β, TIMP1, Desmin) were suppressed posttreatment.

fluorescent imaging, fluorescent immunohistochemistry, and pharmacological experiments.

Fluorescence and immunohistochemistry examinations were conducted to observe the changes of tumor hypoxia state by evaluating markers of hypoxia, immunohistochemical expressions of CAIX (Carbonic Anhydrase Isoform IX) and VEGF (Vascular Endothelial Growth Factor-A), and immunofluorescence expression of GLUT1 (Glucose Transporter 1) were compared between Danshen injection combination group and bevacizumab plus Eloxatin group (**Figure 2**). CAIX and GLUT1 are all markers of hypoxia, suppressions of which represent a reverse of tumor hypoxia state [12-14], while VEGF expression of tumor stroma will also be down-regulated as a result of an improved tumor oxygen supply [15-17]. As a result, expressions of CAIX, GLUT1 and VEGF were suppressed.

In order to evaluate the tumor stroma changes as a result of Danshen injection administration, we found that immunofluorescence expressions of PDGF-β (Platelet-Derived Growth Factor beta), TGF-β (Transforming Growth Factor beta), TIMP1 (Tissue Inhibitor of Metalloproteinase-1) and Desmin were suppressed, which meant a reduction of tumor stroma fibrosis after Danshen injection combination treatment (**Figure 3**).

To evaluate whether Danshen injection treatment enhanced the tumor vasculature normalization effect of bevacizumab, sectioned selected tumors were immunostained with antibodies to CD31 and α-SMA to visualize vasculature (**Figure 4**). We found that tumor vasculatures shown more normalization after Danshen injection combination treatment.

To observe whether Danshen injection treatment generated a rise of the odds of tumor distant metastasis, hematoxylin and eosin staining were conducted, one month, two months and three months posttreatment, respectively. No tumor distant metastases (such as, liver, spleen, kidney, lung and brain) were found after Danshen injection treatment (**Figure 5**).

Discussion

In the present study, we established a patient-derived colorectal cancer xenograft model for the evaluation of Danshen injection in combination with chemotherapy and anti-VEGF therapy. Danshen injection shown significant synergistic effect in combination with bevacizumab and Eloxatin, while Danshen injection shown

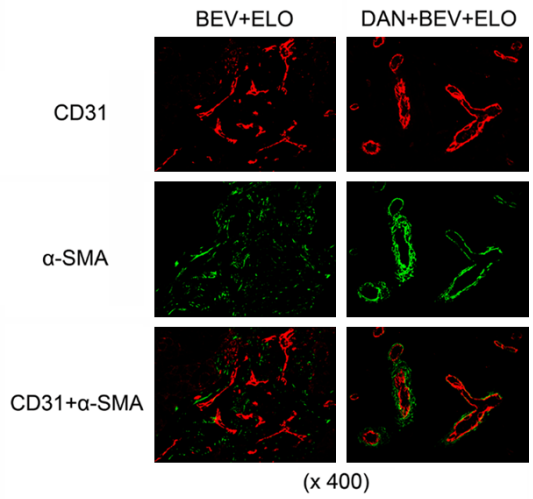

Figure 4. Normalizd vasculature examined by angiography with immunostaining for endothelial cells (using anti-CD31 antibody; bar = 50 μm) and pericytes (using anti-α-SMA antibody; bar = 50 μm). After Danshen injection treatment, tumor vasculatures shown more normalization.

better anti-tumor-growth ability in combination with Eloxatin than single Eloxatin but wihout statistical significance, indicating that Danshen injection might be a novel efficient combination therapy with chemotherapy and anti-VEGF therapy for treatment of metastatic colon carcinoma (**Figure 1**). No significant anti-tumor-growth effect of Danshen was shown as a single agent, indicating that Danshen injection contributes to tumor treatment as a combination therapy but not a single agent.

Then, we got a sight into the changes mainly in terms of tumor vasculature and stroma to explore the mechanism of the synergistic effects of Danshen injection in combination with chemotherapy and anti-VEGF therapy. In our study, we found that expressions of hypoxia markers CAIX, GLUT1 and VEGF were all suppressed after Danshen injection therapy, indicating that Danshen injection reversed tumor hypoxia state to some degree (**Figure 2**). The rectification of tumor hypoxia state manifested that Danshen injection treatment improved the oxygen supply of tumor stroma, indicating that Danshen injection might contribute to an increase in tumor blood flow and oxygen delivery by improving tumor hypercoagulable state, thus the increased tumor blood flow might improve the chemotherapeutics distribution to cancer cells [18, 19]. In addition, transcription of tumor suppressor genes are repressed due to hypermethylation of the promoters of these genes, while about half of hypermethylation are caused by hypoxia [20]. Repressed transcription of tumor suppressor genes is the main cause of tumorigenesis [21-23]. Therefore, the results of our study indicated that Danshen injection might also reduce the morbidity and recurernce rate of malignant neoplasms.

Fibrosis (e.g., collagen fibers) in tumor stroma is one of the determinant factors to influence the kinetics of drug distribution within tumors [24-26]. The pro-fibrogenic cytokines, including TGF-β and PDGF, are strongly associated with stroma fibrosis [27, 28]. TIMP1 and desmin are also contributory factors in the development of tissue fibrosis [29, 30]. To order to evaluate the tumor stroma changes as a result of Danshen injection administration, we found that immunofluorescence expressions of PDGF-β, TGF-β, TIMP1 and desmin were suppressed, which meant a reduction of tumor stroma fibrosis after Danshen injection combination treatment (**Figure 3**). These results indicated that Danshen injection alleviated the tumor stroma fibrosis, thus leading to improvement of drug distribution.

