Chemoprevention of Lung squamous cell carcinoma by Ginseng

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Abstract

Ginseng has been used as a medicinal herb to maintain physical vitality for thousands of years, and it has also been shown to be a non-organ-specific cancer preventive agent by several epidemiological studies. However, the chemopreventive effects of Korea white ginseng (KWG) in lung squamous cell carcinoma (SCC) have not been tested. In this study, we investigated the chemopreventive activity of Korea white ginseng (KWG) in a mouse lung SCC model. N-nitroso-trischloroethylurea (NTCU) was used to induce lung tumors in female Swiss mice, and KWG was given orally. KWG significantly reduced the percentage of lung SCCs from 26.5% in the control group to 9.1% in the KWG group, meantime, increased percentage of normal bronchial and hyperplasia. KWG was also found to greatly reduce squamous cell lung tumor area from an average of 9.4% in control group to 1.5% in the KWG group. Treatment with KWG decreased Ki-67 staining, suggesting that the lung tumor inhibitory effects of KWG were partly through inhibition of proliferation. HPLC/MS identified 10 ginsenosides from KWG extracts, Rb1 and Rd being most abundant as detected in mouse blood and lung tissue. The tumor inhibitory effects of KWG are mediated by inhibition of AP-1, as demonstrated by in vitro study conducted on AP-1/ NFkB dependent mouse NSCLC cell lines. Western blotting of lung tissues also indicated that NTCU upregulated AP-1 through phosphorylation of JNK, which was down regulated by KWG in concurrence with its chemoprevention function. These results suggest that KWG could be a potential chemopreventive agent for lung SCC.

Keywords: ginseng, NTCU, lung, squamous cell carcinoma, mouse, chemoprevention

Introduction
Though great advances have been made in early diagnosis, discovery of chemotherapeutic agents, as well as in molecular oncology, many common forms of epithelial malignancy, especially carcinoma of the lung, remain difficult to cure (1). The five-year survival rate for lung cancer is still around 15%, and from the most recent estimates from the American Cancer Society, there will be about 226,160 new cases of lung cancer with an estimated 160,340 deaths in 2012. In this context, new approaches to the control of cancer are urgently needed. Chemoprevention, centered around the administration of natural or synthetic compounds to inhibit, delay, or reverse the process of carcinogenesis, could be an effective approach to reduce the risk of developing lung cancer, ultimately leading to a decrease in the incidence of lung cancer (2-4).

Over 80% of lung cancer is non-small cell lung carcinoma (NSCLC), with adenocarcinoma and squamous cell carcinoma representing the two major subtypes of NSCLC. The pathology and cause of these two lung cancer subtypes are greatly different; adenocarcinomas tend to occur in the non-smoking population, while squamous cell carcinomas tend to occur in smokers. Despite this fundamental difference, treatment has generally not been tailored to specific tumor subtypes until recently. Over the course of the past several decades, almost all chemopreventive research in lung cancer, especially on primary animal models, have been focused on lung adenoma or lung adenocarcinoma, with no appropriate squamous cell carcinoma mouse model available. Recently, our lab has developed a mouse lung SCC model based on a previous work from another group (5), which induced lung SCCs by skin painting mice with N-nitroso-tris-chloroethylurea. The induced mouse lung SCCs have similar histopathological features and keratin staining to human SCC, therefore providing a valuable preclinical model for lung SCC (6).
Ginseng has been used as medicine plant to maintain physical vitality and prolong life for thousands of years in Eastern Asian, especial in China and Korea. It appears in the pharmacopoeias of several countries including China, Japan, Germany, Austria, the United Kingdom, and France, and is often employed for cancer, diabetes mellitus, and cardiovascular concerns (7). Ginseng has been shown to be a non-organ-specific cancer preventive agent by several epidemiological studies, which involving large populations. These studies have demonstrated a positive association of ginseng intake with a decrease in the risk of several cancer types (8-10), such as head and neck, esophagus, stomach, lung, and ovary. Ginseng has also been demonstrated as a chemopreventive agent in many primary rodent tumor models. For example, in an azoxymethane (AOM) initiated mouse colon tumor model, dietary ginseng significantly inhibited colonic inflammation and tumorigenesis (11). Ginseng has also been reported to inhibit hepatoma (12), leukemia (13), skin tumors (14, 15), as well as multiple different carcinogen induced lung adenomas (16-18). However, the chemopreventive effect of ginseng on lung squamous cell carcinoma has never been explored. In the present study, we investigated ginseng’s effect on a NTCU-induced mouse lung SCCs model, identifying several functional ginsenosides, while also exploring a basic mechanism that may underlie the efficacy of ginsenosides as chemopreventive agents.
Materials and Methods

