Ginsenosides facilitate the anti-cancer efficacy of cyclophosphamide via modulating intestinal bacteria and alleviating intestinal mucositis in mammary carcinoma mice

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Research

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Abstract

Background: Ginsenosides has been reported to facilitate the chemotherapy of cyclophosphamide (CTX), but the underlying mechanism is still elusive. Here, ICR mice bearing subcutaneous 4T1 tumor (mouse mammary carcinoma cells) were used to investigate the enhanced chemotherapeutic effects and underlying the mechanism of concurrent administration of ginsenosides and CTX.

Results: Combinational treatment with ginsenosides in mice enhanced the anti-cancer efficacy of CTX as observed by elevated tumor growth inhibition rate, prolonged survival rate, decreased tumor burden and increased apoptosis in tumor tissues. The mechanism underlying the enhancing effects involved the elevation of the anti-cancer immunity, which manifested as accelerating anti-cancer cytokines (IL-2, IL-6, IL-17, TNF- α and INF- γ) that was further ascribed to the ginsenosides-induced generation of intestinal probiotics including *Bifidobacterium*, *Akkermansia* and *Lactobacillus*. Moreover, through activating Nrf2 and inhibiting NF κ B pathways, ginsenosides reversed CTX-induced intestinal mucositis by maintaining normal intestinal permeability and junction proteins function.

Conclusion: Ginsenosides ameliorate CTX-induced intestinal mucositis and promote anti-cancer immunity via maintaining intestinal homeostasis, consequently enhancing the anti-cancer efficacy of CTX.

Background

Cyclophosphamide (CTX) is one of the first line chemotherapeutic drugs [1, 2], but the usage is often limited by its anti-cancer efficacy and adverse reactions. Recent study indicated that CTX's anti-cancer efficacy is due in part to its anti-cancer immunity [3–5], which is depended by the dosing time and specific intestinal bacteria. After single dosing, CTX triggers species of *Lactobacillus* to activate the secondary immune system and further stimulate the generation of anti-tumor cytokines including interleukin-17 (IL-17) and interferon- γ (IFN- γ) [3, 5–7]. Whereas, continuous treatment with CTX inhibits immunity *via* affecting the diversity and abundance of intestinal bacteria [8], represented by the reduction of serum cytokines such as IFN- γ , interleukin-2 (IL-2), interleukin-6 (IL-6). [9, 10]. Besides, CTX administration has been reported to induce adverse reactions. The most frequently occurred CTX-related complication is intestinal mucositis [11, 12], manifested by symptoms such as nausea, vomiting, abdominal pain, diarrhea and inflammation, as well as histological changes including villus atrophy, enterocytes deficiency, epithelium impairment and barrier dysfunction. Although the mechanisms of intestinal mucositis related to chemotherapeutic drugs remain controversial, upregulating nuclear factor erythroid 2-related factor 2 (Nrf2) and/or downregulating nuclear factor kappa B (NF κ B) proteins in intestinal epithelial cells have been considered as feasible schemes to alleviate intestinal mucositis [13].

The application of herbal medicines has a long history in Asian countries for centuries [14, 15]. Ginseng (derived from the root and rhizome of *Panax ginseng* C. A. Mey.) demonstrated therapeutic effects against various diseases. For cancer treatment, combination use of ginseng as a general health tonic is

popular for enhancing chemosensitivity and modulating immunity [16, 17]. Some ginseng-containing complex prescription have been clinically applied to enhance the anti-cancer efficacy and/or diminish complications of chemotherapeutic drugs [18–20]. Particularly in China, one preparation named Compound Cyclophosphamide, with CTX and ginsenosides (extracts from ginseng) as the main ingredients [21], has been clinically used in China since 1986 [22]. Compared to CTX-only treatment, patients orally administrated with Compound Cyclophosphamide significantly improved the chemotherapeutic efficacy and prolonged the treatment window. However, the underlying mechanism is unexplored.

Previous studies demonstrated that ginseng could enhance the abundance of *Firmicutes* Phylum mainly distributed in *Lactobacillus* genera, and reduce the abundance of *Bacteroidetes* Phylum [23, 24]. Recent metabolomic study exhibited that ginsenosides significantly altered the bacteria biomarkers [25]. All the observation suggested that ginsenosides might have the capability to alter the components of intestinal bacteria. Besides, ginsenosides could alleviate drug-induced oxidative and inflammatory damage via activating Nrf2 antioxidant system and inhibiting NFκB pathway [26, 27].

