Preventive Effect of Actinidia Valvata Dunn Extract on N-methyl-N’-nitro-N-nitrosoguanidine-induced Gastrointestinal Cancer in Rats

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Abstract

Purpose: This study was conducted to assess the preventive effect of Actinidia valvata Dunn (AVD) extract on an animal model of gastrointestinal carcinogenesis on the basis of changes in tumor incidence, cell proliferation, and apoptosis. Materials and Methods: Seventy-five male Wistar rats were divided into five different treatment groups with 15 rats in each group. Group I was given normal feed, whereas Groups II to IV were treated with 10% sodium chloride in the first six weeks and 100ug/mL of N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) in drinking water for 24 weeks. Group II was then given normal feed, whereas Group III was given AVD extract (0.24g/kg/day) for 12 weeks. Group IV was treated with AVD extract alone for 36 weeks. All rats were sacrificed at the end of the 36-week experiment and assessed for the presence of gastrointestinal tumors. The occurrence of cancer was evaluated by histology. Bax, Bcl-2, Caspase-3, and cyclinD1 were determined by immunohistochemical staining and Western blotting. Results: The incidences of gastric cancer were 0% in Group I, 73.3% in Group II, 33.3% in Group III, 26.7% in Group IV, and 0% in Group V. Bcl-2 and cyclinD1 expression was decreased in AVD extract treated groups, whereas Bax and Caspase-3 expression was increased. Comparison with group II revealed significant differences (p<0.01). Conclusions: AVD extract exhibits an obvious preventive effect on gastrointestinal carcinogenesis induced by MNNG in rats through the regulation of cell proliferation and apoptosis.

Keywords: Gastric cancer - chemoprevention - Actinidia valvata Dunn extract - MNNG
of cell lines, including lung, cervix, gastric, ovarian, and colon cells (Xu et al., 2009; Lee et al., 2010; Fujiwara et al., 2013; Nho et al., 2013; Sung et al., 2014). Extract from the root of AVD, also known as Maorenshen in traditional Chinese medicine, has long been used to treat lung carcinoma, hepatoma, and myeloma (Qu et al., 2012).

This study evaluates the use of AVD extract to prevent gastric cancer induced by N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) in rats. In addition, cell apoptosis and proliferation in the stomach epithelium is measured to understand the functional mechanism of AVD extract.

Materials and Methods

Animals

Seventy-five male Wistar rats aged four weeks to six weeks with body weight of ~50 g were obtained from the Laboratory Animal Center of Nanjing Medical University. Three to four rats were kept in each metal cage at 22±2°C temperature, 55%±5% relative humidity, and 12 h light-dark cycle. Rats were fed with regular chow pellets and water ad libitum. After a one-week acclimation period, the rats were used in the experiments. The study protocol was approved by the Animal Ethics Committee of Nanjing Medical University.

Chemicals

MNNG was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). AVDs (Anhui, China) were powdered and refluxed thrice with 80% alcohol for 1.5 h for each time, as described by Zheng et al. (2012) The extract solutions were evaporated to dryness under reduced pressure and crushed into brown powders. The yield of AVD alcohol extract was 9%. This process was supported by Jiangsu Institute of Material Medical.

Study design

Rats were classified into five groups with 15 rats in each group.

Group I: control group with distilled drinking water and given corresponding normal saline daily by oral gavage.

Groups II and III: treated with 0.1 g/L of MNNG with distilled water prepared thrice per week for 24 weeks according to the protocol described in previous reports (Hu et al., 2004; Okazaki et al., 2006; Tomita et al., 2008). The MNNG solution was protected from light and given ad libitum to rats through drinking water. At the first six weeks, 1 mL of 10% sodium chloride was given to rats by oral gavage to enhance gastric cancer development (Tatematsu et al., 1975). From the 25th week to the 36th week, animals in Group II were treated in a manner similar to those in Group I, whereas rats in Group III were given AVD extract (0.24 g/kg body weight daily, dissolved in distilled water by oral gavage). Drug doses between rats and humans were calculated on the basis of body surface area. The regular dosage of AVD for adults was 30 g/day according to the following formula: 30 * 0.018/0.2 = 2.7 g/kg; 2.7 * 9% = 0.24 g/kg/day.

Group IV: treated in a manner similar to Group II for 24 weeks, thereafter treated with AVD extract in a manner similar to group III from the first week to the 36th week.

Group V: control animals treated with AVD extract alone (similar to animals in Group IV) for 36 weeks.

