The herb medicine formula “Yang Wei Kang Liu” improves the survival of late stage gastric cancer patients and induces the apoptosis of human gastric cancer cell line through Fas/Fas ligand and Bax/Bcl-2 pathways☆

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Abstract

The herb medicine formula “Yang Wei Kang Liu” (YWKLF) has been used to inhibit the metastasis of human gastric cancer to prolong patient survival. In this study, we evaluated the effect of combination of chemotherapy with YWKLF on the survival of stage IV gastric cancer patients and the potential mechanisms of YWKLF by focusing on its capacity to activate apoptotic pathways in human gastric cancer cell line MGC-803. We found that combination of chemotherapy with oral administration of YWKLF significantly increased the survival of stage IV gastric cancer patients. In an approach of "serum pharmacology" in which sera were collected from rabbits orally administered with YWKLF and examined for their anti-tumor cell activity in vitro, we observed that sera from rabbits administered with YWKLF induced the apoptosis of MGC-803 cells by causing the loss of mitochondrial membrane potential, increasing the expression of Fas protein and Bax mRNA, as well as down-regulating Fas-L mRNA. Our results suggest that activation of major pro-apoptotic pathways may account for the anti-gastric cancer activity of YWKLF, which may provide a basis for isolation and identification of more highly effective anti-cancer components.

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KEYWORDS
Herb medicine;
Serum pharmacology;
Apoptosis;
Gastric carcinoma;
Bax/Bcl-2;
Fas/Fas ligand

Abbreviations: YWKLF, formula "Yang Wei Kang Liu"; Sub-F1, Sub-Formula 1; Sub-F2, Sub-Formula 2; Sub-F3, Sub-Formula 3; AgNORS, argyrophilic nucleolar organizer regions; AO, acridine orange; Δψm, mitochondrial membrane potential.

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1. Introduction

Gastric cancer is one of the most common malignant tumors in China [1]. Facing the problems of lower rate of early diagnosis, higher possibility of relapse and metastasis after surgery, and poor sensitivity to radiotherapy or chemotherapy, herb medicine plays a role in preventing gastric cancer from relapse and metastasis [2]. A herb medicine formula “Yang Wei Kang Liu” (YWKLF), consisting of six herbs, has been used for a number of years in traditional medicine to promote circulation and human body resistance to pathogens and cancer. Preliminary clinical studies have shown that YWKLF modulates immune function, improves the quality of life, and prolongs survival time of cancer patients [3]. However, the molecular mechanisms underlying the anti-cancer effect of YWKLF are poorly understood.

Apoptosis is an important component of various biological processes, such as development, maintenance of tissue homeostasis and elimination of transformed cells. The aberrant regulation of apoptosis contributes to many types of human diseases including cancer. Apoptosis uses two main signaling mechanisms: the "extrinsic" and the "intrinsic" pathways, to trigger programmed death of cells. The Fas–Fas ligand (FasL) pathway is the most studied in the cells through extrinsic pathway [4–6]. The Bcl-2 family of proteins constitutes a critical intrinsic checkpoint coupled to cell apoptosis. The ratio of anti- to pro-apoptotic molecules, such as Bax/Bcl-2, sets the threshold of cell susceptibility to apoptosis of the Bax/Bcl-2 pathway, which utilizes organelles such as mitochondria to amplify death signal [7].

In the present study, we report the effect of combination of YWKLF with chemotherapy on prolonging survival of stage IV gastric cancer patients. In addition, we report that sera from rabbits administered with YWKLF promote the apoptosis of human MGC-803 gastric cancer cells through Fas/Fas ligand and Bax/Bcl-2 pathways.

