Chemopreventive effects of garlic and mugwort mixture extract on *Helicobacter pylori*-associated mouse gastric carcinogenesis

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Abstract: Garlic and mugwort have long been used in traditional medicine to prevent various diseases. Several *in vitro* studies have reported protective efficacies of garlic and mugwort in cases of gastric cancer. In the present study, we investigated the cancer preventive effects of garlic and mugwort mixture extract (GME) in a *Helicobacter (H.) pylori*-associated gastric carcinogenesis mouse model. To induce gastric cancer, C57BL/6 mice were treated with N-methyl-N-nitrosourea and *H. pylori*. Various concentrations of GME (0, 100, 500, and 1,000 ppm) were then fed to the mice for 38 weeks, after which the tumor tissues were examined for histopathology, mucin histochemistry and β-catenin. The incidence of gastric tumors was significantly lower in the highest dose GME-treated mice (46.7%) than control mice (85.7%) (*p* < 0.05). The multiplicity and size of tumors were also significantly reduced by GME feeding in a dose-dependent manner (*p* < 0.01). Furthermore, GME suppressed the *H. pylori*-associated chronic inflammation measured by histologic grading of *H. pylori* density, chronic gastritis, glandular atrophy and intestinal metaplasia in non-tumorous gastric mucosae. Our data suggest that GME suppresses gastric tumorigenesis via suppression of *H. pylori*-associated chronic inflammation.

Keywords: artemisia, garlic, gastric cancer, *Helicobacter pylori*, mouse

Introduction

Gastric cancer is the second most common cause of cancer-related mortality worldwide. Though the gastric cancer rates have been declining for the last 80 years, it remains still one of the most serious cancers in Korea [15].

Generally, the development of gastric cancer involves a multistep process from normal gastric mucosa to chronic gastritis, gastric atrophy, intestinal metaplasia (IM), dysplasia and neoplasia. Chronic gastritis leads to a loss of glandular lineages in the gastric fundus, especially acid-secreting parietal cells and pepsin-secreting chief cells. Parietal cell loss leads to a loss of glandular lineages in the gastric fundus, especially acid-secreting parietal cells and pepsin-secreting chief cells. Parietal cell loss leads to goblet cell IM and seems to be a prerequisite for the gastric tumorigenesis [10]. Parietal cells are responsible for the secretion of critical growth factors such as TGF-α and amphiregulin [1, 3]. Thus, the loss of parietal cells may eliminate important agents required for appropriate differentiation of gland lineages including mucous neck and chief cells, as well as increase the serum gastrin level as a result of hypochlorhydria [10]. Several studies revealed that the loss of parietal cells with rapid expansion of surface cell numbers (foveolar hyperplasia) and hypergastrinemia leads to mucous cell metaplasia [10, 24]. In this regard, IM has been accepted as the most prominent candidate for the origination of gastric cancer. To confirm IM, the morphologic characteristics by H&E stain with analysis of the types of mucin expressed is necessary [8]. Histochemically, normal gastric mucin is stained with magenta by periodic acid-Schiff (PAS). However, IM produces acidic mucin, of which stain is blue with alcian blue (AB) at pH 2.5. Therefore, AB/PAS staining can discriminate IM from normal gastric mucosa [8].

WHO/IARC classified *Helicobacter (H.) pylori* as a group 1 carcinogen. *H. pylori* causes chronic active gastritis and peptic ulcer disease, and its infection is linked to the gastric cancer development [4]. *H. pylori* directly contacts with gastric epithelial cells and stimulate to release various cytokines such as interleukin-1β (IL-1β), IL-2, IL-6, interferon-γ, and tumor necrosis factor-α [11]. These cytokines activate the pro-inflammatory COX-2, a key enzyme in the formation of inflammatory reaction [24].

In addition, *H. pylori* affects intracellular signal transduction in host cells, leading to the activation of transcriptional
factors. *H. pylori*-host interaction and disease progression depend on different bacterial factors such as VacA and CagA [25, 29]. These genes have effect of the increase phosphorylation of glycogen synthase kinase 3β (GSK3β) via the activation of phosphoinositide-3-kinase/Akt, with subsequent release of β-catenin from a GSK3β/β-catenin complex and its nuclear translocation, which leads to activation of various oncogenes, including c-myc, cyclin D1 and matrix metalloproteinase-3 which have been reported to be target molecules of the unphosphorylated β-catenin [28]. Consistent with these findings, altered expression and intracellular redistribution of β-catenin are important for gastric cancer development as well [6]. Thus, nuclear expression of β-catenin appears to have prognostic value in the evaluation of gastric tumors [20].

