Int J Clin Exp Med 2016;9(10):19064-19073
www.ijcem.com /ISSN:1940-5901/IJCEM0022354

Original Article

Preconditioning treatment with carbocisteine enhance the anti-tumor effect of oxaliplatin on the BRAF-mutant HT29 and metastasis

Zhe-Yuan Wang[1,2*], Qing Zhai[1,2*], Xiao-Le Xu[1,2*], Xuan Ye[1,2], Qiong Du[1,2], Bin Zhu[1,2], Bo Yu[1,2]

[1]Department of Pharmacy, Fudan University Shanghai Cancer Center, 270 Dong-An Road, Shanghai 200032, China; [2]Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China. [*]Equal contributors.

Received December 21, 2015; Accepted March 21, 2016; Epub October 15, 2016; Published October 30, 2016

Abstract: Systematic chemotherapy is an important treatment strategy for colon cancer. However, drug resistance obstructs the improvement of patients' survival and life quality. Since tumor cells invade from oxidative stress for its survival advantage and reactive oxygen species (ROS) has been reported to contribute to the tumor chemo- and radio-resistance. We hypothesized that changes of tumor redox-microenvironment might be associated with the efficacy of chemotherapy drugs. To prove the hypothesis, firstly we verified the anti-tumor formation and metastasis effects of combined usage of oxaliplatin with carbocisteine in BRAF-mutant HT29 colon cancer cells *in vivo*. Here we report that preconditioning treatment with carbocisteine, an antioxidant originally identified as mucoregulatory drug, was more effective than signal usage of oxaliplatin in inhibiting the growth of human colon cancer, while carbocisteine itself had no effects on tumor growth. Besides, carbocisteine could sensitize the colon cancer to oxaliplatin and enhance the anti-tumor effects of chemotherapy in a dose-dependent manner. Also, preconditioning treatment with carbocisteine significantly suppressed colon cancer cell lung metastasis and prolonged the life span compared to the signal usage of oxaliplatin of mice. Consistently, the level of apoptosis on excised xenograft tumors and orthotopic tumors was increased after oxaliplatin administration and enhanced after combination administration of oxaliplatin and carbocisteine. Furthermore, these promigratory effects are related to the epithelia-to-mesenchymal transition (EMT) phenomenon, as evidenced by the up-regulation of vimentin and down-regulation of E-cadherin. Our findings offer new mechanistic insights that carbocisteine might be a useful agent for oxaliplatin-based adjuvant chemotherapy in patients with colon cancer.

Keywords: Carbocisteine, oxaliplatin, ROS, tumor microenvironment, colon cancer, BRAF, apoptosis, epithelia-to-mesenchymal transition (EMT)

Introduction

Colon cancer is one of the most common diagnosed malignances and the fourth leading cause of cancer-related death around the world [1]. In decades, owing to the improvement of targeted drugs, for example the small-molecule inhibitor or antibodies, and clinical treatments, the survival rates and living quality of patient harboring advanced stage colorectal cancer are improved. However, because of the metastasis and relapse, the prognosis of colorectal cancer is still dim, mechanically, mainly due to concomitant genetic alterations including mutations of KRAS, EFGR and BRAF, as well as amplification of HER2 and MET [2-4].

Metastasis is a hallmark of cancer, in which cancer cells lost cell cohesion and acquired migratory and invasion properties. Studies have indicated that epithelial-mesenchymal transition (EMT) is implicated in metastasis of multi-types of cancers. Several key transcriptional regulators, such as SNAI, ZEB and TWIST are largely characterized in colorectal cancer [5, 6]. However, the complex molecular mechanisms underlying the metastasis network are largely unknown. BRAF valine 600 ($BRAF^{V600}$) mutant occur in approximately 10% of colorectal cancers and contribute to the poor prognosis in metastatic stages [7]. BRAF localized directly downstream of RAS, lead to activation of the mitogen-activated protein kinase path-

way, thereby affecting a multitude of key factors involved in cell proliferation and metastasis. Tumor microenvironment is another key factor that confers the cell metastasis, which is called pre-metastasis niche according to the "seed and soil hypothesis" [8]. The conductive microenvironment is needed for disseminating tumor cells to engraft distant site. The pre-metastasis niche is comprised of several inflammatory cytokines and chemokines to recruit immune-suppression cells, causing ROS damage as well [9].

Recently, there is an increasingly recognized linkage - a combination of therapeutic strategy that targeting tumor microenvironment and cell proliferation via using antioxidant and oxaliplatin. Carbocisteine is a mucolytic drug with antioxidative effect [10]. It has been proved that carbocisteine remarkably attenuated hydrogen peroxide-induced inflammatory injury and decreased cytokine expression such as IL-6, IL-8 and TNF [11-13]. We have reported that carbocisteine could reduce the cytotoxicity of oxaliplatin [14]. Oxaliplatin is widely used as the first-line cancer chemotherapy, typically along with folinic acid and 5-fluorouracil in a combination known as oxaliplatin in colorectal cancer [15, 16]. However, oncogene induced tumor resistance to chemotherapy challenges the clinical benefits of treatment. It is thus crucial to know whether the new combination facilitate the translation of them to therapeutic benefits. *In vivo* modeling of lung metastasis in BRAF-mutant colorectal cancer cell HT-29 revealed changing tumor microenvironment could enhance the cancer cells response to chemical drugs and blunt the tumor distal metastasis as well. Finally, the survival analysis of tumor-bearing mice indicated that combination of carbocisteine and oxaliplatin could prolong the mice survival time. Our results provided a theoretical foundation for the further study of the efficacy and molecular mechanisms of combination of carbocisteine and oxaliplatin.