Here we hypothesized that combination use of ginsenosides improved the chemotherapeutic efficacy of CTX by enhancing CTX-induced anti-tumor immunity and alleviating CTX-induced intestinal mucositis. In the present study, we systematically investigated the crosstalk between combination use of ginsenosides-CTX and anti-cancer immunity mediated by intestinal microbiota, as well as alleviating effects on intestinal mucositis mediated by Nrf2 and NFκB pathways

Results

Ginsenosides enhance the anti-cancer effect of CTX in 4T1-bearing mice

The anti-cancer effect of CTX with or without other co-treatments was initially evaluated in breast carcinoma subcutaneous tumor model. The body weight of all the 4T1-bearing mice were not significantly changed in all treated groups (Figure 1A), but the tumor weight and tumor volume were significant inhibited and 20d-survival rate was enhanced in CTX and CTX plus ginsenosides (CTGS) groups compared to Blank (BLK) group (Figure 1B~1D). More importantly, the 20d-tumor volume was significant reduced from 1611 ± 223 to $967 \pm 306\text{mm}^3$ and the 20d-survival rate as well as 20d-tumor inhibition rate were enhanced from 30% and 69.7% to 80% and 75.7% in CTGS mice compared to CTX mice (Figure 1C~1E). In Hematoxylin-eosin (H&E) staining, the tumor tissues in CTX and CTGS groups showed cells with pyknotic nuclei and hypereosinophilic cytoplasm (Figure 1F), which served as an indicator of cell apoptosis, and more apoptotic cells were observed in ginsenosides and CTX co-treated mice than those treated with CTX alone. Western blot analysis revealed that Bax/Bcl-2 ratio and caspase-3 levels significantly increased in both the CTX and CTGS groups compared with that in the BLK group (Figure 1G), which indicated that both CTX and CTGS induce the tumor apoptosis. Moreover,

the up-regulated ratio of Bax to Bcl-2 was observed in CTGS group compared to CTX group. Together, these data demonstrated that ginsenosides sensitize the chemotherapeutic efficacy of CTX.

Ginsenosides strengthen anti-cancer related immune cytokines in CTX-treated 4T1-bearing mice

Harnessing the anti-cancer immunity usually contributes to a satisfactory cancer treatment outcome of CTX. In this regard, the immune organ and anti-cancer related cytokines after different treatment in breast carcinoma subcutaneous tumor model was further evaluated. Spleen index in CTX-treated mice was lower than in the BLK group, while that was reversed when ginsenosides was co-administrated (Figure 2A). The thymus index in CTX-treated mice did not show significant difference compared to the BLK and CTGS mice (Figure 2B). The tendencies of anti-cancer related immune cytokines in CTX and CTGS groups after first dosage were similar, but that after last dosage were different (Figure 2C~F). After first dosage, the serum levels of IFN- γ and IL-17 in CTX and CTGS groups were elevated compared with BLK group. However, after last dosage, the serum levels of IL-2, IFN- γ , IL-6 and IL-17 in CTX group were decreased in CTX group, whereas the serum levels of IFN- γ was increased in CTGS group when compare to BLK group. Besides, the serum levels of IL-2, IFN- γ , IL-6 and IL-17 in CTX and ginsenosides co-treated mice were higher than that in CTX treated mice with significant difference. All those observations suggested that ginsenosides strengthen the anti-cancer immunity in CTX-treated 4T1-bearing mice.