2. Materials and methods

2.1. Patients and the treatment

A total of 123 patients with stage IV gastric cancer were enrolled during the period of 1990 to 1997. The study was permitted by Institutional Review Board (IRB), and informed consents (IC) were obtained from the patients before they were recruited into this study. All patients had Karnofsky performance status >60 and showed adequate hematological, liver and renal functions. Patients were recruited to YMKLF + chemotherapy and chemotherapy alone cohorts by random number tables with informed consent. Patients assigned to chemotherapy group received as MFP chemotherapy (cisplatin 60 mg/m2 on day 1, 5-FU 500 mg/m2/day, on day 1–5 and MMC 6 mg/m2/day, on day 1, 8) every 4 weeks, for 4 cycles. In chemotherapy + YWKLF group: patients received MFP chemotherapy in combination with oral administration of herbal medicine to promote circulation and human body resistance to pathogens and cancer. Preliminary clinical studies have shown that YWKLF modulates immune function, improves the quality of life, and prolongs survival time of cancer patients [3]. However, the molecular mechanisms underlying the anti-cancer effect of YWKLF are poorly understood.

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2.2. Cells and reagents

Human gastric cancer cell line MGC-803 was purchased from Cell Culture Centre, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and cultured in RPMI 1640 (GIBCO/BRL) containing 10% fetal bovine serum (FBS). Rabbit anti-human Fas and FasL polyclonal antibodies and FITC-labeled goat anti-rabbit IgG antibody were obtained from Santa Cruz Corporation (Beijing, China). Annexin V-FITC Apoptosis Detection Kit was from BD Corporation (Shenzhen, China). TRIzol Reagent was purchased from Invitrogen/GIBCO (Beijing, China). Reverse Transcriptase, Taq DNA polymerase and other reagents used in reverse transcription PCR (RT-PCR) were obtained from Promega Corporation (Beijing, China).

2.3. Animals

Japanese flap-eared rabbits were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (CAMS) (Beijing, China) and maintained in the animal facility approved by institutional policies.

2.4. Formula preparation

YWKLF is composed of Astragalus mongholicus Bge (450 g), Panax notoginseng (Burk.) F. H. Chen (40 g), Panax ginseng C. A. Mey (100 g), Paris polyphylla Smith var. chinensis (Franch.) Hare (150 g), Caesalpinia sappan L. (60 g) and Oldenlandia diffusa (Willd.) Roxb (180 g). Based on the principles of herb action, YWKLF was sub-formulated into Sub-Formula1 (Sub-F1): Astragalus mongholicus Bge (450 g), Panax notoginseng C. A. Mey (100 g), that improve the general body conditions [11]; Sub-Formula 2 (Sub-F2): Panax notoginseng (Burk.) F. H. Chen (40 g) and Caesalpinia sappan L (60 g), that improve circulation [12–14]; and Sub-Formula 3 (Sub-F3): Paris polyphylla Smith var. chinensis (Franch.) Hare (150 g) and Oldenlandia diffusa (Willd.) Roxb (180 g), that contain anti-oxidants [15]. All medicinal plants used to prepare formulae were provided by Guang An Pharmacy (Beijing, China), the species, plant parts, and origin used in the formula as Table 1. The plant were homogenized with a Waring blender, then soaked in 12 Liter double distilled water (DDW) for 24 h. The mixture was heated to 100 °C for 1 h, and the decoction was filtrated. The filtrates obtained from 3 cycles of the procedures were mixed, concentrated by heating and granulated by lyophilization. Total

YWKLF prolonged the overall survival time of stage late gastric cancer patients
yield of the YWKLF extract is 125 g lyophilized powder from water extract of 1 kg raw mixed herb. Aqueous solution was prepared by dissolving the granulated formulae in sterile water for animal administration at 1.63 g raw mixed herb/ml for YWKLF, 0.92 g raw mixed herb/ml for Sub-F1, 0.17 g raw mixed herb/ml for Sub-F2 and 0.55 g raw mixed herb/ml for Sub-F3. The quality control on YWKLF preparation, including definition of the correct plants, origin of production, implantation, harvesting, and processing. Good manufacturing practice has been used to produce lyophilized particles. Contamination of hazardous agents such as pesticide residues, arsenic and heavy metals, microorganisms including aflatoxins, and sulfur dioxide residue were excluded. Consequently, YWKLF and its preparations were standardized, regulated and quality controlled according to the guidelines defined by Chinese State Food and Drug Administration (SFDA).