Reagents and animals

N-nitroso-trischloroethylurea (NTCU) was purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). Acetone was purchased from Sigma (St. Louis, MO). Korea white ginseng powder (KWG) was purchased from the Korea Cancer Center Hospital, where it was prepared by peeling, drying and powdering of 4-6 years old ginseng (10).

Mouse lung SCC model induced by NTCU were established as previously reported (6, 19, 20). All studies on animals were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. Female NIH Swiss mice were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed with wood chip bedding in environmentally controlled, clean-air rooms with a 12-hour light–dark cycle and a 50% relative humidity. Drinking water and diet were supplied ad libitum. NIH Swiss mice were randomized into two groups with twenty five mice per group. All mice were treated topically with 0.03 M NTCU in 100-microliter drop, twice a week, with a 3.5-d interval for 28 weeks. Two weeks after the start of NTCU treatment, mice in the control group were given water and mice in test groups were administered KWG in drinking water at concentration of 10 g/L for the duration of the studies. During the studies, the health condition of the mice was monitored daily and body weights were measured weekly (Fig. 1A). Twenty-eight weeks after the initial treatment of NTCU, mice were terminated by CO2 asphyxiation. Lungs were fixed in Tellyesniczky's solution overnight and stored in 70% ethanol for histopathologic evaluation. Unlike lung adenomas/adenocarcinomas, SCC does not form visible solid nodules on the surface of the lung. Serial tissue sections (5-μm each) were made from formalin-fixed lungs, and one in every 20 sections (approximately 100 μm apart) was stained with H&E and examined histologically under a light
microscope to assess severity of tumor development (invasive SCC, SCC in situ, bronchial hyperplasia, metaplasia) as we reported previously (21). All of cross-sectional cuts of bronchiole were counted on all of the slides. The lesions, including invasive SCC, SCC in situ, and the bronchial hyperplasia/metaplasia, were scored. The criteria for histopathologic examination and scoring were described previously (21).

**Histopathology Analysis**

The lesions, including invasive SCC, SCC in situ, and the bronchial hyperplasia/metaplasia, were scored from the H&E-stained sections of each lung by following the guidelines as described below. When hyperplasia occurs (Fig. 1B), a single layer of bronchiolar epithelial cells becomes multiple layers. The cells maintain their normal morphology. When bronchiolar metaplasia occurs (Fig. 1B), the normal columnar epithelium is replaced by flattened squamous epithelium with increased keratin production. When SCC in situ occurs, atypical cells (such as irregular shape, increased nucleus/cytoplasm ratio) with visible mitosis and loss of orderly differentiation replace the entire thickness of the epithelium, although the bronchiole basement membrane remains intact, with no tumor cell invasion into the surrounding stroma. When invasive SCC occurs, general features of SCC such as keratin pearls, multiple nuclei, and increasing mitotic index can be seen. The normal architecture of the lung is disrupted. The lung SCCs area/lung lube area ratio was evaluated using NanoZoomer Digital Pathology Virtual Slide Viewer software (Hamamatsu Photonic Co.). H&E-stained slides were scanned with the NanoZoomer HT slide scanner (Hamamatsu Photonics France SARL) and virtual slides analyzed and quantified.
Immunohistochemical study

Lung tissues, which were fixed in Tellyesniczky’s solution overnight and stored in 70% ethanol, were cut (5 μm each) for future immunohistochemical analysis. All slides (one per mouse) were deparaffinized in xylene and rehydrated in gradient ethanol. Microwave antigen retrieval was carried out for 20 min in citrate buffer (pH 6.0). After blocking in 10% normal goat serum in phosphate buffered saline (PBS), primary antibody was diluted in 10% normal goat serum and incubated at 4°C overnight. Cell proliferation was assessed using primary monoclonal antibody against Ki-67 (1:400 dilution; Labvision Corp., Fremont, CA). Cells undergoing apoptotic changes were assessed using primary monoclonal antibody against cleaved-caspase 3 (Biocare, Cambridge, MA). Negative control slides were processed at the same time. Manual counting of labeled and total cells in high-powered (×400) fields of tumor tissue was conducted.

