Research Article

Radix et Rhizoma Ginseng chemoprevents both initiation and promotion of cutaneous carcinoma by enhancing cell-mediated immunity and maintaining redox homeostasis

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A R T I C L E   I N F O

A B S T R A C T

Background: Radix et Rhizoma Ginseng (thereafter called ginseng) has been used as a medicinal herb for thousands of years to maintain people's physical vitality and is also a non−organ-specific cancer preventive and therapeutic traditional medicine in several epidemiologic and preclinical studies. Owing to few toxic side effects and strong enhancement on body immunity, ginseng has admirable application potential and value in cancer chemoprevention. The study aims at investigating the chemopreventive effects of ginseng on cutaneous carcinoma and the underlying mechanisms.
Methods: The mouse skin cancer model was induced by 7,12-dimethylbenz[a]anthracene/12-O-tetra-decanoylphorbol-13-acetate. Ultraperformance liquid chromatography/mass spectrometry was used for identifying various ginsenosides, the main active ingredients of ginseng. Comprehensive approaches (including network pharmacology, bioinformatics, and experimental verification) were used to explore the potential targets of ginseng.
Results: Ginseng treatment inhibited cutaneous carcinoma in terms of initiation and promotion. The content of Rb1, Rb2, Rc, and Rd ginsenosides was the highest in both mouse blood and skin tissues. Ginseng and its active components well maintained the redox homeostasis and modulated the immune response in the model. Specifically, ginseng treatment inhibited the initiation of skin cancer by enhancing T-cell−mediated immune response through upregulating HSP27 expression and inhibited the promotion of skin cancer by maintaining cellular redox homeostasis through promoting nuclear translocation of Nrf2.
Conclusion: According to the study results, ginseng can be potentially used for cutaneous carcinoma as a chemopreventive agent by enhancing cell-mediated immunity and maintaining redox homeostasis with multiple components, targets, and links.

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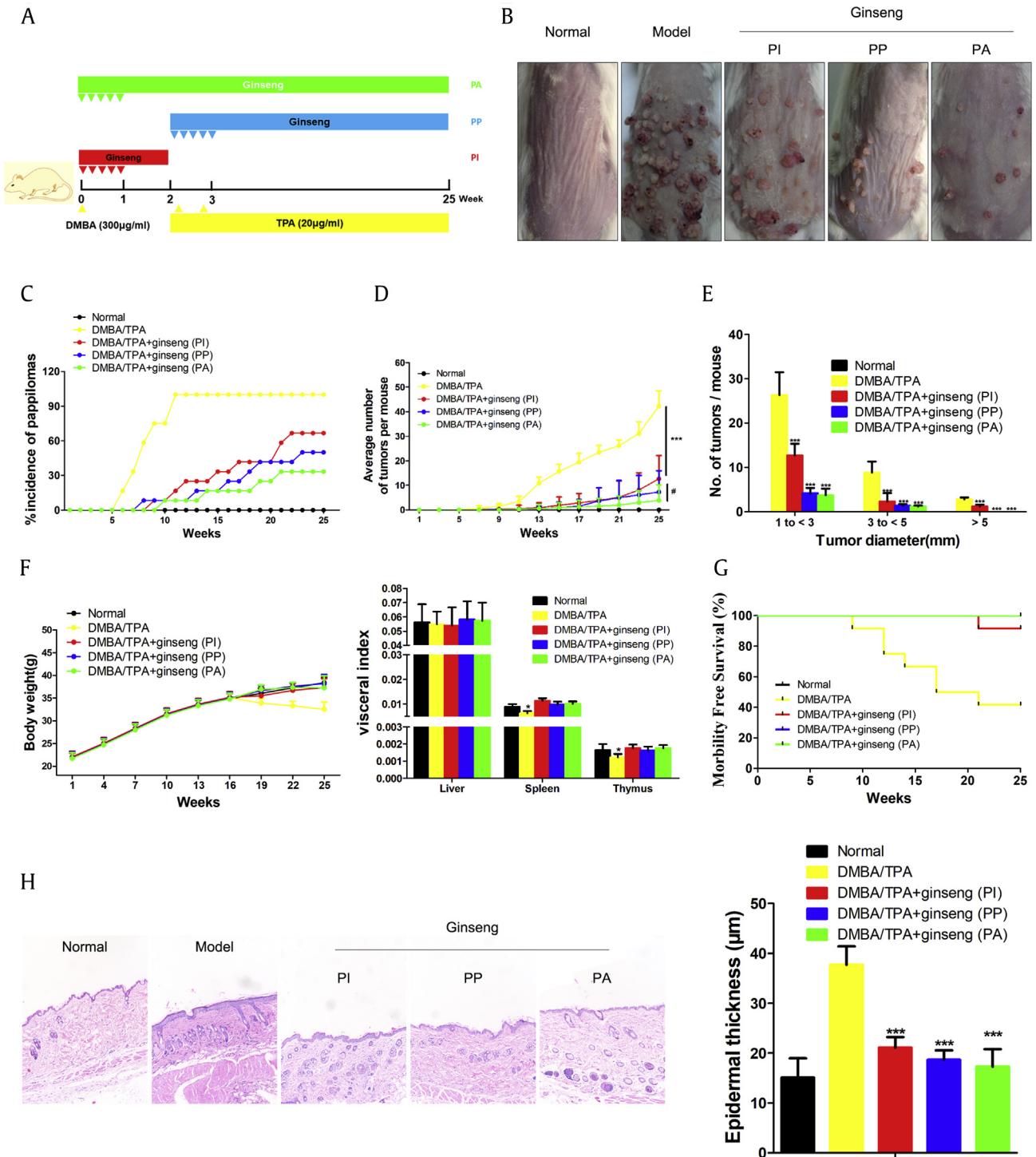☆ These authors contributed equally to this work.

Fig. 1. Chemopreventive effect imposed by ginseng on DMBA/TPA-induced cutaneous carcinoma in vivo. (A) Animal study workflow. (B) Representative images of papillomagenesis in indicated groups at the end of the experiments. (C) Papilloma incidence in different treatment groups (n = 12). (D) Average number of papillomas for each mouse in indicated groups (n = 12). The data are expressed as mean ± SD. ***P < 0.001 (vs DMBA/TPA), #P < 0.05 (PI vs PP). (E) Average number of papillomas for each mouse in different tumor diameter groups (n = 12). The data are expressed as mean ± SD. ***P < 0.001 (vs DMBA/TPA). (F) Left: weekly record of body weights (n = 12). Right: hepatic, thymus, and spleen indices (n = 12). The data are expressed as mean ± SD. *P < 0.001 (vs normal). (G) Survival rates of mice in different treatment groups during 25 weeks. (H) Left: representative images of epidermal development and hyperplasia in indicated groups (40×). Right: quantitative analysis on H&E data (n = 6). The data are expressed as mean ± SD. ***P < 0.001 (vs DMBA/TPA). DMBA, 7,12-dimethylbenz[a]anthracene; H&E, hematoxylin and eosin; PA, prevention of all phases; PI, prevention of initiation; PP, prevention of promotion; SD, standard deviation; TPA, 12-O-tetradecanoylphorbol-13-acetate.

