Original Article

Effects of Jinlongshe granules on gastric precancerous lesions in rats and its mechanism

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Received January 7, 2019; Accepted November 26, 2019; Epub May 1, 2020; Published May 15, 2020

Abstract: Objective: To investigate the anti-gastric precancerous lesions effect and mechanism of Traditional Chinese Medicine Jinlongshe (JLS) granules in ethanol extractive of A. manshuriensis (EEA)-induced gastric precancerous lesions rats. Methods: A rat model with the part typical proliferation of the gastric epithelium mucosa was established by EEA. These rats received different doses of JLS granules treatment for four weeks. Bodyweight, histological and ultrastructural changes of gastric precancerous lesions were evaluated. The expression of Apelin and CD34 mRNA and proteins of the gastric tissue were analyzed by quantitative Realtime PCR, western blot and immunohistochemical staining. Results: We found that the treatment of JLS granules prevented the bodyweight loss and improved behavioral abnormalities of rats that received EEA. The histological and ultrastructural analysis also showed that JLS granules ameliorated EEA induced gastric precancerous lesions in a dose-dependent manner. The expression levels of two critical proteins involved in the angiogenesis of gastric carcinoma, Apelin, and CD34, were significantly reduced by the treatment of JLS granules. Conclusion: Our results indicated that JLS could inhibit the expression of the Apelin and CD34 genes in rat gastric mucosa, which reversed gastric precancerous lesions.

Keywords: Gastric precancerous lesions, Traditional Chinese Medicine, Jinlongshe, Apelin, CD34

Introduction

Gastric carcinoma (GC) is one of the most common malignancies worldwide [1]. GC becomes a major public health problem in China due to the high incidence and mortality [2]. GC usually develops from early neoplastic precursor lesions [3]. Gastric precancerous lesions were gastric epithelial dysplasia and gastric intestinal metaplasia, occurring after chronic atrophic gastritis [4]. Preventing the progression of gastric precancerous lesions to GC represents an effective strategy for GC treatment [5].

Many previous studies have confirmed the anti-tumor effects of Traditional Chinese Medicine (TCM) [6-9]. JLS granules, a widely used oral TCM complex, consist of Chinese herbs (Rhzoma Pinelliae, Radix, Rhizome Arisaemat, Glycyrrhizaepreparata, corium stomachiumgalli, etc.) [10], which functions to dissipate blood stasis and detoxify, inhibit, and reverse gastric precancerous lesions based on lots of clinical effects [10]. In previous studies, we found that JLS granules had anti-tumor effects in GC cell lines and in vivo xenograft model. JLS can inhibit GC cell proliferation and induce apoptosis in GC cells [10]. However, the detailed mechanism of the anti-tumor effects of JLS granules, especially in the gastric precancerous lesions stage, is still unclear. Here, we use an EEA induced gastric precancerous lesion animal model [11] to study how JLS granules prevent the progression of gastric precancerous lesions.

The cluster of differentiation 34 (CD34) is reported to play important roles in angiogenesis of GC [12]. The expression levels of CD34 were correlated with perioperative hemorrhage in GC patients [13]. Moreover, CD34 is an indirect marker of tumorneoangiogenesis [14]. Apelin is an endogenous ligand of the G protein-coupled receptor APJ. Apelin was up-regulated in GC and it was critical in promoting tumor angiogenesis [15, 23]. Patients with high tumor
Apelin levels had a significantly shorter overall survival compared with low tumor Apelin expression group.

In the present study, we examined the expressions of Apelin and CD34 in rats with EEA induced gastric precancerous lesions. In addition, the effects of JLS granules on Apelin and CD34, which are involved in the angiogenesis of GC, were explored to explain the beneficial effects of JLS granules in GC treatment.

Materials and methods

Experimental animals

Sixty four-week-old male Sprague-Dawley (SD) rats, weighing 155-185 g, were provided by the Animal Center of the Second Military Medical University. The animals were housed in groups of six per cage under controlled illumination (12:12 h light/dark cycle, lights on/off: 6 h/18 h), humidity (60%) and temperature (22°C ± 2°C) for one week to acclimatize to the environment. The Animal Care and Use Committee of the Second Military Medical University approved the protocol.