2.5. Method of “serum pharmacology”

“Serum pharmacology” has been used to study the in vitro pharmacological activity of herb medicine. The preparation of “serum pharmacology” was based on the described procedures [16]. Briefly, 18 rabbits were randomly divided into six groups and administered by gavage of YWKLF, Sub-F1, Sub-F2 and Sub-F3. The rabbits of saline and chemotherapy groups were fed with 10 ml saline twice daily for 3 days per rabbit. YWKLF and its sub-formula groups were treated with 10 ml YWKLF or a sub-formula twice daily for 3 days per rabbit. The formula dose was estimated as 10 times of doses given to patients in the clinic. Two hours after the fifth treatment on the third day, blood was obtained from aorta of the rabbits under sterile conditions, and was allowed to coagulate at 25 °C for 4 h. The sera were separated by centrifugation at 2500 rpm for 20 min. After filtration through 0.45 μm cellulose acetate membrane twice, the sera were treated in 56 °C water bath for 30 min then were stored at −20 °C until use.

2.6. Cell viability assay by MTT

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (Sigma, USA). MGC-803 cells were seeded at a density of 1×10^4 cells per well into 96-well plates in RPMI 1640 containing 10% FBS. After 24 h, the cells were washed and further cultured in the following media: the saline groups: RPMI 1640 containing 10%, 20% and 30% sera from rabbits treated with saline; the YWKLF groups: RPMI 1640 containing 10%, 20% and 30% sera from rabbits treated with herb formulae; the chemotherapy groups: 10%, 20% and 30% sera from rabbits treated with chemotherapy. The cells were incubated in 5% CO₂ at 37 °C for 48 h, then with 5 mg/ml MTT at 10 μl/well for 4 h. The culture media were discarded and substituted with DMSO at 100 μl/well. The plates were mounted on a micro-mixer for 5 min to thoroughly dissolve the blue MTT/DNA granules. The plates were then placed in a microplate reader. The OD was measured at 570 nm. The inhibition rate was calculated according to the following formula:

\[
\text{Inhibition rate } (\%) = 100\% \times \left(1 - \left(\frac{\text{mean absorbance of cells in sera from drug treated rabbits}}{\text{mean absorbance for cells in sera from rabbits treated with saline}}\right)\right)
\]

2.7. Morphometrical study of argyrophilic protein associated with the nucleolar organizer regions (AgNORs) in MGC-803 cells

After 24 h treatment with rabbit sera, 200 μl cell suspensions was loaded into a cytopsin chamber and spun for 10 min at 300 rpm. Slides were fixed by a mixture of methanol and glacial acetic acid at 3:1 vol/vol. The silver colloid solution for AgNORs staining was prepared by dissolving gelatin in 1% aqueous formic acid to a concentration of 2%, and this solution was mixed at 1:2 vol/vol with 50% aqueous silver nitrate in which slides were immersed for 45 min at 20 °C in dark [17,18]. After rinsing in H₂O, the slides were immersed in 5% sodium thiosulfate for 45 min at room temperature. Counterstaining was carried out using methyl green.

The parameters of nucleolin and AgNOR were assessed by an image analysis system (KL-2, Beijing). The stained dots of only one focal plane per nucleus, scanned from light microscopy image using a ×100 oil-immersion lens, were converted via a TV camera to appear as dots on a high-resolution monitor. The mean number and area of stained dots were measured using an image analyzer. Measurements were made for 150 cells in random areas of each specimen. The morphometric parameters were means of areas and numbers of AgNORs per nucleus and the ratio of AgNORs area to nucleolin/nucleus area per cell.

2.8. Detection of cell apoptosis by flow cytometry

MGC-803 cells seeded in 6-well plates at 10^5/well were cultured in the presence of sera from rabbits received saline or formulae for 24 h. The cells were then washed and fixed in 70% ethanol at 4 °C overnight. The cells were then re-suspended in 10 μl propidium iodide (PI) solution...
(2 mg/ml) with 50 μl RNAase (10 mg/ml) and incubated in the dark at 37 °C for 30 min, followed by DNA content analysis using a FACSsort flow cytometer (Becton Dickinson). The CellQuest program was used to analyze the results. Different phases of cell cycle were assessed by collecting the signal at channel FL2-A. The percentage of the cell population at a particular phase was estimated by ModFit LT.

