

Antitumor effects of Andrographis via ferroptosis-associated genes in gastric cancer

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Abstract. The overall prognosis of advanced/metastatic gastric cancer (GC) remains poor despite the development of pharmacotherapy. Therefore, other treatment options, such as complementary and alternative medicine, should be considered to overcome this aggressive malignancy. Andrographis, which is a generally unharmed botanical compound, has gained increasing interest for its anticancer effects in multiple malignancies via the regulation of cancer progression-associated signaling pathways. In the present study, a series of *in vitro* experiments (cell proliferation, colony formation and apoptosis assays) was designed to elucidate the antitumor potential and mechanism of Andrographis in GC cells. The present study demonstrated that Andrographis exerted antitumor effects in GC cell lines (MKN74 and NUGC4) by inhibiting proliferation, reducing colony formation and enhancing apoptotic activity. Furthermore, it was demonstrated that the expression levels of the ferroptosis-associated genes

heme oxygenase-1, glutamate-cysteine ligase catalytic and glutamate-cysteine ligase modifier were significantly upregulated after Andrographis treatment in both GC cell lines in reverse transcription-quantitative PCR experiments ($P<0.05$); this finding was further confirmed by immunoblotting assays ($P<0.05$). In conclusion, to the best of our knowledge, the present study was the first to demonstrate that Andrographis possessed antitumor properties by altering the expression levels of ferroptosis-associated genes, thereby providing novel insights into the potential of Andrographis as an adjunctive treatment option for patients with metastatic GC.

Introduction

Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer-related death worldwide (1). Although significant advancements in therapeutic strategies have been achieved, and several chemotherapeutic drugs, such as fluoropyrimidines (5-fluorouracil, S-1, and capecitabine), cisplatin, oxaliplatin, taxanes, and irinotecan, and molecular targeted drugs (trastuzumab or ramucirumab), have improved the treatment of metastatic GC patients (2-5), the overall prognosis of advanced/metastatic GC remains dismal (6,7), and the management of this disease is challenging.

In this context, complementary and alternative medicine, especially dietary compounds, has gained economic and sociological importance because of its cost-effectiveness and reduced toxicity (8-10). Over the past three decades, nearly 100 natural products or direct derivatives from natural remedies have been highlighted in the area of cancer therapy (11). Interestingly, most of these dietary botanicals function by targeting multiple cancer-associated pathways to exert anti-tumorigenic effects on cancer progression (11). Andrographolide is a C20 diterpenoid lactone, which is an active ingredient derived from the traditional Chinese herbal medicine *Andrographis paniculate* (12-14). Because of its ability to circulate in the bloodstream (15-17), Andrographis

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Abbreviations: GC, gastric cancer; FBS, fetal bovine serum; h, hours; ROS, reactive oxygen species; HMOX1, heme oxygenase-1; GCLC, glutamate-cysteine ligase catalytic; GCLM, glutamate-cysteine ligase modifier; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

Key words: GC, Andrographolide, ferroptosis, HMOX1, GCLC, GCLM

exhibits diverse biological activities, such as anti-inflammatory, antiviral, and immunomodulatory effects (18,19). Furthermore, accumulating evidence has shown that Andrographis has anti-tumorigenic properties in multiple malignancies, such as melanoma, leukemia, glioblastoma, breast, lung, esophageal, colorectal, bladder, pancreatic, and liver cancer (14,20-29). Its various underlying mechanisms include the regulation of oxidative stress, apoptosis, necrosis, autophagy, inhibition of cell adhesion, proliferation, migration, invasion, and angiogenesis (13,14,30,31). Moreover, Andrographis influences several cancer-associated and angiogenesis signaling pathways, such as PI3K/AKT/mTOR (20,24), SRC/MAPKs/AP-1 (25), TLR4/NF- κ B/MMP-9 (26), and VEGF/VEGFR2/AKT (29).

A previous study showed that Andrographis enhanced tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in GC cells (32). However, other mechanistic pathways of anti-tumorigenic events induced by Andrographis have not been fully elucidated in GC. Therefore, in this study, we conducted a series of experiments in GC cells and demonstrated that Andrographis exerts its anti-tumorigenic effects via a novel mechanism. Our study indicates that Andrographis may be a potential therapeutic or adjunct option for GC patients.