Material and methods

Cell line and reagents

The human colon cancer HT-29 cell line and HEK-293T cell line were purchased from American Type Culture Collection (ATCC). HT-29 cells and HEK-293T were grown in McCoy's 5a medium (Invitrogen) and Dulbecco's modified Eagle's medium (DMEM) respectively, containing 10% fetal bovine serum (FBS, Gibco) and 100 units/mL of penicillin and streptomycin (Gibco), at 37°C in a humidified incubator containing 5% CO_2. Chemical drugs oxaliplatin and carbocisteine were from sigma and dissolved in normal saline (Sinopharm).

Lentivirus infection

To acquire the HT-29 cell line labeled with luciferase expression, the sequence coding firefly luciferase gene was from GeneBank. The sequence was cloned into the P LKO.1 lentiviral vector (Addgene). Then the constructed plasmid was co-transfected into HEK-293T cells with packaging plasmids to generate lentivirus. HT-29 cells were cultured in 6-well plate at the number of $2*10^5$/well and incubated for 24 h. Lentivirus were applied to HT-29 cells and selected by puromycin.

Xenograft tumor formation and orthotopic transplantation

For xenograft tumor formation assay, 1×10^7 HT-29-Luc cells were injected subcutaneously into the flanks of BALB/C nude mice. For orthotopic transplantation, after 10 days, the subcutaneous tumors were resected. Then the cubes of the tumor (1 mm^3) were implanted orthotopically in the end of caecum of the nude mice. Implantation was carried out by pinching the tumor pieces into the caecum with sharp fine-end forceps. Stabilization of the tumor pieces was performed by suturing the tumors and closing the abdominal skin with surgical needles. Bioluminescent signals were detected by the IVIS 100 Imaging System (Xenogen). Mice were injected with 100 mg/kg D-luciferin intraperitoneally before imaging. Mice were killed and examined 9 weeks after the orthotopic implantation. Experiments were performed under the institute's guidance on animal experimentation. For histological analysis, xenograft tumors, metastasis lungs of the mice were harvested and fixed in 10% formalin before paraffin embedding, then were sectioned and stained in HE and TUNEL assay.

Experimental protocol

Sixty 5- to 6-week-old athymic nude mice were randomized three days after orthotopic trans-

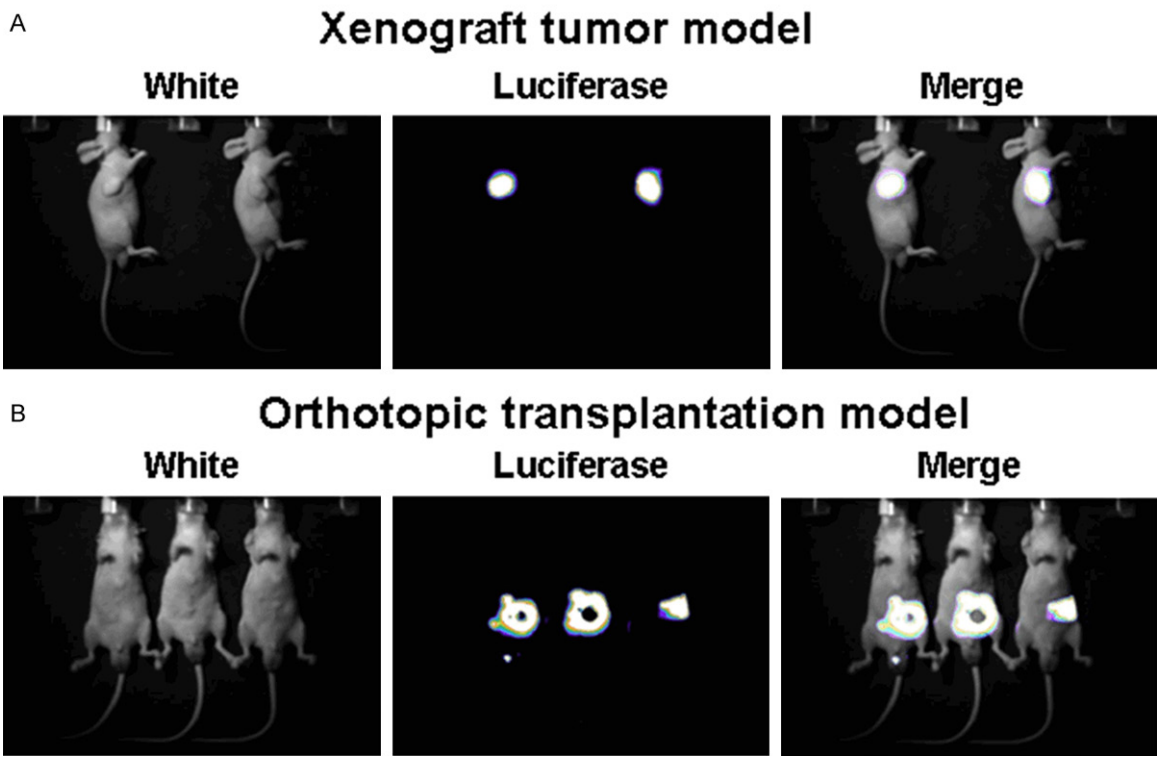

Figure 1. HT-29 cells xenograft tumor and orthotopic tumor. Tumor was recognized by luciferase and indicated as white light, luciferase and merge. Representative images were showed as (A) and (B).

plantation into one of four treatment groups: Group 1: normal saline (NS) + NS; Group 2: NS+ oxaliplatin 10 mg/kg·day (week 5-9); Group 3: Carbocisteine 4 mg/kg·day (week 1-5) + oxaliplatin 10 mg/kg·day (week 5-9); Group 4: Carbocisteine 8 mg/kg·day (week 1-5) + oxaliplatin 10 mg/kg·day (week 5-9).

This resulted in 4 groups of 15 animals each. To control for variance in initial tumor volumes, the mice were randomized according to estimated initial tumor volume.