HPLC-MS instrumentation and chromatographic conditions

The concentrations of the ginsenosides in the KWG powder extracts, mouse lungs and blood were determined by liquid chromatography-electrospray ionization-mass spectrometry (Agilent 6460 triple quad LC/MS). The samples were separated on a Jupiter 300 C18 column, 250 x 2.0mm, 5μm (Phenomenex, Torrance, CA) using a mobile phase of (A) water containing 0.3 % formic acid and (B) acetonitrile containing 5% methanol and 0.3% formic acid. The mobile phase gradient increased from 20% (B) to 40% (B) in 30 min, increased to 50% (B) in 10 min, increased to 80% (B) in 10 min and then increased to 100% (B) in 5 min. The flow rate was 200 μl/min. The retention times were Re = 12.4 min, F3 = 25.7 min, Rb1 = 25.8 min, Rg2 = 26.2 min, Rc = 27.0 min, Rb2 = 28.6 min, Rb3 = 29.1 min, F1 = 29.2 min, Rd = 31.5 min, F2 = 40.5 min and CK = 49.4 min. The detection was made in the positive mode. For quantization,
m/z 969, 793, 1131, 807, 1101, 1101, 661, 969, 807 and 645 were used for Re, F3, Rb1, Rg2, Rc, Rb2, Rb3, F1, Rd, F2, and CK, respectively. The concentrations were calculated by comparing the ratio of peak areas to the standard curve.

Sample preparation

The internal standard solution (Rg2) was added to the samples, and they were processed to remove proteins and other related substances. One volume of 200 µl of methanol was added into a 20 µl aliquot of mouse plasma sample. The sample mixtures were vortexed for approximately 30 s, and precipitates were removed by centrifugation at 15,000 rpm for 15 min. The supernatant was transferred into a clean glass vial and evaporated to dryness with nitrogen. The residue was reconstituted with 30 µl of 100% methanol (v/v), and a 5 µl aliquot of the resulting solution was injected into the LC/MS system for analysis.

Cell lines

Human NSCLC cell lines H226 and H520 were purchased in the last 2 years from the American Type Culture Collection, where they are regularly authenticated. Mouse NSCLC line LM2 is a metastatic line established from urethane-induced lung tumors in the A/J strain; the SPON10 cell line was derived from spontaneous lung tumors in the A/J mouse (22). No authentication was done by the authors on both SPON 10 and LM2 cells. All cells were stored in liquid nitrogen and used within 6 months after thawing. H226 and H520 were maintained in RPMI-1640 medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin (P/S), LM2 was grown in MEMα medium (Gibco) with 10% FBS and 1% P/S, and the SPON10 cells were maintained in RPMI-1640 medium (Gibco) supplemented with 2% FBS and 1% P/S.
Inducible dominant negative cell lines, DOX-inducible Tam67 or IκBα cells were generated using the Retro-X Tet-On Advanced Inducible Expression System (Clontech). pRetroX-Tet-On-Advanced, pRetroX-Tight-Pur-Luc Control Vector, pRetroX-Tight-Pur-TAM67 or pRetroX-Tight-Pur- IκBα were transfected into GP2-293T packaging cells (Clontech) using lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. Mouse NSCLC lines LM2 and SPON10 were first infected with pRetroX-Tet-On-Advanced virus with polybrene (Sigma-Aldrich). After G418 selection, individual colonies were expanded and screened with pRetroX-Tight-Pur-Luc Control Vector. High-inducible clones were then subsequently infected with pRetroX-Tight-Pur-TAM67 or pRetroX-Tight-Pur- IκBα. 48 h after virus infection, puromycin was added to a final concentration of 1 µg/ml. Individual colonies were expanded and screened for the expression of TAM67 or IκBα by induction with doxycycline and Western blotting with anti-c-JUN (C-terminal, Santa Cruz) or anti-IκBα. The resulting cell lines, which inducible expressed TAM67 or IκBα were maintained in medium supplemented with G418 and puromycin.