A

Blood   Tissue

Rb1 Rb2 Rh1 Rg1 Rc Re Rd Rf

B

PCNA

DMBA/TPA   DMBA/TPA +Ginseng(PI)   DMBA/TPA +Ginseng(PP)   DMBA/TPA +Ginseng(PA)

C

Cleaved caspase-3

DMBA/TPA   DMBA/TPA +Ginseng(PI)   DMBA/TPA +Ginseng(PP)   DMBA/TPA +Ginseng(PA)
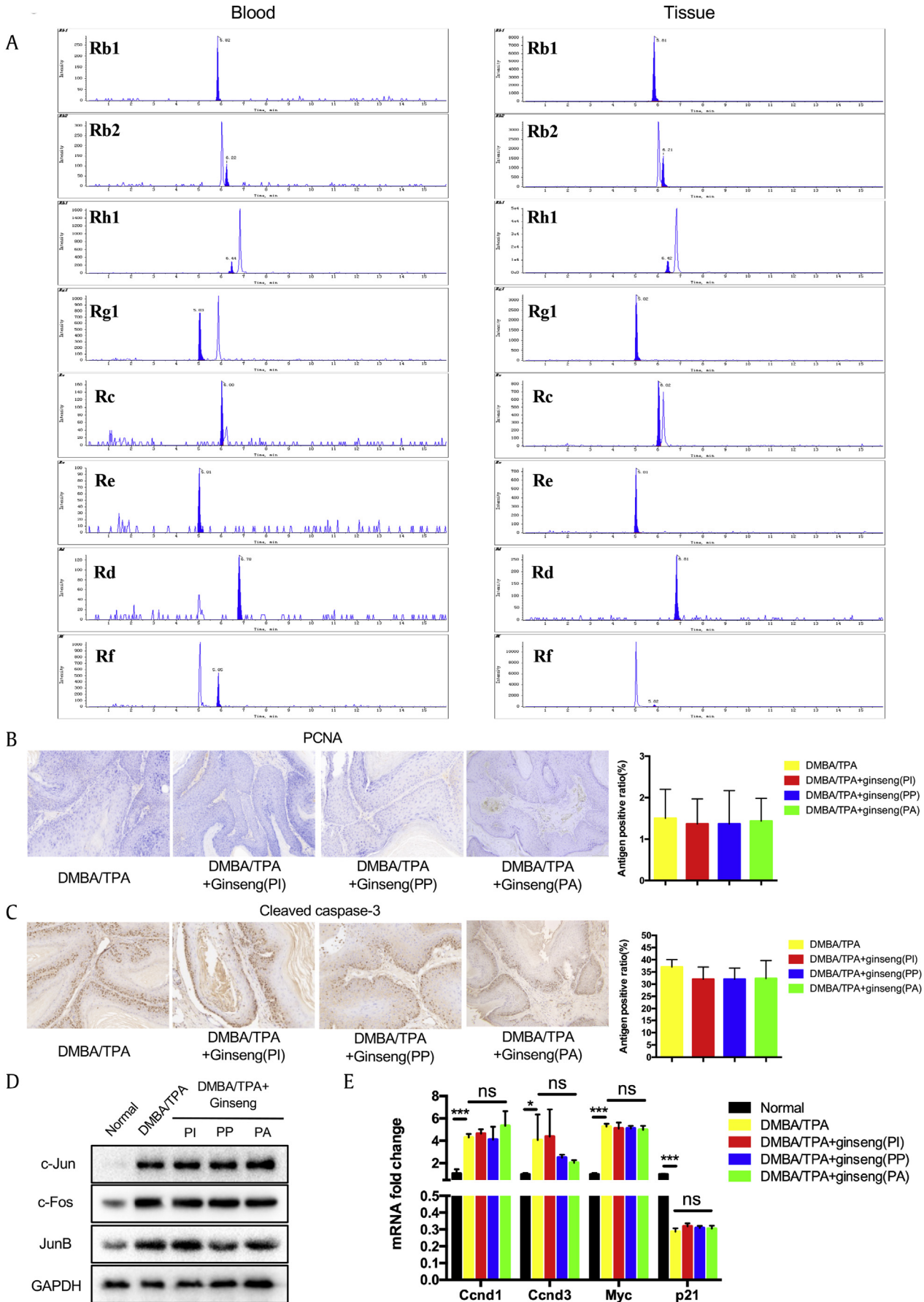
D

c-Jun
c-Fos
JunB
GAPDH

E

Table 1
UPLC/MS quantification analysis of the concentration of the 8 ginsenosides in mouse cutaneous and blood samples after 24 weeks of treatment.

Groups	Rb1	Rb2	Rc	Rd	Re	Rf	Rg1	Rh1
Blood (μg/ml)	32.3 ± 7.2	22.7 ± 6.3	29.7 ± 7.2	56.1 ± 11.7	7.2 ± 2.3	0.4 ± 0.2	1.0 ± 1.1	2.1 ± 1.3
Tissue (μg/mg)	51.1 ± 11.1	15.6 ± 6.1	38.2 ± 9.1	71.1 ± 19.7	16.7 ± 3.1	0.9 ± 0.3	1.3 ± 0.7	8.1 ± 6.2

MS, mass spectrometry; UPLC, ultraperformance liquid chromatography.

1. Introduction

Cutaneous carcinoma is a malignancy for human beings, and about millions of people around the world suffer from cutaneous carcinoma each year [1,2]. Most of the patients with this disease can be successfully treated with multimodalities involving surgery, along with radiotherapies, chemotherapies, photodynamic therapies, targeted therapies, and immune therapies [3,4]. However, when the carcinoma metastasizes, this will lead to severe morbidity and mortality [5]. Alternatively, chemoprevention has been widely accepted to lower the incidence rate and the death rate of cutaneous carcinoma [6,7]. Radix et Rhizoma Ginseng [commonly called 人参 (Ren Shen) in Chinese; ginseng in English] has been widely used as a medicinal herb for thousands of years in China to boost people's physical vitality and prolong people's life [8]. It has been used for treatment of diabetes mellitus and cardiovascular disease and is known as a non−organ-specific cancer preventive and therapeutic traditional Chinese medicine (TCM) [9−12]. Ginseng intake has been positively associated with decreased risk of development of head and neck, breast, stomach, colon, and lung cancers [13−15]. Ginseng and its active ingredients have also been demonstrated as chemopreventive agents for multiple tumors in various preclinical research studies [16−18]. However, there is no relevant study on the chemopreventive effect of ginseng on cutaneous carcinoma.

The two-stage 7,12-dimethylbenz[a]anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA)−induced model has been widely used as a mouse cutaneous carcinoma model [19]. It exhibits the organ-specific characteristic and captures the explicit pathological development from normal status to papilloma and from deterioration to squamous cell carcinoma in humans [20]. This mouse model is identical to the multistep process (mostly initiation and promotion) of cutaneous carcinogenesis, in which DMBA mediates the initiation phase and TPA initiates the promotion phase [21−23]. It is thus useful for studying the cancer preventive and therapeutic capacities of active compounds and their underlying mechanisms.

The term of chemoprevention was coined by Michael in 1976 [24]. Vitamin A, nicotinamide, and some other components have been reported as the chemopreventive agents for cutaneous carcinoma [25]. Existing studies have shown that ginseng may exert chemopreventive effect mainly by inhibiting cell proliferation and inducing apoptosis [26,27]. In addition to the aforementioned mechanism, chemoprevention also mainly focuses on antioxidative and antiinflammatory activity, increased capacity of the immune system, Phase II enzyme induction, and so on [28]. However, the underlying mechanisms of ginseng on cutaneous carcinoma are unknown. Relevant research indicated that heat shock proteins (HSPs), especially HSP27 and HSP70, can promote cell-mediated immune response to DMBA, while TPA can evidently accelerate the malignant transformation of preneoplastic cells induced by DMBA by causing redox imbalance [29,30]. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), heme oxygenase 1 (HO-1), and other antioxidant enzyme systems can maintain cell redox homeostasis to a large extent [31−34].