During the study, general conditions were applied to all rats. The body weights were recorded weekly. Rats that died before the end of the experiment were autopsied to determine the cause of death and to assess the presence of gastric tumors. At the end of the 36-week experiment, all animals were sacrificed by cervical dislocation after overnight fasting. The stomach tissues were subdivided and underwent various processes for distribution to each experiment.

Histopathology

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, mounted on polylysine-coated slides, and stained with hematoxylin and eosin. Histological analysis was performed in duplicate by two different pathologists who were blinded to the study design. The defining characteristics for atypical hyperplasia and adenocarcinoma were adapted from the existing literature (Ikezaki et al., 1996).

Immunohistochemistry

Immunohistochemistry expressions of Bax, Bcl-2, cyclinD1, and Caspase-3 were analyzed by using the streptavidin-peroxidase method. After deparaffinization, 5 μm thick sections were rehydrated in graded alcohol and then washed with water. The slides were placed in a microwave and incubated in citrate buffer (pH 6.0). Antigen retrieval was performed by steam heating for 10 min. The sections were naturally cooled and rinsed with phosphate buffered saline (PBS, pH 7.4), after which they were treated with 3% H2O2 for 20 min to inhibit the endogenous peroxidase activity. After blocking with normal goat serum for 20 min for nonspecific antibody binding, the sections were treated overnight at 4°C in a humid chamber with primary antibodies for Bcl-2, Bax, Caspase-3, and cyclinD1, as well as rabbit polyclonal antibody (Santa Cruz, CA, USA). The slides were washed with PBS and then incubated with biotin-labeled goat secondary antibody (1:100 dilution). Then samples were incubated with peroxidase-conjugated streptavidin for 20 min at room temperature (RT). The reaction was developed by using 0.024% diaminobenzidine (Sigma, USA) solution and 0.16% hydrogen peroxide. Finally, the slides were dehydrated, cleared, and counter-stained with Harris’ hematoxylin. As a negative control, non-immunized rabbit serum was substituted for the primary antibody. The known positive gastric cancers were used as positive controls.

Image-Pro Plus 6.0 was used to analyze the immunohistochemical images. Accumulated integrated optical density was applied to determine the positive value of every image.

Western blot

Proteins were separated on 12% sodium dodecyl sulfate-polyacrylamide gels at 80 V. The gels were then transferred onto a poly-vinylidene difluoride membrane,
Table 1. Cause of Death and Gastric Cancer Incidence in Different Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Number (number)</th>
<th>Cause of death incidence (%)</th>
<th>Gastric cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>15</td>
<td>Lung congestion (1)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>II</td>
<td>15</td>
<td>Gastric cancer (5), esophageal cancer (1), Lung congestion (1)</td>
<td>11 (73.3%)</td>
</tr>
<tr>
<td>III</td>
<td>15</td>
<td>Gastric cancer (4), Lung congestion (1)</td>
<td>5 (33.3%)</td>
</tr>
<tr>
<td>IV</td>
<td>15</td>
<td>Gastric cancer (3), Small bowel cancer (1)</td>
<td>4 (26.7%)</td>
</tr>
<tr>
<td>V</td>
<td>15</td>
<td>0 (0)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

*p=0.000 (x2); p=0.028, III versus II; p=0.011, IV versus II; p=0.000, V versus II

Figure 1. Body Weight of Rats in Different Groups. the Body Weight of Group II Was Lower Than that of any Other Group at the End of the Experiments (*p<0.05). However, no significant difference in body weight was observed among Groups I and III to V (*p>0.05)

incubated in a blocking solution containing 5% milk proteins in Tris-buffered saline (TBST) with Tween 20 (TBST: 10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.6) for 2h at RT and then probed overnight at 4°C with primary antibodies (Bcl-2, Bax, cyclinD1, Santa Cruz, CA, USA). The blots were washed thrice with TBST and incubated for 2h at RT with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Beyotime Technology) and then again washed thrice in TBST. The membrane was incubated with ECL substrate solution for 5 min according to manufacturer’s instructions and visualized with autoradiography film. The imaging program Quantity One (Bio-Rad) was used for quantification. Human β-actin (cell signaling technology, MA, USA) was used as the control of protein integrity.

Statistical analysis

The data were presented as means and standard deviations from triplicate experiments performed in parallel, unless otherwise indicated. Data statistical analyses were performed with SPSS software (Version 13.0, SPSS Inc. NC, USA). Differences among groups were tested by one-way ANOVA. Groups were compared by using unpaired Student’s t test. A value of *p<0.05 was considered to be statistically significant.

