Research Article

Effects of Huaier Polysaccharide SP1 on Gastric Cancer Cell Proliferation, Apoptosis, Migration, and Invasion by Regulating TGF-β/SMAD Signaling Pathway

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Objective. To study the effects of Huaier polysaccharide SP1 on the proliferation, apoptosis, migration, and invasion of gastric cancer cell line MGC-803 and the underlying mechanism.

Methods. MGC-803 cells were cultured in vitro and treated with SP1. The effects of SP1 on the proliferation, apoptosis, migration, and invasion of MGC-803 cells were detected by CCK-8 assay, flow cytometry analysis, and Transwell assay, respectively. Western blot and qRT-PCR were used to detect the expression of related genes.

Results. Our study showed that Huaier polysaccharide SP1 could inhibit proliferation, migration, invasion, and promote the apoptosis of MGC-803 cells in vitro in a dose-dependent manner. Huaier polysaccharide SP1 could inhibit the activation of TGF-β/SMAD signal pathway by upregulating SMAD7 expression, thereby downregulating the expression of SOX4, ZEB2, MMP9, Snail, and Slug.

Conclusion. Huaier polysaccharide SP1 can regulate the proliferation, apoptosis, migration, and invasion of gastric cancer cells by promoting the expression of SMAD7 and inhibiting the activation of TGF-β/SMAD signal pathway as well as the expression of the downstream oncogenes.

1. Introduction

Gastric cancer, one of the common malignancies in the world, has seen great development in its therapeutic strategies such as radical surgery, traditional chemotherapy, radiation therapy, and targeted therapy in recent years. However, it still has a high mortality rate [1, 2], which is mainly because there is no better treatment for patients with advanced and/or metastatic gastric cancer, and systemic chemotherapy remains the main option [3, 4]. As conventional chemotherapeutics are causing higher drug resistance, and cytotoxic and serious side effects [5, 6], it is of great significance to find a novel, highly effective anticancer drug with few side effects for treating gastric cancer and reducing the chemotherapy-induced adverse effects.

Trametes robiniophila Murr, also called Huaier, is a Chinese medicinal fungus [7]. Recent studies have found that polysaccharides extracted from Huaier have inhibitory effects on various tumor cells such as those of breast cancer [7, 8], liver cancer [9, 10], and clear cell renal cell carcinoma (CCRCC) [11]. Moreover, Huaier polysaccharides exert anticancer effects in various ways. For example, Luo et al. reported that Huaier polysaccharide SP1 caused apoptosis in breast cancer MCF-7 cells by downregulating MTDH protein [8]; Li et al. proved that Huaier polysaccharides suppressed liver cancer growth and metastasis by inhibiting angiogenesis [9]; Fang et al. found that Huaier polysaccharide HP-1 inhibited the progression of CCRCC by inhibiting epithelial-mesenchymal transition and improved the efficacy of sunitinib [11]. So far, there have been no
studies on Huaier polysaccharides in gastric cancer, and the underlying mechanism remains unknown.

According to the method of Luo et al. [8], we extracted Huaier polysaccharide SP1 from Trametes robiniophila Murr, explored its function in gastric cancer cell line MGC-803, and further explained the corresponding mechanism, thus providing a theoretical basis for the application of Huaier polysaccharide SP1 as a chemotherapeutic agent in the treatment of gastric cancer.

2. Materials and Methods

2.1. Extraction and Identification of Huaier Polysaccharide SP1. Huaier polysaccharide SP1 was extracted according to the method reported by Luo et al. [8]. First, sandy and beige powdered water-soluble crude polysaccharide (SCP) was isolated and purified from the fruiting bodies of Trametes robiniophila Murr. Next, SCP was further purified by DEAE cellulose and agarose CL-6B gel filtration chromatography to obtain polysaccharides with different average molecular weight distributions. The water-soluble neutral fraction SP1 was collected and lyophilized for further study, and the products were identified by high-performance liquid chromatography. A single peak indicated the homogeneity of the products, with an average molecular weight of approximately 56 kDa (Figure 1). Gas chromatography analysis showed that SP1 was composed of galactose, arabinose, and glucose in a molar ratio of 3.1:1.1:1.1:3.3.