Materials and methods

Cell culture and materials. The GC cell lines MKN74 and NUGC4 were provided by the Cell Resource Center of Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). All cell lines were authenticated using a panel of genetic and epigenetic markers and tested for mycoplasma regularly. The cells were cultured in RPMI-1640 medium (Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS; Biowest) and an antibiotic-antimycotic mixed stock solution (Nacalai Tesque) and maintained at 37°C in a humidified incubator at 5% CO₂. Andrographis (EP80 Andrographis extract standardized to 80% andrographolide content, dissolved in DMSO) was purified by EuroPharma-USA and kindly provided by Professor Ajay Goel at the City of Hope Comprehensive Cancer Center. Andrographis was diluted to appropriate experimental concentrations in culture medium.

Cell viability and proliferation assays. For WST assays, cells were plated in 96-well tissue culture plates (TPP Techno Plastic Products AG) at a density of 5,000 cells/well in RPMI-1640 medium supplemented with 10% FBS and antibiotics and allowed to adhere overnight. First, we treated GC cells with various doses of Andrographis (10, 20, 40, 60, 80 and 100 μ g/ml) for 72 h to evaluate its cytotoxic effects and then measured cell proliferation using WST-8 (Dojindo Laboratories) in accordance with the manufacturer's instructions. Subsequently, based on the IC₅₀ concept (33), we evaluated cell proliferation after treatment with 40 μ g/ml Andrographis for 24, 48 and 72 h. The absorbance in each well was measured at a wavelength of 450 nm using SoftMax Pro (Molecular Devices).

Cell colony formation assays. Colony formation activity was measured according to established procedures (34). Briefly,

2x10³ MKN74 cells/well and 5x10³ NUGC4 cells/well were seeded in 6-well tissue culture plates (TPP Techno Plastic Products AG) in the same culture medium as described above and incubated for 24 h in Andrographis-free culture medium. We then added 20 μ g/ml Andrographis to the culture medium and incubated the cells for 72 h. Subsequently, the medium was replaced with Andrographis-free culture medium, and the cells were maintained at 37°C/5% CO₂ for 5 days in a humidified atmosphere. The number of colonies was counted using Image J software ver.1.52 (NIH) (35) and compared between Control and Andrographis treatment groups.

Cell apoptosis assays. Apoptosis assays were conducted using PI/Annexin V double staining and flow cytometry. Cells were plated in a 6-well plate (MKN74: 1.2x10⁵/well; NUGC4: 1.5x10⁵/well) for 24 h, followed by treatment with 40 μ g/ml Andrographis for 48 h. The apoptotic cells were harvested and measured using a Muse[®] Annexin V and Dead Cell Assay (Luminex) on a Muse[™] Cell Analyzer (Millipore) in accordance with the manufacturer's instructions.

Quantitative mRNA expression analysis. For the quantification of mRNA expression, cells were plated in 6-well dishes (MKN74: 2.5x10⁵/well; NUGC4: 2.0x10⁵/well), incubated for 24 h, and then treated with 40 μ g/ml Andrographis or DMSO. Total RNA from cells in the treatment and control groups was extracted using an RNA extraction miRNeasy Mini kit (Qiagen). cDNA was synthesized from 5.0 ng total RNA using a Reverse Transcription kit (Toyobo), and RT-qPCR was performed using the Power SYBR[®] Green PCR Master Mix (Life Technology). Quantitative real-time reverse transcription (RT)-PCR analysis was conducted using the StepOne[™] Real-time PCR System (Applied BiosystemsA). The primer sequences were as follows: heme oxygenase-1 (*HMOX1*): forward, 5'-AAGACTGCGTTCCTGCTCAAC-3' and reverse, 5'-AAAGCCCTACAGCAACTGTCTG-3'; glutamate-cysteine ligase catalytic (*GCLC*): forward, 5'-AGGCCAACATGCGAAAC-3' and reverse, 5'-CGGATATTTCTTGTTAAGGTA CTGG-3'; glutamate-cysteine ligase modifier (*GCLM*): forward, 5'-GGGGAACCTGCTGAACTG-3' and reverse, 5'-AGATACAGTGCAATCCAAGACATC-3'; and β -actin (*ACTB*): forward, 5'-CATGTACGTTGCTATCCAGGC-3' and reverse, 5'-CTCCTTAATGTACGCACGAT-3'. The relative expression of target genes was calculated using the 2^{- $\Delta\Delta$ C_q} method (36) and normalized against the housekeeping gene *ACTB*.