Ginsenosides alleviate CTX-induced intestinal damage in 4T1-bearing mice

Repeated administration of CTX for 20d to 4T1-bearing animals caused diarrhea, which was alleviated by co-administrated ginsenosides (Figure 3A). Serum Fluorescein isothiocyanate dextran 4kDa (FD-4) was used to compare the intestinal permeability. As shown in Figure 3B, the serum level of FD-4 in CTX-treated group was markedly increased compared with BLK group, while co-administration with ginsenosides reversed the CTX-induced FD4 elevation. Histological changes of the colon of each mouse was analyzed (Figure 3B and 3C). Complete and slender intestinal villi with tight and order arrangements and clear brush borders were observed in BLK and ginsenosides (GS) groups mice. In comparison, CTX-treated mice showed shorten and thick intestinal villus with loose arrangements, destructed crypts, reduced villus length/crypt depth ratio and atrophied epithelial cells. In contrast, in CTX and ginsenosides co-treated mice, the villus arrangements, crypts, length and length/crypt depth ratio appeared to be restored and the mucosal architecture was preserved. Besides, the intestinal tight junction proteins were investigated by immunohistochemistry and western blot methods (Figure 3C and 3D). Comparing with BLK mice, CTX-treated mice had lower intestinal protein levels of ZO-1, ZO-2, occludin and E-cadherin. While co-administration with ginsenosides significantly reversed the CTX-induced damage in tight junction proteins. Moreover, compared to BLK group mice, CTX-treated mice had downregulated Nrf2 signaling and upregulated NF κ B signaling, indicating oxidative and inflammatory damage induced by CTX treatment. Interestingly, the CTX-induced oxidation and inflammation were reversed when co-administrated with ginsenosides. The results suggested that CTX damaged the integrity of the intestinal

mucosa, while ginsenosides alleviated the intestinal mucosa injury *via* regulating NF κ B and Nrf2 pathways.

Ginsenosides alter the taxonomic composition of the gut microbiota in CTX-treated 4T1-bearing mice

To access the impact of ginsenosides consumption on gut microbiota in CTX-treated 4T1-bearing mice, high-throughput pyrosequencing of fecal samples was performed. A total of 1196799 bacterial sequences belonged to 29782 operational taxonomic units (OUT) were obtained from across all samples. First, α - and β -diversity analyses on whole microbiota profiling at OTU levels were performed. The α -diversity was evaluated from community richness (sobs, chao and ace indexes) and community diversity (shannon index), which were slightly upregulated compared with BLK group, but were significantly restored in CTGS group (Figure 4A). The β -diversity was investigated from Hierarchical cluster analysis (HCA), non-metric multidimensional scaling analysis (NMDS) and Principal co-ordinates analysis (PCoA) based on Bray-Curtis distance, as well as Principal Component Analysis (PCA). HCA indicated microbial communities in CTX group were clustered not to CTX and GS groups but to BLK group, and NMDS, PCoA and PCA suggested bacterial OTU in 4 groups were clearly separated from each other (Figure 4B). These results indicated that ginsenosides altered intestinal microbial communities in CTX-treated 4T1-bearing mice.

The average relative abundances of bacterial taxa at the phylum level are shown in Figure 4C. The major phyla in experimental mice were *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Verrucomicrobia*. No significant difference was observed in the relative abundances of the four phyla between BLK and CTX groups. However, compared to BLK and CTX groups, the relative abundances of *Actinobacteria* and *Verrucomicrobia* were significantly increased in CTGS group ($p < 0.05$), and *Actinobacteria* were significantly increased in GS group ($p < 0.05$). Besides, repeated administration of ginsenosides combined with or without CTX decreased *Bacteroidetes*, and ginsenosides alone increased *Firmicutes*, but the *Firmicutes/Bacteroidetes* ratio among four groups didn't show significant difference. The average relative abundances of bacterial taxa at the genus level are shown in Figure 4D. The bacteria ranked at the top 12 of relative abundances at genus level were further investigated. Except the decreased *Lactobacillus*, the relative abundances of top 12 genera were not significantly altered in CTX treated 4T1 bearing mice. Whereas compared to CTX group, 7 and 5 of 12 top genera were significantly changed in CTGS and GS groups, respectively. Among the altered genera, *Lactobacillus*, *Akkermansia*, *Bifidobacterium* and *Enterorhabdus* were increased and *Prevotellaceae UCG-001*, *Lachnospiraceae NK4A136 group* and *Odoribacter* were decreased in CTGS group, as well as *Lactobacillus* and *Bifidobacterium* were increased and *Lachnospiraceae NK4A136 group*, *Alloprevotella* and *Odoribacter* were decreased in GS group compared with CTX group. Collectively, our findings suggested that ginsenosides could alter the composition of the intestinal bacteria in CTX treated 4T1 bearing mice.