Based on the aforementioned information, how ginseng affects a standard two-stage mouse skin cancer model induced by DMBA/TPA is evaluated, and several functional ginsenosides (Rb1, Rb2, Rc, and Rd) are identified. In addition, the mechanisms underlying the chemopreventive effects of ginseng on tumor initiation and promotion may be associated with cell-mediated immunity and redox homeostasis.

2. Materials and methods

2.1. Reagents

Ginseng powder was purchased from Jiangsu Provincial Hospital of TCM (Nanjing, China), where it was prepared by peeling, drying, and powdering with 4 to 6-year-old Radix et Rhizoma Ginseng. The Delta Information Center for Natural Organic Compounds (Xuancheng, Anhui, China) and Indofine Chemical Company (Somerville, NJ, USA) together provided the standards of ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, Rh1, and Rg1. TPA was purchased from Cayman Chemical (Ann Arbor, MI, USA), whereas DMBA was provided by Sigma-Aldrich (St. Louis, MO, USA). Many assay kits used in the study, such as GSH and GSSG Assay Kit, CAT assay kit, GR assay kit, ROS assay kit, GPx assay kit, and SOD assay kit, were provided by Beyotime from Shanghai, China. The HNE ELISA kit and 8-OhdG ELISA kit were bought from Cell Biolabs (San Diego, CA, USA). For Western blot or immunohistochemistry, the following antibodies were used: rabbit polyclonal antibodies to HSP27 and HSP70 (BioVision, Milpitas, CA, USA), HO-1 and NQO1 (ABclonal, Woburn, MA, USA), Nrf2 and JunB (Abcam, Cambridge, MA, USA), c-Jun, c-Fos GPX-1 SOD1, and CAT (Cell Signaling Technology, Danvers, MA, USA), GAPDH (Bioworld Technology, Nanjing, China), and the combination of goat anti−rabbit IgG and horseradish peroxidase (Bioworld Technology, Nanjing, China). For the animal study, the normal goat IgG and the goat polyclonal antibody to HSP27 were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Establishment of the DMBA/TPA-induced carcinogenesis model

Female mice (6−7 weeks old) used at the Institute of Cancer Research were supplied by Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). These mice were raised in

Fig. 2. Chemopreventive effect imposed by ginseng on skin cancer has no relation with cell proliferation or apoptosis, as well as AP-1. (A) Left: UPLC/MS was used to test Rb1, Rb2, Rb3, Rg1, Rc, Re, Rd, and Rf in blood samples. Right: UPLC/MS was used to test Rb1, Rb2, Rb3, Rg1, Rc, Re, Rd, and Rf in mouse cutaneous samples. (B) Left: IHC analysis of cleaved caspase-3 expression in the mouse epidermis in different treatment groups (200×). Right: quantitative analysis of IHC data (n = 9). The data are expressed as mean ± SD. (C) Left: IHC analysis of PCNA expression in the mouse epidermis in different treatment groups (200×). Right: quantitative analysis of IHC data (n = 9). The data are expressed as mean ± SD. (D) Western blot analysis of c-Fos, c-Jun, and JunB expression in the mouse epidermis in different treatment groups. (E) Real-time PCR analysis of mRNA expression of myc, Ccnd1, Ccnd3, and p21 in different treatment groups. *P < 0.01, ***P < 0.001, ^{ns}P > 0.05 (vs DMBA/TPA). AP-1, activator protein 1; DMBA, 7,12-dimethylbenz[a]anthracene; IHC, immunohistochemical; MS, mass spectrometry; PA, prevention of all phases; PCNA, proliferating cell nuclear antigen; PI, prevention of initiation; PP, prevention of promotion; PCR, polymerase chain reaction; SD, standard deviation; TPA, 12-O-tetradecanoylphorbol-13-acetate; UPLC, ultraperformance liquid chromatography.

A

B

- immune response
- positive regulation of response to stimulus
- inflammatory response
- ion binding
- small molecule binding
- chemokine receptor binding
- carbohydrate derivative binding
- circulating immunoglobulin
- transcription factor activity, sequence-specific DNA binding
- others

C

- protein binding
- oxidative stress
- Fluid shear stress and atherosclerosis
- Ubiquitin mediated proteolysis
- Pathways in cancer
- antioxidase activity
- hydrolase activity
- macromolecular complex binding
- transferase activity
- others

temperature-controlled chambers on a 12-h light/12-h dark cycle. This research obtained the approval from the Animal Care and Use Committee of the Nanjing University of Chinese Medicine. All experimental procedures related to mice were carried out following the Regulations for the Administration of Affairs Concerning Experimental Animals that have been approved by the State Council of the People's Republic of China (PRC). Experimenters divided the Institute of Cancer Research mice into 5 groups randomly, with 12 mice for each group. Fig. 1A shows the workflow and the grouping associated with the *in vivo* study. Particularly, the naked backs of the mice in all the groups were treated with DMBA (60 μg, dissolved in 0.2 mL of acetone). One week after the treatment with DMBA, the mice were further exposed to TPA (4 μg, dissolved in 0.2 mL of acetone) for 25 weeks (twice per week). The mice were intragastrically (i.g.) administrated with ginseng (1 mg suspended in 1 mL of physiological saline) five times a week, following the schedule as illustrated in Fig. 1A. The number of tumors, the diameter of which was more than 1 mm, was counted each week. The Nrf2$^{-/-}$ mice were provided by Professor Peng Cao from the Jiangsu Province Academy of Chinese Medicine.

2.3. Histological evaluation

The skin tissues of the mice were separated after sacrificing the mice, with some fresh tissues fixed in paraformaldehyde at a concentration of 4% and stained with hematoxylin and eosin. The obtained sections were visualized, and photographs were taken under a Zeiss invert microscope (40×) equipped with a digital camera, followed by the analysis using ZEN 2011 imaging software (Zeiss, Göttingen, Germany).

2.4. Ultraperformance liquid chromatography/mass spectrometry

Analyses were performed on an ultraperformance liquid chromatography (UPLC) system (Waters Corp., Milford, MA, USA). An ACQUITY UPLC T3 C18 column (2.1 mm × 100 mm, internal diameter (i.d.), 1.8 μm) from Waters was used. The column temperature was maintained at 30°C. The standards and samples were separated using a gradient mobile phase consisting of water and acetonitrile with 0.1% formic acid. The flow rate was at 0.4 mL/min. The injection volume of sample was 2 μL. Mass spectrometry was carried out on a Micromass Quattro Micro API mass spectrometer (Waters Corp.) using an electrospray ionization source. The temperatures of source and desolvation were 120°C and 300°C, respectively. The flow rate of desolvation gas was set at 600 L/h.

2.5. Analyses of 8-OhdG, 4-NHE, ROS, GSH, GSSG, and antioxidant enzyme activity

The obtained fresh skin tissues were collected after the mice were sacrificed, with some being immediately frozen in liquid nitrogen for further analyses. The levels of 8-OhdG, 4-NHE, ROS, GSH, and GSSG and the activity exhibited by antioxidant enzymes were determined using corresponding commercial kits, following the instructions of the suppliers.