TUNEL assay

Terminal deoxyribonucleotide transferase-mediated nick-end labelling (TUNEL) staining was performed using the In situ Cell Death Detection kit (Roche Applied Science, Indianapolis, IN, USA) following the instructions of the manufacturer. Briefly, 4-μm-thick formalin-fixed, paraffin-embedded tissue sections were deparaffinised and re-hydrated. Endogenous peroxidase activity was quenched by hydrogen peroxide and tissue protein was hydrolysed with proteinase K. Positive control sections were treated with DNase I 1000 U/ml. Negative control sections were incubated with label solution (without terminal deoxynucleotidyl transferase enzyme). All other sections were incubated with TUNEL reaction mixture (fluorescein-labelled nucleotides) at 37°C for 1 h in a humid chamber, incubated with converter-POD solution (anti-fluorescein antibody conjugated with POD) for 30 min at 37°C, treated with DAB and counterstained with haematoxylin.

qPCR analysis

Total RNA from tumor tissues was isolated with Ultraspec (Biotecx) according to the manufacturer's instructions. Quantitative PCR analysis was done on the ABI PRISM 7900 Sequence Detector System (Applied Biosystems) as previously described [14]. For determination of gene expression, Assay-on-demand (Applied Biosystems) was used. Transcript levels were normalized to those of GAPDH, which was used as endogenous control. Each experiment and every determination were done at least in triplicate, and the levels of gene expression were calculated using the ΔΔCt method.

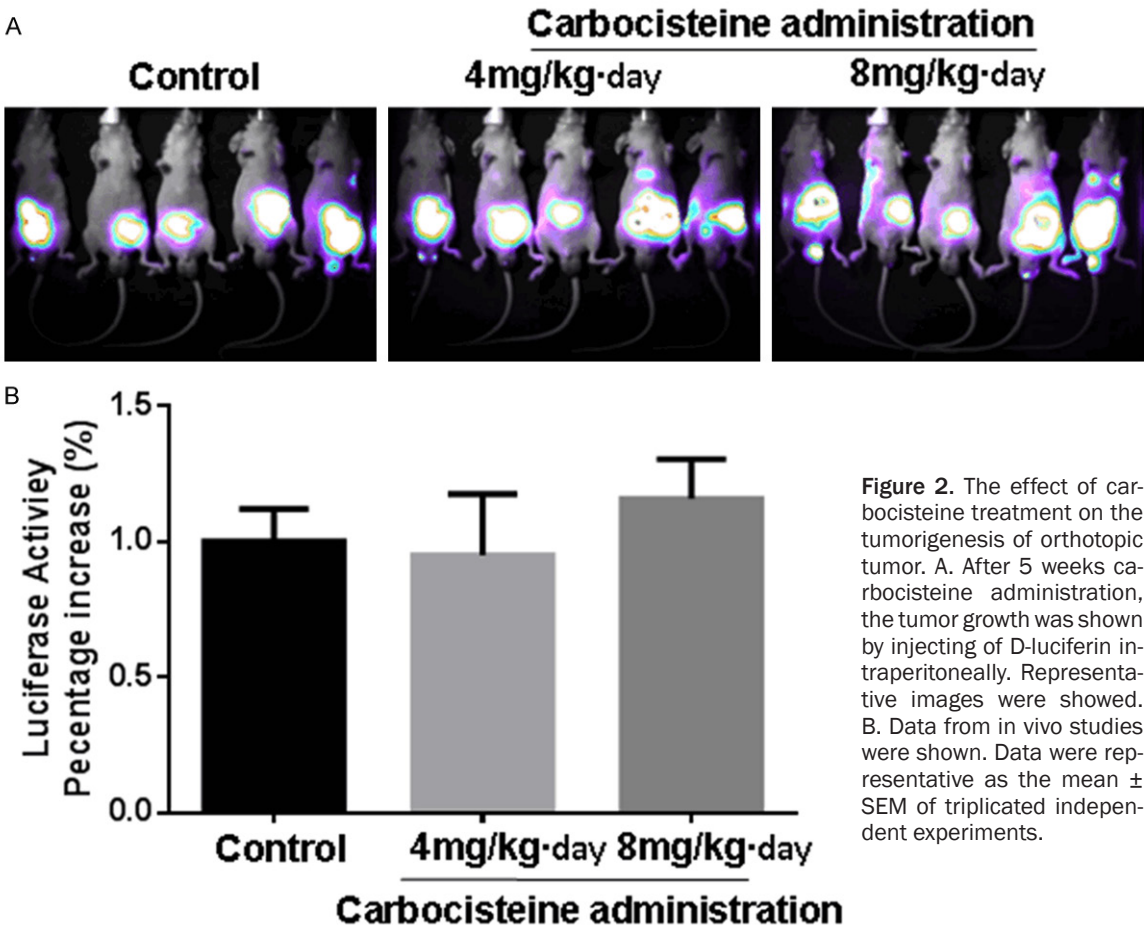

Figure 2. The effect of carbocisteine treatment on the tumorigenesis of orthotopic tumor. A. After 5 weeks carbocisteine administration, the tumor growth was shown by injecting of D-luciferin intraperitoneally. Representative images were showed. B. Data from in vivo studies were shown. Data were representative as the mean ± SEM of triplicated independent experiments.

Statistical analysis

Statistical analyses were conducted using SPSS 21.0 (IBM). Overall survival (OS) was calculated using Kaplan-Meier method. The survival distributions were compared through log-rank test. All statistical tests were two-sides. One-way analysis of variance (ANOVA) was used to compare groups. $P<0.05$ was considered statistically significant.

Results

Carbocisteine itself has no effect on the growth of orthotopic tumorgenesis

Recently, it has been demonstrated that BRAF mutation could induced chemotherapy resistance in colon cancer [17, 18]. To explore the effectiveness of the novel treatment strategy, we used BRAF-mutant colon cancer cell line HT29 to perform xenograft and orthotopic transplation, so we could test the efficacy of chemical drugs. As shown in **Figure 1**. HT-29 cells could form the xenograft tumor (**Figure 1A**) and orthotopic tumor (**Figure 1B**) successfully and when we injected D-luciferin intraperitoneally, the tumor could be apparently recognized and quantificated as the intensity of luciferase activity. Then we performed the tumor model to test the effect of carbocisteine on the growth of human colon cancer. After 5 weeks treatment with carbocisteine alone, representative tumor image (**Figure 2A**) and luciferase intensity (**Figure 2B**) of tumors sizes was similar compared with the control group. These data demonstrated that carbocisteine itself has no effect on the growth of BRAF mutant colon cancer cells.