Intestinal bacteria drive the enhanced effect of CTX and ginsenosides in 4T1-bearing mice

Fecal transplantation (FT) and antibiotics were further used to evaluate the synergetic anti-cancer effect of CTX and ginsenosides in 4T1-bearing mice. Intriguingly, compared to CTGS group, Fecal transplant CTX (FT-CTX) group exhibited similar anti-cancer potency, while antibiotics treated CTGS (anti-CTGS) group showed same intestinal protective effect. Specifically, the anti-cancer efficacy (Figure 5A and Figure 5B) and the anti-cancer related immune cytokines (Figure 5C) were significantly reduced in anti-CTGS group compared to CTGS or FT-CTX group, but the intestinal permeability (Figure 5D and Figure 5E) was significantly reduced in FT-CTX group compared to CTGS or anti-CTGS group. Our findings indicated that promoted anti-cancer immunity, not intestinal protective effect, could be ascribed to the intestinal bacteria in 4T1-bearing mice.

Correlation of intestinal bacteria with the anti-cancer immunity of CTX and ginsenosides in 4T1-bearing mice

Redundancy analysis/Canonical Correlation Analysis (RDA/CCA) was used to directly measure the correlation between intestinal bacteria and the anti-cancer efficacy as well as anti-cancer immunity of CTX and ginsenosides in 4T1-bearing mice. Generally, *Bifidobacterium*, *Enterorhabdus*, *Akkermansia* and *norank_f_Muribaculaceae* were positively correlated with the anti-cancer effect (Figure 6A), while *Lactobacillus*, *Bifidobacterium*, *Enterorhabdus* and *Akkermansia* were positively correlated with the anti-cancer related immune cytokines (Figure 6B). Combine the statistical analyses of intestinal bacteria among groups, *Bifidobacterium*, *Enterorhabdus*, *Akkermansia* and *Lactobacillus* hold the most promising potency in promoting anti-cancer immunity of CTX and ginsenosides in 4T1-bearing mice.

Discussion

Anti-cancer immunity is recently considered as one of the most promising oncotherapy strategies, and many immuno-chemotherapeutic drugs have been developed in the near decade [28]. However, immuno-chemotherapeutic drugs had limitations in terms of their unstable therapeutic efficacies and adverse reactions [29-31]. Taking clinically applied immuno-chemotherapeutic agents as examples, investigating feasible protocols to enhance their anti-cancer immunity might grasp keys to accelerate the development of immuno-chemotherapeutic agents. CTX is one of the oldest chemotherapeutic drugs which could date back to the end of 1950s [32]. Till now, CTX remains a mainstay in the therapy of haematological malignancies and breast carcinomas partly for its immune anti-cancer response [4, 33] which is dependent by the anti-cancer immunity. However, its anti-cancer immunity was not constant with the dosing time [3, 34]. Ginsenosides from ginseng are a clinically applied immunomodulator, and has been regularly combined with chemotherapeutic drugs which shows better anti-cancer efficiency than monotherapy [20]. As expected in our study, ginsenosides in combination with CTX showed facilitating anti-cancer immunity in 4T1-bearing mice through suppressing the tumor burden, prolonging the survival time and promising tumor apoptosis. Besides, CTX usually causes serious intestinal mucositis and finally induces bacterial translocation [35]. Here ginsenosides alleviated CTX-induced intestinal mucositis evidenced by decreased permeability and enhanced junction protein expression. Therefore, the present

study delineated the mechanism underlying the immune-related sensitization and intestinal mucositis reduction of co-administration with ginsenosides and CTX. Our findings provide a feasible strategy to improve the anti-cancer efficacy of immuno-chemotherapeutic agents via enhancing the anti-cancer immunity and alleviating related adverse reactions.

Spleen and thymus are important secondary immune organs for anti-cancer immunity [10] through the generation of anti-cancer related immune cytokines. Combination use of CTX and ginsenosides for 20 days could restore spleen index but not thymus index, indicating that ginsenosides promoted the anti-cancer related immune cytokines of CTX mainly in the spleen. IL-2, IFN- γ , IL-17 and IL-6, secreted by the spleen, are considered as the uppermost cytokines implicated in anti-tumor immunity [3, 36, 37]. IL-2 is the first cytokine which has been successfully applied in cancer treatment since 1992 [37]. IL-2 induces T cell proliferation and differentiation and promotes natural killer cell cytolytic activity in response to antigen, which further initiates the immune anti-cancer effect [38]. IFN- γ initiates an “immune-exposing” program in the tumor cells, where the genes implicated in the antigen presentation pathways are upregulated [39, 40]. IL-17, produced by Th17 cells, is one of the major cytokines in response to the anti-cancer immunity of CTX [3, 5-7, 41]. IL-6 is now viewed as major therapeutic targets for clinical intervention [42]. Reduced IL-6 level is frequently observed in cancer patients and often associated with poor clinical outcome [43]. Here ginsenosides were found to keep IL-2, IFN- γ and IL-17 at higher level, and IL-6 at a normal level during co-administration of CTX, indicating that ginsenosides enhanced the anti-tumor immunity.

