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*Original Article*

# Effect of the traditional Chinese medicine Weifuchun on regulating atrophic gastritis through RUNX3/TGF-beta/Smad signaling pathway

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**Abstract:** Chronic gastritis was a chronic inflammatory condition of gastric mucosa, which has been considered as one of the most common disease in the general population of China. The traditional Chinese medicine Weifuchun was widely used in treatment of chronic gastritis in China. However, the mechanisms underlying the treatment of atrophic gastritis by Weifuchun remain poorly understood. In the present study, we have collected 10 non-atrophic gastritis, intestinal atrophic gastritis and dysplasia atrophic gastritis gastric samples expectedly by gastroscope. Compared to non-atrophic gastritis, the methylation level of RUNX3 in intestinal atrophic gastritis and dysplasia atrophic gastritis was much higher. Next, we found the mRNA and protein level of RUNX3, Smad2, p-Smad3/4, TGF-beta2, and p21 were up-regulated while Bim and Foxo3 were down-regulated in above groups. Further, we collected gastric samples from 10 atrophic gastritis patients before and after they took Weifuchun. The result shown that the mRNA and protein level of RUNX3, smad2, p-smad3/4, TGF-beta2 and p21 were downregulated and bim, foxo3 were upregulated after taking Weifuchun. These findings may be utilized in developing novel therapeutic tools for atrophic gastritis.

**Keywords:** Weifuchun, atrophic gastritis, RUNX3, TGF-beta, smad

## Introduction

Chronic gastritis [1], a chronic inflammatory condition of gastric mucosa, was considered as one of the most common disease in the general population of China [2]. In addition, *Helicobacter pylori* induced atrophic gastritis is epidemiologically associated with the occurrence of gastric cancer [3, 4]. It is estimated the patients with premalignant gastric lesions carry a significant risk of gastric cancer within 10 years of follow-up, and the annual incidence of gastric cancer was 0.1% for patients with atrophic gastritis within 5 years after diagnosis [1, 5]. Though there seems to be a worldwide decline in the overall incidence of gastric cancer, the public health burden of gastric cancer remains significant, and it is still the fourth in cancer incidence and the second leading cause of cancer-related mortality worldwide. Erosive gastritis is caused by damage to the gastric mucosa with a depressed area due to erosion on the hemispheric nodule. Although the pathophysiology of erosive gastritis has not been clarified yet, it is thought to occur because of an imbalance between the defenses of the stomach wall and offensive factors in the stomach. The defenses of the stomach wall include mucus, bicarbonate, tissue regeneration ability and mucosal blood flow, while the offensive factors include gastric acid, pepsin, nonsteroidal anti-inflammatory drugs, alcohol and Helicobacter pylori [6].

Runt-related transcription factor 3 (RUNX3) is a functionally important transcription factor of the TGF-β-mediated signaling pathway [7, 8]. RUNX3 binds directly to Smads, major mediators in the transforming growth factor-beta (TGF-β)/BMP signaling pathway, which leads to synergistic activation of the Immunoglobulin Cα promoter [9]. upon stimulation by TGF-β, RUNX3 cooperates with Smads to directly upregulate targets related to cell-growth inhibi-

**Table 1.** Primer sets for qPCR analysis

| Gene | Forward | Reverse | bp |
|---|---|---|---|
| RUNX3 | GCAGGCAATGACGAGAACTA | CAGTGATGGTCAGGGTGAAA | 136 |
| Smad2 | CACACCGAGATCCTAACAGAAC | AGGTGGCGTTTCTGGAATATAA | 120 |
| Smad3 | CCTGAGTGAAGATGGAGAAACC | GGCTGCAGGTCCAAGTTATTA | 117 |
| Smad4 | TCCAGCATCCACCAAGTAATC | GCAGTGCTGGTAGCATTAGA | 91 |
| p21 | GCGATGGAACTTCGACTTTG | GTGGGAAGGTAGAGCTTGG | 99 |
| Bim | CAGATATGCGCCCAGAGATATG | GTCTTCGGCTGCTTGGTAAT | 113 |
| Foxo3 | CGTGCCCTACTTCAAGGATAAG | ATTCTGGACCCGCATGAATC | 106 |
| TGF-β2R | CGTGTGCCAACAACATCAAC | TGCTTCAGCTTGGCCTTATAG | 106 |
| GAPDH | CAGGGCTGCTTTTAACTCTGGTAA | GGGTGGAATCATATTGGAACATGT | 101 |