Annexin V-FITC/PI staining was performed after the cells were treated with rabbit sera for 24 h in vitro. Rabbit sera treated cells (2×10^6 / group) were rinsed with ice-cold PBS and then re-suspended in 200 μl of reaction buffer. Annexin V stock solution (10 μl) was added to the cells and incubated for 30 min at 4 °C. The cells were further mixed with 5 μl PI and immediately analyzed on a FACSsort equipped with CellQuest software.

2.9. Assessment of morphological changes by acridine orange (AO) staining

Cell slides were fixed in 95% ethanol for 10 min on ice and air dried for 10 min. The slides were stained with AO (6 μg/ml; sigma), then examined by laser scanning confocal microscopy (Radiance2000, BIO-RAD) using a 488 nm argon laser excitation beam, a 515 nm beam filter for DNA, and a 630 nm beam filter for RNA. Apoptotic cells were characterized by morphological alterations such as condensed nuclei and cell shrinkage. AO is an intercalating, nucleic acid specific fluorochrome, which emits green fluorescence when bound to DNA. Under fluorescence microscopy, viable cells show bright green nuclei with intact structure while apoptotic cells exhibit bright green nuclei with condensed chromatin in dense green areas. Three to five randomly chosen fields were viewed in each slide with a minimum of 300 cells for each group.

2.10. Analysis of mitochondrial membrane potential

Mitochondrial membrane potential (Δψm) was assessed by rhodamine 123, a mitochondrial potential sensor (Molecular Probes). Cells were seeded in six-well plates for 6 h. The medium of each well was discarded and treated with 1 ml of medium containing rhodamine 123 at 25 μM for 30 min at 37 °C and 5% CO2 in the dark. The cells were washed three times in PBS, then were analysed analyzed by flow cytometry.

2.11. Fas and FasL detection by flow cytometry

Sera-treated cells washed twice in PBS buffer were incubated with a rabbit anti-human Fas antibody or a rabbit anti-human FasL antibody at 4 °C for 30 min. FITC-labeled goat anti-rabbit IgG antibody was then added to cells for 30 min at 4 °C. Flow cytometric analysis was performed using a Becton Dickinson FACSsort and CellQuest software. A total of 10,000 events were acquired for each analysis.

2.12. Detection of FasL, Bax, Bcl-2 mRNA in MGC-803 cells by RT-PCR

After rabbit sera treatment for 24 h, MGC-803 cell RNA was extracted with TRizol Reagent, and used for reverse transcription RT-PCR. For PCR amplification, specific primers used were as follows: FasL (299 bp): 5′-GGAGCTGGCAGAACTCAGGAG-3′ and 5′-GGCTCAGGGCGAGGTTGTTG-3′; Bax (312 bp): 5′-GGCTCACCAGAAAGCTGATCC-3′ and 5′-ACCACCTGGTCTTTGGATCC-3′; Bcl-2 (231 bp): 5′-CAGCTGACGTCGACCCCTT-3′ and 5′-GCCTCAGGTATGGATCC-3′; β-actin primers: 5′-CGTCTGGACCTGGCTGGCCGG-3′ and 5′-CTAGAAGCATTTGCGGTGGACGATG-3′ (600 bp). The PCR conditions for FasL were: denaturation at 94 °C for 50 s, annealing at 63 °C for 50 s, and extension at 72 °C for 1 min, 40 cycles. For Bcl-2 and Bax: denaturation at 94 °C for 50 s, annealing at 62 °C for 50 s, and extension at 72 °C for 1 min, 35 cycles. The PCR products were separated by electrophoresis on 2% agarose gels which were stained with ethidium bromide.

2.13. Statistical analysis

All in vitro experiments were performed for at least 3 times and the mean±SD of representative results are presented. Significant differences among the groups were determined using two way analysis of variance. Comparison of the rate of the patient survival was analyzed by Kaplan–Meier survival analysis method. P<0.05 was considered as an indication of statistical significance.

