Chemoprevention of Lung Squamous Cell Carcinoma by Ginseng

Jing Pan1, Qi Zhang1, Kezhen Li1, Qian Liu1, Yian Wang2, and Ming You1

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 nonorgan-specific cancer preventive agent by several epidemiologic 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 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 and in the meantime, increased the 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. High-performance liquid chromatography/mass spectrometry identified 10 ginsenosides from KWG extracts, Rb1 and Rd being the most abundant as detected in mouse blood and lung tissue. The tumor inhibitory effects of KWG are mediated by inhibition of activator protein (AP-1), as showed by in vitro study conducted on AP-1/NF-kB–dependent mouse non–small cell lung carcinoma cell lines. Western blotting of lung tissues also indicated that NTCU upregulated AP-1 through phosphorylation of c-jun-NH2-kinase, which was downregulated by KWG in concurrence with its chemoprevention function. These results suggest that KWG could be a potential chemopreventive agent for lung SCC. Cancer Prev Res; 6(6); 530–9. ©2013 AACR.

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 5-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).

More than 80% of lung cancers are non–small cell lung carcinoma (NSCLC), with adenocarcinoma and squamous cell carcinoma (SCC) representing the 2 major subtypes of NSCLC. The pathology and cause of these 2 lung cancer subtypes are greatly different; adenocarcinomas tend to occur in the nonsmoking population, whereas SCCs 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, has been focused on lung adenoma or lung adenocarcinoma, with no appropriate SCC 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 histopathologic 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 used for cancer, diabetes mellitus, and
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cardiovascular concerns (7). Ginseng has been shown to be a nonorgan-specific cancer preventive agent by several epidemiologic studies involving large populations. These studies have shown 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 shown as a chemopreventive agent in many primary rodent tumor models. For example, in an azoxymethane-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 SCC has never been explored. In the present study, we investigated ginseng’s effect on a N-nitroso-trichloroethyleurea (NTCU)-induced mouse lung SCCs model, identifying several functional ginsenosides, whereas also exploring a basic mechanism that may underlie the efficacy of ginsenosides as chemopreventive agents.

Materials and Methods

Reagents and animals

NTCU was purchased from Toronto Research Chemicals, Inc. Acetone was purchased from Sigma. Korean white ginseng powder (KWG) was purchased from the Korea Cancer Center Hospital (Seoul, Korea), where it was prepared by peeling, drying, and powdering of 4- to 6-year-old ginseng (10). Ginsenoside standards were purchased from LKT Laboratories, Inc. (St. Paul, MN). Mouse lung SCC models 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 (Milwaukee, Wisconsin). Female NIH Swiss mice were obtained from Charles River Laboratories. 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 2 groups with 25 mice per group. All mice were treated topically with 0.03 mol/L NTCU in 100-microliter drop, twice a week, with a 3.5-day interval for 24 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-six 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.

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 bronchiolue basement membrane remains intact, with no tumor cell invasion into the surrounding stroma. When invasive SCC occurs, general features of SCC, such as keratin pearl, 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 calculated. The lung SCCs area/lung lube area ratio was 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).

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 minutes in citrate buffer (pH 6.0). After blocking in 10% normal goat serum in 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.). Cells undergoing apoptotic changes were assessed using primary monoclonal antibody against cleaved-caspase 3 (Biocare). 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 × 2.0 mm, 5 μm (Phenomenex) 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 minutes, increased to 50% (B) in 10 minutes, increased to 80% (B) in 10 minutes and then increased to 100% (B) in 5 minutes. The flow rate was 200 μL/min. The retention times were Re = 12.4 minutes, F3 = 25.7 minutes, Rb1 = 25.8 minutes, Rg2 = 26.2 minutes, Rc = 27.0 minutes, Rb2 = 28.6 minutes, Rb3 = 29.1 minutes, F1 = 29.2 minutes, Rd = 31.5 minutes, F2 = 40.5 minutes, and CK = 49.4 minutes. The detection was made in the positive mode. For quantization, m/z 969, 793, 1131, 807, 1101, 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 with 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 seconds and precipitates were removed by centrifugation at 15,000 rpm for 15 minutes. 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 liquid chromatography/mass spectrometry (LC/MS) system for analysis.