**Table 2.** Primer sets for MSP analysis

| Gene | Forward | Reverse | bp |
|---|---|---|---|
| RUNX3-M | ATAATAGCGGTCGTTAGGGCGTCG | GCTTCTACTTTCCCGTTCTCGCG | 115 |
| RUNX3-U | ATAATAGTGGTTGTTAGGGTGTTG | ACTTCTACTTTCCCACTTCTCACA | 115 |

tion [10-12]. TGF-β2 is a secreted protein known as a cytokine that performs many cellular functions and has a vital role during embryonic development [13]. Much of the cellular response to TGF-β2 involves the Smad-dependent signaling pathway, which is activated by receptor-activated Smad (R-Smad, Smad2, and Smad3) phosphorylation [14]. The Smad2/3 complex combines with Smad4 and is then translocated into the nucleus, where target gene expression regulation is initiated [15]. Furthermore, cell cycle arrest by TGFβ involves suppression of the oncogene Myc, a repressor of the CDK inhibitors p21 [16].

RUNX3 acts as a tumor suppressor in many cancers, including stomach, bladder, breast, lung, brain, colorectal, pancreatic, and hepatocellular carcinoma. In these cancers, RUNX3 inhibits oncogenic Wnt signaling by interacting with β-catenin and promotes TGF-β-induced growth inhibition by interacting with SMAD3/SMAD4 [11]. In contrast, RUNX3 reportedly plays an oncogenic role in basal cell carcinoma and epithelial ovarian cancer. In head and neck cancers, the role of RUNX3 is controversial. Several studies have demonstrated that RUNX3 expression correlates with cancers [17], and the survival rate of patients with lower RUNX3 expression is significantly decreased compared with that of patients with higher expression [18]. Other studies have indicated an oncogenic function for RUNX3 in head and neck cancer. RUNX3 expression correlates well with poor differentiation, invasiveness, metastasis, and resistance to chemotherapeutic drugs. However, the mechanism of RUNX3 hypermethylation in intestinal atrophic gastritis and dysplasia atrophic gastritis was still unclear.

The Chinese medicine Weifuchun was a traditional medicine in the treatment of chronic gastritis and gastric cancer. It mainly contains *Radixginsengrubra* and *Rabdosia amethystoides* Hara [19]. However, the mechanisms underlying the treatment of atrophic gastritis by Weifuchun remains unclear.

In the present study, we have investigated the mechanism of traditional Chinese medicine Weifuchun involved in atrophic gastritis. The results showed that symptoms were significantly relieved after taking Weifuchun. We further found that Weifuchun alleviate the progress of atrophic gastritis by regulating the RUNX3 methylation and RUNX3/TGF-beta/smad signaling pathway.

## Materials and methods

### *The tissues from patients*

Gastric tissue samples were obtained from 10 non-atrophic gastritis, Intestinal atrophic gastritis and dysplasia atrophic gastritis patients at Wuxi hospital of TCM, affiliated to Nanjing University of TCM. Gastric tissue samples were collected from 10 atrophic gastritis patients before and after taken traditional Chinese medicine Weifuchun for 2 months by gastroscope. Written informed consent was obtained from each patient, and the use of clinical specimens was approved by the Institutional Ethics Committee. Weifuchun was purchased from Hu Qingyutang (Hangzhou, China).

### *Immunohistochemistry*

The patient tissue samples were fixed in 10% neutral buffered formalin for 48 h. The samples were then embedded in paraffin and cut into 6

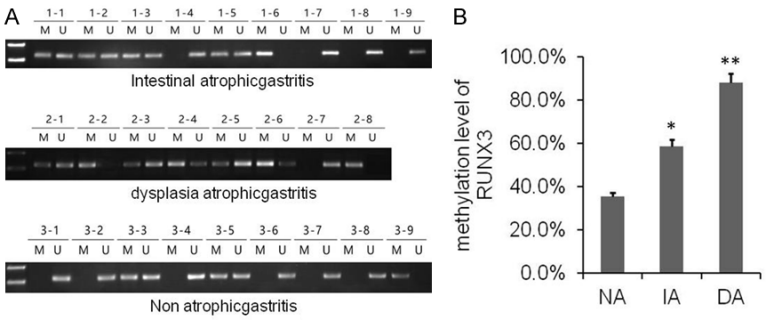


**Figure 1.** The methylation level of RUNX3 in atrophic gastritis tissues. A. The methylation level of runx3 detected by MSP. B. The methylation level of Intestinal atrophic gastritis was 57.5% and dysplasia atrophic gastritis was 87.5%. The methylation of Non atrophic gastritis tissues was 32.5%. NA: Non-atrophic gastritis; IA: Intestinal atrophic gastritis; DA: dysplasia atrophic gastritis. *means $P<0.05$, **means $P<0.01$.