<table>
<thead>
<tr>
<th>Table 2 Clinical characteristics of patients with stage IV gastric cancer</th>
<th>Chemotherapy</th>
<th>Chemotherapy + YWKLF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of patients</td>
<td>46</td>
<td>77</td>
</tr>
<tr>
<td>Sex: male/female</td>
<td>35/11</td>
<td>59/18</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>32</td>
<td>53</td>
</tr>
<tr>
<td>Mucinous adenocarcinoma</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Disease status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relapse after surgery</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>Bypass surgery</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Laparotomy</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>No surgery</td>
<td>12</td>
<td>19</td>
</tr>
</tbody>
</table>
3. Results

3.1. The effect of combination therapy of YWKLF with chemotherapy on the survival of stage IV gastric cancer patients

77 patients were assigned to treatment with chemotherapy plus YWKLF and 46 patients were assigned to chemotherapy alone. The background factors of patients as Table 2. There is no statistically significant difference of the background in the patients between chemotherapy alone and chemotherapy with YWKLF cohorts in terms of age, sex and tumor histology. The rate of response (RR) as reduction by at least 50% in the size of primary tumor in 46 patients receiving chemotherapy alone was 13.04% (6/46), and 33.77% (26/77) of patients receiving chemotherapy and YWKLF showed responses. The median survival time was 14 months for chemotherapy alone and 21.5 months for YWKLF + chemotherapy patients (P < 0.001) (Fig. 1). These results indicate the advantage of combination of chemotherapy with YWKLF in prolonging the survival of stage IV gastric cancer patients over chemotherapy alone.

3.2. The effect of YWKLF rabbit serum on MGC-803 cell viability

We next examined the effect of YWKLF on the biological behavior of human gastric cancer cells. Pervious studies of serum pharmacology suggested an eight to eighteen times of drug doses should be needed to administer into rabbits as compared to humans. Based on our clinical data and preliminary experiments, we chose a rabbit administration dose that equaled to 10 times of doses given to patients. Administration with herbal mixtures did not cause the loss of body weight, decreased activity, and change in fur quality of the rabbits (data not shown). By using “serum pharmacology”, we harvest sera from rabbits administered with YWKLF and sub-formulae and tested their effects on the growth of the MGC-803 human gastric cells in vitro. As shown in Fig. 2A, after treatment for 48 h, with serum from rabbits received YWKLF, MGC-803 cells showed significantly reduced rate of growth. The maximal cell growth inhibition was observed when serum from YWKLF rabbits was used at 20% concentration in the cell culture with slightly decreased effect at 30% concentration reflecting biological responses seen in many drug experiments. The results also suggest that serum harvested 2 h after last gavage administration of YWKLF was optimal in inhibiting cell growth. When sera from rabbits administered with sub-formulae were tested, Sub-F1 and Sub-F3 sera showed significant inhibition of cell growth, although the efficacy was lower than the intact formula serum. In contrast, Sub-F2 serum showed little inhibitory activity on cell growth. Thus, components in both Sub-F1 and F3 contributed to the overall activity of YWKLF in “serum pharmacology” test. It should be noted that YWKLF and its sub-formulae did not affect the viability of normal gastric cells in primary culture or endothelial cells (data not shown).

3.3. YWKLF serum inhibited AgNORs activity in MGC-803 cells

The levels of argyrophilic nucleolar organizer regions (AgNORs) represent cell cycle kinetics and the shorter the cell cycle, the greater rate of the synthesis of rRNA, therefore, the increased quantity of AgNORs present in the nucleoli. Thus, AgNOR value was used to measure the rate of cell proliferation. Previous
studies showed a linear increase in both the area and the number of AgNOR stainings in each tumor cell with increasing degree of malignancy [18]. We found AgNORs as distinct black–brown dots on a yellow pale background (Fig. 3A), and the black–brown dots in the YWKLF serum treatment cell group were smaller than cells treated with serum from saline administered rabbits (Fig. 3B). The ratio of AgNORs area to nucleolin/nucleus area per cell (I/S) is significantly decreased in cells treated by YWKLF serum and saline serum containing cisplatin (⁎P < 0.05). Sub-F2 and Sub-F3 sera did not affect I/S in MGC-803 cells, while Sub-F1 serum decreased I/S in MGC-803 cells, but with the efficacy lower than YWKLF serum (Fig. 3C). Thus, YWKLF rabbit serum inhibited MGC-803 cell proliferation as shown by reduced AgNORs activity in the nuclei.