2.6. Immunohistochemical staining

Experimenters collected the skin tissue samples of the mice and fixed them in paraformaldehyde for the convenience of immunohistochemical (IHC) analysis of Nrf2 protein. The sections embedded with paraffin (4-μm thick) were mounted on the 2-aminopropyltriethoxysilane—coated slides, followed by a series of treatment, such as baking, deparaffinization, rinsing with hydrogen peroxide (3%), proteinase K (concentration: 0.5 mg/mL) incubation, washing, 5-min blocking with StartingBlock blocking buffer purchased from Pierce, Rockford, Il, USA, and 30-min incubation at room temperature with anti-Nrf2 (1:100, Abcam) polyclonal antibody. At last, the streptavidin-biotin complex (Solarbio, Beijing, China) was used to incubate these obtained sections at room temperature for 30 min, and then, hematoxylin (counter stain) together with 3,3-diaminobenzidine tetrahydrochloride solution (chromogen) (ZSGB-BIO, Beijing, China) was used to detect them. The sections were then mounted with neutral gums. Mantra 1.01 provided by PerkinElmer, Waltham, MA, USA, was used for photographing the IHC sections.

2.7. RNA isolation and quantitative real-time polymerase chain reaction

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from the tissue cells and samples. The Hiscript® II QRTSuperMix (Vazyme, Nanjing, China) was used to synthesize the first-strand cDNA using total RNA (500 g). An SYBR Green Master kit provided by Bio-Rad, Hercules, CA, USA, was used for carrying out the quantitative real-time polymerase chain reaction based on the instruments from the manufacturer. The expression level was quantified with the comparative cycle threshold (Ct) method by calculating $2^{(-\Delta\Delta Ct)}$. Supplementary Table S1 shows primers for the polymerase chain reaction.

2.8. Contact hypersensitivity to DMBA

In short, sensitization on the shaved abdominal skin of C3H/HeN mice was carried out on Day 0 with 100 mL of 0.1% DMBA solution (w/v in acetone). Five days later, baseline ear thickness was measured, followed by applying 20 mL of 0.1% DMBA (a challenge dose) on the ear. The measurement was performed after the ear swelling increased for each 24 h, aiming at quantitating the volume of contact hypersensitivity response.

2.9. Assessment of immunological tolerance to DMBA

Fourteen days before the experiments (Day 14), 2 mg of isotype control antibody or a combination of anti-HSP27 with or without ginseng (40 mg/kg, i.g., once per day (qd)) was used to treat the abdominal skin of the mice under occlusion with a bio-occlusive dressing. Soon afterward, 100 mL of 0.1% DMBA was locally applied on the site treated with the antibody. On Day 0, 100 mL of 0.1% DMBA was applied to resensitize the Antibody (Ab)-untreated dorsal skin of the mice. After 5 days, 20 mL of 0.1% DMBA was injected in the ear of the mice. The measurement interval was the same as mentioned previously.

Fig. 3. Different mechanisms are involved in ginseng prevention of skin cancer initiation and promotion. (A) The flowchart for identifying potential targets of ginseng using pharmacophore mapping, bioinformatics, and network analysis. (B–C) Gene ontology (GO) and enrichment analysis of the candidate targets in the (B) initiation or (C) promotion phase. Enriched categories were functionally grouped in the form of network for target genes. Only terms that are the most significant were labeled. Interactions of the representative enriched pathway (P < 0.05) among candidate targets. CAT, catalase; PPI, protein—protein interaction.
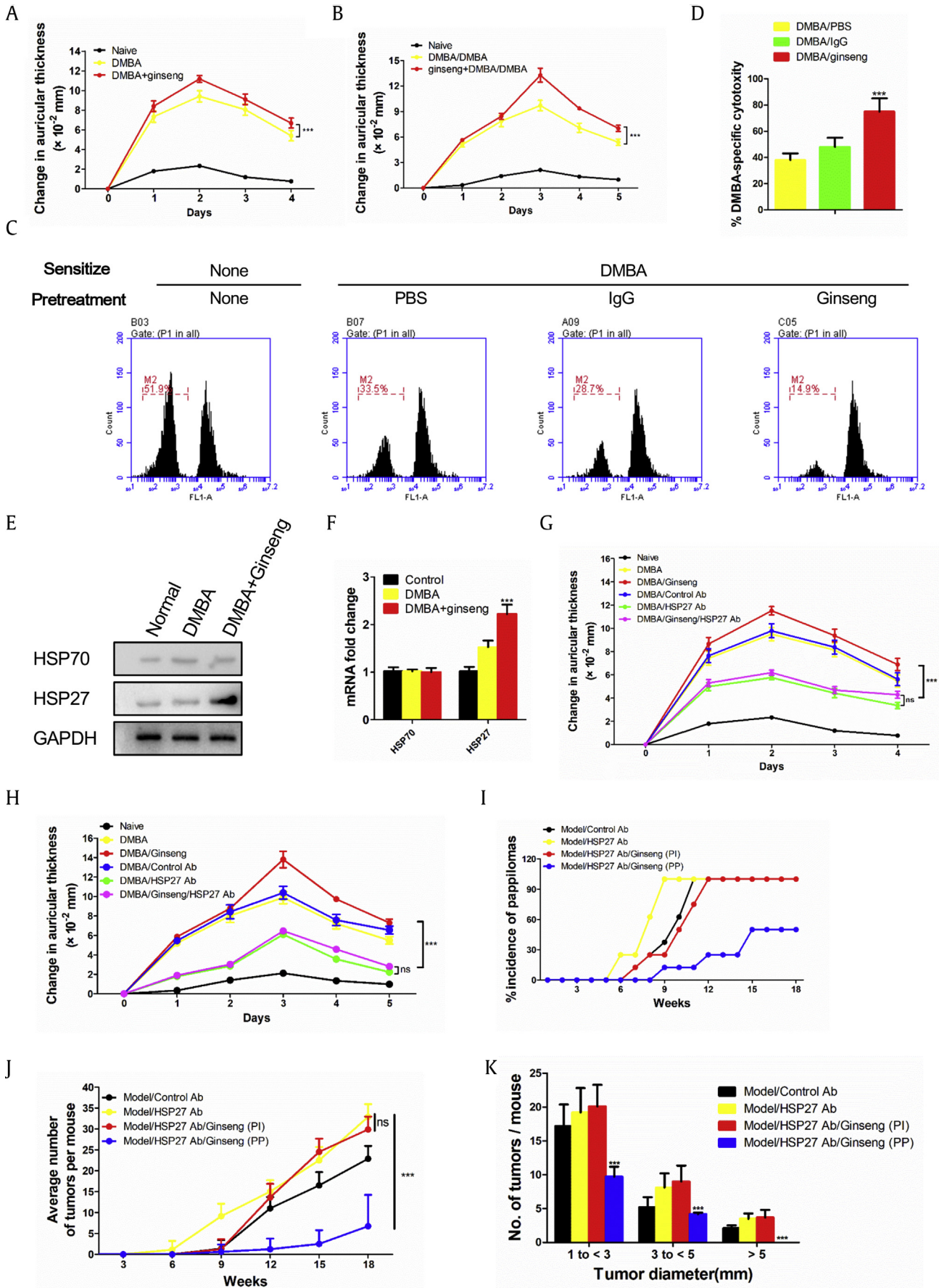
2.10. In vivo cytotoxic T lymphocyte activity

Cytotoxic T lymphocyte (CTL) activity was assessed using an *in vivo* antigen-specific cytotoxicity assay. The mice were topically treated with anti-HSP27 antibody with or without ginseng (40 mg/kg, i.g., qd) as described previously and were then sensitized at the same site with DMBA. Eight days later, the mice received an intravenous injection of 5×10^6 CFSE (Life Technologies, Carlsbad, USA)-labeled target spleen cells composed of two populations: CFSE low (6-mM)-labeled cells that were prepulsed with 50 mM DMBA for 25 min, washed, and mixed in the ratio of 1:1 with unpulsed CFSE high (12-mM)-labeled cells. In 16 h, the spleens were harvested and processed into single-cell suspensions, followed by flow cytometric analysis to quantify CFSE high and low cells. Flow cytometric acquisition of 2000 CFSE high cells had been detected in each sample. The calculation of percent DMBA specific cytotoxicity was expressed as follows: $100 \times [1 - (\text{percent CFSE low/naive})]$. The calculation of percent inhibition is expressed as follows: $100 \times [1 - (\text{percent cytotoxicity}) \text{ average percentage of cytotoxicity IgG}]$.