2.2. Cell Culture and Treatment. Human gastric cancer cell line MGC-803 was purchased from ATCC, USA. The MGC-803 cell line was routinely cultured with DMEM high glucose medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin. The cells were cultured in a humidified incubator containing 5% carbon dioxide at 37°C.

2.3. Cell Viability Test. MGC-803 cells were seeded in 96-well plates at a density of 3000 cells/well and preincubated for 24 hours. Different concentrations of SP1 were then added, and CCK-8 reagent (Dojindo, Japan) was added at 24, 48, and 72 h respectively. The absorbance (A) at 450 nm was measured on a spectrophotometer. Relative cell activity = (the treatment group value – the blank background value)/(the control group value – the blank background value) × 100%.

2.4. Cell Migration Assay. Cell migration ability was detected using a scratch assay. MGC-803 cells treated with different concentrations of SP1 were cultured to a confluency rate of 95%. A straight line was scratched on the cells perpendicular to the culture plate with a tip, followed by PBS washing to remove detached cells. Serum-free DMEM high glucose medium with different concentrations of SP1 was then added. The cells were cultured in a cell culture incubator and photographed at 24 hours. Cell mobility was calculated.

2.5. Apoptosis Detection. MGC-803 cells were seeded in 12-well plates and treated with different concentrations of SP1 for 72 h in an incubator. Cells were collected, washed twice with PBS, and stained using the Annexin V-FITC/PI Apoptosis kit. Samples were analyzed on a flow cytometer. Apoptosis rate (%) = early apoptosis rate (%) + late apoptosis rate (%).

2.6. Cell Invasion Assay. Cell invasion ability was measured using a Transwell assay. A 100 μl mixture of matrix gel (Corning, USA) and serum-free DMEM medium (1:8) was coated on the upper transwell chamber (Corning). The cells (5 × 10⁴) were seeded in the upper chamber with DMEM containing no serum 4 h after the matrix gel had solidified, and DMEM medium containing 10% fetal bovine serum was added to the lower chamber. The cells were cultured for 24 hours, fixed with methanol and stained with crystal violet, and cells on the lower surface of the membrane were photographed and counted using a microscope.

2.7. Western Blot. The proteins extracted with RIPA lysate were quantified using a BCA protein assay kit, and equal amounts of proteins were electrophoresed on 10% SDS-PAGE followed by immunoblotting on nitrocellulose membranes. Membranes were blocked with 5% skim milk for 2 h at room temperature and then incubated with primary antibodies overnight at 4°C. After incubation with HRP-conjugated secondary antibodies, the proteins were visualized on an ECL gel imaging system. Primary antibodies were purchased from Abcam.

2.8. qRT-PCR. Total cellular RNA was extracted with TRIZol reagent (Takara, Japan), and cDNA was synthesized using Prime Script RT reagent kit (Takara). All reactions were performed with the use of TaqMan Gene Expression Assay system (Applied Biosystems, USA) and TaqMan Universal PCR Master Mix reagents (Thermo Fisher Scientific, USA). The relative gene expression levels were calculated using the 2^(-ΔΔct) method with β-actin as an internal reference. All experiments were repeated at least three times. All primers used in the experiments are listed in Table 1.
Table 1: qRT-PCR Primer sequence.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primers (5′–3′)</th>
<th>Reverse primers (5′–3′)</th>
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<tbody>
<tr>
<td>SMAD7</td>
<td>TTCCCTCCGCTGAAACAGGG</td>
<td>CCTCCCCGATATGCCACACAC</td>
</tr>
<tr>
<td>SOX4</td>
<td>GACCTGCTGACGTCGAAA</td>
<td>CCGGGCTCGAGAGTTAAATCC</td>
</tr>
<tr>
<td>ZEB2</td>
<td>CAAGAGGCGGAAACACAGCC</td>
<td>GGTGTCGATACGCCGCTATCC</td>
</tr>
<tr>
<td>MMP9</td>
<td>GGGACGCAGACATCGTCACAT</td>
<td>TGCTCATGTCAGAAATGGGC</td>
</tr>
<tr>
<td>Snail</td>
<td>TCGGAAGCCTAATCAGCGA</td>
<td>AGATGAGCATTGGGACAGGAG</td>
</tr>
<tr>
<td>Slug</td>
<td>CGAATCGACACACACACAGTG</td>
<td>CTGAGATTCTGTGTTGTTGTT</td>
</tr>
<tr>
<td>β-actin</td>
<td>CATGTACGTGTCATCCAGGC</td>
<td>CTCCTAATGTACGCACGAT</td>
</tr>
</tbody>
</table>