3.4. YWKLF rabbit serum induced MGC-803 cell apoptosis

We evaluated sub-diploid DNA content (sub-G1) in MGC-803 cells using flow cytometry. As shown in Fig. 4A, YWKLF rabbit serum treatment resulted in an increase in the sub-G1 phase of MGC-803 cells after 24 h (19.58 ± 2.1% versus 2.66 ± 1.2% cells with saline rabbit serum). We did not observe significant effect on sub-G1 peak by Sub-F2 and Sub-F3 sera (Fig. 4A). However the sub-G1 peak in cells treated by Sub-F1 rabbit serum was 12.36 ± 2.3%, lower than YWKLF serum, but the effect was statistically significant. The number of cells in the G2/M phase was significantly decreased after treatment with YWKLF rabbit serum for 24 h with an increase in S-phase cell number. These results indicate MGC-803 cell cycle arrest at S phase after treatment with YWKLF rabbit serum (Fig. 4B). The chemotherapy agent cisplatin has been reported to increased the fraction of S-phase cells in prostate cancer cells [19] and induced S-phase arrest of human ovarian carcinoma cells [20]. In this study, Fig. 4A shows cisplatin (2 μg/ml) in saline rabbit serum induced S-phase arrest of MGC-803 cells after 24 h treatment, with an increase in cells in apoptotic sub-G1 phase (25.86 ± 3.1%). These results that indicate YWKLF rabbit serum was able to prevent gastric cancer cell division.

We then evaluated MGC-803 cell apoptosis by using annexin V-FITC and PI staining. Fig. 4C showed that YWKLF rabbit serum treated cells increased Annexin V+/PI− staining. In contrast, cisplatin in saline rabbit serum induced Annexin V+/PI+ cells. Cisplatin caused both the early and late stage apoptosis in gastric cancer cells, while YWKLF rabbit serum preferentially induced early stage cancer cell apoptosis. Sub-F2 and Sub-F3 rabbit sera did not increase Annexin V+/PI− and Annexin V+/PI+ cells (Fig. 4C).

We further determined morphological changes in MGC-803 cells by confocal scanning laser microscopy. Fig. 5A-1 shows the

Figure 3  Argyrophilic nucleolar organizer regions (AgNORs) in MGC-803 cells. A. Cells cultured in the presence of 20% serum from rabbits given saline. B. Cells cultured in the presence of 20% serum from rabbits given YWKLF. X axis indicates cell numbers, Y axis defines I/S%. C. The ratio of AgNORs area to nucleolin/nucleus area per cell. A total of 150 random cells were counted under a light microscope (×400). *P < 0.05 compared with cells cultured with serum from rabbits given saline.
change in gastric cancer cell morphology after exposure to saline-treated rabbit serum, which failed to induce apoptosis. Meanwhile, MGC-803 cells showed features of apoptosis, including cell shrinkage, nuclear condensation, DNA fragmentation, break down of the cells and formation of apoptotic bodies, after 24 h exposure to YWKLF rabbit serum and Sub-F1 rabbit serum (Fig. 5A). After YWKLF rabbit serum treatment, the nuclear structure of MGC-803 cells became incomplete (Fig. 5C), supporting the notion that YWKLF rabbit serum induced cell apoptosis.