Combination of oxaliplatin with carbocisteine suppresses tumor formation and metastasis in a dose-dependent manner

In order to explore the function of carbocisteine precondition on the anti-tumor effect of oxaliplatin, we performed orthotopic transplanta-

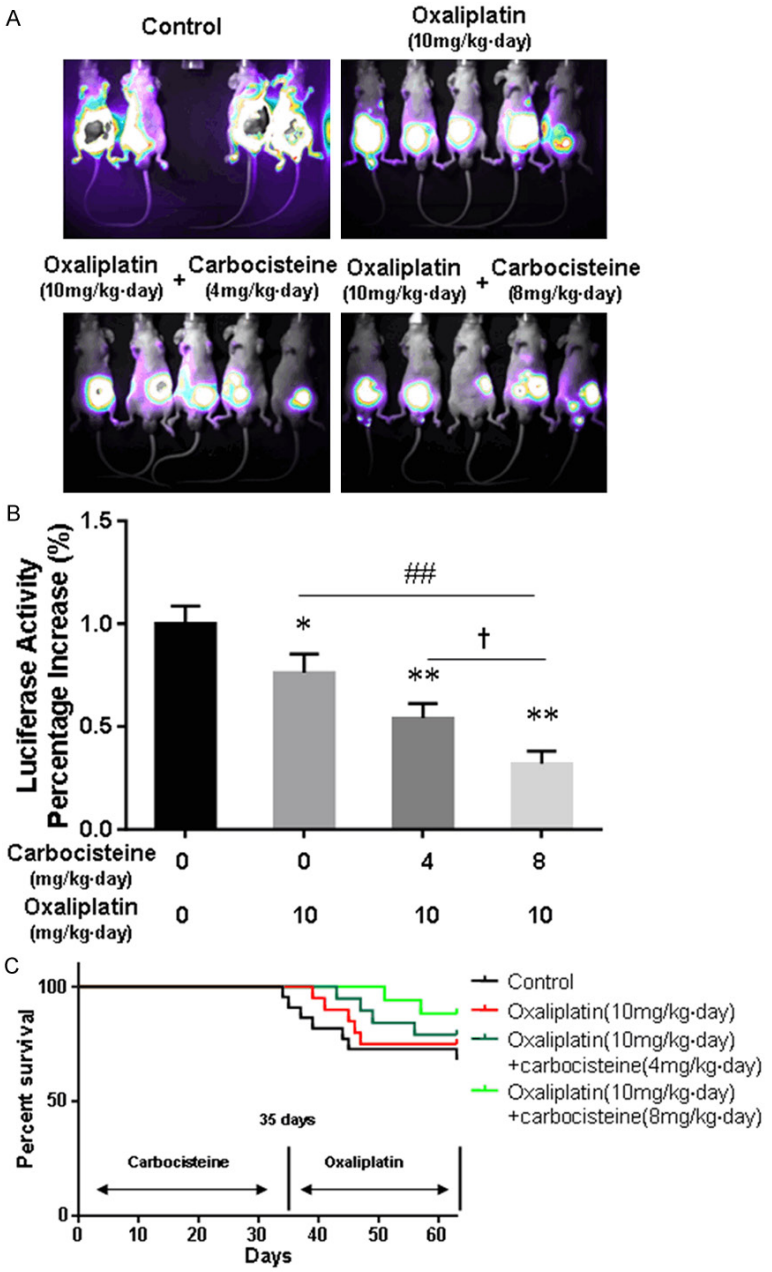

Figure 3. Preconditioning treatment with carbocisteine enhances the suppression of tumor formation and metastasis and the restored survival rate of orthotopic transplanting mice aftrer oxaliplatin chemotherapy. After subsequent 4 weeks oxaliplatin administration. The orthotopic transplantation of HT-29 cells caused lung metastasis and each group of mice was treated as described. The orthotopic tumorigenesis and lung metastasis site was measured by injecting D-luciferin intraperitoneally. Representative images (A) and analysis data (B) were showed. The survival rates (C) were analyzed by the Kaplan-Meier method, and survival was determined from the time of operation to mortality or the last follow-up. Data were representative as the mean ± SEM of triplicated independent experiments. *P<0.05, **P<0.01 vs. control group; ##P<0.01 vs. oxaliplatin (10 mg/kg·day) group; †P<0.05 vs. oxaliplatin (10 mg/kg·day) + carbocisteine (4 mg/kg·day) group.

tion. The carbocisteine and saline were administered once daily from Monday to Friday (5 times) for 5 weeks. After 5 weeks' administration of carbocistenine, orthotopic tumor and lung metastasis were observed. The oxaliplatin and saline were administered once daily from Monday to Friday (5 times) for another 4 weeks. We found that the treatment of 10 mg/kg oxaliplatin led to an inhibition effect on orthotopic tumor growth and lung metastasis (**Figure 3**). Meanwhile, when combining with 4 mg/kg carbocisteine, the anti-growth and anti-metastasis effect of oxaliplatin was significantly enhanced (**Figure 3**). These phenomena implied that combination of oxaliplatin and carbocisteine could elevate the efficacy of chemotherapy, and high dose of carbocisteine could more significantly induce the anti-cancer effect of oxaliplatin. Furthermore, we found that treatment with oxaliplatin and carbocisteine could increase the mice survival rate than signal usage of oxaliplatin. Survival rate of group 1 (none treatment) was about 40% (6/15); and with combination of oxaliplatin and carbocisteine, the survival rate was up to 80% (12/15).