2.11. Statistical analysis

The obtained value is the mean ± standard deviation of no less than three experiments performed independently. The comparison within groups was carried out via the one-way analysis of variance. When P value is less than 0.05, it is considered that the study has statistical significance.

3. Results

3.1. Ginseng exhibits preventive effects on both initiation and promotion of cutaneous carcinoma in mice

The two-stage DMBA/TPA-induced model has been successfully established and used in the laboratory as a mouse cutaneous carcinoma model [14]. The study adopts the two-stage DMBA/TPA-induced model for exploring how ginseng affects skin cancer with respect to the initiation stage and promotion stage. In addition to the normal control group (no treatments) and the model group (DMBA/TPA treatment only), three ginseng treatment groups [prevention of initiation (PI), prevention of promotion (PP), and prevention of all phases (PA)] were set up. As shown in Fig. 1A, the PI group was administered with ginseng from Day 0 (starting treatment with DMBA) to Day 14 (starting treatment with TPA); the PP group was administered with ginseng from Day 14 (starting treatment with TPA) to the end of the experiment (the end of Week 25); and the PA group was administered with ginseng from Day 0 (starting treatment with DMBA) to the end of the experiment. As expected, treatment with DMBA/TPA successfully induced the generation of papillomas, compared with the normal control group (Fig. 1B). At the end of the experiments, the mice treated with DMBA/TPA had a 100%

prevalence of cutaneous papillomas. Interestingly, treatment with ginseng exerted preventive effects on the initiation phase, the promotion phase, and the whole procedure (Fig. 1B). Ginseng treatment profoundly reduced both the incidence (Fig. 1C) and multiplicity (Fig. 1D) of the formation of cutaneous papilloma, as well as prolonged the incubation from six weeks to eight weeks in a substantial manner. Besides, the size distribution of papilloma also indicates how ginseng inhibits tumorigenesis (Fig. 1E). Furthermore, ginseng neither caused any visible toxicity or unhealthy sign nor significantly affected the mice's body weight. In the last stage of the experiments, the model animals lost the body weight by about 16%, which was rescued in all of the three groups treated with ginseng (Fig. 1F). Moreover, ginseng treatments dramatically improved the survival rate of animals under the treatment with DMBA/TPA (Fig. 1G). At the end of this study, only 41.67% of animals in the model group were alive, whereas almost all the ginseng-treated animals survived with cutaneous carcinoma at the same time. Surprisingly, the incidence, multiplicity, and latency period of cutaneous papilloma formation and the survival rate of the animals were further improved in the mice of the PA group, compared with the animals of the PP and PI groups. Histological analysis further revealed that ginseng treatments significantly suppressed the DMBA/TPA-induced epidermal thickness or hyperplasia increase (Fig. 1H). Collectively, the results indicate that ginseng possesses potent preventive effects on skin cancer with regard to initiation and promotion.

3.2. Ginsenosides Rb1, Rb2, Rc, and Rd, the principal active components of ginseng, were responsible for its chemopreventive capacity

As we know, compounds that can be absorbed into the blood and reach lesions may be the active ingredients of TCM for disease treatment. Thus, to identify the possible ingredients of ginseng possibly causing the chemopreventive effects, UPLC/mass spectrometry (MS) was used to test eight main ginsenosides (Rb1, Rb2, Rb3, Rg1, Rc, Re, Rd, and Rf) of the ginseng extracts, and the blood and cutaneous samples of the mice were also treated. According to the comparison in terms of the MS data and the UPLC retention time, the aforementioned eight ginsenosides, Rb1, Rb2, Rb3, Rg1, Rc, Re, Rd, and Rf, were all well separated and identified in the mouse blood and cutaneous samples (Fig. 2A). UPLC/MS quantification analysis showed that of the 8 ginsenosides, Rb1, Rb2, Rc, and Rd occupied the largest proportion in ginseng, as well as in mouse cutaneous and blood samples (Table 1). Therefore, these 4 ginsenosides may be responsible for the chemopreventive effect of ginseng on cutaneous carcinoma.

3.3. Chemopreventive effect of ginseng on skin cancer is independent of cell proliferation and apoptosis

Studies have shown that ginseng may exert chemopreventive effect by inhibiting cell proliferation and inducing apoptosis of many types of cancerous cells. To determine whether this occurs in

