Trametes robiniophila may induce apoptosis and inhibit MMPs expression in the human gastric carcinoma cell line MKN-45

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Abstract. Gastric carcinoma (GC) is one of the most common malignant tumors and is mainly treated by invasive surgeries. The present study aimed to investigate the treatment potential of *Trametes robiniophila* on GC using the human GC cell line MKN-45. Cells were incubated with *Trametes robiniophila* at a concentration of 0, 5 and 10 mg/ml for 24 h. The apoptosis of the cell line was examined with acridine orange/ethidium bromide staining and flow cytometry. The expression of B-cell lymphoma (Bcl)-2, Fas, caspase-3, matrix metalloproteinase (MMP)-2 and MMP-9 was analyzed using reverse transcription-polymerase chain reaction and western blotting. With increasing drug concentrations, the proportion of apoptotic and necrotic cells increased. For a certain concentration, the apoptotic ratio also increased with increasing response times. Compared with the control group, the Bcl-2, MMP-2 and MMP-9 expression levels in the MKN-45 cell line decreased, while the expression levels of Fas and caspase-3 increased ($P<0.05$), and the expression patterns were strengthened with increasing drug concentrations. The present study revealed that *Trametes robiniophila* had treatment potential on GC, and it may act on gastric cells through apoptotic induction and MMPs expression inhibition. Based on the present results, *Trametes robiniophila* may be considered as an alternative approach for noninvasive therapy of GC. However, future studies should be performed to clarify this further.

Introduction

Gastric carcinoma (GC) remains a major health issue worldwide, with 1 million newly diagnosed cases and 700,000 mortalities each year (1). Occurrence of GC varies with geography, and in Asia or the Pacific Islands, the incidences and mortality rates of GC can be twice as high as those in Western countries (2). GC is considered as a multifactorial disease due to numerous inherited and environmental factors, including genetic background, infectious agents and dietary habits (3). Surgery remains the only curative therapy for GC treatment, and perioperative and adjuvant chemotherapy can improve the outcome (4,5). However, no clear superiority of one strategy over others has been observed, and serious side effects and dose-limiting toxicities of chemotherapy treatments are common (6). In addition, >50% of resected GC patients experience reccurrence and metastases (7). Safe, natural and non-toxic agents that can interfere with the essential steps of cancer development are increasingly used for cancer therapy (8,9). Traditional Chinese medicines (TCMs) have recently drawn great attention as possible anticancer agents with few side effects.

Trametes robiniophila Murr. (Huaier) is a fungal species in China that has been applied in TCM for >1,600 years (10). In recent years, the antitumor effect of Huaier has been demonstrated, and *Trametes robiniophila* extracted from the fungus in hot water to eliminate the free proteins and amino acids has been increasingly applied in clinical cancer therapy (10,11). The major active ingredient of *Trametes robiniophila* is proteoglycan, including 41.53% polysaccharides, 12.93% amino acids and 8.72% water (12). However, the inhibition effect on cancer of proteoglycan and other isolated ingredients was much lower than that of *Trametes robiniophila* (13,14). The antitumor effect of *Trametes robiniophila* involves various mechanisms, including induction of apoptosis, anti-angiogenesis, drug resistance reversal, anti-metastasis and system immune activation (15). The current study attempted to investigate the antitumor effect of *Trametes robiniophila* on GC using the human GC cell line MKN-45. The apoptosis of the cell line was examined with acridine orange (AO)/ethidium bromide (EB) staining and flow cytometry, and the expression level of molecules involved in the apoptotic and metastatic processes of tumors were analyzed using reverse transcription-polymerase chain reaction (RT-PCR) and western blotting.

Materials and methods

Preparation of Trametes robiniophila. Trametes robiniophila was purchased from Qidong Gaitianli Pharmaceutical Co., Ltd.

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Table I. Detailed information of primers.