Western immunoblotting. For western immunoblotting experiments, cells (MKN74: 3.5x10⁵/well; NUGC4: 2.5x10⁵/well) were treated with 40 μ g/ml Andrographis (treatment group) or DMSO (control group) for 48 h, followed by cell lysis using RIPA buffer (BioDynamics) supplemented with a proteinase inhibitor cocktail (Sigma-Aldrich; Merck KGaA). The protein concentration of cells in each group was measured using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). The proteins were mixed with loading buffer and boiled for 5 min. Then, they were subjected to electrophoresis on 5%-20% gradient e-PAGE HRMINI gels (ATTO) for 85 min for protein separation and transferred to Clear Blot Membrane-P plus (ATTO) using an EB RAPID for 10 min.

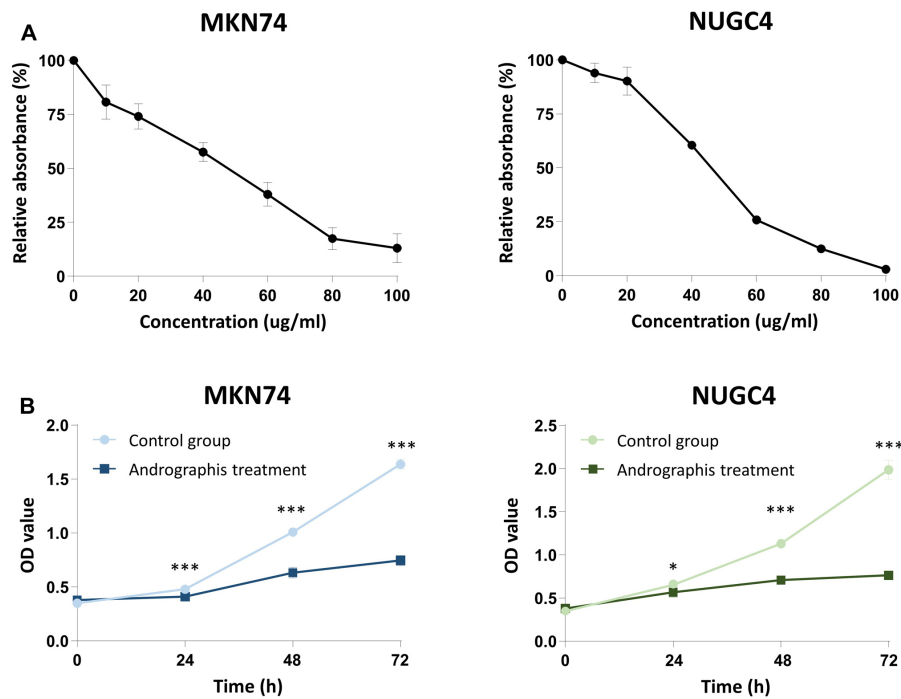


Figure 1. Antiproliferative effects of Andrographis in gastric cancer cells. (A) WST assay results revealing the dose-dependent effects of Andrographis on cell viability in MKN74 and NUGC4 cell lines. (B) WST assay results demonstrating the effects on cell viability following treatment with 40 µg/ml Andrographis in MKN74 and NUGC4 cell lines. * $P < 0.05$ and *** $P < 0.001$ (two-tailed Student's t-test; control group vs. Andrographis treatment group). OD, optical density; WST, water-soluble tetrazolium salts.