 Constitutive AP-1 active cell line SPON 10-JNKK2 was generated by transduction SPON 10 cells with the virus particale produced in pWZL-MAP2K7 (Addgene 20516) (23) and pVSVG co-transfected GP2-293T cells. Constitutive NFkB active cell line LM2-IKKβ was generated by transfection of LM2 cells with pCR- IKKbeta (Addgene 15465) (24). Both cell lines were then selected with G418 for stable expression.

**Cell proliferation assay**

Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, according to standard protocols. Briefly, cells were
seeded onto 96-well tissue culture plates at 10,000 cells per well. Twenty-four hours after seeding, cells were exposed to various concentrations of Rb1, Rb2, Rc or Rd for 48 h for the H226 and H520 cells, or 1µg/ml of DOX and/or various concentrations of KWG for 48 h for the dominant negative SPON 10 and LM2 cells, or various concentrations of KWG for 48 h for the constitutive active SPON 10 and LM2 cells, while that of the control group was replaced with fresh medium. MTT (0.5 mg/ml) was added after the exposure period. The formazan crystals that formed were dissolved in DMSO after 4-hour incubation and the absorbance was measured at 490 nm by Infinite M200 Pro plate reader (Tecan, Durham, NC). All assays were performed in triplicate.

**Antibodies and Western Blotting**

Cell lysates were collected and lysed in M-PER (Pierce, Rockford, IL) with proteinase and phosphatase inhibitor cocktails (Pierce, Rockford, IL). Mouse lung tissue lysates were lysed in N-PER (Pierce, Rockford, IL) with proteinase and phosphatase inhibitor cocktails (Pierce, Rockford, IL). Lysates were separated by polyacrylamide gel electrophoresis, transferred to a PVDF membrane and blotted with primary antibodies against c-Fos (Cell signal), Jun B (Cell signal), c-Jun (Santa Cruz), ph-JNK (Cell signal), ph-p38 (Santa Cruz) and β-Actin (Santa Cruz). Signals were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Sci, IL).

**Statistical analysis**

Data are presented as mean ± standard error (SE). The data was analyzed by two-tailed Student t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Results

Inhibitory effect of KWG on lung tumorigenesis in NTCU-induced lung squamous cell carcinoma in Swiss mice

The chemopreventive activity of ginseng in other primary tumor models has been investigated, such as in B(a)P or urethane induced lung adenoma. However, the efficacy of ginseng on lung squamous cell carcinoma has never been explored. In this study we used a NTCU-induced lung SCC model, which has been used for years in our lab as a successful mouse lung SCC model, is organ specific and captures well-defined pathologic development from normal to bronchiolar hyperplasia, metaplasia, SCC \emph{in situ}, and finally, SCC as seen in human\cite{19, 21, 25}. During the study, KWG did not cause any visible sign of toxicity or ill health, nor have any significant effect on body weight in mice. NTCU treatment caused different kinds of lesions in mouse lungs as shown in Fig. 1B, normal (22.6%), hyperplasia (46.0%), metaplasia (4.9%), SCC \emph{in situ} (0.1%), and invasive SCC (26.5%). Drinking KWG significantly reduced the percentage of lung SCCs to 9.1%, while also increasing the percentage of normal bronchial architecture to 26.8% and hyperplasia to 60.3%. By calculating lung SCC area (as shown in Fig. 1C), KWG was found to significantly reduce lung SCC area from an average of 9.4% in control group to 1.5% in the treated mice. These results suggest that KWG effectively blocked the progression of hyperplasia to invasive SCC and could be a potential chemopreventive agent for lung SCC.