Garlic has been intensively studied for the past few decades for their ability to impart beneficial effects on several human diseases including antimicrobial [5, 12], anti-inflammatory and various antitumor effects on hepatic cancer [30], and colorectal cancer [2, 19]. *Artemisia montana* is a kind of mugwort belonging to the family Asteraceae, which has 500 or more species of Artemisia in the world. In Korea, it has traditionally been used for human health possibly as home remedies. Some *Artemisia* spp. have been reported to have some beneficial effects such as anti-inflammation, anti-microbial, anti-cancer, and anti-oxidant activities [7, 13, 18]. Some components such as terpenoids, flavonoids, coumarins, glycosides, sterols and polyacetylenes were known to be responsible for those effects [27].

In this study, therefore, we investigated the chemopreventive effects of garlic and mugwort (*Artemisia montana*) extract on *H. pylori*-associated gastric cancer in a mouse model.

**Materials and Methods**

**Animals**

Specific pathogen-free, 6-week-old male C57BL/6 mice were obtained from Japan SLC (Shizuoka, Japan). All animal experiments were performed in accordance with Standard Operation Procedures of Laboratory Animals that were approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Research Center of Chungbuk National University, Cheongju, Korea. All mice were given a Teklad Global 18% protein rodent diet (Harlan Laboratories, USA) *ad libitum* and were maintained under specific pathogen-free conditions.

**Reagents and *H. pylori* culture**

*N*-methyl-*N*-nitrosourea (MNU) was purchased from Sigma-Aldrich (USA) and a solution was prepared by dissolving MNU in distilled water at a concentration of 200 ppm. Mouse-adapted *H. pylori* (Sydney strain 1, SS1) was inoculated on Brucella agar (Becton and Dickinson company, USA) containing 10% heat-inactivated fetal bovine serum and Skirrow medium (Difco, USA). They were kept at 37°C under micro-aerobic conditions using GasPak jar and CampyPak (Becton and Dickinson company).

**Preparation of garlic and mugwort extract**

The garlic and *Artemisia montana* were purchased from Kyungdong Market in Seoul. Voucher specimens are deposited at College of Agriculture, Life and Environmental Sciences, Chungbuk National University, Cheongju, Korea. Fresh garlic (20 g) and dried leaves of *Artemisia montana* (40 g) were mixed with 800 mL of 70% ethanol and extracted three times with 30 min ultrasonication. The extracts were then filtered through Whatman No. 2 paper to remove the debris. The filtrates were concentrated using rotary evaporation (Rotovapor R-114, BÜCHI Labortechnik, USA) and then freeze-dried. The yield of the ethanolic extract was measured to be 22.14% of the raw material. For solvent fractionation, it was suspended in water, and then extracted successively with equal volumes of *n*-hexane, chloroform, and ethylacetate, leaving residual aqueous fraction. Each fraction was evaporated in vacuo to yield the residues of *n*-hexane (4.39%), chloroform (6.52%), ethylacetate (3.94%), aqueous (85.15%) fractions, respectively. The chloroform fraction of the ethanol extract was used for the experiment.

**Mouse gastric cancer model**

The gastric cancers were induced by co-treatment of MNU and *H. pylori* [21]. The experimental protocol was illustrated in Fig. 1. All mice were given *N*-methyl-*N*-nitrosourea (MNU) that was mixed in their drinking water at concentration of 200 ppm for a total five cycles one-week regimens with a one-week pause. After completion of the MNU administration, they were further inoculated with *Helicobacter pylori* three times every other day. Animals of groups 1, 2, 3, 4 were then given a basal diet containing garlic and mugwort mixture extract (GME) 0 ppm, 100 ppm, 500 ppm, 1,000 ppm, respectively. Mice were sacrificed after 38 week GME treatment.
in Fig. 1. Briefly, 80 mice were randomized into 4 groups, which were positive control group (group 1) and GME administration groups (group 2, group 3, group 4). The total mice were given the solution of MNU (200 ppm) ad libitum in place of drinking water every other week for 10 weeks. One week after the completion of the MNU administration, they were further inoculated with 0.1 mL of *H. pylori* suspension containing 1 × 10⁶ colony-forming units/mL by intragastric intubation three times every other day. Then, the mice were given a basal diet containing GME at the concentrations of 0 ppm (group 1), 100 ppm (group 2), 500 ppm (group 3), 1,000 ppm (group 4) throughout the experiment. All mice were sacrificed at 50th week from the start of the experiment.