Results

General observation

Seventeen deaths occurred during the study period one in Group I, seven in Group II, five in Group III, and four in Group IV. Three animals died of lung congestion. Twelve rats died of gastric cancer. One rat died of esophageal cancer and one of small bowel cancer. The causes of death are listed in Table 1.

Body weights of Group II were significantly lower than those of the other groups (*p<0.05). However, no significant difference was observed among other groups during the whole study period (*p>0.05) (Figure. 1).

Twenty animals developed gastric cancer at the end of this study: none in Group I, 11 in Group II, five in Group III, and four in Group IV (Table 1). All gastric tumors were histologically confirmed to be adenocarcinomas. Tumors usually occurred near the lesser curvature of the gastric antrum, which was similar to human gastric cancer.

Immunohistochemical and Western blot data for markers of apoptosis and proliferation in stomach tissue

Bax and Caspase-3 expressions were significantly lower, whereas Bcl-2 and cyclinD1 expressions were significantly higher in MNNG-treated rats (Group II) than those in other groups. Upon pre- and post-treatment with AVD extract (Groups III and IV), Bax and Caspase-3 increased, whereas Bcl-2 and cyclinD1 decreased with a significance of *p<0.05 in Group II. The administration of AVD extract alone did not affect the expression of the analyzed markers when compared with the control (Group I) (Figure. 2).

Discussion

Gastric cancer remains one of the most frequent gastrointestinal cancers. However, WHO recognized that most of cancers and deaths can be prevented. Traditional medicine and therapies can be considered in research on cancer prevention, early detection, and management strategies (World Health Organization, 200). AVD has
been widely used to treat cancers. However, recent reports on experimental data regarding specific agents that prevent or retard gastric carcinogenesis are limited.

In this study, the protective action of AVD extract against MNNG-induced gastric carcinogenesis has been discussed. With the use 0.24 g/kg/day of AVD extract, an approximately 40% reduction in tumor incidence was observed. However, no significant difference was observed between the pre-treatment group (Group IV) and the post-treatment group (Group III). To our knowledge, this study is the first to report an inhibitory effect of AVD extract on MNNG-induced gastric carcinogenesis.

Hyperproliferation and apoptosis evasion are interlinked relative to cancer cell survival. The potential mechanism of apoptosis avoidance for gastric carcinogenesis has been related to the increase in Bcl-2 to Bax ratio with decreased expression of Caspase-3 (Manikandan et al., 2008). According to a previous report, cyclinD1 expression is an early event in human gastric carcinogenesis and may serve as one of the early molecular markers for gastric cancers (Motohashi et al., 2011). In this study, high Bcl-2 and cyclinD1 expressions and low Bax and Caspase-3 expressions were observed in the pyloric gastric epithelium of the MNNG-treated group. AVD extract acted as a suppressing agent by inhibiting cell proliferation and inducing apoptosis to reduce the incidence of gastric cancer. The results provide evidence for the differential sensitivities of tumor and normal cells to apoptosis induction by AVD extract. No significant changes were observed in the expression of apoptosis-related proteins in animals administered with AVD extract alone compared with control animals. This finding indicates that AVD extract drives carcinogen-exposed cells to apoptosis while protecting normal cells.

In summary, we demonstrated that the administration of AVD extract can suppress MNNG-induced gastrointestinal cancer in rats. AVD extract can be considered as a promising chemopreventive agent against the occurrence of human gastric cancers. The possible mechanism might be related to the modulation of several key molecules that regulate cell growth.

Acknowledgements

This work was supported by the Traditional Chinese Medicine Project of Jiangsu Province (LZ13247) and the Education Bureau of Jiangsu Province (12KJB36002).

References


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<table>
<thead>
<tr>
<th>Group</th>
<th>Newly diagnosed without treatment</th>
<th>Newly diagnosed with treatment</th>
<th>Persistence or recurrence</th>
<th>Remission</th>
<th>None</th>
<th>Chemotherapy</th>
<th>Radiotherapy</th>
<th>Concurrent chemoradiation</th>
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<tr>
<td>Control</td>
<td>10.3</td>
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<td>0</td>
<td>0</td>
<td>51.7</td>
<td>10.3</td>
<td>10.3</td>
<td>12.8</td>
</tr>
<tr>
<td>Actinidia valvata Dunn</td>
<td>12.8</td>
<td>10.3</td>
<td>30.0</td>
<td>30.0</td>
<td>51.1</td>
<td>12.8</td>
<td>12.8</td>
<td>33.1</td>
</tr>
</tbody>
</table>