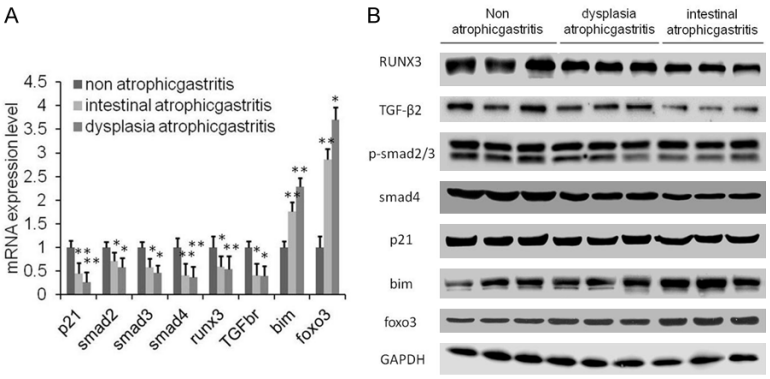


**Figure 2.** RUNX3/TGF-beta/smad signaling was inhibited in atrophic gastritis. A. mRNA expression level of RUNX3, smad2, smad3, smad4, TGF-beta2, p21 in intestinal atrophic gastritis and dysplasia atrophic gastritis downregulated compared to non-atrophic gastritis. And the bim and foxo3 were upregulated. B. The protein expression levels of RUNX3, smad2, p-smad3/4, TGF-beta2, p21 were decreased compared to non-atrophic gastritis. And the expression levels of bim and foxo3 were upregulated. *means $P<0.05$, **means $P<0.01$.

μm sections and stained with haematoxylin and eosin (H&E) to visualize general morphology. For stomach metastasis analysis, five step sections of the entire stomach separated by 50 μm were examined and metastases were counted. Stomach metastasis was identified by microscopic analysis of H&E sections of 6 μm. For immunohistochemistry, the sections were deparaffinized and incubated in 0.3% hydrogen peroxide in methanol for 10 min at room temperature to block endogenous peroxidase activity. After incubation in normal blocking serum for 30 min, sections were incubated with primary antibody at 37°C overnight. Followed by washing three times in phosphate-buffered saline supplemented with Triton X-100 (PBST) for 3-5 min, and finally incubated with secondary antibody (Dako EnVision + peroxidise Rabbit; Code: K40-02; Carpentaria, CA) at room temperature for 30 min. The bound antibodies were detected using DakoCytomation Envision + system-HRP labelled polymer. The sections were washed, saturated with 3, 30-diaminobenzidine tetra hydrochloride for 3 min, and then counterstained with haematoxylin. Antibodies used in the experiment includes: smad4 (ab40579, abcam), bim (ab32158, abcam), foxo3 (ab12162, abcam), p-smad 2/3 (8828, cell signaling), RUNX3 (ab11905, abcam), p21 (ab109520, abcam), TGF-beta2 (ab36495, abcam).

*Realtime quantitative RT-PCR*

Total RNA was isolated from human tissue samples by using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The first-strand complementary miRNA was synthesized using oligo dT from total RNA using the PrimeScript RT master Mix Perfect Real Time (Takara, Dalian, China). mRNA expression level was detected by real time PCR using SYBR green (Takara, Dalian, China) on ABI stepine plus real time PCR system. GAPDH served as loading control. The primers were shown in **Table 1**.