**Histological analysis**

At sacrifices, the stomachs were inflated with buffered formalin and opened along the greater curvature. The numbers of neoplastic nodules were counted under a stereoscopic microscope, and the sizes of the nodules were measured with a vernier-caliper. The excised stomachs were fixed in neutral buffered 10% formalin and processed by routine methods for paraffin embedment. The tissues were sectioned at a thickness of 4 µm and stained with hematoxylin and eosin (H&E). Histopathologic typing of gastric tumors was determined by Lauren’s classification [17].

**Histologic analysis of chronic inflammation**

To determine the effects of GME on chronic inflammation, histologic grades for chronic gastritis, glandular atrophy, IM and *H. pylori* density were examined. H&E and May Grünwald Giemsa stains were used for histopathology of chronic inflammation. Warthin-Starry silver stain and AB/PAS were performed for *H. pylori* colonization and the presence of IM, respectively. The histologic grades including *H. pylori* density were scored according to the histologic classification of the updated Sydney system [10] into four grades (0–3; 0, none; 1, mild; 2, moderate; 3, severe).

**Immunohistochemical staining**

Immunohistochemistry for β-catenin was performed with monoclonal antibody against β-catenin (clone 14, 1 : 200 dilution; BD Transduction Laboratories, USA) after antigen retrieval protocol [26]. The reactivity of β-catenin in nucleus was classified into negative, scattered, focal positive and diffuse positive as described by Kobayashi et al. [16]. The negative pattern was defined as localization limited to cytoplasmic membranes. The scattered pattern presented as nuclear staining less than 5% of the cells without any cluster. The focal positive pattern was defined as positive cells clustered in focal area and diffuse positive as positive cells distributed diffusely. Cytoplasmic immunoreactivity was not considered in the present study because this expression was variable and not clearly related to the shift from membranous to nuclear staining [22].

**Statistical analyses**

The data were analyzed with SAS software (ver. 9.1.2; SAS Institute, USA). Tests for statistical analyses were Fisher’s exact test for tumor incidence, malignancy and nuclear β-catenin expression data, Tukey t test after ANOVA for tumor multiplicity and size, Dunn’s multiple comparison test after Kruskal-Wallis’ nonparametric ANOVA for histologic scores of chronic inflammation. For all comparisons, p values < 0.05 were considered statistically significant.

**Results**

**Tumor incidence, multiplicity and sizes**

Table 1 summarizes the incidence, multiplicity and sizes of gastric tumors. Mice from group 1 showed 85.7% in the incidence, 7 in the multiplicity and 6.8 mm in the sizes of the gastric tumors induced by the MNU and *H. pylori* regimen. Treatment of GME 1,000 ppm (group 4) significantly impaired the incidence to 46.7% (p < 0.05). In tumor multiplicity, the treatment of GME 500 ppm and 1,000 ppm significantly decreased the number of tumors in tumor bearing mice (p < 0.05). In addition, tumor sizes significantly impaired in all GME treatment groups (p < 0.01) when compared to group 1.

**Histological classification of gastric tumors**

As shown in Table 2, the incidences of adenocarcinoma in the all GME treatment groups were significantly reduced when compared to group 1 (p < 0.01). Histological typing of adenocarcinoma was mainly intestinal type in group 1 (81.8%). Gastric adenocarcinoma showed an irregular glandular proliferation and stromal invasion of tumor cells (Fig. 2A). The hyperplastic irregular gland was composed of hyperchromatic atypical tumor cells showing loss of columnar orientation and cell stratification (Fig. 2B). In this lesion, there were increased intestinal type acidic mucins (blue) in

<table>
<thead>
<tr>
<th>Table 1: Tumor incidence, multiplicity and tumor size</th>
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<tbody>
<tr>
<td><strong>Groups</strong></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