Gastric precancerous lesions rat model

The animals were then randomly divided into six groups (10 in each group): normal group (the rats were able to eat regular food and drinking water), model group (the rats were able to eat regular food and drinking water, given EEA (corresponding to Aristolochic Acid I 10.0 mg.kg⁻¹ every other day for 10 weeks), high-dose, medium-dose and low-dose JLS granules groups (the rats were able to eat regular food and drinking water, given EEA (corresponding to Aristolochic Acid I 10.0 mg.kg⁻¹) every other day for 10 weeks, besides, 20 g/kg, 60 g/kg, 120 g/kg JLS granules were respectively given to high-dose, medium-dose, and low-dose group rats every other day from 6th week for 4 weeks), positive control group (the rats were able to eat regular food and drinking water, given EEA (corresponding to Aristolochic Acid I 10.0 mg.kg⁻¹) every other day for 10 weeks, besides, 20 mg/kg 5-Fluorouracil (5-Fu) was respectively given to positive control group rats every other day from 6th week for 4 weeks, i.g.). The rats were weighed per week to evaluate the toxicity of the treatment. The general condition such as the diet, water intake, mental state, activity, stool, and urine output were observed twice every day and noted body weights per week.

Electron microscopy

At the end of the 10th week, rats in each group were sacrificed and the stomachs were weighed. The stomachs were fixed in 4% neutral formalin, and examination by transmission electron microscope was performed.

Histology

Stomachs tissues were fixed in 10% formaldehyde solution fixation, paraffin embedding, continue freezing section and stained with hematoxylin-eosin (H&E staining).

Immunohistochemical staining

The expressions of Apelin and CD34 in gastric mucosa in all groups were evaluated by using immunohistochemistry. Stomachs tissue were cut into 1 cm blocks. Blocks were placed in buffered PFA (pH 7.2) (Sigma, St. Louis, MO, United States). After 48 h, the stomach tissues were paraffin-embedded for immunohistochemistry. 3% H₂O₂ in methanol was used to block endogenous peroxidase activity for 15 min. Stomachs tissue were washed in H₂O, then PBS and stained with a rabbit polyclonal antibody against Apelin and CD34 (Abcam, Cambridge, British) diluted 1:1000. Primary antibody binding was detected using the Bond Polymer Refine Detection Kit (Leica, Wetzlar, Germany), then counterstained with hematoxylin, dehydrated and mounted. All slides were observed under a microscope.

Western blot analysis

Western blot analysis was performed. Briefly, the treated tissues were lysed with lystate. The protein concentration was measured with the BCA Protein Assay kit. 50 μg tissue lysates were denatured in 5 × sample loading buffer by heating at 100°C for 5 min. The samples were then separated by 12.5% SDS polyacrylamide gels and blotted onto nitrocellulose membranes. These membranes were incubated in TBST-milk (Tris-buffered saline containing 0.05% Tween-20 and 5% non-fat milk) for 40 min, followed by primary antibody for Apelin and CD34 at 4°C overnight. The blots were then washed
Table 1. the PCR primer sequences

<table>
<thead>
<tr>
<th>Primer name</th>
<th>primer sequences (5'-3')</th>
</tr>
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<tbody>
<tr>
<td>β-actin</td>
<td>left TGCTATGTTGCCCTAGACTTCG right GTTGGCATAGAGGTCTTTACGG</td>
</tr>
<tr>
<td>ki67</td>
<td>left TGGCAATCTGGCTTCTGAGG right TGCAATGTCCTCGTTTCTG</td>
</tr>
<tr>
<td>Apelin</td>
<td>left AGCCCGAGAACTCTGAGGACTG right GAGCCCTTCACCTGCTTTAGA</td>
</tr>
<tr>
<td>CD34</td>
<td>left CAGCCAACGTTTCAACTCCA right CTTAAACTCGCAGCCTGG</td>
</tr>
</tbody>
</table>

with TBST 4 times and subsequently incubated with secondary antibodies for 1.5 h. The blots were then washed 4 times with TBST and added a chemiluminescent ECL Advance western blotting detection kit before quantified using the Quantity One system (Bio-Rad Inc., Hercules, CA, United States).