HE staining and TUNEL assay of orthotopic transplantation tumor and metastasis site

To further confirm the tumor development and progression of orthotopic transplantation tumor and metastasis site, we performed the HE staining (**Figure 4**) and TUNEL assay (**Figure 5**) in each group. As shown in **Figure 5**, along with the usage of oxaliplatin, the tumor cell growth was repressed and drug

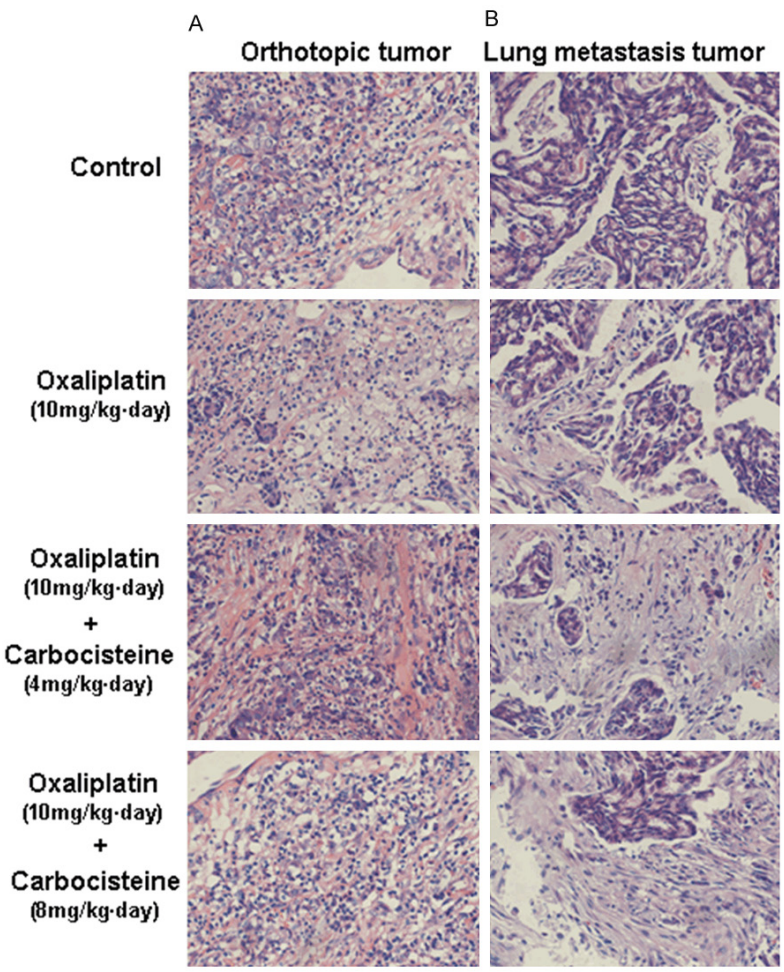

Figure 4. HE staining for orthotopic transplantation tumor and metastasis site. HE staining for the xenograft tumor (A) and metastasis site (B) indicated the effects of different treatment strategy.

induced apoptosis was increased. Furthermore, preconditioning treatment with carbocisteine, the tumor sizes was further decreased while the representative image (**Figure 5A**) and analysis data (**Figure 5B**) of aopotosis was further increased. Collectively, that the data demonstrated that preconditioning treatment with carbocisteine strongly enhanced the anti-tumor effect of oxaliplatin chemotherapy on tumorigenesis and metastasis.

Preconditioning treatment with carbocisteine enhance the inhibitory effect of oxaliplatin on epithelia-to-mesenchymal transition

Because preconditioning treatment with carbocisteine enhance the inhibitory effect of oxaliplatin on orthotopic tumor metastasis, we further determined whether the metastasis change was consistent with changes of epithelial-mesenchymal transition (EMT) biomarker. Clearly, restored preconditioning treatment increased the expression of E-cadherin (**Figure 6A**) but decreased the expression of (**Figure 6B**) vimentin and caused the morphologic change from mesenchymal to epithelial transition in orthotopic transplantation tumor.

Discussion

Recently, with the development of systemic chemotherapy as neoadjuvant or adjuvant setting, colon cancer treatment has been largely improved. However, the chemotherapy drug resistance has become the obstruction of cancer chemotherapy, limiting the efficiency of chemotherapy and restricting the application of chemotherapy. Moreover, therapy in colon cancer is still insufficient and short of theoretical foundation. Here, we intended to reveal the effectiveness of a new potential combination with existing drugs in colon cancer.

Cellular metabolism is crucial for the generation of energy to supply cell survival; however, ROS could be also generated during electron transfer reactions. Tumor cells undergo abnormal rate of metabolism, and the hypoxia-responsive genes are activated in tumor cells [19]. Not only is there a mount of host cells including fibroblasts, but there is immune cells infiltrated into the tumor locus, making the tumor microenvironment more complicated. Macrophages and granulocytes could survive in the hypoxic environment and contribute to it by hyperproduction of ROS upon local activation [20]. In the tumor milieu, cells are rapidly apoptosis and expanding and phagocytes receive activation signal and produce quantity of ROS.