The membranes were blocked in 5% milk at room temperature and then incubated with the indicated primary antibody at room temperature for 30 min. The detailed information and dilutions of primary antibodies were as follows: mouse monoclonal anti-HMOX1 (sc-136960; Santa Cruz Biotechnology, Inc.; 1:500), mouse monoclonal anti- γ -GCLM (sc-55586; Santa Cruz Biotechnology, Inc.; 1:1,000), and rabbit polyclonal anti-GCLC (ab53179; Abcam; 1:2,000). The membranes were then washed with cold PBS three times and incubated with anti-mouse IgG (W4028; Promega; 1:5,000) and anti-rabbit IgG (W4018; Promega; 1:10,000) secondary antibodies at room temperature for 30 min. A mouse monoclonal β -actin antibody (691001, 691002; MP Biomedicals) was used as the loading control. Chemiluminescence detection was performed using Immobilon® Western (Millipore), and protein signals were detected using a chemiluminescent imaging system (ATTO). Band intensity was quantified using Image J software ver.1.52 (NIH) (35) and shown as a ratio of the B-actin band intensity.

Statistical analysis. All experiments were repeated in triplicate. The data were expressed as the mean \pm SD. Statistical comparisons were determined by a two-tailed unpaired Student's t-test. P-values less than 0.05 were considered statistically significant. Statistical analyses were performed using MedCalc Statistical Software version 19.1.2 (MedCalc Software bv) and GraphPad Prism Ver.7.0 (GraphPad Software, Inc.).

Results

Andrographis exhibits antiproliferative effects in GC cells. To evaluate the potential antiproliferative effects of Andrographis in GC cells, we first treated two GC cell lines (MKN74 and

NUGC4) with Andrographis at concentrations of 10, 20, 40, 60, 80 and 100 µg/ml. As expected, Andrographis suppressed the proliferation of MKN74 and NUGC4 cell lines in a dose-dependent manner (Fig. 1A). Next, we treated GC cells with 40 µg/ml Andrographis for 24, 48 and 72 h and compared the proliferative ability of the Andrographis treatment group with that of the control group. Intriguingly, the results showed that Andrographis treatment significantly inhibited the growth of both cell lines (MKN74: 24 h, $P < 0.0001$; 48 h, $P < 0.0001$; 72 h, $P < 0.0001$ and NUGC4: 24 h, $P = 0.03$; 48 h, $P < 0.0001$; 72 h, $P < 0.0001$) (Fig. 1B).

Andrographis inhibits the colony formation activity of GC cells. We next investigated the colony forming ability of two GC cell lines. After treatment of MKN74 and NUGC4 cells with Andrographis, we observed a significant reduction in the size and number of colonies compared with the corresponding controls (Fig. 2A). These results also indicated that Andrographis exhibits anti-tumorigenic effects on the phenotype of GC cells.

Andrographis treatment enhances the apoptosis of GC cells. To verify and strengthen the results of previous studies showing the apoptosis-enhancing activity of Andrographis in GC (32,37,38), we next investigated whether Andrographis treatment influences apoptosis in MKN74 and NUGC4 cells via an Annexin V binding assay. Apoptosis was clearly enhanced in the Andrographis-treated group compared with the control group in both cell lines (Fig. 2B). More specifically, compared with the control group, the percentage of apoptotic cells in the Andrographis-treated group significantly increased to $5.45 \pm 0.95\%$ for MKN74 cells ($P = 0.001$) and $6.18 \pm 1.6\%$ for