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA extraction of tissues was carried out following the one-step method of Trizol (Invitrogen Corp., USA), and the high-quality total RNA was tested and verified by the ultraviolet analysis technology and formaldehyde gel electrophoresis. RNA (2 μg) was used for obtaining cDNA via AMV reverse transcriptase. The PCR primer sequences were designed and synthesized by Invitrogen Corp (Table 1). The condition of PCR amplification was pre-degeneration at 95°C for 10 minutes, degeneration at 95°C for 15 seconds, annealing at 60°C for 60 seconds and extension at 60°C for 5 minutes, which were cycled 40 times. The data were analyzed by adopting $2^{\Delta\Delta Ct}$ method, with $2^{\Delta\Delta Ct}$ demonstrating the relative expression multiple of the target gene of the experiment group to the normal group ($\Delta Ct = \Delta Ct$ experiment group - $\Delta Ct$ normal group, $\Delta Ct = Ct$ target gene - Ct internal reference gene). This experiment was repeated three times and average value was selected for use.

Statistical analysis

All statistical tests were performed with the SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). Continuous data were presented as means ± standard deviation (Mean ± SD), and one-way analysis of variance (ANOVA) for multi-group comparisons. Besides, the comparison between two groups that obey the normal distribution measurement data was conducted by t-test. A P value of <0.05 indicates statistically significant.

Results

The effects of JLS granules on bodyweight loss and behavioral changes caused by EEA

As shown in Figure 1, after 10 weeks, rat weight in the control group was 517.50 ± 23.28 g, in contrast, the bodyweight of EEA treated model group was significantly lower compared to control group (419.90 ± 39.91 g). The treatment of JLS improved EEA induced bodyweight loss in a dose-dependent manner. The high dose JLS showed similar beneficial effects on rat bodyweight with positive control 5-Fu group. In addition, compared with rats in EEA model group, all rats in JLS treated groups showed improved behavioral abnormalities such as lack of grooming, decrease in activity and diminished food consumption compared with EEA model group.

The effects of JLS granules on EEA induced histological changes in rat stomach tissues

To compare alterations in stomach histology of different groups of rats. H&E staining was performed. As shown in Figure 3, a significant increase in dysplasia of gastric mucosa in the EEA model group was observed compared with control group. Moreover, the levels of mucosal
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Figure 2. Histopathological analysis of stomach tissue. H&E staining of the sections of stomach tissue in different groups as indicated. 400 × magnification.

Figure 3. Ultrastructural changes in rat gastric mucosa. Electron micrographs of the sections of stomach tissue in different groups as indicated. 5000 × magnification.

Figure 4. The Ki67 mRNA levels in the stomach tissue of each group were measured by quantitative Realtime PCR. The values represent mean ± SEM. *P<0.05, **P<0.01.

atypical hyperplasia were significantly lower in the high-dose JLS granules treated group and a less extent of improvement of histological change was found in the low-dose and medium-dose JLS granules treated groups. These results suggested that the histopathological structure for precancerous gastric lesions be successfully induced by EEA and JLS treatment could reverse the atypical hyperplasia in dose dependent manner (Figure 2).
The effects of JLS granules on EEA induced ultrastructural changes in rat stomach cells

After 10 weeks, samples from the stomachs from each group were obtained for transmission electron microscope analysis. As shown in Figure 3, compared with the control group, irregular morphology such as collagen deposition, endoplasmic reticulum damage, mitochondria swelling and vacuolar degeneration were observed in EEA model group. JLS granules treatment dose-dependently improved these changes induced by EEA. Notably, similar extent of improvement of the ultrastructural was found in high dose JLS with positive control 5-Fu group. In addition, we also found that the treatment of JLS was able to inhibit the proliferation of gastric precancerous lesions in a dose dependant manner (Figure 4).

Figure 5. Apelin and CD34 mRNA expression. Apelin (A) and CD34 (B) mRNA levels in the stomach tissue were measured by quantitative Realtime PCR. The values represent mean ± SEM. *P<0.05 compared with Apelin or CD34 protein levels in the model rats.