3.5. YWKLF rabbit serum induced mitochondrial dysfunction in tumor cells

Mitochondria play a critical role in apoptosis induced by cancer therapeutic agents [21,22], which cause mitochondrial membrane potential (Δψm) loss and cytochrome c release into the cytosol, inducing caspase-9-dependent activation of caspase-3 and cleavage of DNA reparatory protein PARP. We examined the effect of YWKLF rabbit serum on Δψm using rhodamine 123, a mitochondrial potential sensor. A marked loss of Δψm was observed after exposure of MGC-803 cells to YWKLF rabbit serum for 4 to 24 h. The Sub-F2 and Sub-F3 rabbit sera did not affect cell Δψm, whereas, Sub-F1 rabbit serum caused Δψm loss in cancer cells at the levels similar to YWKLF rabbit serum (Fig. 6).

3.6. Fas/Fasl expression in MGC-803 cells

The Fas–FasL interaction is a key physiological regulator of programmed cell death [23]. We investigated the expression of Fas/Fasl in MGC-803 cells by flow cytometry and found that...
Figure 5  Changes in nuclear shape in MGC-803 cells. A. The change of gastric cancer cell morphology after exposure to sera in the fluorescent microscope: 1, saline serum treated cells; 2, YWKLF serum treated cells; B. YWKLF serum treated cells: 1, green fluorescence indicating DNA; 2, red fluorescence indicating RNA; 3, superimposed DNA and RNA fluorescence. Sub-F1 serum treated cells: 4, DNA in green fluorescence; 5, RNA in red fluorescence; 6, superimposed DNA and RNA fluorescence. C. Changes in the fluorescence intensity of nuclei: saline serum treated cells: 1, cell with normal morphology; 2, topographic map of nuclear DNA fluorescence distribution. YWKLF serum treated cells: 3, DNA fragmentation and formation of apoptotic bodies. 4, incomplete nuclear structure.
treatment of the cells by YWKLF rabbit serum and Sub-F1 rabbit serum increased Fas expression by two fold (Fig. 7). The FasL expression on MGC-803 was low and was not significantly changed by any treatment (Fig. 7).

Since recent studies have suggested that resistance to apoptosis is associated with the loss of Fas function in several malignancies [24–27], up-regulating Fas expression on MGC-803 cells by YWKLF rabbit serum may increase cell sensitivity to FasL killing.

3.7. FasL and Bax/Bcl-2 mRNA in MGC-803 cells

RT-PCR showed that the level of FasL mRNA in MGC-803 declined significantly after treatment by YWKLF rabbit serum for 24 h. However, the level of Bax mRNA was markedly increased (Fig. 8). There was no quantitative difference in Bcl-2 mRNA levels in cells after rabbit sera treatment (Fig. 8). Sub-F1 rabbit serum also markedly increased the level of Bax mRNA in MGC-803 cells.

4. Discussion

Alternative medicine has been considered as a complementary therapy for late stage gastric cancer for a number of years in Southeast Asia because the lack of effective means to prolong the life span of patients with this deadly tumor type [28–31]. In this study, we provided evidence that combination of chemotherapy with herbal medicine formula YWKLF prolonged the overall survival time of stage IV gastric cancer patients. Although the therapeutic regime still requires multi-centered clinical evaluation with lager patient cohorts, YWKLF may represent a basis on which specific herb components possessing anti-cancer effect could be identified by further screening.

Medicinal herbs constitute challenges to clinicians and basic researchers for their complexity and the potential of active components generated after metabolism in vivo. It is now realized that adding herb extracts to cancer cell culture may not be able to better evaluate the pharmacological activity of the herbs. Therefore, "serum pharmacology" has recently been proposed and utilized in Southeast Asian, in which sera collected from small animals administered with herb extracts would retain the major components of the extracts and also potential metabolites of the components [10]. Based on the "serum pharmacology" principle, in this study, we used sera from rabbits administered YWKLF and its sub-formulae to show that YWKLF rabbit serum significantly inhibited the growth of human gastric cancer cells in vitro by triggering the apoptosis signaling pathways. These findings were supported by the induction of tumor cell cycle arrest, loss of mitochondrial membrane potential and increased expression of Fas and Bax in tumor cells. Previous studies indicate that gastric carcinomas express Fas ligand but low level Fas to escape killing from host immune system. Clinical data also showed that apoptosis in gastric carcinoma may be an independent indicator of better prognosis [32]. This apoptosis...
is associated with tumor Fas expression. Thus up-regulating Fas expression on MGC-803 cells by YWKLF rabbit serum may increase gastric cancer cell sensitivity to apoptotic killing by FasL positive cells (such as T lymphocytes).