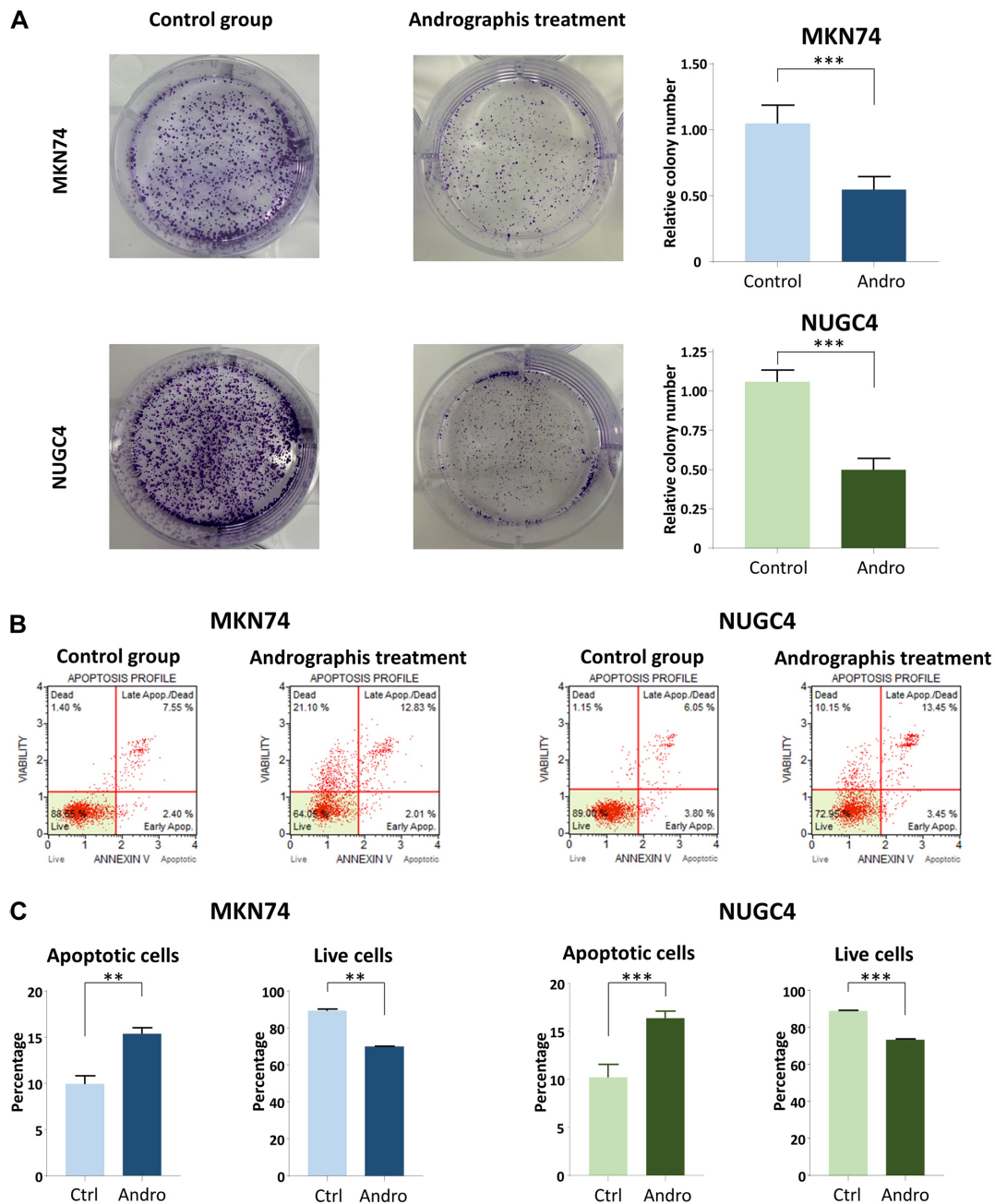


Figure 2. Inhibition of colony formation and enhancement of apoptotic activity induced by Andrographis in gastric cancer cells. (A) Colony formation assay to assess clonogenicity in MKN74 and NUGC4 cells following treatment with Andrographis. (B) Representative images illustrating the percentage of MKN74 and NUGC4 cells undergoing apoptosis, as indicated by positive staining for Annexin V. (C) Bar graphs showing the percentage of live and apoptotic cells in each treatment group in the apoptosis assay. ** $P < 0.01$ and *** $P < 0.001$ (two-tailed Student's *t*-test). Andro, Andrographis treatment group; Ctrl, Control group; Apop, apoptosis.

NUGC4 cells ($P = 0.002$), and Andrographis treatment significantly reduced the percentage of live cells to $19.43 \pm 1.13\%$ for MKN74 cells ($P = 0.0001$) and $15.42 \pm 0.65\%$ for NUGC4 cells ($P < 0.0001$) (Fig. 2C). Together, our results confirmed the previous finding that Andrographis exhibits anti-cancer potential through the enhancement of apoptosis using MKN74 and NUGC4 cells.