*Western blot*

The gastric tissues were lysed in RIPA lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF and protease inhibitor cocktail) for 30 min on ice, protein was quantificated with BCA protein assay kit (Beyotime, Shanghai, China), then 1/6 volumes of 6 × SDS-loading buffer (Beyotime, Shanghai, China) was added to denaturing the protein. Equal amounts of protein were sepa-

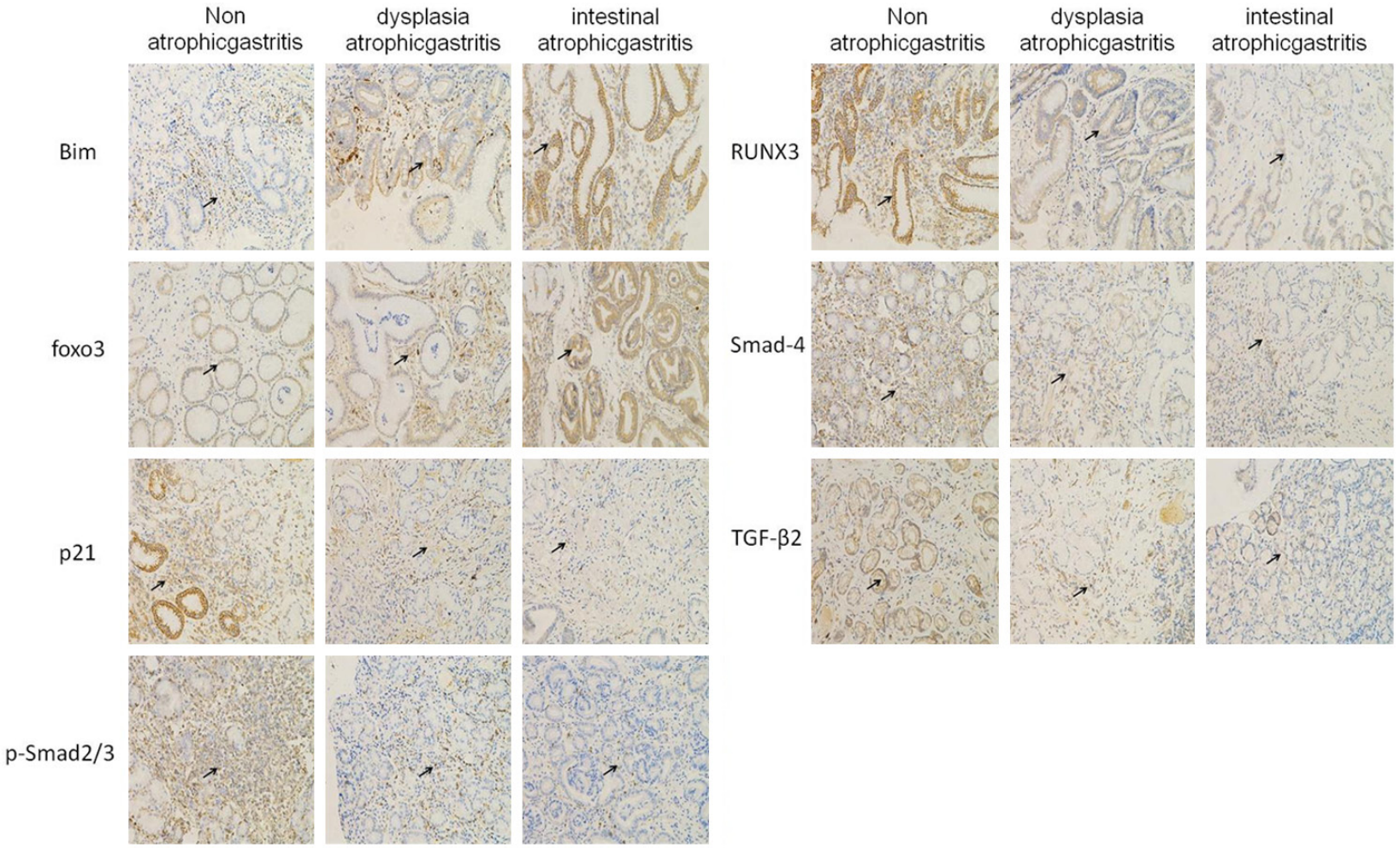


**Figure 3.** The immunohistochemistry result showed the RUNX3, smad2, p-smad3/4, TGF-beta2, p21 expression levels of atrophic gastritis were decreased compared to non-atrophic gastritis. And the expression levels of bim and foxo3 were upregulated.