Consequences of the production of ROS are complicated in cancer cells. At present, the entire network of reactions and effects after

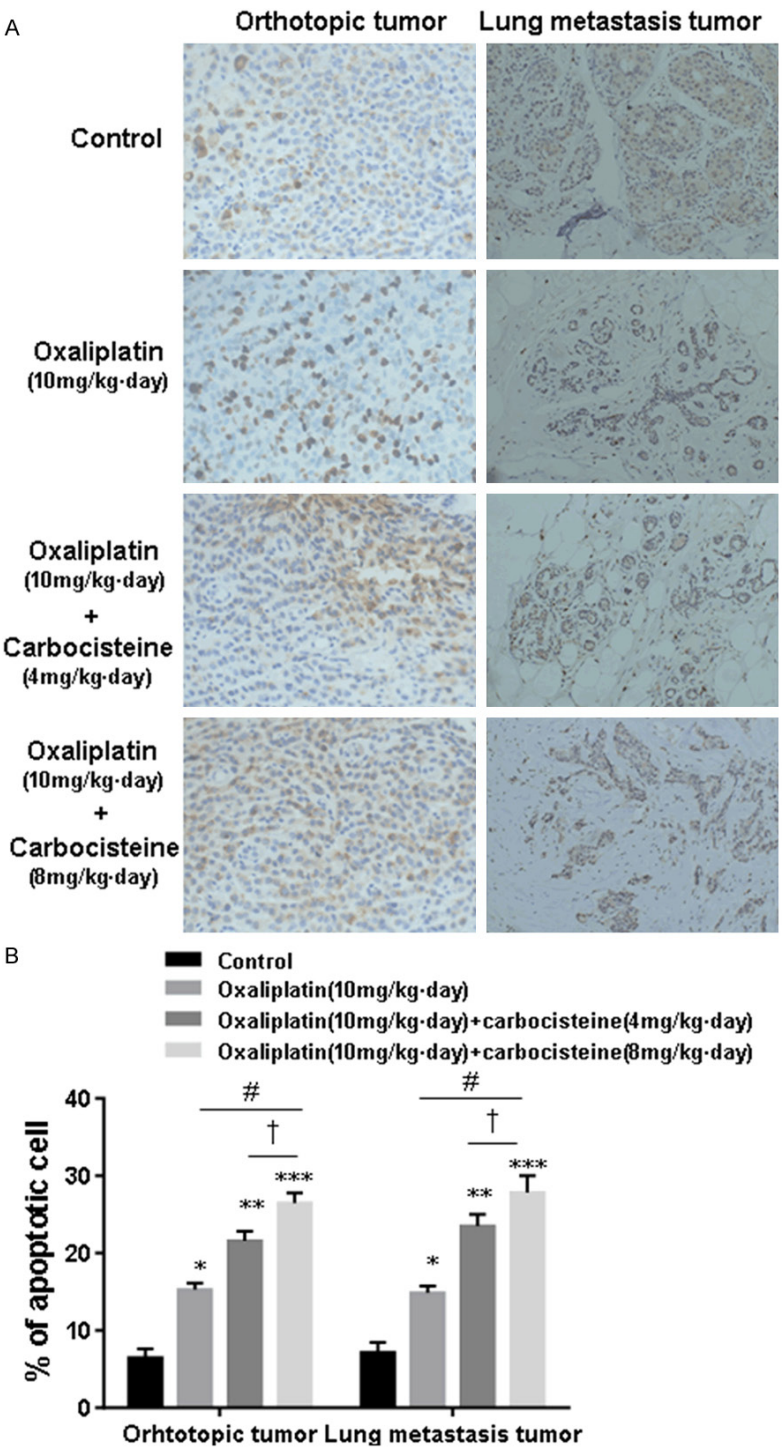

Figure 5. TUNEL assay for orthotopic transplantation tumor and metastasis site. Representative imagers and analysis data of TUNEL assay for the xenograft tumor and metastasis site indicated the level of apoptosis after the treatment of oxaliplatin with or without carbocisteine preconditioning treatment. *P<0.05, **P<0.01 vs. control group; #P<0.01 vs. oxaliplatin (10 mg/kg·day) group; †P<0.05 vs. oxaliplatin (10 mg/kg·day) + carbocisteine (4 mg/kg·day) group.

redox-based anti-cancer treatments was quietly unclear and implemented. In fact, ROS causes gene mutation and genomic instability which contribute to cancer development and progression. Also, ROS acts as the downstream signal messengers to induce abnormal signaling activation and transduction, leading to cell invasion and malignant phenotypes [21, 22]. Furthermore, ROS was reported to be involved in tumor chemo- and radio-resistance [23].

Carbocisteine, known as S-carboxymethylcysteine (S-CMC), has been applied as mucoregulatory drug. Recently, it has been reported that carbocisteine could improve the quality of life of patients with COPD or upper respiratory inflammation and chronic sinusitis [6, 24]. In addition, carbocisteine was reported to inhibit IL-1β-induced release of IL-8 and IL-6 in lung cancer cell lines [25]. Given the reason that cytokines IL-6 and IL-8 were mainly responsible for the abnormal tumor microenvironment, this finding suggested the potential mechanisms of carbocisteine. Yasuda [26] et al. also found that carbocisteine inhibited the release of IL-6 and IL-8 via the activation of NF-κB signaling when the virus infection in airway epithelial cells. Collectively, inhibition of the release of IL-8 and IL-6 by carbocisteine might be involved in the inhibition of NF-κB activation through scavenging ROS and together usage of carbocisteine and oxaliplatin might be effective.

In our study, there are significant effects on suppressing tumor formation and metastasis