Andrographis mediates its anti-cancer activity by activating ferroptosis-associated genes. Because previous evidence revealed that Andrographolide induces the upregulation of ferroptosis-associated genes, such as *HMOX1*, *GCLC*, and

GCLM, in both non-cancer (39–43) and cancer cells (44–46), we investigated whether this finding applies to GC by performing RT-qPCR and western blot assays. Intriguingly, the RT-qPCR results demonstrated that all target genes were significantly upregulated ($P < 0.0001$) at the mRNA level following Andrographis treatment compared with the corresponding control in both cell lines (Fig. 3A). Furthermore, western blot experiments confirmed a substantial increase in the expression of HMOX-1 ($P < 0.05$), GCLC ($P < 0.05$), and GCLM ($P < 0.05$) at the protein level in both cell lines after Andrographis treatment compared with the corresponding control (Fig. 3B and C). Collectively, these results suggested

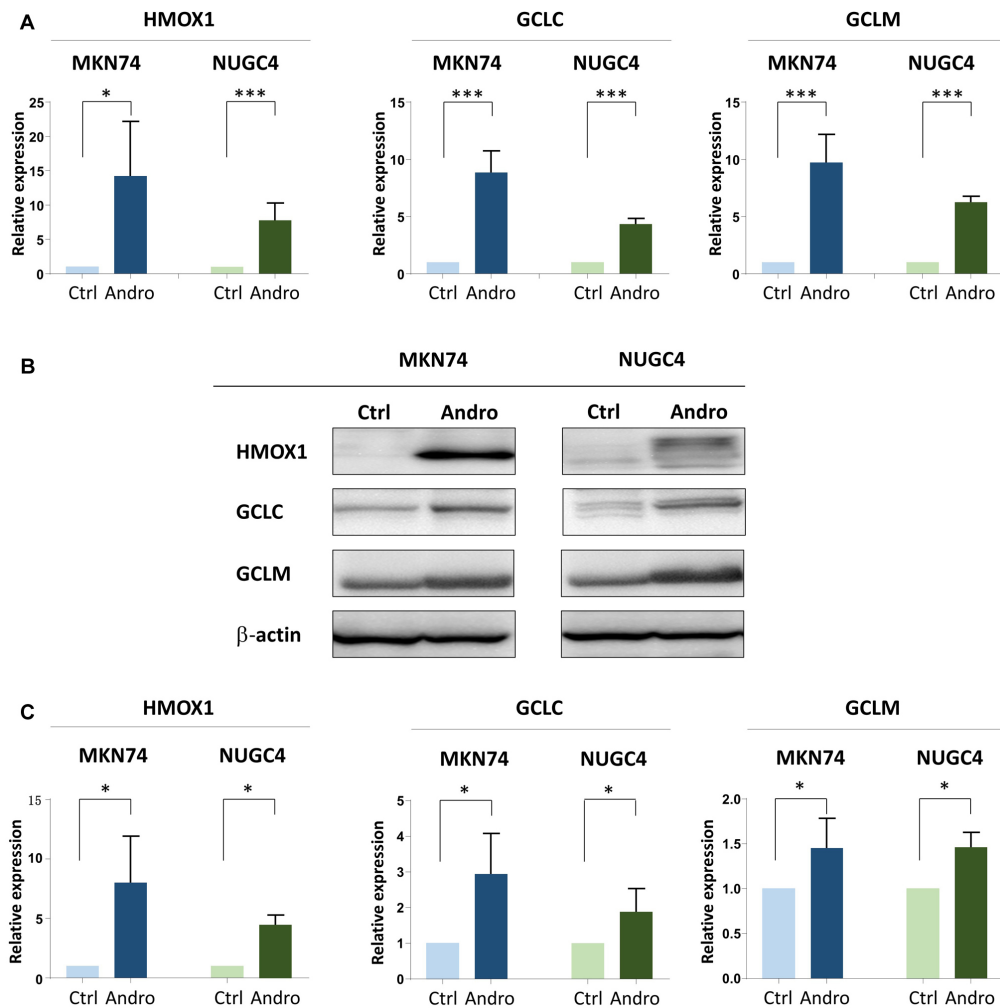


Figure 3. Altered mRNA and protein expression levels of the ferroptosis-associated targets *HMOX1*, *GCLC* and *GCLM* after Andrographis treatment in gastric cancer cells. (A) Changes in *HMOX1*, *GCLC* and *GCLM* mRNA expression after Andrographis treatment in MKN74 and NUGC4 cells. (B) Representative image of immunoblotting assays for each group in MKN74 and NUGC4 cells. (C) Changes in HMOX-1, GCLC and GCLM protein expression after Andrographis treatment in MKN74 and NUGC4 cells. * $P < 0.05$ and *** $P < 0.001$ (two-tailed Student's t-test). GCLC, glutamate-cysteine ligase catalytic; GCLM, glutamate-cysteine ligase modifier; HMOX1, heme oxygenase-1; Andro, Andrographis treatment group; Ctrl, Control group.

that the alteration of ferroptosis-associated genes may be one of the possible mechanisms by which Andrographis exerts its anti-tumorigenic potential in GC cells.

Discussion

Accumulating evidence has shown that dietary botanical compounds, such as herbal medicines, have increasingly important roles in the field of cancer treatment, both as therapeutic agents and adjunctive treatments to traditional therapies (10,47). Andrographolide is a C20 labdane diterpenoid derived from the traditional Chinese medicine *Andrographis paniculate* (12-14), which