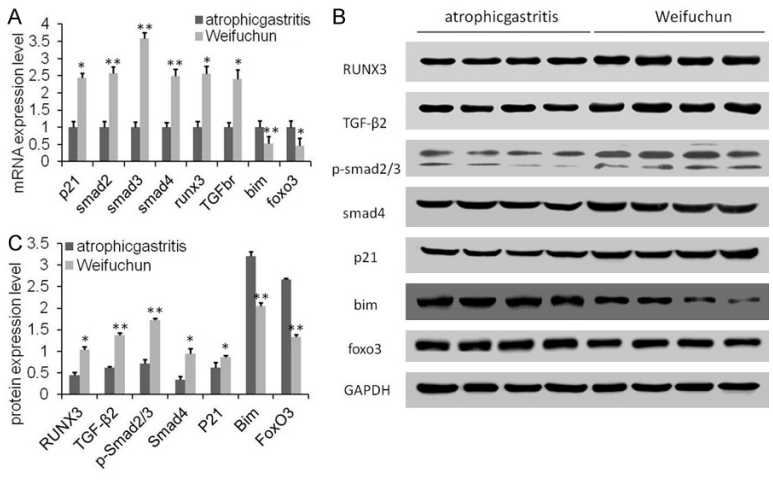


**Figure 4.** After taking Weifuchun, atrophic gastritis patients RUNX3/TGF-beta/smad signal was activated. A. The mRNA expression level of smad2, smad3, smad4, RUNX3, TGF-beta2, p21 was upregulated when the atrophic gastritis patient took Weifuchun. And bim and foxo3 were downregulated. B. The protein of smad2, smad3, smad4, RUNX3, TGF-beta2 and p21 were also increased and bim, foxo3 decreased after taken Weifuchun. C. Quantitative protein expression level of smad2, smad3, smad4, RUNX3, TGF-beta2 and p21. *means $P<0.05$, **means $P<0.01$.

rated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). The blots were blocked with 5% BSA (Albumin from bovine serum, Beyotime, Shanghai, China) and incubated with indicated antibodies. The Immobilon Western blot detection system (Millipore, Billerica, MA) was used to detect bound antibodies. Antibodies used in the experiment includes: smad4 (ab40579, abcam), bim (ab32158, abcam), foxo3 (ab12162, abcam), p-smad2/3 (8828, cell signaling), RUNX3 (ab11905, abcam), p21 (ab109520, abcam), TGF-beta2 (ab36495, abcam), GAPDH (ab8245, abcam), goat anti-mouse IgG HRP (m21001, Abmart, Shanghai, China), goat anti-rabbit IgG HRP (m21002, Abmart, Shanghai, China).

*Bisulfite modification and methylation-specific PCR*

Bisulfite modification was performed by using a DNA modification kit (Intergen). DNA (1 μg) in a volume of 100 μl was denatured by 0.2 M NaOH for 10 min at 37°C. Salmon sperm DNA (1 μg) (Sigma) was added as a carrier before modification. In all, 550 μl of freshly prepared 3 M sodium bisulfite (pH 5) was added and mixed with the samples, which were then incubated at 50°C for 16 h. Modified DNA was purified using Wizard DNA purification resin according to the manufacturer's instructions (Promega). Samples were eluted with 50 μl of water, and modification was completed by 0.3 M NaOH treatment for 5 min at room temperature, followed by ethanol precipitation. The DNA was resuspended in water and used immediately or stored at -20°C. The sequences of the primers and annealing temperatures are summarized in **Table 2**. Negative control samples without DNA were included for each set of PCR. Each PCR product (10 μl) was directly loaded onto 2% agarose gel, stained with ethidium bromide, and directly visualized under UV illumination.

*Statistics analysis*

The results were determined as mean ± SD of three independent experiments, in which each assay was performed in triplicate. *P*-values calculated by student's *T* test using SPSS 16.0 software and less than 0.05 were considered statistically significant. The statistical analysis of differences between discrete data was analyzed by student's *T* test. $P<0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS 16.0 software.