Intestinal bacteria can interact with the host *via* modulating gut epithelium [44], immune system [45], oncogenesis [46] and even oncotherapy [7]. Recently, intestinal bacteria targeted drugs and treatment have been developed [47]. Thus, in our study, the intestinal bacteria structures between groups were further evaluated. The overall structure of the gut microbiota in CTX-treated 4T1-bearing mice was significantly changed, which was consistent to CTX-treated normal and pathological animals in previous studies [3, 10, 48-50]. Interestingly, ginsenosides-treated mice restored their gut microbiota structure despite the co-treatment effects of CTX, showing significant increments of *Lactobacillus*, *Akkermansia*, *Bifidobacterium* and *Enterorhabdus*. Among them, *Lactobacillus*, *Akkermansia* and *Bifidobacterium* are considered as probiotics and tightly linked with the host health, including intestinal protection, immunomodulation and anti-cancer effect [31, 51]. Our findings suggest that ginsenosides induce more robust overall structure of the intestinal probiotics.

FT and antibiotics protocols were further used to evaluate the correlation between intestinal bacteria and intestinal protection and immune anti-cancer effect in CTX and ginsenosides co-treated 4T1-bearing mice. We found that ginsenosides indirectly drives the immune anti-cancer effect *via* influencing the composition of intestinal bacteria, but directly shows intestinal protective effect of CTX in 4T1-bearing mice. Further statistical analysis suggested that *Lactobacillus*, *Bifidobacterium* and *Akkermansia* held the most contribution in the anti-cancer immunity of CTX and ginsenosides in 4T1-bearing mice, respectively. *Bifidobacterium* could ameliorate the toxicity of a checkpoint blockade antibody and finally mitigate the autoimmunity caused by anti-CTLA-4 [28], anti-PD-1 [29, 52] and other checkpoint inhibitors. Recent

studies revealed the important role of *Bifidobacterium* in the gut as a modulator positively associated with antitumor T cell responses and anti-cancer related immune cytokines (IFN- γ and IL-17) production [28, 29, 52]. Our findings showed an increase of *Bifidobacterium* in ginsenosides-treated mice, suggesting that the beneficial effect of combined treatment of ginsenosides and CTX in delaying tumor growth was related to the increase production of IFN- γ and IL-17. *Akkermansia* is a strict anaerobe belonging to the *Verrucomicrobia* and specialized in the degradation of mucin. *Akkermansia*, as a conserved intestinal symbiont with probiotic properties, has been proposed as the gatekeeper of our mucosa promoting immune responses and beneficial interactions in the intestinal tract [53], however, in our study, *Akkermansia* is not the key factor to alleviate the intestinal mucositis. Previous studies have indicated that *Akkermansia* has an active cross-talk with host cells [54] and thus mediate many acute and chronic diseases [55, 56]. Notably, *Akkermansia* has recently been found to be beneficial in clinical cancer treatment, which sensitized the patients' response to anti-PD-1 anti-cancer immunity [30]. Consistent to the previous reports, ginsenosides and CTX co-treated mice displayed a higher relative abundance of *Akkermansia* than other tumor bearing mice, indicating that ginsenosides treatment favored the dominance of selected *Akkermansia* genus. *Lactobacillus* has also been reported to enhance anti-cancer immunity via promoting T lymphocyte proliferation and stimulating the secretion of cytokines (IL-17) in the spleen of mice [3, 10, 57]. However, in this study, *Lactobacillus* is positively correlated with anti-cancer immunity, but negatively correlated with the anti-cancer efficacy in ginsenosides and CTX co-treated 4T1-bearing mice. Recent study indicated that *Lactobacillus* memory T lymphocyte immune responses [3], which indicated that slight *Lactobacillus* could active the anti-cancer immunity after the first activation. Therefore, ginsenosides facilitate the anti-cancer immunity of CTX *via* promoting the generation of intestinal probiotics and thus modulating spleen-derived anti-cancer related immune cytokines (shown in Figure 7).