Ginsenosides of KWG were well separated and identified by HPLC-PDA/ESI-MS

To identify possible ginsenosides in KWG extracts that may be responsible for the chemopreventive effects, 17 major ginsenosides, F1, F2, F3, Rb1, Rb2, Rb3, Re, Rd, Re, Rg1,
Rg2, Rg3, Rh1, Rh2, Ginsenoside x, Ginsenoside R1 and compound K, were tested using HPLC/MS for the KWG extracts, as well as the treated mouse lungs and blood samples. Representative chromatogram is shown in Fig. 2A. By comparing with the MS data and HPLC retention time of the 17 standard ginsenosides, 10 ginsenosides were well separated and identified, including compound K, ginsenoside F1, F2, F3, Rc, Rd, Re, Rb1, Rb2, and Rb3. The ginsenosides were also quantified by HPLC/MS, as shown in Table.1, Rb1, Rb2, Rc and Rd were identified as the most abundant ginsenosides in the KWG extracts. To test whether these more abundant species are potentially responsible for the chemoprevention effect, two human NSCLC cell lines H226 and H520 were treated with different concentrations of Rb1, Rb2, Rc and Rd for 48 hours, all these four ginsenosides showed strong inhibition on cell survival at μM grade (Fig. 2B), suggesting possible chemopreventive effect of these ginsenosides for lung SCC.

KWG inhibits proliferation in vivo

To determine the extent of proliferation and apoptosis in lung SCC, immunohistochemical assays using —Ki-67 antibody for proliferative index and cleaved caspase-3 antibody for apoptotic index were done (Fig. 3) in paraffin embedded lung tissue slides from both control (only NTCU treated) and KWG (NTCU + KWG treated) groups. Staining for Ki-67 was present in 26% of tumor cells in the control group, which decreased significantly to 14% in KWG treatment group (Fig. 3A, B, and C). There was no significant change in the number of cleaved caspase-3–positive cells in the lungs receiving KWG compared with control mice (Fig. 3D, E, and F). These results indicate that treatment with KWG decreased the proliferative index.

Inhibition of AP-1 abrogates the chemoprevention effects of KWG in vitro

Many mechanisms have been reported to contribute to the chemopreventive activity of
ginseng, some reports also suggest that the chemopreventive effect of ginseng may be mediated through down-regulation of certain transcription factors, such as NF-κB \(^{(26)}\) and AP-1\(^{(27)}\). To identify the key pathway responsible for the chemopreventive function of KWG, we used two NSCLC cell lines for studying the role of AP-1 or NF-κB in survival; SPON10 stably expressing inducible TAM 67, the dominant negative of c-Jun, and LM2 expressing inducible IκB super suppressor (IκB SR), an IκBα mutant that is resistant to phosphorylation and degradation. In these cell lines, basal AP-1 or NFκB activity is high, once Tam67 or IκB super suppressor expression is induced by DOX, the transcription of AP-1 and NFκB respectively is significantly inhibited, which is also correlated with significantly reduced cell proliferation rate (unpublished data).

We treated SPON10-Tam67 and LM2-IκB SR cells with KWG. As shown in Fig. 4B, KWG significantly inhibited cell proliferation in LM2-IκB SR cells, regardless of NFκB activity(Fig. 4B, active-open bars; inactive-closed bars). In contrast, KWG only inhibited cell proliferation in SPON10-Tam67 cells when AP-1 was active (Fig. 4A, open bars), however, when AP-1 activity been inhibited by DOX induced TAM67 expression, KWG no longer suppressed proliferation (Fig. 4A, closed bars), indicating that KWG may depend on AP-1 to inhibit tumor cell growth \textit{in vitro}. To confirm the involvement of AP-1 in KWG’s chemoprevention action, we also created cell lines stably expressing constitutive activator of both pathways, overexpression of IKKβ for the activation of NFkB and overexpression of JNKK2 for AP-1. As shown in Fig. 4C, overexpression of JNKK2 in SPON 10 cells partially overrode the suppression effect of KWG, however, overexpression of IKKβ showed no difference on the inhibition effect of KWG (Fig. 4D). The effects of KWG treatment on the expression of AP-1 subunits as well as upstream activators were also been examined in the
SPON 10 cells, as shown in Fig. 4E, the level of c-Fos and c-Jun were dramatically decreased dose dependly after KWG treatment, and phosphorylated JNK and ph-p38 were also downregulated by KWG treatment, suggesting KWG may function through AP-1 pathway \textit{in vitro}.