2.9. Statistical Analysis. Data were presented as mean ± standard deviation, and each experiment was independently repeated at least three times. Statistical analysis was performed using SPSS 20.0 software. Comparisons between the experimental and control groups were performed using Student’s t-test and one-way ANOVA. P < 0.05 was considered statistically significant.

3. Results

3.1. SP1 Inhibited MGC-803 Cell Proliferation. Figure 2 showed that MGC-803 cells had a dose-dependent inhibition on the growth of MCF-7 cells after exposure to different concentrations of SP1 (25, 50, 100, 200, 400, and 800 µg/ml) for 24, 48, and 72 hours (P < 0.05). As the SP1 concentration increased to 50 µg/ml, cell viability decreased significantly over all time periods. However, the decreasing trends of cell viability were similar at a concentration above 200 µg/ml. Therefore, SP1 could suppress MGC-803 cell proliferation.

3.2. SP1 Promoted Apoptosis in MGC-803 Cells. As shown in Figure 3, flow cytometric analysis revealed that MGC-803 cells after exposure to different concentrations of SP1 (25, 50, 100, and 200 µg/ml) for 72 hours showed significantly increased proportion of apoptosis with increasing doses of SP1 (P < 0.05), indicating that SP1 could promote MGC-803 cell apoptosis.

3.3. SP1 Inhibited Migration of MGC-803 Cells. As shown in Figure 4, the scratch assay revealed that MGC-803 cells treated with 25, 50, 100, and 200 µg/ml of SP1 showed a marked decrease in cell migration in a dose-dependent manner (P < 0.05), suggesting that SP1 could inhibit MGC-803 cell migration.

3.4. SP1 Inhibited MGC-803 Cell Invasion. As shown in Figure 5, the transwell assay revealed that MGC-803 cells treated with 25, 50, 100, and 200 µg/ml of SP1 showed a significant decrease in cell invasion in a dose-dependent manner (P < 0.05), demonstrating that SP1 could inhibit MGC-803 cell invasion.

3.5. SP1 Inhibited the Activation of the TGF-β/SMAD Signaling Pathway. After treatment of MGC-803 cells with 200 µg/ml of SP1 for 72 h, we found that SP1 could significantly elevate SMAD7 mRNA and protein levels (Figures 6(a) and 6(b)), which in turn inhibited the expression of TGF-βRI. Meanwhile, SP1 could significantly reduce the phosphorylation levels of SMAD2 and SMAD3, and downstream proteins of TGF-βRI (Figure 6(b)).

3.6. SP1 Inhibited the Expression of Downstream Oncogenes in the TGF-β/SMAD Signaling Pathway. We examined mRNA and protein expressions of the proliferation, apoptosis, migration, and invasion-related downstream genes in the
TGF-β/SMAD signaling pathway including SOX4, ZEB2, MMP9, Snail, and Slug and found that these gene expression levels were inhibited by SP1 (Figure 7).