Fig. 4. Ginseng inhibits the initiation of skin cancer by enhancing cell-mediated immune response through upregulating HSP27. (A) The effect of ginseng on contact hypersensitivity to DMBA (n = 5). The data are expressed as mean ± SD. ***P < 0.001 (vs DMBA). (B) The effect of ginseng on tolerance to DMBA. The data are expressed as mean ± SD. ***P < 0.001 (vs DMBA/DMBA). (C–D) The effect of ginseng on development of DMBA-specific CTLs. (C) Representative images of flow cytometry data in the indicated groups. (D) Quantitative analysis on flow cytometry data (n = 5). The data are expressed as mean ± SD. ***P < 0.001 (vs DMBA/phosphate buffer saline (PBS)). (E) How ginseng affects protein expression of HSP27 and HSP70, as detected by Western blot. (F) How ginseng affects mRNA expression of HSP27 and HSP70, as determined by real-time qPCR. ***P < 0.001 (vs DMBA). (G) How ginseng affects contact hypersensitivity to DMBA with or without HSP27 antibody (Ab) (n = 5). The data are expressed as mean ± SD. ***P < 0.001 (DMBA/ginseng/HSP27 Ab versus DMBA/ginseng); nsP > 0.05 (DMBA/HSP27 Ab versus DMBA/ginseng/HSP27 Ab). (H) How ginseng affects tolerance to DMBA with or without HSP27 antibody (Ab) (n = 5). The data are expressed as mean ± SD. ***P < 0.001 (DMBA/ginseng/HSP27 Ab versus DMBA/ginseng); nsP > 0.05 (DMBA/HSP27 Ab vs DMBA/ginseng/HSP27 Ab). (I) Papilloma incidence in different treatment groups (n = 12). (J) Average number of papillomas for each mouse in indicated groups (n = 12). The data are expressed as mean ± SD. ***P < 0.001 [model/HSP27 Ab vs DMBA/HSP27 Ab/ginseng (PP)]; nsP > 0.05 [model/HSP27 Ab vs DMBA/HSP27 Ab/ginseng (PI)]. (K) Average number of papillomas for each mouse in different tumor diameter groups (n = 12). The data are expressed as mean ± SD. ***P < 0.001 [model/HSP27 Ab vs DMBA/HSP27 Ab/ginseng (PP)]. CTL, cytotoxic T lymphocyte; DMBA, 7,12-dimethylbenz[a]anthracene; HSP, heat shock protein; PI, prevention of initiation; PP, prevention of promotion; PCR, polymerase chain reaction; SD, standard deviation.
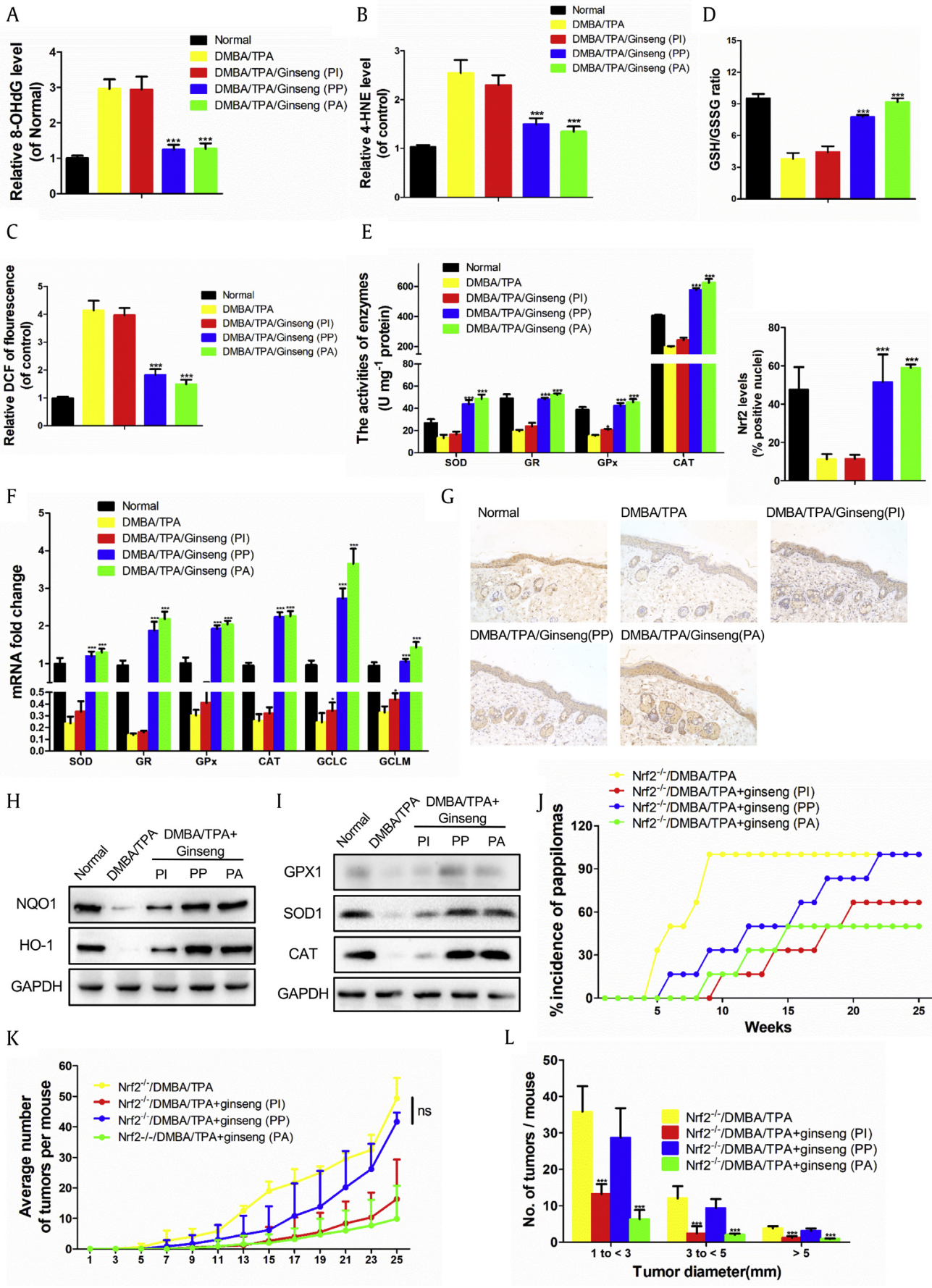
our skin cancer model, paraffin-embedded skin tissue sections from each group were subjected to IHC staining with antibodies to proliferating cell nuclear antigen (for detection of cell proliferation) and cleaved caspase-3 (for detection of cell proliferation). As shown in Fig. 2B and C, ginseng treatments (PI, PP, and PA) did not significantly affect cell proliferation and apoptosis in skin samples of the DMBA/TPA-treated mice.

Activator protein 1 (AP-1), which comprises homodimers or heterodimers among members of the Jun family and Fos family and can regulate cell cycle and apoptosis [35], participates in the preventive action of ginseng in other cancer types. For identifying how DMBA/TPA exposure and ginseng treatment affect the AP-1 subunit expressions, the protein levels of members of the Jun family and Fos family in mouse skin tissues from both control and ginseng groups were detected by Western blotting (Fig. 2D). All the family members dramatically increased in the skin tissues exposed to DMBA/TPA compared with those in the normal controls, which was barely reversed with ginseng treatment in the PI, PP, and PA groups. To further identify this, we detected the mRNA expression of cell cycle regulators including myc, Ccnd1, Ccnd3, and p21 in these groups. As shown in Fig. 2E, the expression of myc, Ccnd1, and Ccnd3 all dramatically increased in the skin tissues exposed to DMBA/TPA compared with those in the normal controls, whereas p21 expression decreased, which was barely reversed with ginseng treatment in the PI, PP, and PA groups. These results suggest that the mechanism of ginseng's chemoprevention against skin cancer is totally different from that in other cancer types.

3.4. Ginseng prevents initiation and promotion of skin cancer through different mechanisms

To elucidate how ginseng prevents initiation and promotion of skin cancer, we used comprehensive approaches involving pharmacophore mapping, bioinformatics, and network analysis (Fig. 3A). Having identified Rb1, Rb2, Rc, and Rd ginsenosides as the principal active ingredients of ginseng by UPLC/MS, we conducted pharmacophore mapping using a Web server to identify the potential targets of these active ginsenosides. Totally, 185 putative targets of Rb1, Rb2, Rc, and Rd after the duplicates were wiped off and top 100 putative targets for the four ginsenosides were selected and sorted according to the fit scores (submission IDs: 16108160603, 16108121539, 16108121559, and 16108121617, Supplementary Table S2).

The pharmacological action of a drug is closely related to its targets, which play critical roles in the pathogenesis of a disease. It is equally significant to make clear the key points of the initiation stage and promotion stage of skin cancer. To obtain the preventive signature of ginseng for these phases, we analyzed the gene expression profiles using the microarray data available in ArrayExpress (E-MEXP-188). In brief, we conducted two sets of comparison. The first was to compare DMBA-initiated skin (D)

with normal control skin (C) to pinpoint the effect of DMBA exposure on gene expressions in skin tissue for cancer initiation. The second was to compare D with DMBA-initiated/TPA-promoted skin to find the effect of TPA treatment on gene expressions associated with cancer promotion after DMBA-induced initiation. Differentially expressed genes with top 250-fold changes of logFC were obtained and treated as significantly altered in the two phases (Supplementary Tables S3, 4). Based on this, we constructed two networks, that is, drug initiation network and drug promotion network, that showed the interactions of the putative targets of ginseng with initiation- or promotion-related preventive signature of skin cancer, respectively, in a protein−protein interaction manner. In addition, using the plugin CytoNCA, topological features were calculated for each node in the networks. The nodes with values exceeding the median were all selected as the pivotal ones of these two networks and regarded as the potential key targets of ginseng chemoprevention in the initiation and promotion phases of skin cancer. For further clarifying how these key targets work, enrichment analysis was used to find out the interrelations between functional groups and the way they affect the biological networks. As shown in Fig. 3B, the targets with topological importance in the drug initiation network are significantly enriched in the molecular functions/biological processes and pathways of immune function. In contrast, the key targets in the drug promotion network were significantly associated with antioxidative responses (Fig. 3C). Because the preventive mechanism of ginseng for these two processes of skin cancer has not been fully elucidated, we further performed experimental validation based on *in vitro* and *in vivo* systems (see the following section). The validation experiment was carried out on the basis of the *in vitro* system and *in vivo* system considering the fact that how ginseng prevents skin cancer in the initiation and promotion stage remains unclear.