\textbf{KWG downregulates AP-1 through JNK}

AP-1 is composed of either homo- or hetero-dimers between members of Jun and Fos families. Expression of AP-1 subunits is differentially regulated in response to various stimuli. To examine the effects of NTCU exposure as well as KWG treatment on the expression of AP-1 subunits, the nuclear protein levels of Jun and Fos family members were measured by Western blot analysis in mouse lung tissue from both control and KWG groups. Among all the family members, as shown in Fig. 5, the level of c-Fos, JunB, and c-Jun were dramatically increased in animals exposed to NTCU when compared with normal tissue, and it was significantly decreased in the animals treated with NTCU and subsequently exposed to KWG. Activation of AP-1 is triggered through distinct pathways in response to various stimuli. In order to better understand the underlying mechanism of KWG, AP-1 activation pathways were examined (Fig. 5 and data not shown). NTCU significantly upregulated phosphorylated JNK over normals, and KWG significantly downregulated it to the same level as in normals. In contrast, phosphorylated p38 was not significantly different from the normal group, indicating that NTCU’s carcinogenic effect may be mediated through upregulation of AP-1, leading to phosphorylation of JNK, while this is directly antagonized by KWG’s ability to downregulate JNK, possibly explaining KWG’s chemopreventive effects.
Discussion

Lung cancer has typically been classified into four major subtypes: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma, and the small cell carcinoma (28). Approximately, 25-30% of NSCLC lung cancers are SCC. Histological and cytological studies have revealed a series of changes that occur over many years and represent a morphological progression to bronchogenic carcinoma (21). Early changes include a basal cell hyperplasia followed by a squamous metaplasia, dysplasia, carcinoma in situ, and invasive SCC. There is strong evidence that tobacco smoke plays a major role in the pathogenesis of lung cancer, especially in lung SCC.

Historically, approaches to the treatment of NSCLC were determined solely by disease stage regardless of the histological subtypes. Fortunately, some progress has been made with a number of molecular therapies currently available or in development for the treatment of adenocarcinoma. One of the most prominent examples is EGFR tyrosine kinase inhibitors Gefitinib and Erlotinib, which is developed based on the frequent EGFR mutations found in lung adenocarcinoma patients and exhibit good efficacy upon treatment of adenocarcinoma but not in patients with squamous cell carcinoma where EGFR mutation rarely occurs. Therefore, it is critical to identify more appropriate therapeutic intervention for SCC. In the present study, Ginseng was found to remarkably decrease lung SCC percentage (65%) accompanied with an increased percentage of hyperplasia, suggesting an inhibition of progression of bronchial cell hyperplasia and squamous metaplasia to SCC lesions, thus, providing a rationale for its further development as a chemopreventive agent for lung cancer prevention (Fig. 1). At the same time, based on our immunohistochemistry study, we noted that decreased staining for Ki-67 assay but
not for cleaved caspase-3 in lung tumors in KWG-treated group (Fig. 3). Our data suggests that KWG’s inhibitory effect on proliferation within mouse lung tumors, which likely contributes to the observed chemopreventive effect.

Among the complex constituents of ginseng, ginsenosides have been found to be the major components responsible for its biochemical and pharmacological actions, especially in the chemoprevention of lung cancer (29-31). With the development of modern technology, more than 150 ginsenosides have been isolated (32), and Rb1, Rb2, Rb3, Rc, Rd, Re and Rg1 have been identified as the main naturally occurring constituents, representing 80% of ginsenosides (our unpublished review). In our current study, we found that Rb1 and Rd are the two most abundant ginsenosides present in the mouse after drinking KWG extracts (Fig. 2 and Table 1). This finding is in accord with a previous report that Rd and Rb1 have relatively longer half-lives (33). Rb2, Rb3, Rc and Re are all ginsenosides more commonly prevalent in white ginseng compared to red ginseng, and with high abundance in mountain ginseng (34) as well as in Chinese medicine Panax ginseng C. A. Meyer (35). Rd was previously found as a potential drug for cancer prevention due to its specific 26S proteasome inhibitory effect(36), and Rb2 was reported to be able to inhibit tumor angiogenesis and metastasis in a mouse melanoma model (37). Other than direct inhibition on various aspects of tumorigenesis of these ginsenosides, their metabolites also exhibit anti-tumor effect, such as the bacterial metabolit of Rb1 was found to be able to significantly inhibit lung carcinoma metastasis in vivo (38). Therefore, though we did not detect, but other metabolites of these ginsenosides could also contribute to this chemoprevention effect.