YWKLF is composed of six herbs that include 3 medicinal principles, and on this basis, we divided the formula into 3 sub-formulae, and tested the effect of each sub-formula on generation of rabbit serum that may affect the biological behaviour of tumour cells. We found Sub-F1 composed of herbs that strengthen the host defense [11], also inhibited MGC-803 cell proliferation and induced apoptosis, albeit with lower efficacy as compared with the intact YWKLF. Therefore, combination of therapeutic principles represented by multiple herbs may yield better results in cancer treatment.

In-depth studies are needed to clarify the components contained in rabbit sera that induce the apoptosis of gastric cancer cells. The "serum pharmacology" preserves that some herb components will be retained in sera in their original or metabolized form [9]. In fact, some components of the YWKLF and metabolites have been showed to be capable of strengthening the host defense [11,37,41], also inhibited MGC-803 cell proliferation and induced apoptosis, albeit with lower efficacy as compared with the intact YWKLF. Therefore, combination of therapeutic principles represented by multiple herbs may yield better results in cancer treatment.

Table 3 Major components contained in YWKLF with known biological activities

<table>
<thead>
<tr>
<th>Herb</th>
<th>Known components</th>
<th>Biological activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astragalus mongholicus Bge</td>
<td>Polysaccharides</td>
<td>Stimulation of cell-mediated immunity; improvement of cardiovascular and neuroendocrine systems; anti-oxidation; inhibition of tumor growth; anti-inflammation</td>
<td>[11,37,41]</td>
</tr>
<tr>
<td>Panax ginseng C. A. Mey</td>
<td>More than forty kinds of saponin (ginsenosides Rg3, Rh2, Rb1, Rb2, etc)</td>
<td>Anti-allergy; anti-tumor; improvement of learning and memory anti-aging</td>
<td>[11,33,38]</td>
</tr>
<tr>
<td>Panax notoginseng (Burk.) F. H. Chen</td>
<td>Ginsenosides Rb1, Rg1, Rd; Notoginsenoside R1,Rb1, Re, Rg1 and Rh1; Dencichine, flavonoid and polysaccharide</td>
<td>Anti-thrombosis; anti-atherosclerosis; anti-inflammation; liver and kidney protection; immunological adjuvant activity and immunostimulatory action; anti-tumor; chemopreventive effect</td>
<td>[12,13,39]</td>
</tr>
<tr>
<td>Caesalpinia sappan L</td>
<td>Triterpenoids, Flavonoids, Phenolics, Steroids, Brazilian</td>
<td>Anti-oxidation; anticonvulsant; anti-complement; anti-inflammation</td>
<td>[14]</td>
</tr>
<tr>
<td>Oldenlandia diffusa (Willd.) Roub.</td>
<td>Ursolic acid, Oleanolic acid, Iridoid glucosides flavone</td>
<td>Anti-cancer; improvement of immune function; anti-oxidation</td>
<td>[15,36,40]</td>
</tr>
<tr>
<td>Paris polyphylla Smith var. chinensis (French) Hare</td>
<td>Steroidal saponins, Diosgenin, Pennogenin</td>
<td>Anti-cancer; analgesic; support immune function</td>
<td>[34,35]</td>
</tr>
</tbody>
</table>

Despite abundance of evidence for the capacity of herbal components to inhibit tumor cell growth, it remains to be elucidated whether in "serum pharmacology" the herbal formula might induce host factors such as TNF α or other cytostatic molecules that could be "indirectly" suppress cancer cells [41]. Also in human, it is unknown whether herbal formula acts directly on tumor cells through absorption by gastrointestinal tract or indirectly through mobilizing the host defense system or both. Our present study calls for more thorough investigation into the mechanisms of herbal medicine and its potential in the treatment of cancer and other human diseases.

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References