*, **Significantly different from group 1 at p < 0.05 and p < 0.01, respectively. Data represent the mean ± SD.
Since GME treatment suppressed the development of gastric tumors, the gastric mucosae of non-neoplastic lesion were examined histologically to determine whether GME affected the development of precursor lesions of dysplasia (glandular atrophy, chronic inflammation and intestinal metaplasia). GME also significantly suppressed the histologic grades of those lesions in high dose groups determined by quantitative histopathological scoring ($p < 0.05$) (Table 3). The density of $H. pylori$ colonization was also significantly impaired by GME treatment (Table 3, Fig. 2D). Chronic gastritis was characterized by moderate-to-severe infiltration of lymphocytes, plasma cells and neutrophils (Fig. 2E) and the loss of parietal cells and chief cells, and replacement with proliferating epithelial cells (Fig. 2F). The histochemical pattern of IM was different between group 1 and GME treatment groups. In group 1, gastric mucosa was exhibited the change of mucin type to the acidic and mixed mucin stained by AB/PAS (Figs. 3C and D) and GME treated groups are tend to exhibit PAS positive (neu-

**Table 2. Incidences of gastric tumors according to histopathologic classification**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Adenoma (%)</th>
<th>Adenocarcinoma (%)</th>
<th>Adenocarcinoma subtype</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intestinal (%)</td>
</tr>
<tr>
<td>1</td>
<td>1/14 (7.1)</td>
<td>11/14 (78.6)</td>
<td>9/11 (81.8)</td>
</tr>
<tr>
<td>2</td>
<td>2/12 (16.7)</td>
<td>4/12 (30.8)**</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>3</td>
<td>3/12 (25.0)</td>
<td>3/12 (25.0)**</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>4</td>
<td>4/15 (26.7)</td>
<td>3/15 (20.0)**</td>
<td>3/3 (100)</td>
</tr>
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</table>

**Significantly different from positive control group (group 1) at $p < 0.01$.**

**Table 3. Scoring of histologic grade of gastritis according to the updated Sydney system**

<table>
<thead>
<tr>
<th>Groups</th>
<th>$H. pylori$ density</th>
<th>Chronic inflammation</th>
<th>Glandular atrophy</th>
<th>Intestinal metaplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.50 ± 0.65</td>
<td>2.43 ± 0.51</td>
<td>2.50 ± 0.52</td>
<td>2.29 ± 0.47</td>
</tr>
<tr>
<td>2</td>
<td>1.29 ± 0.76</td>
<td>2.14 ± 0.69</td>
<td>2.14 ± 0.69</td>
<td>1.74 ± 0.95</td>
</tr>
<tr>
<td>3</td>
<td>1.00 ± 0.81*</td>
<td>1.86 ± 0.76</td>
<td>1.83 ± 0.76</td>
<td>1.43 ± 0.53*</td>
</tr>
<tr>
<td>4</td>
<td>0.74 ± 0.76**</td>
<td>1.43 ± 0.41*</td>
<td>1.74 ± 0.41*</td>
<td>1.00 ± 0.63*</td>
</tr>
</tbody>
</table>

*, **Significantly different from positive control group (group 1) at $p < 0.05$ and $p < 0.01$, respectively. Data represent the mean ± SE.
Fig. 3. Mucin changes in non-neoplastic gastric lesions by AB/PAS staining. (A) Neutral mucin was predominant in normal gastric mucosa. (B) High magnification of rectangle in A. (C) A representative lesion of group 1. Note the reduced parietal cell zone and change of mucin type from neutral mucin to the acidic and mixed mucin. (D) High magnification of rectangle in A shows marked acidic mucin. (E) A representative lesion of group 4. The acidic mucin was almost disappeared by GME treatment. (F) High magnification of rectangle in C reveals almost normal condition in the gastric cell lineage. ×100 (A, C and E). ×200 (B, D and F).

Nuclear β-catenin expression

A variation of the nuclear staining was detected in the epithelial cells of the neoplastic mucosa (Table 4). The strong cytoplasmic and nuclear β-catenin immunoreactivity was observed in the epithelial cells of polyps (Fig. 4B) in group 1 (57.14%). On the other hand, following GME treatment, the expression of β-catenin was more tend to be observed in the cell-to-cell border (Fig. 4D) and then the number of cells with nuclear β-catenin expression was significantly lower in group 3 (16.66%) and group 4 (13.33%) compared with group 1.