Gene	Sequence (5'-3')	T_a (˚C)	Length (bp)
Bcl-2	Forward GCTGTCGCAGAGGGGCTAC Reverse ATCCTCCCCCAGTTCACCC	55	375
Fas	Forward CTGCCATAAGCCCTGTCCTC Reverse GGTGTTGCTGGTGAGTGTGC	56	316
Caspase-3	Forward CAAATGGACCTGTTGACCTGA Reverse ATTCTGTTGCCACCTTTCGG	56	351
MMP-2	Forward GATGCCGCCTTTAACTGG Reverse TCAGCAGCCTAGCCAGTCG	55	278
MMP-9	Forward CAGTACCGAGAGAAAGCCTATTTCTG Reverse TAGGTCACGTAGCCCACTTGGT	54	101
β-actin	Forward GGGAAATCGTGCGTGACATT Reverse GGAAGGAAGGCTGGAAGAGTG	55	183

MMP, matrix metalloproteinase; Bcl, B-cell lymphoma; T_a, annealing temperature.

(Qidong, China). *Trametes robiniophila* (1.0 g) was dissolved in 10 ml RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and sterilized using a 0.22-μm filter to obtain the 100 mg/ml stock solution that was suitable for long-term storage at 37˚C.

Preparation of the MKN-45 cell line. The GC cell line MKN-45 was obtained from the Shanghai Institute of Cellular Biology of Chinese Academy of Sciences (Shanghai, China) and was routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Bioengineering Material Co., Ltd., Hangzhou, Zhejiang, China), 100 U/ml penicillin and 100 mg/ml streptomycin (Biyuntian, Jiangsu, China) under the conditions of 5% CO_2 at 4˚C.

AO/EB staining to detect apoptosis. The cell concentrations were adjusted to $5x10^4$ cells/ml, and cells were incubated on slides in 24-well plates for 24 h. For experimental use, the *Trametes robiniophila* stock solution was diluted to a final concentration of 5 or 10 mg/ml, with 0 mg/ml serving as control. AO/EB (4 μg; Amresco, LLC, Solon, OH, USA) was added to each well after 48 h. The apoptosis of the cell line was detected with a fluorescence microscope at 510 nm. Each treatment was represented by three replicates.

Flow cytometric analysis of apoptosis. Cells were cultured with *Trametes robiniophila* for 24, 48 and 72 h as aforementioned. Annexin V-fluorescein isothiocyanate (5 μl; Kaiji Bio Co., Nanjing, China) was added to different wells and incubated for 10 min at room temperature. The cells were resuspended with 1X binding buffer, and 5 μl propidium iodide (PI) was added. Next, the apoptosis of the cells was analyzed by flow cytometry. Each treatment was represented by three replicates.

RT-PCR. Total RNA was isolated from cells treated with *Trametes robiniophila* for 24 h using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) (16). RT-PCR was used to detect the gene expression level of matrix metalloproteinase *(MMP)-2*, *MMP-9*, B-cell lymphoma *(Bcl)-2*, *Fas*, *caspase-3* and the reference gene *β-actin*. Primers and annealing temperature information is shown in Table I. The RNA was reversely transcribed into complementary DNA using an RT-PCR kit (Thermo Fisher Scientific, Inc.), and the final reaction mixture (20 μl) contained 4 μl of 5X reaction buffer, 1 μl RNase inhibitor, 1 μl oligo$(dT)_{18}$ primer, 2 μl 10 mM deoxynucleotides, 1 μl Moloney-murine leukemia virus reverse transcriptase, 2 μl RNA template and 9 μl double distilled H_2O. The thermal cycling parameters for the amplification were as follows: A denaturation step at 95˚C for 3 min, followed by 30 cycles at 95˚C for 15 sec, the annealing temperature indicated in Table I for 30 sec and 72˚C for 45 sec. The RT-PCR products were semi-quantified with the UVP Gel Imaging System (UVP, Inc., Upland, CA, USA).