4. Discussion

There is increasing evidence that herbs have antitumor effects. Huaier is a common medicinal fungus that has been used in traditional Chinese medicine for more than 1000 years [12]. Moreover, it has attracted more and more attention due to its antitumor and immune-promoting functions as well as negligible side effects. In this study, we isolated purified polysaccharide SP1 from Huaier and demonstrated that SP1 could exert antitumor effects by inhibiting the TGF-β signaling pathway.

In vitro studies revealed that Huaier polysaccharide SP1 could significantly inhibit the proliferation, migration, and invasion while it could promote the apoptosis of MGC-803...
cells. However, there have been no studies on the effects of Huaier polysaccharides in gastric cancer, so we have proved for the first time that Huaier polysaccharide SP1 has a significant anticancer effect on gastric cancer and can be used as a chemotherapeutic or adjuvant drug in the treatment of gastric cancer. Through oral administration, Huaier polysaccharide SP1 can directly enter the stomach and act on gastric cancer, which may have better antitumor effects.

So far, the explanation for the mechanism of Huaier polysaccharides against cancer is still insufficient. In this study, we found that SP1 could inhibit the activation of TGF-β/SMAD signaling pathway by upregulating the expression of SMAD7. TGF-β, transforming growth factor β, is a pleiotropic cytokine that signals via membrane receptors and intracellular SMAD proteins, which then enter the nucleus after receptor activation and act as transcription regulators that control gene expression.

Figure 5: The invasion ability of MGC-803 cells treated with different concentrations of SP1 (0, 25, 50, 100, and 200 μg/ml) for 72 h was detected by transwell assay. Compared with 0 μg/ml SP1-treated (control) group, *P < 0.05; & P < 0.05 for comparison between groups under different concentrations of SP1 treatment.

Figure 6: (a) The expression of SMAD7 mRNA in MGC-803 cells of the control group (SP1 0 μg/ml) and SP1 treatment group (200 μg/ml) for 72 h was detected by qRT-PCR. (b) The protein level of SMAD7 in MGC-803 cells of the control group (SP1 0 μg/ml) and SP1 treatment group (200 μg/ml) for 72 h was detected by Western blot. Compared with the control group, *P < 0.05.
The TGF-β/SMAD signaling pathway plays a critical role in such malignant progressions of cancer cells as survival, EMT, migration, and invasion [13, 14]. Upon activation of transforming growth factor beta receptor 1 (TGF-βR1), the receptor-regulated SMAD proteins, SMAD2, and SMAD3 are recruited and phosphorylated for activation. Activated SMAD2 and SMAD3 continue to form oligomeric SMAD complexes with SMAD4, which together translocate into the nucleus, bind to DNA, and regulate the expression of target genes [15]. SMAD7 as a negative regulator of the TGF-β/SMAD signaling pathway can interact with TGF-βR1 in the cytoplasm, leading to TGF-βR1 degradation [16]. In this study, we found that SP1 could significantly increase the levels of SMAD7 mRNA and proteins and inhibit the expression of TGF-βRI, thereby regulating the proliferation, apoptosis, migration, and invasion of gastric cancer cells by inhibiting the TGF-β/SMAD signaling pathway and the expression of downstream molecules Sox4, Zeb2, Mmp9, Snail, and Slug (Figure 8).

In this study, we extracted the polysaccharide SP1 from Huaier and confirmed by in vitro experiments that SP1 could inhibit the activation of TGF-β/SMAD signaling pathway and the expression of downstream oncogenes SOX4, ZEB2, MMP9, Snail, and Slug by facilitating the expression of SMAD7, thereby regulating the proliferation, apoptosis, migration, and invasion of gastric cancer cells, which provided a theoretical basis for the use of SP1 in the treatment of gastric cancer.

**Data Availability**

The data used to support the findings of this study are available from the corresponding author upon request.

**Conflicts of Interest**

The authors declare that there are no conflicts of interest regarding the publication of this paper.

**Authors’ Contributions**

Miaoliang Chen and Ying Lu contributed equally to this work.
Acknowledgments

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References