3.5. Ginseng inhibits the initiation of skin cancer by enhancing cell-mediated immune response through upregulating HSP27 expression

Accumulating evidence has shown that administration of carcinogenic DMBA to skin tissue results in an antigen-specific response mediated by T lymphocytes [36], raising the possibility that such an immune response may effectively eradicate mutant cells and prevent these cells from developing a tumor in the first place. To verify whether ginseng can enhance this cell-mediated immune response, we first tested its effect on the development of contact hypersensitivity to DMBA. Being exposed to DMBA activates the humoral and cell-mediated immunity, and the resulting sustainable immunosuppression offers an epigenetic mechanism that contributes to the development or metastasis of tumor [37]. As shown in Fig. 4A, DMBA contact hypersensitivity is significantly enhanced by pretreatment with ginseng. We further demonstrated that ginseng together with

Fig. 5. **Ginseng inhibits promotion of skin cancer by maintaining cellular redox homeostasis through promoting nuclear translocation of Nrf2.** (A and B) The levels of (A) 8-OhdG and (B) 4-HNE in mouse skin tissues of the indicated groups (n = 6). The data are represented as mean ± SD. ***P < 0.001 (vs DMBA/TPA). (C) 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) staining was applied to detect ROS levels in indicated groups (n = 6). The data are expressed as mean ± SD. ***P < 0.001 (vs DMBA/TPA). (D) The GSH/GSSG ratio in mouse skin tissues of the indicated groups (n = 6). The data are expressed as mean ± SD. ***P < 0.001 (vs DMBA/TPA). (E) The activities of SOD, GR, GPx, and CAT in mouse skin tissues of the indicated groups (n = 6). The data are expressed as mean ± SD. ***P < 0.001 (vs DMBA/TPA). (F) Total mRNA was isolated and analyzed for identifying the levels of Cat, Sod1, Gpx1, Gsr, Gclc, and Gclm expression with real-time qPCR in mouse skin tissues of indicated groups (n = 6). The data are expressed as mean ± SD. *P < 0.05, ***P < 0.001 (vs DMBA/TPA). (G) IHC analysis of Nrf2 expression in the mouse epidermis in different treatment groups (100×). Top: quantitative analysis of IHC data (n = 9). The data are expressed as mean ± SD. ***P < 0.001 (vs DMBA/TPA). (H) Western blot analysis of HO-1 and NQO1 expression in the mouse epidermis in different treatment groups. (I) Western blot analysis of GPX1, SOD1, and CAT expression in the mouse epidermis in different treatment groups. (J) The papilloma incidence in different treatment groups (n = 12). (K) Average number of papillomas for each mouse in indicated groups (n = 12). The data are expressed as mean ± SD. ns P > 0.05 (vs DMBA/TPA) (L) Average number of papillomas for each mouse in different tumor diameter groups (n = 12). The data are expressed as mean ± SD. ***P < 0.001 (vs DMBA/TPA). CAT, catalase; DMBA, 7,12-dimethylbenz[a]anthracene; GR, glutathione reductase; GSH, reduced glutathione; GPx, glutathione peroxidase; GSSG, oxidized glutathione; HO-1, heme oxygenase 1; IHC, immunohistochemical; NQO1, quinone 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PA, prevention of all phases; PI, prevention of initiation; PP, prevention of promotion; PCR, polymerase chain reaction; SD, standard deviation; SOD, superoxide dismutase; TPA, 12-O-tetradecanoylphorbol-13-acetate.

DMBA can enhance the following behavior to make mice sensitive to such a carcinogen (Fig. 4B). Further experiments were performed to determine whether ginseng was involved in the development of DMBA-specific CTLs. DMBA-specific cytotoxicity was intensified by 37% in the animals pretreated with ginseng compared with the controls (Fig. 4C and D). These results suggest that ginseng prevents DMBA-initiated skin cancer through enhancing host T-cell immune response to this carcinogen and thereby eliminating the mutant preneoplastic cells.

Recently, it has been described that HSP27 and HSP70 can greatly promote cell-mediated immune response to DMBA, which predominantly prevents skin cancer initiated by DMBA and promoted by TPA [29]. Here, we tested whether HSP27 and HSP70 are involved in the preventive effect of ginseng on DMBA-induced initiation. The results showed that ginseng was able to remarkably upregulate the protein and mRNA levels of HSP27, but not HSP70 (Fig. 4E and F). Besides, HSP27 antibody potently reversed the enhancing effect of ginseng on the contact hypersensitivity to DMBA (Fig. 4G). Similarly, HSP27 antibody also interfered with the effect of ginseng on DMBA-specific tolerance (Fig. 4H). Preconditioning with HSP27 antibody reversed the preventive effects of ginseng on the incidence, multiplicity, and size of skin cancer induced by DMBA/TPA, considerably in the initiation phase rather than in the promotion phase (Fig. 4I−K). Thus, the results suggest that ginseng hinders DMBA-induced initiation of skin cancer by strengthening cell-mediated immune response through upregulating HSP27 expression.

3.6. Ginseng inhibits the promotion of skin cancer by maintaining cellular redox homeostasis through promoting nuclear translocation of Nrf2

TPA, a well-known tumor promoter, can accelerate the malignant transformation of preneoplastic cells induced by DMBA by causing redox imbalance [30]. Next, we investigated whether treatment with ginseng can reverse this stress. As shown in Fig. 5A−C, ginseng treatment in the promotion phase (PP ginseng) significantly inhibited ROS accumulation, as well as oxidative damage to DNA (8-OHdG) and lipid (4-HNE), compared with the model group or the PI ginseng group. In addition, ginseng treatment rebalanced the reduced glutathione/oxidized glutathione (GSH/GSSG) ratio in the PP or PA group, which partly represented the cellular redox condition (Fig. 5D). SOD, CAT, GPx, HO-1, and some other antioxidant enzyme systems are related to cell redox homeostasis. Therefore, we suspected that ginseng treatment maintains cellular redox homeostasis also partly by preventing TPA from reducing the activities of these enzymes. As predicted, ginseng treatment indeed rescued the TPA-downregulated activities of these enzymes in the PP or PA group (Fig. 5E). Moreover, ginseng treatment also reversed the TPA-downregulated mRNA level exhibited by antioxidant enzymes and subunits such as glutamate−cysteine ligase catalytic subunit, glutamate−cysteine ligase modifier subunit, SOD, CAT, GPx, and glutathione reductase (Fig. 5F). The results suggest a close relationship between the preventive effect of ginseng on promotion of skin cancer and its impact on the maintenance of cellular redox homeostasis by regulating the antioxidant system.