Western blot analysis. The cells and *Trametes robiniophila* were cultured as aforementioned. Total protein was extracted from the cells using a 400 μl single detergent lysis buffer (including phenylmethylsulfonyl fluoride) and radioimmunoprecipitation buffer (Sangon Biotech, Shanghai, China). The lysis mixture was then added to a homogenizer, and centrifuged at 13,500 x *g* and 4˚C for 5 min. The supernatant was subpackaged into Eppendorf tube and stored at -20˚C. The extracts were boiled with loading buffer for 5 min and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels. Targeted proteins were transferred onto polyvinylidene difluoride membranes. The membranes were washed with Tris-buffered saline containing Tween 20 three times, for 20 min each time. Then, the membranes were incubated overnight with antibodies against Bcl-2 (catalog no. 15071), caspase-3 (catalog no. 9668) and Fas (catalog no. 8023) (all Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C. Following an additional three washes, anti-mouse immunoglobulin G, horseradish peroxidase-linked secondary antibodies (catalog no. 7076; 1:3,000 dilution; Cell Signaling Technology, Inc.) were added and incubated at room temperature for 1 h. Upon three final washes, the blots were developed using Beyo ECL Plus reagent (Beyotime Institute of Biotechnology, Haimen, China) and the results were detected in the

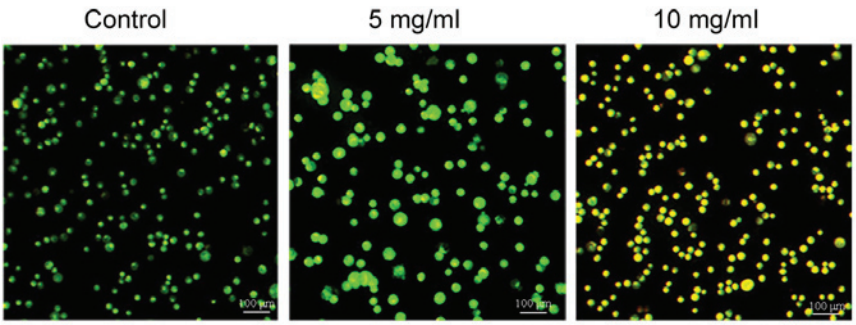

Figure 1. Morphological changes in MKN-45 cells treated with *Trametes robiniophila* (magnification, x100). Green coloration represents normal cells with an intact structure; yellow coloration represents apoptotic cells with an irregular morphology; and red coloration represents necrotic cells.