has been found to have cytotoxic/anti-tumorigenic potential in various malignancies (14,20,22,24-26,28). Intriguingly, Andrographolide was demonstrated to alter multiple cancer-associated signaling pathways, such as BAX-dependent apoptotic signaling in breast cancer (22), PI3K/AKT/mTOR-dependent signaling in leukemia (20) and glioblastoma (24), p38 signaling in melanoma (14), STAT3/AKT activation in pancreatic cancer (28), and the Src/MAPKs/AP-1 axis (25) and TLR4/NF- κ B/MMP-9

pathway (32) in colorectal cancer. Moreover, Andrographolide modulates chemosensitization in multiple tumors (28,46,48). In this study, we demonstrated that Andrographis exerts anti-tumorigenic effects by suppressing cell proliferation and colony formation and enhancing apoptotic activity in MKN74 and NUGC4 GC cells. Intriguingly, we also revealed that Andrographis treatment altered the expression of ferroptosis-associated genes, including *HMOX1*, *GCLC*, and *GCLM*, which might offer novel mechanistic insight into the pathogenesis of GC. Collectively, our findings may provide additional evidence supporting the anti-tumorigenic potential of Andrographis as an adjunctive treatment in GC.

In our study, we showed that cell viability was significantly reduced and apoptotic activity was enhanced in Andrographis-treated GC cells compared with the control cells, which is consistent with previous reports. Lim *et al* (32) reported that Andrographolide dose-dependently decreased the proliferation and viability of GC cells, which was accompanied by increased apoptotic and non-apoptotic cell death. They also demonstrated that Andrographolide enhanced recombinant human TRAIL-induced apoptotic cell death

mediated by the TRAIL-RS (DR5) pathway (32). Furthermore, Li *et al* (37) verified that Andrographolide inhibited cell proliferation and induced apoptosis by altering the expression of BAX, caspase-3, and BCL-2. In a study by Dai *et al* (38), Andrographolide inhibited cell proliferation, invasion, and migration in a dose-dependent manner and promoted apoptosis. They also showed that Andrographolide influenced the expression of various targets, including the upregulation of TIMP-1/2, cyclin B1, p-Cdc2, BAX, and BIK and downregulation of MMP-2/9 and BCL-2 (38).