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) acts as a transcription factor that regulates the expression of various antioxidant enzymes and can well control the redox status. In our enrichment analysis, Nrf2 was found to be a major signaling molecule involved in prevention of promotion of skin cancer by ginseng (Fig. 3C). Therefore, whether Nrf2 signaling plays a role in the preventive effect of ginseng was further determined. Fig. 5G shows that ginseng treatment exhibits an obvious induction effect on the accumulation of Nrf2 in the epidermis in the PP and PA groups. In line with this, ginseng treatment completely rescued DMBA/TPA-induced downregulation of HO-1 and NAD(P)H dehydrogenase, quinone 1 (NQO1), which are two specific target genes of Nrf2 in the PP and PA groups (Fig. 5H). Generally, Nrf2 is a redox-sensitive transcription factor that is the primary cellular defense against the cytotoxic effects of oxidative stress and is the most prominent pathway contributing to the upregulation of such antioxidant enzymes. Therefore, we present the protein expression of other antioxidant enzymes such as SOD1, CAT, and GPX1. As predicted, ginseng treatment also reversed the TPA-downregulated protein expression level of these antioxidant enzymes (Fig. 5I). To explore whether Nrf2 dominantly participates in the preventive effect of ginseng against the promotion of skin cancer, the Nrf2$^{-/-}$ mice were used. Interestingly, the preventive effects of ginseng on the incidence, multiplicity, and size of skin cancer induced by initiation of DMBA and promotion of TPA were reversed considerably in the PP group and partially in the PA group of the Nrf2$^{-/-}$ mice (Fig. 5J−L *versus* Fig. 1D−E). However, knockout of Nrf2 did not obviously affect the preventive effect of ginseng treatment in the initiation phase (Fig. 5J−L *versus* Fig. 1D−E). All these prove that ginseng treatment suppresses the promotion of skin cancer by maintaining the cellular redox homeostasis through upregulating Nrf2.

4. Discussion

Skin cancer is a result of the abnormal development of skin cells, which originated from the epidermis. Apart from hereditary defects (mutations of genes, e.g., TP53, EGFR, CDKN2A, and RAS), exposure to ultraviolet radiation and carcinogens (e.g., arsenic and benzo[a] pyrene) may cause DNA damage, leading to skin cancer [38,39]. It is estimated that the number of Americans who suffer skin cancer is nearly 3.5 million each year, and 13,460 deaths will be attributed to skin cancers (including melanoma and nonmelanoma) in 2018 (source: https://www.aad.org/media/stats/conditions/skin-cancer). Thus, development of a novel and effective chemopreventive intervention against skin cancer would have a great impact on the public health care. In this study, we present evidence that Radix et Rhizoma Ginseng (ginseng) is a potential chemopreventive agent for cutaneous carcinoma in a DMBA/TPA-induced mouse skin cancer model.

Generally, carcinogenesis involves a series of processes, such as the initiation stage, the promotion stage, and the progression stage [40]. For chemoprevention, chemical agents are used to inhibit, to reverse, and/or to retard the pathological processes of carcinogenesis [41]. In general, it is most effective to intervene with the promotion stage of cancer because initiation is an irreversible stage [42]. According to the study results, ginseng remarkably lowered papilloma incidence, multiplicity, and size by suppressing not only promotion but also initiation in the DMBA/TPA-induced skin cancer model. Thus, ginseng may be an effective chemopreventive agent for skin cancer.

Among the complex constituents of ginseng, the ginsenoside is characterized by its pharmacologic and biochemical function, particularly the chemopreventive function for different kinds of cancers. As modern technology develops, there are 150 kinds of ginsenosides; Rb1, Rb2, Rb3, Rc, Rd, Re, and Rg1 ginsenosides account for approximately 80% of naturally occurring constituents of ginseng [43]. For treatment of disease, the active ingredients of TCM need to be taken up by blood to reach the lesions. The ginsenosides Rb1, Rb2, Rc, and Rd were the most abundant in the blood and skin tissue samples of the mice administered orally with ginseng, as shown in the study. Thus, likely, Rb1, Rb2, Rc, and Rd ginsenosides are responsible for the chemopreventive effect of ginseng on cutaneous carcinoma.

The chemopreventive activity of ginseng has been described in many studies, most of which suggest that ginseng acts as a

chemopreventive agent by inhibiting cell proliferation and inducing apoptosis. Here, we found that ginseng neither inhibited cell proliferation nor induced apoptosis. In addition, AP-1 consists of homodimers or heterodimers among members of the Jun family and Fos family, can regulate cell cycle and apoptosis [44], and has also been implicated in the chemopreventive action of ginseng in other types of cancer [18,45]. Ginseng treatment failed to significantly reverse carcinogen-induced increase in c-Jun, c-Fos, and JunB. Hence, the mechanisms of ginseng's chemoprevention for skin cancer differ from those for other types of cancer.

As ginseng has complex active components and the human body has diverse modulated targets, it is not easy to directly unravel how ginseng prevents skin cancer in the two distinct phases (initiation and promotion). To clarify the preliminary mechanism without blind searching, we used a comprehensive method combining pharmacophore mapping, bioinformatics, and network analysis. To further annotate and to clarify the network, the candidate targets were functionally analyzed by using ClueGO to find out the action mechanisms of ginseng. According to the results, ginseng prevented skin cancer in the initiation phase possibly by regulating T-cell−mediated immune function and HSPs. On the other hand, the preventive effect of ginseng on the promotion phase of skin cancer may be associated with antioxidant responses and Nrf2.

In experimental validation, pretreatment with ginseng dramatically enhanced DMBA contact hypersensitivity and accelerated the development of DMBA-specific CTLs. Consistent with our enrichment analysis and literature investigation, ginseng prevented skin cancer in the initiation phase, highly depending on the levels of HSP27 and HSP70. By using transgenic mice, we also proved that ginseng exerted regulatory effects on redox status basically by accelerating the nuclear translocation of Nrf2. Accordingly, ginseng well inhibited skin cancer from the initiation to the promotion phase because of multitarget, multilink synergistic enhancing effects, being completely consistent with the action mode and theory of TCM.

The DMBA/TPA-induced skin cancer model is closely associated with potential clinical experiments through exposing patients who suffer precancerous lesions, thus exhibiting stronger clinical relevance. As far as we know, for the first time, the study shows that ginseng effectively prevented skin cancer in both initiation and promotion phases, which meanwhile cannot lead to weight loss or other obvious side effects. Hence, ginseng is a potential skin cancer chemopreventive agent, which deserves future clinical trials. Clearly, it would be interesting to further study (1) whether and how the trace metabolites produced by parent ginsenosides, after oral administration of ginseng water decoction, benefit the prevention of skin cancer; (2) how the ginsenosides (Rb1, Rb2, Rc, and Rd) regulate HSPs and Nrf2; and (3) whether combining the screened active compounds in a natural ratio can have a comparable preventive activity to that of ginseng water decoction.

Author contributions

Yin Lu and Wenxing Chen conceived the project and designed the research. Suyun Yu, Siliang Wang, Wei Wang, and Shuai Huang performed experiments. Suyun Yu, Siliang Wang, Zhonghong Wei, Yushi Ding, and Aiyun Wang analyzed the data. Suyun Yu, Siliang Wang, and Shile Huang wrote the manuscript. The final version of the manuscript has been read by the authors and acquired the approval of the authors.

Conflicts of interest

The authors declare that no conflicts of interest exist in relation to the article.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2019.05.004.

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