Cell lines

Human NSCLC cell lines H1226 and H1520 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; LM2 was grown in MEMa medium (Gibco) with 10% FBS and 1% penicillin/streptomycin, and the SPON10 cells were maintained in RPMI-1640 medium (Gibco) supplemented with 2% FBS and 1% P/S.

Inhibitory dominant-negative cell lines, Doxycycline (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. Forty-eight hours 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 DOX 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-JNK2 was generated by transduction SPON 10 cells with the virus particulate produced in pWZL-MAPK7 (Addgene 20516; ref. 23) and pVSVG cotransfected GP2-293T cells. Constitutive NF-κB active cell line LM2-IκKB was generated by transfection of LM2 cells with pCR- IKKbeta (Addgene 15465; ref. 24). Both cell lines were then selected with G418 for stable expression.

Cell proliferation assay

Cell proliferation was assessed using the 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, Rr, or Rd for 48 hours for the H226 and H520 cells, or 1 μg/mL of DOX and/or various concentrations of KWG for 48 hours for the dominant-negative SPON 10 and LM2 cells, or various concentrations of KWG for 48 hours for the constitutive active SPON 10 and LM2 cells, whereas 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 dimethyl sulfoxide after 4-hour incubation and the absorbance was measured at 490 nm by Infinite M200 Pro plate reader (Tecan). All assays were conducted in triplicate.

Antibodies and Western blotting

Cell lysates were collected and lysed in M-PER (Pierce) with protease and phosphatase inhibitor cocktails (Pierce). Mouse lung tissue lysates were lysed in N-PER (Pierce) with protease and phosphatase inhibitor cocktails (Pierce). Lysates were separated by polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride 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).

Statistical analysis

Data are presented as mean ± SE. The data was analyzed by 2-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 in situ, and finally, SCC as seen in human (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 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, Rc, Rd, Re, Rg1, Rg2, Rg3, Rh1, Rh2, Ginsenoside x, Ginsenoside R1, and compound K, were tested using high-performance liquid chromatography (HPLC/MS) for the KWG extracts, as well as the treated mouse lungs and blood samples. Representative chromatogram is shown in Fig. 2A. 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, Rh, 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, 2 human NSCLC cell lines H226 and H520 were treated with

<table>
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<tr>
<th>Groups</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>Rb1</th>
<th>Rb2</th>
<th>Rb3</th>
<th>Rc</th>
<th>Rd</th>
<th>Re</th>
<th>Compound K</th>
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<tr>
<td>KWG extracts (mg/g dry weight)</td>
<td>0.9</td>
<td>1.6</td>
<td>1.9</td>
<td>23.3</td>
<td>10.7</td>
<td>8.7</td>
<td>15.9</td>
<td>16.5</td>
<td>1.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Lung (µg/g)</td>
<td>0.8 ± 0.8</td>
<td>2.1 ± 0.3</td>
<td>1.1 ± 1.0</td>
<td>53.1 ± 13.7</td>
<td>18.3 ± 7.1</td>
<td>8.1 ± 6.3</td>
<td>43.1 ± 13.2</td>
<td>77.4 ± 22.8</td>
<td>14.2 ± 8.4</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Blood (µg/ml)</td>
<td>0.1 ± 0.03</td>
<td>2.3 ± 0.6</td>
<td>0.2 ± 0.1</td>
<td>36.0 ± 9.1</td>
<td>21.8 ± 5.5</td>
<td>15.8 ± 7.7</td>
<td>42.0 ± 9.3</td>
<td>53.6 ± 13.3</td>
<td>9.3 ± 8.0</td>
<td>0.02 ± 0.02</td>
</tr>
</tbody>
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Figure 2. Pharmacokinetics of KWG. A, typical chromatogram of ginsenosides identified from KWG extracts.
different concentrations of Rb1, Rb2, Rc, and Rd for 48 hours, all these 4 ginsenosides showed strong inhibition on cell survival at μmol/L 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–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–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 downregulation 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 2 NSCLC cell lines for studying the role of AP-1 or NF-κB in survival; SPON10 stably expressing inducible TAM67, 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 NF-κB activity is high, once Tam67 or IκB super suppressor expression is induced by DOX, the transcription of AP-1 and NF-κB 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 NF-κB 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 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 IκB for the activation of NF-κB 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 IκB 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 was dramatically decreased dose-dependently after KWG treatment, and phosphorylated JNK and ph-p38 were also downregulated by KWG treatment, suggesting KWG may function through AP-1 pathway in vitro.