## Results

*The methylation level of RUNX3 in atrophic gastritis tissues*

Intestinal atrophic gastritis and dysplasia atrophic gastritis were two main types of atrophic gastritis. 40 Intestinal atrophic gastritis and dysplasia atrophic gastritis patients' tissues were collected with endoscope. Non-atrophic gastritis tissues were used as internal control. We conducted Bisulfite modification and Methylation-Specific PCR to detect the methylation level of RUNX3. The methylation level of Intestinal atrophic gastritis was 57.5% and dysplasia atrophic gastritis was 87.5%. However,

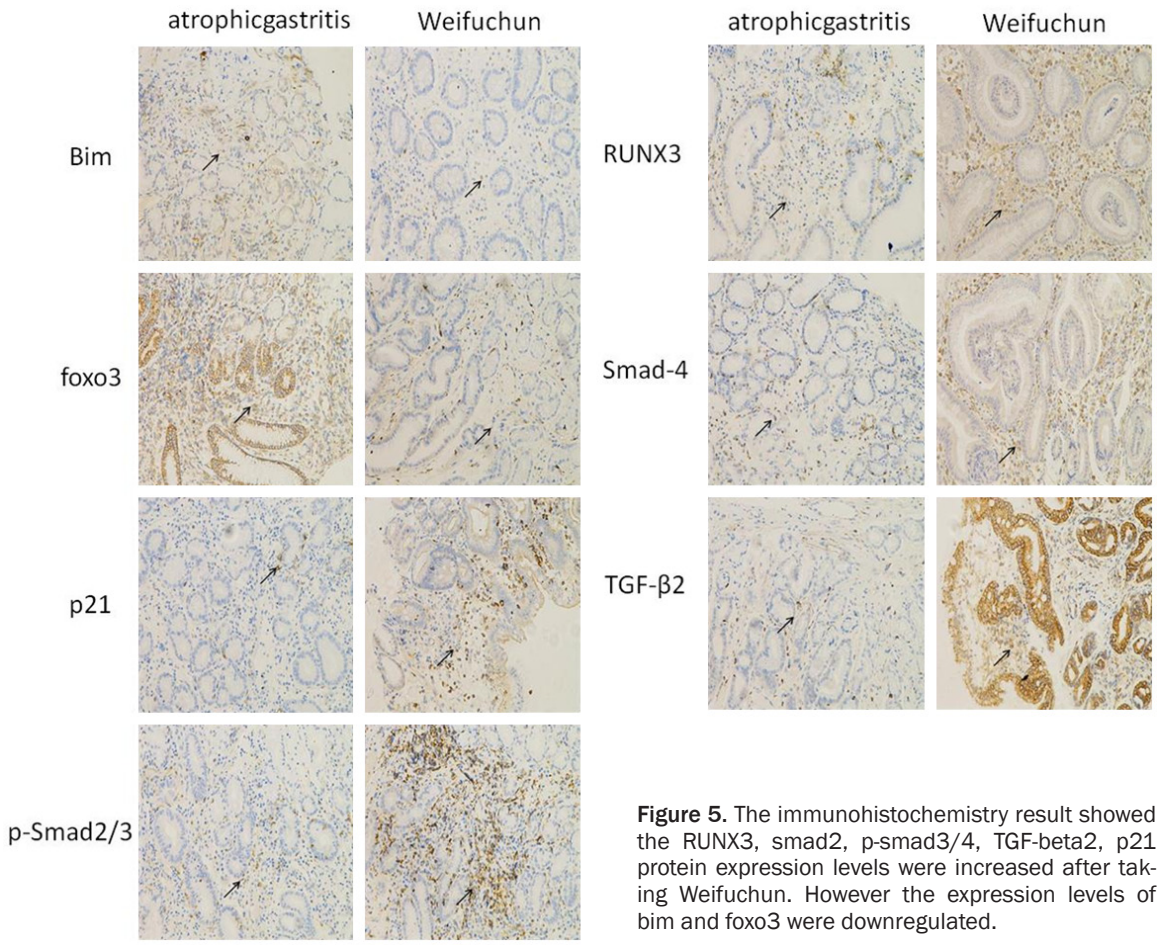


**Figure 5**. The immunohistochemistry result showed the RUNX3, smad2, p-smad3/4, TGF-beta2, p21 protein expression levels were increased after taking Weifuchun. However the expression levels of bim and foxo3 were downregulated.

the methylation of non-atrophic gastritis tissues was 32.5% (**Figure 1A** and **1B**).