The most striking result of this study was that Andrographis treatment remarkably upregulated the expression of the ferroptosis-related genes *HMOX1*, *GCLC*, and *GCLM*. Ferroptosis is a recently defined form of regulated cell death that differs from apoptosis, necrosis, and necroptosis and is characterized by iron-dependent reactive oxygen species (ROS) generation, lipid peroxidation, and iron accumulation (49-51). Because ferroptosis is often associated with resistance to chemotherapeutic drugs, several types of malignancies, including large B-cell lymphoma, leukemia, head and neck cancer, renal cell carcinoma, osteosarcoma, prostate adenocarcinoma, hepatocellular carcinoma, cholangiocarcinoma, ovarian cancer, pancreatic carcinoma, and lung cancer (52-61), exhibit sensitivity to ferroptosis inducers. For instance, ferroptosis inducers (e.g., erastin and sorafenib) may be considered a novel treatment regimen for non-small cell lung cancer patients with cisplatin failure (62). Furthermore, sulfasalazine depletes paclitaxel-resistant tumor cells by inducing ferroptosis in uterine serous carcinoma, which may be an effective treatment for patients with recurrent paclitaxel-resistant uterine serous carcinoma (63).

In our study, significant upregulation of *HMOX-1* was observed after Andrographis treatment in GC cells. Heme oxygenase is a rate-limiting enzyme that catalyzes the oxidative degradation of cellular heme, which produces carbon monoxide, bilirubin, and free iron (64). *HMOX-1* is a subtype of heme oxygenase that maintains cellular homeostasis and reduces tissue oxidative damage and the inflammatory response (65,66). Additionally, *HMOX-1* regulates cellular iron and ROS levels during ferroptosis (67-70). *HMOX-1* was reported to play an anticancer role in various types of human malignancies, such as fibrosarcoma, breast cancer, and prostate cancer (45,71-73). Moreover, some evidence indicates that Andrographolide induces the upregulation of *HMOX-1* in breast cancer, fibrosarcoma, and colorectal cancer (44-46). In this study, we revealed that Andrographis treatment induced significant upregulation of *HMOX-1* in GC cells, which strengthens the idea that Andrographis may have potential as an adjunctive treatment by enhancing ferroptotic activity mediated by *HMOX-1*.

Our experimental findings also demonstrated that Andrographis treatment upregulated the expression of *GCLC* and *GCLM*. *GCLC* and *GCLM* are involved in the synthesis of GSH in the oxidative stress response and metabolism of intracellular labile iron, and *GCLC* and *GCLM* are critical genes in the ferroptosis-associated pathway (74,75). Several dietary botanical compounds, such as chrysin, apigenin, and luteolin, can upregulate *GCLC* and *GCLM* in addition to *HMOX1* gene transcription via the ERK2/NRF2/ARE signaling pathway (76). Consistent with this study, our findings demonstrated that the altered expression of *HMOX-1* was

accompanied by the upregulation of *GCLC* and *GCLM* following treatment of GC cells with Andrographis.

There are several limitations to our current study. First, although we showed the anti-tumorigenic potential of Andrographis, we demonstrated this using just two GC cell lines. In addition, although we revealed the upregulation of *HMOX-1*, *GCLC*, and *GCLM* after Andrographis treatment, we did not perform detailed mechanistic studies of ferroptosis pathways. In the future, we plan to further identify the molecular mechanisms underlying the effects of this dietary compound on ferroptosis.

Collectively, our study demonstrated the anti-tumorigenic properties of Andrographis through the alteration of the ferroptosis-associated genes *HMOX1*, *GCLC*, and *GCLM* in GC cells. Although further mechanistic validation is warranted, our study may provide substantial evidence for the use of Andrographis as a potential adjunctive treatment in patients with GC.

In conclusion, we demonstrated that Andrographis exerts its anti-tumorigenic effects by altering the expression of ferroptosis-associated genes, indicating that Andrographis could serve as an adjunctive therapeutic option in patients with GC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

RM, TS, CY, Yoku and YT conceived and designed the study. RM, TS, CY, Yoku, TK, YK, YT, AG, LY and XZ acquired, analyzed and interpreted the data. RM, TS, CY, Yoku, AG, LY, XZ and YT drafted the manuscript. RM, TS, CY, Yoku, MO and KU performed statistical analysis. YT supervised the study. RM, TS, CY and Yoku confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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