Multiple mechanisms have been implicated in the chemopreventive action of ginsenosides, including inhibition of NF-kB, AP-1, and induction of p53 and p21 suppressor gene. However,
the exact mechanisms, mediating ginseng’s chemopreventive function remain unclear. Our current study demonstrates that KWG functions as a chemopreventive agent through pathways involving AP-1. In the present study, we utilized both NF-kB, and AP-1 dependent cell lines to uncover the possible connections between these two pathways and KWG treatment, and found that KWG may partially dependent on AP-1 for its chemopreventive function (Fig. 4), possibly through inhibition of JNK phosphorylation (Fig 5).

The use of the lung tumor progression model is more clinically relevant because it closely parallels potential clinical trials by exposing individuals with established precancerous lesions. To our knowledge, this is the first time that KWG has been shown to be effective for lung cancer prevention in SCC models without causing weight loss or any other observable side effects. Therefore, our results suggest that KWG is a potential chemopreventive agent, which should be explored in future clinical trials.

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References


Figure legends

**Fig. 1:** Efficacy of KWG on development of lung SCC. **A**, Design of the experiment. Chemoprevention by KWG of NTCU-induced lung SCC in NIH Swiss mice is illustrated. Mice at age 6 to 8 weeks were treated topically with 0.03 mol/L NTCU (arrow) twice a week for 24 consecutive weeks. Two weeks after the start of NTCU treatment, mice were fed either regular water or water with 10 g/L KWG (arrowhead). Twenty-six weeks after the initial treatment of NTCU, mice were terminated by CO2 asphyxiation. Horizontal lines, the time, by weeks, that mice were treated with carcinogen-control (solid line), or carcinogen + KWG (shaded line). **B**, Efficacy of KWG on lung SCC development based on the percentage of different histopathology. As mouse SCC does not form visible solid nodules on the surface of the lung, serial tissue sections were made from each formalin-fixed lung and 1 in every 20 sections was stained with H&E. To assess specific effects of these agents on each histopathologic stage, all of the bronchial in each given slides were counted and grouped into 5 categories based on normal, hyperplasia, metaplasia, SCC in situ (dysplasia was included in this category), and invasive SCC. The number in each category was then converted into percentage. *, P < 0.05. Upper panel, histopathology of mouse normal bronchial, lung squamous hyperplasia, metaplasia, carcinomas in situ, and carcinoma is shown. Lower panel, efficacy of KWG on lung SCC development. **C**, Efficacy of KWG on lung SCC development based on the area of lung SCC. Top, typical area distribution of lung SCC in control group and KWG group. Bottom, percentage of the squamous lesions area.

**Fig. 2:** Pharmacokinetics of KWG. **A**, Typical chromatogram of Ginsenosides identified from KWG extracts. **B**, Quantification of ginsenosides identified from KWG extracts, mouse blood and lung samples. **C**, Growth inhibition of the most abundant ginsenosides on H226 and H520 cells. Cells were treated with different concentrations of Rb1, Rb2, Rc and Rd for 48 hours, cell proliferation rate was measured with MTT assay, and relative proliferation rate was shown as the percentage of control.

**Fig. 3:** Effect of KWG on Ki-67 and cleaved caspase-3 staining in NTCU-induced lung SCC model. Lungs harvested from mice on the 26 weeks in NTCU study (n≥5 mice/group) were fixed, paraffin embedded, and stained using specific antibodies as detailed in Materials and Methods. **A**, **B**, Representative picture from immunohistochemistry for Ki-67 (**A**, control group, **B**, KWG group). **C**, Proliferation index as determined by Ki-67. **D**, **E**, Representative picture from
immunohistochemistry for cleaved caspase-3 (D, control group, E, KWG group). F, Apoptosis index as determined by cleaved caspase-3. ***, P<0.001, Control group versus KWG group.