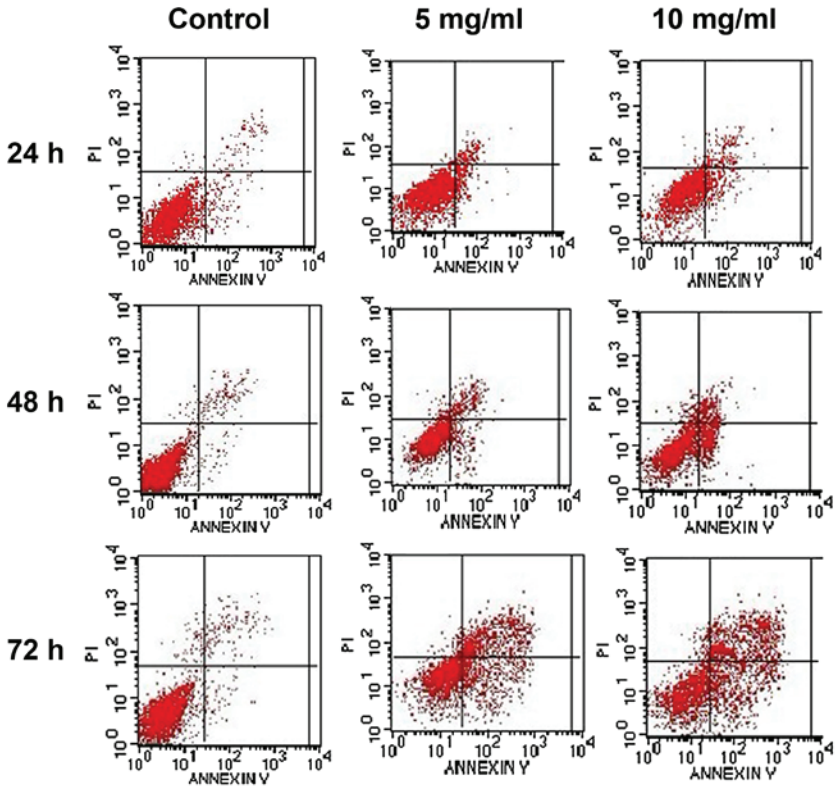

Figure 2. Flow cytometric analysis of *Trametes robiniophila*-induced apoptosis in the gastric carcinoma cell line MKN-45. PI, propidium iodide.

UVP Gel Imaging System. β-actin was used as reference. The expression levels were calculated with Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All the data were expressed as the mean ± standard deviation. Differences between the groups were calculated using one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were conducted using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA).

Results

Cell apoptosis is induced by Trametes robiniophila. Morphological changes in the human GC cell line MKN-45 following treatment with different concentrations of *Trametes robiniophila* (0, 5 and 10 mg/ml) for 24 h are shown in Fig. 1. Compared with the control group, the majority of the *Trametes robiniophila*-treated cancer cells became enlarged and irregular-shaped, and exhibited vacuolated changes in the cytoplasm. These morphological changes demonstrated cell damage subsequent to *Trametes robiniophila* treatment. MKN-45 cells treated with 5 mg/ml *Trametes robiniophila* were yellow-dyed, indicating the occurrence of apoptosis (Fig. 1).

Flow cytometry was used to detect intact cells, early apoptotic cells, late apoptotic cells and dead cells. Following treatment with *Trametes robiniophila*, the cell death rate [as indicated by the upper right (UR) quadrant, which represents the percentage of cells in advanced-stage apoptosis] and the early apoptosis rate [as indicated by the lower right (LR) quadrant, which represents the percentage of cells in prophase apoptosis] of the MKN-45 cell line increased in a time- and dose-dependent manner (Fig. 2).

Table II. Proportion of apoptotic cells in the MKN-45 cell line.

	MKN-45		
Apoptotic cells (%)	24 h	48 h	72 h
Control	6.5	7.3	7.5
5 mg/ml	14.6[a]	18.3[a]	50.2[a]
10 mg/ml	18.0[a]	24.5[a]	58.0[a]

[a]P<0.05 vs. the control group.

Table III. Expression changes in MMP-2, MMP-9, Bcl-2, Fas and caspase-3 genes induced by *Trametes robiniophila* in the MKN-45 cell line.

	MKN-45		
Gene	Control	5 mg/ml	10 mg/ml
MMP-2	0.64±0.02	0.49±0.01[a]	0.36±0.02[a,b]
MMP-9	0.71±0.01	0.54±0.02[a]	0.47±0.02[a]
Bcl-2	1.20±0.06	0.83±0.05[a]	0.64±0.05[a,b]
Fas	0.67±0.06	0.83±0.06[a]	0.90±0.04[a]
Caspase-3	0.65±0.01	0.70±0.03[a]	0.99±0.08[a,b]

[a]P<0.05 vs. the control group; [b]P<0.05 vs. cells treated with 5 mg/ml *Trametes robiniophila*. MMP, matrix metalloproteinase; Bcl, B-cell lymphoma.

Apoptotic cells (as indicated by the sum of the UR and LR quadrants, which represents the percentage of cells in all apoptosis stages) of the MKN-45 cell line were significantly different from those of the control group after being exposed to *Trametes robiniophila* for 24, 48 and 72 h at drug concentrations of 5 and 10 mg/ml (all P<0.001) (Table II).