*RUNX3/TGF-beta/smad signaling was inhibited in atrophic gastritis*

TGF-beta/smad signaling was a key signaling pathway in cell proliferation, metastasis, tumor formation et al. In **Figure 2A**, we found mRNA expression level of RUNX3, smad2, smad3, smad4, TGF-beta2 in intestinal atrophic gastritis and dysplasia atrophic gastritis downregulated compared to non-atrophic gastritis. In **Figure 2B**, western blot was conducted, the protein expression levels of RUNX3, smad2, p-smad3/4, TGF-beta2 were decreased compared to non-atrophic gastritis. We also found RUNX3, smad2, p-smad3/4, TGF-beta2 were downregulated by Immunohistochemistry in intestinal atrophic gastritis and dysplasia atrophic gastritis (**Figure 3**).

In **Figure 4A**, real time PCR was conducted, compare to gastritis, the Chinese medicine treated atrophic gastritis patients showed lower expression of RUNX3, smad2, smad3, smad4, TGF-beta2. And Western blot (**Figure 4B** and **4C**), immunohistochemistry (**Figure 5**) were also showed downregulated expression of RUNX3, smad2, p-smad3/4, TGF-beta2 in Chinese medicine treated atrophic gastritis patients.

*The role of Chinese medicine in apoptosis regulation in atrophic gastritis*

Foxo3 was an important transcription factor and participated apoptosis regulation. Bim and p21 were both key protein in the process of proapoptosis. We found the mRNA expression of foxo3, bim, p21 were downregulated in intestinal atrophic gastritis and dysplasia atrophic gastritis compared to atrophic gastritis, detected by real time PCR (**Figure 2A**). In **Figures 2B** and **3**, the protein expression level of foxo3, bim and p21 were decreased in intestinal atrophic gastritis and dysplasia atrophic gastritis by Western blot and immunohistochemistry. The Chinese medicine treated gastritis patients

showed lower expression of foxo3, bim and p21 compared to gastritis patients (**Figures 4**, **5**).

## Discussion

RUNX3 was a functionally important transcription factor of the TGF-β-mediated signaling pathway and acted as a tumor suppressor in many cancers [20-22]. RUNX3 promoter hypermethylation were associated to lung cancer [23], breast cancer [21], and esophageal squamous cell carcinoma [24] progression. In gastric cancer, step cumulation of RUNX3 methylation mediated by Helicobacter pylori infection contributed cancer progression. Here we showed, the methylation level of intestinal atrophic gastritis was 57.5% and dysplasia atrophic gastritis was 87.5%, the methylation of non-atrophic gastritis tissues was 32.5%. Atrophic gastritis patients showed hypermethylation compared to non-atrophic gastritis patients. The TGF-β superfamily consists of highly pleiotropic molecules including activins, inhibins, BMPs (Bone morphogenic proteins), GDFs (Growth differentiation factors) and GDNFs (Glial-derived neurotrophic factors), and exerts multiple biological functions in renal inflammation, fibrosis, cell apoptosis, and proliferation. In this paper we found in intestinal atrophic gastritis and dysplasia atrophic gastritis RUNX3/TGF-beta/smad signal was inhibited compared to non-atrophic gastritis.

Apoptosis was a highly regulated and controlled process that confers advantages during an organism's lifecycle [25]. Apoptosis was a self-protective mechanism, and Foxo3, Bim and p21 were play significant roles in apoptosis. In this paper, we found in the Chinese medicine treated gastritis patients showed lower expression of foxo3, bim and p21 compared to gastritis patients. The Chinese medicine Weifuchun inhibited atrophic gastritis cell apoptosis, protected and promoted to cell survival.

The Chinese medicine Weifuchun was a traditional medicine in the treatment of chronic gastritis and gastric cancer. Huang group had found Weifuchun on inhibiting inflammation of Helicobacter pylori-infected GES-1 cells and by inhibiting NF-kappaB signaling pathway [19]. In this paper, we have found, in atrophic gastritis, Weifuchun activates RUNX3/TGF-beta/smad signal.

In conclusion, we compared the RUNX3 methylation and RUNX3/TGF-beta/smad signal pathways and apoptosis pathway related protein expression in vivo in this study. We found in intestinal atrophic gastritis and dysplasia atrophic gastritis, the methylation of RUNX3, TGF-beta/smad signal decreased expression compared to non-atrophic gastritis. And in Chinese medicine Weifuchun treated patients, RUNX3, TGF-beta/smad signal related protein was up-regulated compared to atrophic gastritis.

## Disclosure of conflict of interest

None.

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