TJ proteins that formed by integral membrane proteins (claudins and occludins) and peripheral membrane proteins (ZO-1 and ZO-2 etc.) are crucial indicators to evaluate the intestinal mucositis. In this study, the expressions of TJ proteins were investigated, and the results showed that ginsenosides alleviated the expressions of all these junction proteins in CTX-treated 4T1 bearing mice. Nrf2 activation has been shown to affect epithelial TJ protein expression in animal models [58], and NF κ B activation has been thought to elicit the inflammatory and apoptotic responses that lead to the intestinal mucosal injury [59]. Here we found that the expression of Nrf2 was upregulated and that of NF κ B was downregulated in ginsenosides and CTX co-administrated mice, which indicated that ginsenosides alleviated CTX-induced intestinal mucositis *via* activating Nrf2 and inhibiting NF κ B pathways (shown in Figure 7).

Conclusion

In summary, ginsenosides alleviate intestinal mucositis *via* activating Nrf2 and inhibiting NF κ B pathways. Besides, ginsenosides enhanced the anti-cancer immunity via modifying the components of intestinal bacteria. Therefore, ginsenosides improve anti-cancer efficacy of CTX through alleviating adverse reactions and maintaining intestinal microbial homeostasis.

Methods

Chemicals and reagents

CTX was purchased from Jiangsu Hengrui Medicine Co., Ltd. (Lianyungang, China). The ginseng sample (JSPACM-03-1) was collected from Jilin province, the authentic origin of China, and authenticated by Dr. Song-Lin Li. The voucher specimen was deposited in Department of Metabolomics, Jiangsu Province Academy of Traditional Chinese Medicine. Reference compounds, including ginsenoside Re, Rg1, Rf, Ro, Rb1, Rc, Rb2 and Rd, were purchased from Sichuan Victory Co. Ltd. (Chengdu, China). Murine 4T1 (mouse mammary carcinoma) cell was purchased from Keygen Biotech Co., Ltd. (Nanjing, China), and FD-4 were obtained from Sigma (St. Louis, USA). Antibodies for ZO-1, Occludin and E-Cadherin were obtained from Abcam Inc. (CA, USA), and for ZO-2, NFκB and Nrf2 were purchased from Santa Cruz Biotechnology (CA, USA). All other reagents were obtained from commercial sources.

Preparation of CTX and ginsenosides

CTX was dissolved in physiological saline for intravenous (i.v.) administration, and ginsenosides sample was prepared according to our previous protocol [18] and suspended in 2% CMC-Na solution for intragastric (i.g.) administration. The composition and structural information of the ginsenosides were determined by our published UPLC-QTOF-MS method [60]. The contents of Re, Rg1, Rf, Ro, Rb1, Rc, Rb2 and Rd were 2837μg/g, 6784μg/g, 1043μg/g, 1935μg/g, 1937μg/g, 1043μg/g, 811μg/g and 1225μg/g, respectively.

Animal experimental design

Female ICR Mice (weight 18–22g) were supplied by The Chinese University of Hong Kong (Hong Kong SAR, China) or Comparative Medicine Centre of Yangzhou University (Yangzhou, China) and maintained in a pathogen-free environment, air-conditioned at $24 \pm 2^{\circ}\text{C}$ with a standard 12h light–dark cycle. Five mice were housed in per cage and allowed access to distilled water and standard pellet diet *ad libitum*. Animal experiments were approved and performed in accordance with the guidelines of Institutional Animal Care and Use Committee at the Chinese University of Hong Kong (Ref No. (16-710) in DH/HA&P/8/2/1 Pt.63) or Animal Ethics Committee and Institutional Animal Care and Use Committee at Jiangsu Province Academy of Traditional Chinese Medicine (AEWC-20150705-4).