Discussion

In the present study, we investigated the preventive effects of GME on gastric cancer with MNU-treated H. pylori-associated gastric cancer mouse model. The incidence of tumors, multiplicity and tumor sizes were lowered by GME treatment. Histological typing of adenocarcinomas was mostly intestinal type according to the Lauren’s classification.

The development of gastric carcinoma involves multiple processes from chronic gastritis to atrophy, intestinal metaplasia, dysplasia, and, finally, adenocarcinoma. Therefore, to examine the preventive effect of GME on gastric cancer, non-neoplastic lesions of the gastric mucosa were evaluated according to the Updated Sydney System to confirm the pre-neoplastic changes. As results, GME feeding significantly alleviated the severity of H. pylori colonization, chronic gastritis, glandular atrophy, and intestinal metaplasia. Extensive inflammation of the infected mucosa and submucosa by various immune cell populations observed in group 1 was significantly reduced by 1,000 ppm of GME treatment. Also

Table 4. The localization of β-catenin

<table>
<thead>
<tr>
<th>Groups</th>
<th>Nuclear β-catenin expression (%)</th>
<th>Patterns of nuclear β-catenin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative/scattered</td>
</tr>
<tr>
<td>1</td>
<td>8/14 (57.14)</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>6/12 (50.00)</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2/12 (16.66)*</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>2/15 (13.33)*</td>
<td>1</td>
</tr>
</tbody>
</table>

The nuclear pattern of β-catenin expression was subdivided into negative/scattered, no or few scattered positive cells; focal, positive cells clustered in focal area; diffuse, positive cells distributed diffusely. *Significantly different from positive control group (group 1) at \( p < 0.05 \).
severe atrophy characterized by the loss of the parietal cell and chief cell lineages was impaired by treatment of GME. In addition, alcian blue-positive metaplasia mostly found in group 1 was significantly reduced by 500 and 1,000 ppm of GME treatment.

The protective effect of GME against chronic inflammation of the gastric mucosa was paralleled by significant inhibition of gastric atrophy and IM. These data strongly support that GME inhibits gastric carcinogenesis by suppressing the Helicobacter pylori colonization, the attendant gastric atrophy and chronic inflammation. In line with this, GME reduces the risk of metaplastic change of the gastric mucosa. In addition, GME inhibited the nuclear translocation of β-catenin in adenocarcinomas. The intense β-catenin expression in nuclear and cytoplasm of epithelial cells in tumors of group 1 was significantly reduced in 500 and 1,000 ppm of GME treatment groups. β-catenin is known to have important roles related with cell adhesion. It has been documented that mutation of β-catenin closely associated with the development of gastric cancer occurs at a higher rate in intestinal type gastric cancer rather than the diffuse type [23]. Furthermore, recent studies demonstrated that β-catenin is selectively activated by Helicobacter pylori cytotoxin-associated gene (Cag)-A dependent manner [9] and abnormal expression of β-catenin is correlated with poor prognosis [14]. Thus, considering the changes of β-catenin expression, it is anticipated that GME reduces Helicobacter pylori-associated β-catenin regulation. Therefore, the suppression of gastric cancer in our study may be the result of suppressed gastric inflammation caused by Helicobacter pylori infection. This suggestion is consistent with the observed impaired oxyntic atrophy and metaplastic changes by GME.

Therefore, it can be postulated that regression of Helicobacter pylori activity by GME inhibits β-catenin nuclear translocation, leading to the suppression of malignancy. In conclusion, GME inhibited the development of gastric tumorigenesis via suppression of Helicobacter pylori infection-associated chronic inflammation.

GME treatment inhibited the gastric carcinogenesis in MNU and Helicobacter pylori-associated mouse gastric cancer model. In addition to the tumor incidence, multiplicity and sizes, GME treatment also suppressed Helicobacter pylori colonization and the development of preneoplastic lesions such as chronic gastritis, glandular atrophy and intestinal metaplasia. These results suggest that GME inhibited mouse gastric tumorigenesis via the suppression of Helicobacter pylori infection-associated chronic inflammation.

Acknowledgments

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References