Vasculature normalization brings an improvement of the cytotoxic agents distribution by reversing tumor irregular vasculature to a normal one, which is the mechanism underlying the synergistic effects of chemotherapy in combination with anti-VEGF therapies [31]. To evaluate whether Danshen injection treatment enhanced the tumor vasculature normalization effect of bevacizumab, sectioned selected tumors and immunostained with antibodies to CD31 and α-SMA to visualize vasculature (**Figure 4**). We found that tumor vasculatures shown more normalization after Danshen injection combination treatment.

In addition, no tumor distant metastases were found one month, two months and three months after Danshen injection treatment (**Figure 5**). Our results demonstrated anticoagulation, in combination with chemotherapy, doesn't contribute to tumor distant metastases. Similar conclusion were reported by several other studies focusing on the synergistic effects of aspirin as a combination therapy for malignant tumour treatment [32-34]. While in our study, we demonstrated Danshen injection,

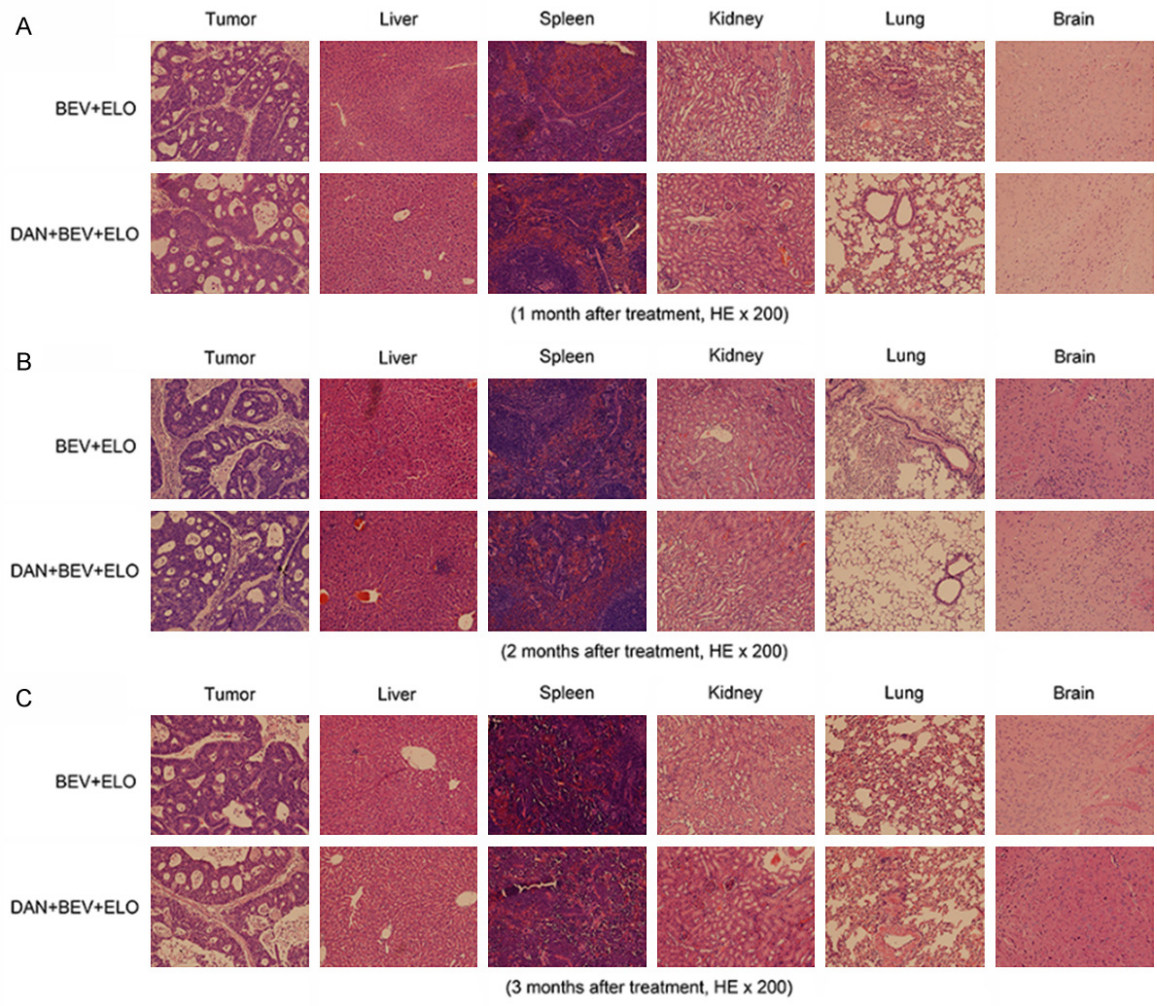

Figure 5. Hematoxylin and eosin staining shown no tumor metastases found in distant organs (such as, liver, spleen, kidney, lung and brain) after Danshen injection treatment (bar = 100 μm). A. One month after treatment; B. Two months after treatment; C. Three months after treatment.

as a novel anticoagulation therapy strategy in combination with chemotherapy and anti-VEGF targeted therapy, shown no side effect of tumor distant metastases.

In conclusion, we first reported Danshen injection as a novel efficient combination therapy with chemotherapy and anti-VEGF therapy for treatment of metastatic colon carcinoma through several possible mechanisms of improving drug distribution, such as by improving tumor hypercoagulable state and blood flow, by alleviating the tumor stroma fibrosis state, and by enhancing the tumor vasculature normalization effect of anti-VEGF therapy. In addition, Danshen injection showed no side effect of tumor distant metastases.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Grant No. 81374014), Zhejiang Provincial Science and Technology Projects (grants no. 2015C33264, 2017C33212 and 2017C33213), and Zhejiang Provincial Medical and Healthy Science and Technology Projects (Grants No. 2013KYA228 and 2016KYA180).

Disclosure of conflict of interest

None.

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