Mice were randomly divided into 6 groups, which were BLK, CTX, CTGS, GS, FT-CTX and anti-CTGS groups. Every group contains 10 animals, all mice were subcutaneously inoculated murine mastadenoma 4T1 cells (2×10^6) in the right armpit, and treatments were performed when tumors reached $200 \pm 30\text{mm}^3$. The dosage regimens of all groups were shown in Figure 8. The fresh feces solution of GS was prepared referring to the published protocol [61], and broad-spectrum antibiotics including vancomycin (100mg/L), neomycin (200mg/L), ampicillin (200mg/L), and metronidazole (200mg/L) were dissolved in sterile drinking water according to previous protocol [3] and changed day.

Tumor Growth Evaluation and Immunity Assessment

Mouse behavior was observed every day, body weight and tumor size were measured every 5d. On the first day of the treatment, blood samples of the first four groups mice were collected for analysis at 4h after CTX administration, and on the twentieth day of the treatment, blood, feces, intestine, 4T1 mastadenoma tissues, spleen and thymus of all mice were collected after humanely sacrificed. The tumor volume, survival rate, tumor inhibition rate as well as thymus and spleen indexes were calculated. Besides, serum IL-2, IL-6, INF- γ and IL-17 were measured using an assay kit according to the manufacturers' recommendations.

Histological and Immunohistochemical Assessments

Fixed hepatic tissues were embedded in paraffin, cut into 5 μ m sections, stained with H&E or stained with primary antibodies and mounted with HRP conjugated secondary antibody, and observed using a light microscope.

Diarrhea Assessment and Intestinal Permeability Assay

For diarrhea assessment, stool was scored on day 20, with 0 indicating normal, 1 indicating slight diarrhea (slightly wet and soft stool), 2 indicating moderate diarrhea (wet and unformed stool), and 3 indicating severe diarrhea (watery stool with severe perianal staining) as previously described [62]. For intestinal permeability assay, each animal was gavaged with 6mg/kg FD-4 after last dosing. Serum samples were obtained by cardiac puncture at 4h after oral administration [63], and fluorescence intensity of each serum sample was measured by DTX 880 Multimode Detector (Beckman Coulter, CA, USA).

Western Blot Analysis

Intestinal or tumor tissue homogenates were prepared with RIPA Buffer. Denatured total protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked by 5% fetal bovine serum and incubated with appropriate dilutions of primary antibody, followed by incubation with rabbit anti-mouse or goat anti-mouse HRP-conjugated secondary antibody. The intended protein was detected using ECL kit and normalized to the corresponding GAPDH expression.

Intestinal microbiota analysis

Changes of intestinal microbiota profiles in response to treatments were determined by analyzing bacterial DNA of feces following our protocols [15]. Fastq files generated through Illumina were demultiplexed and quality filtered, OTUsGS assigned using SILVA database, and the data processed through Mothur 1.40.1.

Statistical Analysis

Data are expressed as means $\pm$ standard deviation. To compare samples collected from any groups, a non-parametric paired Student's t-test (Wilcoxon matched-pairs signed rank test) was performed. All the univariate statistical analyses were performed using either with SPSS software (version 24.0) or GraphPad Prism (version 7) for windows. All statistical tests were two-sided and differences were considered statistically significant if $p < 0.05$.

Abbreviations

CTX: Cyclophosphamide

FD-4: Fluorescein isothiocyanate dextran 4kDa

FT: Fecal transplantation

H&E: Hematoxylin-eosin

HCA: Hierarchical cluster analysis

i.g.: Intragastric

i.v.: Intravenous

IFN- γ : Interferon- γ

IL-17: Interleukin-17

IL-2: Interleukin-2

IL-6: Interleukin-6

NF κ B: Nuclear factor kappa B

NMDS: Non-metric multidimensional scaling analysis

Nrf2: Nuclear factor erythroid 2-related factor 2

operational taxonomic units (OTUs)

PCA: Principal Component Analysis

PCoA: Principal co-ordinates analysis

RDA/CCA: Redundancy analysis/Canonical Correlation Analysis

Declarations

Ethics approval and consent to participate

All experimental procedures were approved by Institutional Animal Care and Use Committee at the Chinese University of Hong Kong (Ref No. (16-710) in DH/HA&P/8/2/1 Pt.63) and Animal Ethics Committee as well as Institutional Animal Care and Use Committee at Jiangsu Province Academy of Traditional Chinese Medicine (AEWC-20150705-4).

Consent for publication

Not applicable.

Availability of data and materials

All 16S rRNA gene sequencing reads data has been deposited to the National Center for Biotechnology Information's Sequence Read Archive under accession number PRJNA590096.