**Fig. 4:** Inhibition of AP-1 abrogates the chemoprevention effects of KWG in vitro. A. Relative cell proliferation rate of SPON 10-Tam67 cells treated with KWG with or without Tam67 expression (DOX). B. Relative cell proliferation rate of LM2-IkBα cells treated with KWG with or without IkBα super suppressor expression (DOX). Open bars, without DOX treatment, AP-1/or NFκB is active. Closed Bars, with DOX treatment, AP-1/or NFκB is inactive. C. Relative cell proliferation rate of SPON 10 cells treated with KWG with or without JNK2 expression. D. Relative cell proliferation rate of LM2 cells treated with KWG with or without IKKβ expression. Open bars, control cells. Closed Bars, JNK2/or IKKβ overexpressed cells, AP-1/or NFκB is constitutive active. Cell proliferation rate was measured with MTT assay, and relative proliferation rate was shown as the percentage of control. E. KWG down-regulated AP-1 pathway *in vitro*. Left panel, western blot analysis of c-Fos, Jun B, c-Jun, ph-p38 and ph-JNK expression in SPON 10 cells. Right panel, quantification of western blot. Values are presented as mean ± SE. *P<0.05.

**Fig. 5:** KWG decreases c-Jun expression and ph-JNK pathway *in vivo*. Upper panel, western blot analysis of c-Fos, Jun B, c-Jun, ph-p38 and ph-JNK expression in mouse lungs. Lanes 1-3, normal control mice; lanes 4-8, NTCU mice; lanes 9-12, NTCU mice treated with KWG. Lower panel, quantification of western blot. Values are presented as mean ± SE. *P<0.05.
A. Experiment design for screening chemoprevention agent in SCC mouse model
Fig. 2A

KWG – total ion chromatogram

Compound K

F1

F3

F2

Rd

Re

Rc

Rb2

Rb3

Rb1
Fig. 2B

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Author manuscripts have been peer-reviewed and accepted for publication but have not yet been edited.
Fig. 3

A B

C

Control KWG

D E

F

Control KWG

Proliferation Index (%)

Apoptotic Index (%)

0 10 20 30 40

0 1 2

***
Fig. 4

A  

SPON10-Tam67

\[
\begin{align*}
\text{KWG concentration (µg/ml)} & = 0, 300, 400, 500 \\
\text{Cell Proliferation Rate} (%) & = 0, 20, 40, 60, 80, 100
\end{align*}
\]

B  

LM2-IKBα

\[
\begin{align*}
\text{KWG concentration (µg/ml)} & = 0, 700, 800, 900 \\
\text{Cell Proliferation Rate} (%) & = 0, 20, 40, 60, 80, 100
\end{align*}
\]

C  

SPON10-Vector  
SPON10-JNKK2

\[
\begin{align*}
\text{KWG concentration (µg/ml)} & = 0, 300, 400, 500 \\
\text{Cell Proliferation Rate} (%) & = 0, 20, 40, 60, 80, 100
\end{align*}
\]

D  

LM2-vector  
LM2-IKKβ

\[
\begin{align*}
\text{KWG concentration (µg/ml)} & = 0, 300, 400, 500 \\
\text{Cell Proliferation Rate} (%) & = 0, 20, 40, 60, 80, 100
\end{align*}
\]

E  

KWG  

C-Fos  
JunB  
c-Jun  
Ph-p38  
Ph-JNK  
β-Actin

\[
\begin{align*}
\text{Relative expression} & = 0, 0.5, 1, 1.5, 2, 2.5, 3 \\
\end{align*}
\]
Fig. 5

![Diagram showing protein expression levels in normal, SCCs, and SCCs + KWG conditions.](image)

- **C-Fos**: The expression levels of C-Fos are shown for each condition.
- **JunB**: Similar expression pattern as C-Fos.
- **c-Jun**: Expression levels are indicated for each condition.
- **Ph-p38**: Phosphorylated p38 expression levels are displayed.
- **Ph-JNK**: Phosphorylated JNK expression levels.
- **β-Actin**: Loading control for all samples.

**Graphical Representation**

A bar graph showing relative expression levels for each protein under different conditions:

- **c-Fos**
- **JunB**
- **c-Jun**
- **Ph-p38**
- **Ph-JNK**

Each bar is labeled with significance markers (*), indicating the statistical significance of the differences in expression levels.
Chemoprevention of Lung Squamous Cell Carcinoma by Ginseng

Jing Pan, Qi Zhang, Kezhen Li, et al.

Cancer Prev Res Published OnlineFirst April 2, 2013.

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