Trametes robiniophila significantly influences the transcription of MMP-2, MMP-9, Bcl-2, Fas and caspase-3. The expression of MMP-2, MMP-9, Bcl-2, Fas and caspase-3 was significantly influenced by *Trametes robiniophila*. Analysis of the optical density value revealed that, for the MKN-45 cell line, the expression of MMP-2, MMP-9 and Bcl-2 was significantly downregulated (at 5 mg/ml and 10 mg/ml, all P<0.001 vs. control; at 10 mg/ml, P<0.001, P=0.003 and P=0.007 vs. 5 mg/ml for MMP-2, MMP-9 and Bcl-2, respectively), while the expression of Fas and caspase-3 was upregulated (at 5 mg/ml, P=0.326 and P=0.100 vs. control for Fas and caspase-3, respectively; at 10 mg/ml, P<0.001 and P=0.020 vs. control, and P=0.017 and P=0.028 vs. 5 mg/ml for Fas and caspase-3, respectively) (Table III). The regulation was dose-dependent.

Trametes robiniophila significantly influences the protein expression of Bcl-2, Fas and caspase-3. The production of Fas and caspase-3 was enhanced following treatment with *Trametes robiniophila* in the MKN-45 cell line; however, the synthesis of Bcl-2 decreased. These results were consistent with the pattern of RT-PCR validation. The regulation was dose-dependent (Fig. 3).

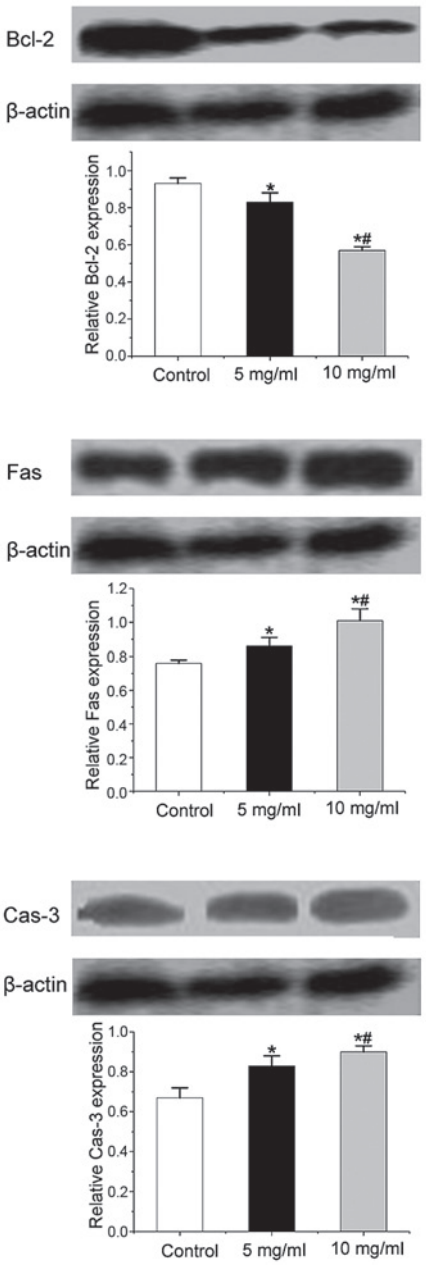

Figure 3. Influence of *Trametes robiniophila* on the protein expression of Bcl-2, Fas and Cas-3 in the gastric carcinoma cell line MKN-45. *P<0.05 vs. the control group; #P<0.05 vs. cells treated with 5 mg/ml *Trametes robiniophila*. Bcl, B-cell lymphoma; Cas, caspase.