Figure 6. Apelin and CD34 proteins levels. A. Apelin and CD34 protein levels were determined by western blot. B. Quantitation of the Apelin and CD34 proteins bands. The values represent mean ± SEM. *P<0.05 compared with Apelin or CD34 protein levels in the model rats.
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The effects of JLS granules on EEA induced Apelin and CD34 mRNA and protein overexpression

Since Apelin and CD34 played critical roles in the angiogenesis of GC, we tested whether the beneficial effects of JLS granules were mediated by blocking the expression of Apelin and CD34 in gastric precancerous lesions. As shown in Figure 5A and 5B, EEA treatment significantly increased both Apelin and CD34 mRNA levels in the stomach, JLS granules blocked the elevation of Apelin and CD34 mRNA induced by EEA in a dose-dependent manner. Inconsistent with mRNA results, the increased protein levels of Apelin and CD34 induced by EEA were also prevented by JLS granules treatment (Figure 6A and 6B). Moreover, we performed immunohistochemical staining for Apelin and CD34. As shown in Figure 7A and 7B, EEA treatment substantially increased the Apelin and CD34 expression in gastric epithelium, while the treatment of JLS dose-dependently reduced the overexpression of Apelin and CD34 induced by EEA treatment. The inhibitory effects of high dose JLS were comparable with positive control 5-Fu group.

Discussion

In the study, we showed that JLS granules significantly improved precancerous lesions by down-regulating of Apelin and CD34 in a rat EEA induced precancerous lesions model.

We have reported that JLS granules significantly inhibit the metastasis of GC cells and induced apoptosis of MKN-45 cells. The tumor inhibition rate was 58.46% in vivo [10]. However, the effects of JLS granules on precancerous lesions were still unknown. Since GC is preceded by a series of the precancerous lesion, it is believed that the intervention of precancerous lesions was critical in the prevention of GC. So, we used an EEA induced precancerous lesions model to evaluate the beneficial effects of JLS granules. Our results indicated that JLS granules not only targeted GC cells, but also prevented the formation of precancerous lesions evidenced by improved histological and ultrastructural changes.

Apelin, a ligand for the G-protein-coupled Apelinangiotension receptor-like 1 (APJ) receptor [17]. Apelin stimulates the maturation of tumor blood capillaries and increase the growth and vascularization of tumors by binding to APJ receptor [18, 19]. Previous studies suggested that high expression of Apelin in GC samples was associated with poor differentiation and high incidence of distant metastases [20]. CD34 is a specific marker of endothelial cells as a scaffold for the attachment of lineage-specific glycans [13, 21], which allows stem cells to bind to lectins expressed by stromal cells or other marrow components. Sun et. al. [22] reported that CD34 could facilitate the progression of gastric cancer as a prognostic indicator for GC. Here, we showed that JLS significantly inhibited the expression of Apelin and CD34 in rat gastric precancerous lesions which may explain the beneficial effects of JLS granules on the development of gastric precancerous lesions and GC.

In addition, we observed that JLS granules reversed collagen deposition and gastric epithelial cell disorder induced by EEA. High dose (120 g/kg) JLS granules treatment showed better effect than 20 mg/kg 5-Fu, a common used
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chemotherapeutic drug for the treatment of many types of human cancers, including GC [23]. As we observed in this study, the general condition and behavior of the rats in all JLS treated groups were better than the EEA model group. In addition, there was no significant weight loss in the JLS-treated rats. In contrast, significant weight loss got in the model group rats.

Taken together, our results demonstrated that JLS significantly prevented gastric precancerous lesions and inhibited the expressions of Apelin and CD34 levels. Our studies thus provide a rationale for the development of JLS granules against GC in the clinical setting.

Acknowledgements

This study was supported by the initial funding of Changzheng Hospital for Young Scholars (2016CZQN14).

Disclosure of conflict of interest

None.

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References

[19] Sorli SC, Le Gonidec S, Kniebiehler B and Audigier Y. Apelin is a potent activator of tumour...
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