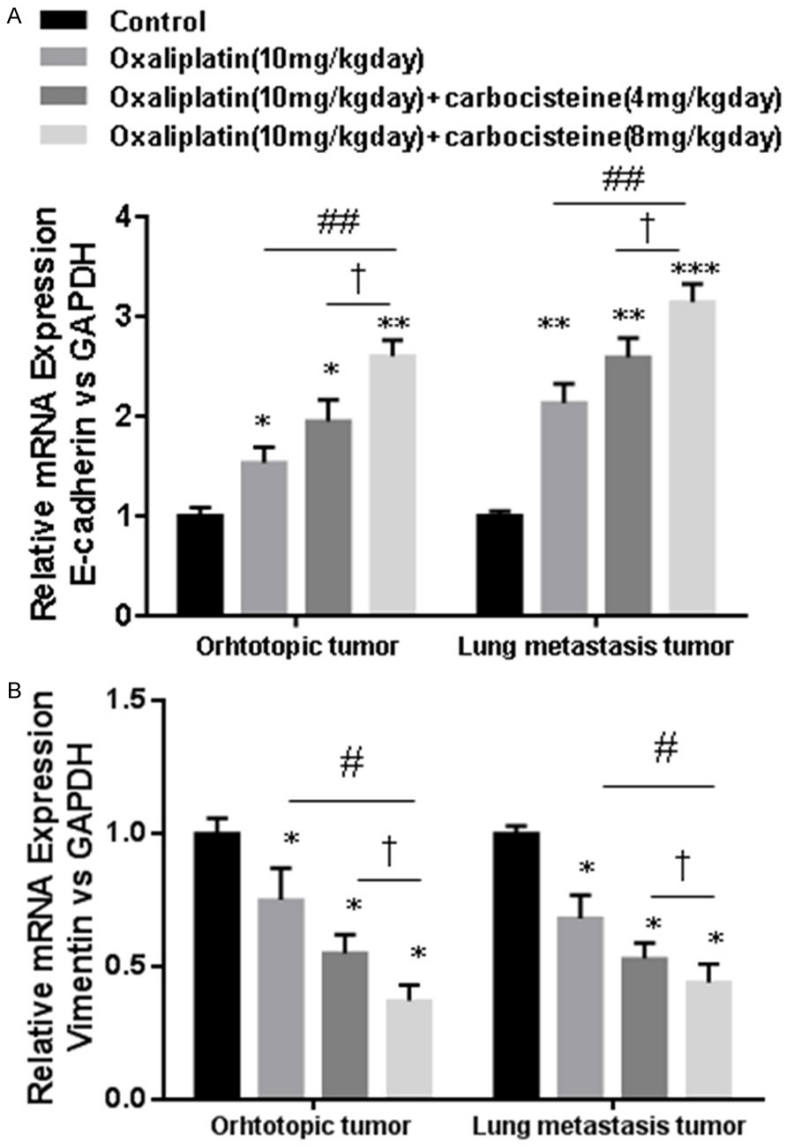

Figure 6. Effects of carbocisteine preconditioning treatment on epithelial (E-cadherin) and mesenchymal (vimentin) markers in orthotopic transplantation tumor tissue and metastasis tumor tissue. (A) E-cadherin and vimentin (B) mRNA expression was determined using real-time PCR. GAPDH was used as an internal control. Data were representative as the mean ± SEM of triplicated independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. control group; #P<0.05, ##P<0.01 vs. oxaliplatin (10 mg/kg·day) group; †P<0.05 vs. oxaliplatin (10 mg/kg·day) + carbocisteine (4 mg/kg·day) group.

when combining oxaliplatin and carbocisteine to treat CRC in mice model. The mice injected with oxaliplatin of 10 mg/kg oxaliplatin could notably suppress the tumor growth and metastasis. We also found that treatment with carbocisteine has none effect on tumor. However, carbocisteine could increase the sensitivity and efficacy of oxaliplatin, which was consistent with previous study [27]. The apparent discrepancy between combination and single chemical drugs provided additional insight into the new clinical treatment strategy targeting the tumor microenvironment which will allow for more effective application of therapeutics targeting this pathway, as well as additional targets for the development of effective drugs.

In conclusion, our results from in vivo experiments demonstrated that changing tumor microenvironment with antioxidant could cooperate with chemotherapy drugs in terms of tumor suppression. We proposed that inhibition of ROS related-pathway and pre-metastasis niche may represent a novel therapeutic strategy for controlling tumor growth and metastasis in colon cancer. Apart from mice experiments, we further performed gene expression array to explore possible genes which would unequivocally addressed into pathways associated with clinical treatments and increasing survival rate. Notably, in this circumstance, using antioxidant and oxaliplatin together offered a new insight into potential clinical application.

Acknowledgements

This study is supported by National Natural Science Foundation (81201807 to BY) and by Science and Technology Commission of Shanghai Municipality (14411970300 & 14401972400 to QZ).

Disclosure of conflict of interest

None.

Address correspondence to: Bo Yu, Department of Pharmacy, Fudan University Shanghai Cancer Center, 270 Dong-An Road, Shanghai 200032, China; Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China. E-mail: miguelboyu@msn.cn

References

[1] Losert A, Lötsch D, Lackner A, Koppensteiner H, Peter-Vörösmarty B, Steiner E, Holzmann K, Grunt T, Schmid K, Marian B, Grasl-Kraupp B, Schulte-Hermann R, Krupitza G, Berger W, Grusch M. The major vault protein mediates resistance to epidermal growth factor receptor inhibition in human hepatoma cells. Cancer Lett 2012; 319: 164-172.

[2] Rome L, Kedersha N and Chugani D. Unlocking vaults: organelles in search of a function. Trends Cell Biol 1991; 1: 47-50.

[3] Scheper RJ, Broxterman HJ, Scheffer GL, Kaaijk P, Dalton WS, van Heijningen TH, van Kalken CK, Slovak ML, de Vries EG, van der Valk P, et al. Overexpression of a Mr 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Res 1993; 53: 1475-1479.

[4] Scheffer GL, Wijngaard PL, Flens MJ, Izquierdo MA, Slovak ML, Pinedo HM, Meijer CJ, Clevers HC, Scheper RJ. The drug-resistance-related protein LRP is the human major vault protein. Nat Med 1995; 1: 578-82.

[5] List AF, Spier CS, Grogan TM, Johnson C, Roe DJ, Greer JP, Wolff SN, Broxterman HJ, Scheffer GL, Scheper RJ, Dalton WS. Overexpression of the major vault transporter protein lung-resistance protein predicts treatment outcome in acute myeloid leukemia. Blood 1996; 87: 2464-2469.

[6] Pasillas MP, Shields S, Reilly R, Strnadel J, Behl C, Park R, Yates JR 3rd, Klemke R, Gonias SL, Coppinger JA. Proteomic analysis reveals a role for Bcl2-associated athanogene 3 and Major Vault Protein in resistance to apoptosis in senescent cells by regulating ERK1/2 activation. Mol Cell Proteomics 2015; 14: 1-14.