Conflict of interest

The authors declare that they have no competing interests.

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Author's contributions

HZ, GL and SLL contributed to the conception of the study. HZ, YSH, JZ, JM, GL and SLL contributed to the design of the study. HZ, YSH, JZ and CYW contributed to the acquisition of the data. HZ, YSH, JZ, JM, MK, QM, GL and SLL contributed to the analysis of the data. HZ contributed to the drafting of the manuscript. HZ, YSH, JM, GL and SLL contributed to the revising of the manuscript.

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Figures

Figure 1

Effects of ginseng association with CTX on the antitumor activity in 4T1-bearing mice. (A) Body weight, (B) tumor weight, (C) tumor volume, (D) survival rate, (E) tumor inhibition rate, (F) pathological structure in the tumor of 4T1-bearing mice (H&E staining, 200X), and (G) apoptosis proteins levels in tumor. Values are means $\pm$ SDs. (# $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs BLK group, and * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs CTGS group).

Figure 2

Effects of ginseng association with CTX on immune organ and the serum cytokine levels in 4T1-bearing mice. (A) thymus index, (B) spleen index, (C) IL-2, (D) IFN-γ, (E) IL-6, (F) IL-17. Values are means $\pm$ SDs. ($\#P < 0.05$, $\##P < 0.01$ and $\###P < 0.001$ vs BLK group, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ vs CTX group, and $^{\wedge}P < 0.05$ and $^{\wedge\wedge\wedge}P < 0.001$ vs CTGS group).

Figure 3

Effects of ginseng association with CTX on the intestine toxicity in 4T1-bearing mice. (A) diarrhea score, (B) FD4 concentration in blood, (C) intestinal villus length, (D) intestinal villus number per field, (E) H&E staining, ZO-1 IHC and ZO-2 IHC of intestine, and (F) junction proteins and NFκB, Nrf2 expressions in intestine. Values are means $\pm$ SDs. (# $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs BLK group, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs CTX group, and ^ $P < 0.05$ and ^^ $P < 0.001$ vs CTGS group).

Figure 4

Effect of ginseng association with CTX on the composition of intestinal microflora in 4T1-bearing mice. (A) α -diversity estimates at OTU level (sobs, chao, ace and shannon indexes), (B) β -diversity estimates at OTU level (HCA, NMDS, PCoA and PCA), (C) relative abundances of bacterial taxa at the phylum level, and (D) relative abundances of bacterial taxa at the genus level. (# $P < 0.05$ and ## $P < 0.01$ vs BLK group, and * $P < 0.05$ vs CTX group).

Figure 5

Effect of intestinal bacteria on the synergistic effects of combination of CTX and ginseng. (A) survival rate, tumor inhibition and tumor volume, (B) apoptosis proteins levels in tumor, (C) immune cell proliferation, (D) 20 d serum cytokine level, (E) FD4 concentration in blood and (F) junction proteins and NFkB, Nrf2 expressions in intestine. ($\wedge P < 0.05$ and $\wedge\wedge P < 0.01$ vs CTGS group, and $\emptyset P < 0.05$ and $\emptyset\emptyset P < 0.01$ vs FT-CTX group).

Figure 6

Correlation of intestinal bacteria with the anti-cancer and immunomodulatory effects of CTX and ginseng in 4T1-bearing mice. (A) correlation of intestinal bacteria and anti-cancer efficacies, (B) correlation of intestinal bacteria and immune cytokine.

Figure 7

Schematic illustration of ginseng facilitating CTX efficacy in tumor bearing mice. Combination treatment with ginseng and CTX could markedly trigger tumor suppression in subcutaneous tumor bearing mice, which was stronger than CTX treated alone. The enhanced efficiency of the combinational therapy was accompanied by improving immunity in vivo, which may be the result of cross-talk between intestinal commensal bacteria and alleviation of CTX-induced intestinal damage.

Figure 8

Experimental scheme of animal protocol. BLK: blank; CTX: cyclophosphamide; CTGS: cyclophosphamide plus ginsenosides; GS: ginsenosides; FT-CTX: fecal transplant cyclophosphamide; anti-CTGS: antibiotics treated cyclophosphamide plus ginsenosides; i.v.: intravenous injection; i.g. intragastric administration.