Figure 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-IκBα cells treated with KWG with or without IκBα super suppressor expression (DOX). Open bars, without DOX treatment, AP-1/ or NF-κB is active. Closed Bars, with DOX treatment, AP-1/ or NF-κB is inactive. C, relative cell proliferation rate of SPON 10 cells treated with KWG with or without JNKK2 expression. D, relative cell proliferation rate of LM2 cells treated with KWG with or without IκB expression. Open bars, control cells. Closed Bars, JNKK2/ or IκBα overexpressed cells. AP-1/ or NF-κB is constitutive active. Cell proliferation rate was measured with MTT assay and relative proliferation rate was shown as the percentage of control. E, KWG downregulated AP-1 pathway in vitro. Left, Western blot analysis of c-Fos, Jun B, c-Jun, ph-p38, and ph-JNK expression in SPON 10 cells. Right, quantification of Western blot. Values are presented as mean ± SE. **, P < 0.05.
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 was 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. 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, whereas 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 4 major subtypes: SCC, adenocarcinoma, and large cell carcinoma, and the small cell carcinoma (28). Approximately, 25%–30% of NSCLC lung cancers are SCC. Histologic and cytologic studies have revealed a series of changes that occur over many years and represent a morphologic 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 histologic 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 EGF receptor (EGFR) tyrosine kinase inhibitors gefitinib and erlotinib, which is developed on the basis of frequent EGFR mutations found in patients with lung adenocarcinoma and exhibit good efficacy upon treatment of adenocarcinoma but not in patients with SCC 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 the decreased staining for Ki-67 assay but not for cleaved caspase-3 in lung tumors in KWG-treated group (Fig. 3). Our data suggest that KWG’s inhibition on proliferation within mouse lung tumors 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 pharmacologic 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 2 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 with red ginseng, and with high abundance in mountain ginseng (34) as well as in Chinese medicine Panax ginseng C. A. Meyer (35). Rd was reported to be a potential drug for cancer prevention due to its specific 26S proteasome inhibitory effect (36), and Rd and Rb2 were 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 antitumor effect, such as the bacterial metabolite 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-κB, AP-1, and induction of p53 and p21 suppressor gene. However, the exact mechanisms, mediating ginseng’s chemopreventive function remain unclear. Our current study shows that KWG functions as a chemopreventive agent through pathways involving AP-1. In the present study, we used both NF-κB and AP-1-dependent cell lines to uncover the possible connections between these 2 pathways and KWG treatment, and found that KWG may partially dependent on AP-1 for its chemopreventive function (Fig. 4), possibly through inhibition of INK 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J. Pan, M. You

Development of methodology: J. Pan, Q. Zhang, Y. Wang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Pan, Q. Zhang, K. Li

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Pan, Q. Liu

Writing, review, and/or revision of the manuscript: J. Pan, Q. Zhang, M. You

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Li, Q. Liu, Y. Wang

Study supervision: Y. Wang, M. You

Acknowledgments

The authors thank Dr. William T. Hahn for the pWZL-MAP2K7 donated to Addgene, Hiroyasu Nakano for the pCR-IKKB donated to Addgene, Taik-Koo Yun from Korea Cancer Center Hospital in South Korea for providing KWG.

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Received August 25, 2012; revised February 14, 2013; accepted February 16, 2013; published OnlineFirst April 2, 2013.

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