[7] Celestino AT, Levy D, Maria Ruiz JL, Bydlowski SP. ABCB1, ABCC1, and LRP gene expressions are altered by LDL, HDL, and serum deprivation in a human doxorubicin-resistant uterine sarcoma cell line. Biochem Biophys Res Commun 2015; 457: 664-668.

[8] DeSantis C, Siegel R, Bandi P, Jemal A. Breast cancer statistics, 2011. CA Cancer J Clin 2011; 61: 408-418.

[9] Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005; 365: 1687-1717.

[10] Zheng JP, Kang J, Huang SG, Chen P, Yao WZ, Yang L, Bai CX, Wang CZ, Wang C, Chen BY, Shi Y, Liu CT, Chen P, Li Q, Wang ZS, Huang YJ, Luo ZY, Chen FP, Yuan JZ, Yuan BT, Qian HP, Zhi RC, Zhong NS. Effect of carbocisteine on acute exacerbation of chronic obstructive pulmonary disease (PEACE Study): a randomised placebo-controlled study. Lancet 2008; 371: 2013-2018.

[11] Nogawa H, Ishibashi Y, Ogawa A, Masuda K, Tsubuki T, Kameda T, Matsuzawa S. Carbocisteine can scavenge reactive oxygen species in vitro. Respirology 2009; 14: 53-59.

[12] Wang W, Zheng JP, Zhu SX, Guan WJ, Chen M, Zhong NS. Carbocisteine attenuates hydrogen peroxide-induced inflammatory injury in A549 cells via NF-κB and ERK1/2 MAPK pathways. Int Immunopharmacol 2015; 24: 306-313.

[13] Lötsch D, Steiner E, Holzmann K, Spiegl-Kreinecker S, Pirker C, Hlavaty J, Petznek H, Hegedus B, Garay T, Mohr T, Sommergruber W, Grusch M, Berger W. Major vault protein supports glioblastoma survival and migration by upregulating the EGFR/PI3K signalling axis. Oncotarget 2013; 4: 1904.

[14] Zhai Q, Bian XL, Lu SR, Zhu B, Yu B. Carbocisteine reduces the cytotoxicity of oxaliplatin. Z Naturforsch C 2012; 67: 215-21.

[15] Liu B, Wang T, Wang L, Wang C, Zhang H, Gao GD. Up-regulation of major vault protein in the frontal cortex of patients with intractable frontal lobe epilepsy. J Neurol Sci 2011; 308: 88-93.

[16] Chen ZJ, Le HB, Zhang YK, Qian LY, Sekhar KR, Li WD. Lung resistance protein and multidrug resistance protein in non-small cell lung cancer and their clinical significance. J Int Med Res 2011; 39: 1693-1700.

[17] De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G, Cappuzzo F, Frattini M, Molinari F, Saletti P, De Dosso S, Martini M, Bardelli A, Siena S, Sartore-Bianchi A, Tabernero J, Macarulla T, Di Fiore F, Gangloff AO, Ciardiello F, Pfeiffer P, Qvortrup C, Hansen TP, Van Cutsem E, Piessevaux H, Lambrechts D, Delorenzi M, Tejpar S. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 2010; 11: 753-762.

[18] Di Nicolantonio F, Martini M, Molinari F, Sartore-Bianchi A, Arena S, Saletti P, De Dosso S,

Mazzucchelli L, Frattini M, Siena S, Bardelli A. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. J Clin Oncol 2008; 26: 5705-5712.

[19] Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massagué J. Genes that mediate breast cancer metastasis to lung. Nature 2005; 436: 518-524.

[20] Bartelink H, Horiot JC, Poortmans P, Struikmans H, Van den Bogaert W, Barillot I, Fourquet A, Borger J, Jager J, Hoogenraad W, Collette L, Pierart M; European Organization for Research and Treatment of Cancer Radiotherapy and Breast Cancer Groups. Recurrence rates after treatment of breast cancer with standard radiotherapy with or without additional radiation. N Engl J Med 2001; 345: 1378-1387.

[21] Yuan JH, Yang F, Wang F, Ma JZ, Guo YJ, Tao QF, Liu F, Pan W, Wang TT, Zhou CC, Wang SB, Wang YZ, Yang Y, Yang N, Zhou WP, Yang GS, Sun SH. A long noncoding RNA activated by TGF-β promotes the invasion-metastasis cascade in hepatocellular carcinoma. Cancer Cell 2014; 25: 666-681.

[22] Zhou F, Drabsch Y, Dekker TJ, de Vinuesa AG, Li Y, Hawinkels LJ, Sheppard KA, Goumans MJ, Luwor RB, de Vries CJ, Mesker WE, Tollenaar RA, Devilee P, Lu CX, Zhu H, Zhang L, Dijke PT. Nuclear receptor NR4A1 promotes breast cancer invasion and metastasis by activating TGF-β signalling. Nat Commun 2014; 5: 3388.

[23] Ma XJ, Dahiya S, Richardson E, Erlander M, Sgroi DC. Gene expression profiling of the tumor microenvironment during breast cancer progression. Breast Cancer Res 2009; 11: R7.

[24] Siegel PM and Massagué J. Cytostatic and apoptotic actions of TGF-β in homeostasis and cancer. Nat Rev Cancer 2003; 3: 807-820.

[25] Li P, He Q, Luo C, Qian L. Diagnostic and prognostic potential of serum angiopoietin-2 expression in human breast cancer. Int J Clin Exp Pathol 2015; 8: 660.

[26] Berger W, Steiner E, Grusch M, Elbling L, Micksche M. Vaults and the major vault protein: novel roles in signal pathway regulation and immunity. Cell Mol Life Sci 2009; 66: 43-61.

[27] Kim SK, Jung WH and Koo JS. Yes-associated protein (YAP) is differentially expressed in tumor and stroma according to the molecular subtype of breast cancer. Int J Clin Exp Pathol 2014; 7: 3224.