Discussion

Generally, treatments for GC patients include surgery, radiotherapy and chemotherapy (17). Certain alternative treatments such as gene therapy and targeted therapy have also been proposed as optional treatment methods (18,19). However, these therapies are usually costly for the majority of patients and have limited curative effect and serious side effects (20). In the past years, TCM, which has been widely used in China for thousands of years, has shown an anticancer potential

effect (21). TCM has been demonstrated to reduce toxic side effects, improve the quality of life of patients, enhance the immune function, and prevent recurrence and metastasis in cancer patients, with few side effects (22). In the current study, AO/EB staining, flow cytometry and RT-PCR were conducted to detect the effect of *Trametes robiniophila* on the apoptosis and metastasis of the human GC cell line MKN-45. The results indicated that *Trametes robiniophila* changed the morphology and number of regular MKN-45 cells in a time- and dose-dependent manner, suggesting cell damage due to treatment with *Trametes robiniophila* (Figs. 1 and 2, Table II). Our results also demonstrated that *Trametes robiniophila* acts as an inducer of the apoptotic process in GC cells by influencing the expression of *Fas*, *caspase-3* and *Bcl-2* (Figs. 2 and 3, Table III). However, the effect was significantly different between 5 and 10 mg/ml. Considering the potential toxic effect of high doses of *Trametes robiniophila* on normal cells, 5 mg/ml should be taken as a reference for future clinical application.

Generally, apoptosis can be classified into two types: Apoptotic process mediated by the mitochondrial pathway and apoptotic process mediated by a membrane receptor signaling pathway (23). Caspase-3 is one of the key apoptosis executors, since the majority of factors that trigger apoptosis ultimately lead to apoptosis through the caspase-3-mediated signaling pathway (24,25). Bcl-2, which is mainly distributed in the mitochondrial membrane and the cytoplasm, is an intracellular anti-apoptotic factor that can stabilize the mitochondrial membrane, prevent mitochondrial caspase release and inhibit the oxygen free radical-induced apoptosis signaling pathway (26). Fas can initiate the apoptotic process mediated by a membrane receptor by binding to Fas ligand, and can also recruit caspase-3 to induce apoptosis (27-30). Treatment with *Trametes robiniophila* suppressed *Bcl-2* expression and increased *caspase-3* expression, which suggested a mitochondrial-mediated apoptosis. Additionally, the expression of *Fas* was also upregulated, indicating activation of the death receptor pathway of apoptosis. The results in the present study demonstrated that *Trametes robiniophila* could induce the apoptosis of GC cells by different mechanisms, whereas previously, only apoptosis induced by *Trametes robiniophila* via the mitochondrial pathway was reported (11).

The apoptosis observed in the present study was associated with downregulation of the expression of *MMP-2* and *MMP-9*. Members of the MMP family are an integral part of the extracellular matrix's enzymatic arsenal and have been regarded as major critical molecules that assist tumor cells during metastasis (31). The expression of various MMPs has been observed to be upregulated in almost every type of human cancer, and correlates with advanced stage, invasive and metastatic properties, and, in general, with poor prognosis (32). Upon treatment with *Trametes robiniophila*, the expression of *MMP-2* and *MMP-9* was suppressed. The effect was dose dependent. Ji and Mai have revealed that the expression level of *MMP-2* and *MMP-9* was positively correlated with the metastatic ability of GC cells (33). In addition, a previous study has reported the inhibition of metastasis of lung cancer via the suppression of *MMP-2* and *MMP-9* (22). Therefore, *Trametes robiniophila* has the potential to regulate MMPs and can be considered a promising target for the therapeutic intervention in human cancer. Similarly to the majority of TCMs, *Trametes robiniophila* also has the advantage of few side effects and low cost compared with surgery, radiotherapy and chemotherapy (34).

Based on the present study, treatment with *Trametes robiniophila* could markedly induce the apoptosis of the human GC cell line MKN-45. The effect acted through both the mitochondrial and the member receptor signaling pathways. In addition, *Trametes robiniophila* could also suppress the metastatic ability of GC cells via downregulating the expression of *MMP-2* and *MMP-9*. In conclusion, we recommend that *Trametes robiniophila* is taken into consideration as a noninvasive therapy of GC in future clinical treatment.

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