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

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Received February 14, 2021; Accepted March 31, 2021

DOI: 10.3892/ol.2021.12784

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 unharmful 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.

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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

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

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-κB/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% CO_2. 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 μg/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 IC_{50} concept (33), we evaluated cell proliferation after treatment with 40 μg/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, $2x10^3$ MKN74 cells/well and $5x10^3$ 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 μg/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% CO_2 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: $1.2x10^5$/well; NUGC4: $1.5x10^5$/well) for 24 h, followed by treatment with 40 μg/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: $2.5x10^5$/well; NUGC4: $2.0x10^5$/well), incubated for 24 h, and then treated with 40 μg/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'-AAAGCCCTACAGCAACTGTCG-3'; glutamate-cysteine ligase catalytic (*GCLC*): forward, 5'-AGGCCAACATGCGAAAAC-3' and reverse, 5'-CGGATATTTCTTGTTAAGGTACTGG-3'; glutamate-cysteine ligase modifier (*GCLM*): forward, 5'-GGGGAACCTGCTGAACTG-3' and reverse, 5'-AGATACAGTGCATTCCAAGACATC-3'; and β-actin (*ACTB*): forward, 5'-CATGTACGTTGCTATCCAGGC-3' and reverse, 5'-CTCCTTAATGTCACGCACGAT-3'. The relative expression of target genes was calculated using the $2^{-\Delta\Delta Cq}$ method (36) and normalized against the housekeeping gene *ACTB*.

Western immunoblotting. For western immunoblotting experiments, cells (MKN74: $3.5x10^5$/well; NUGC4: $2.5x10^5$/well) were treated with 40 μg/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-PAGEL 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.

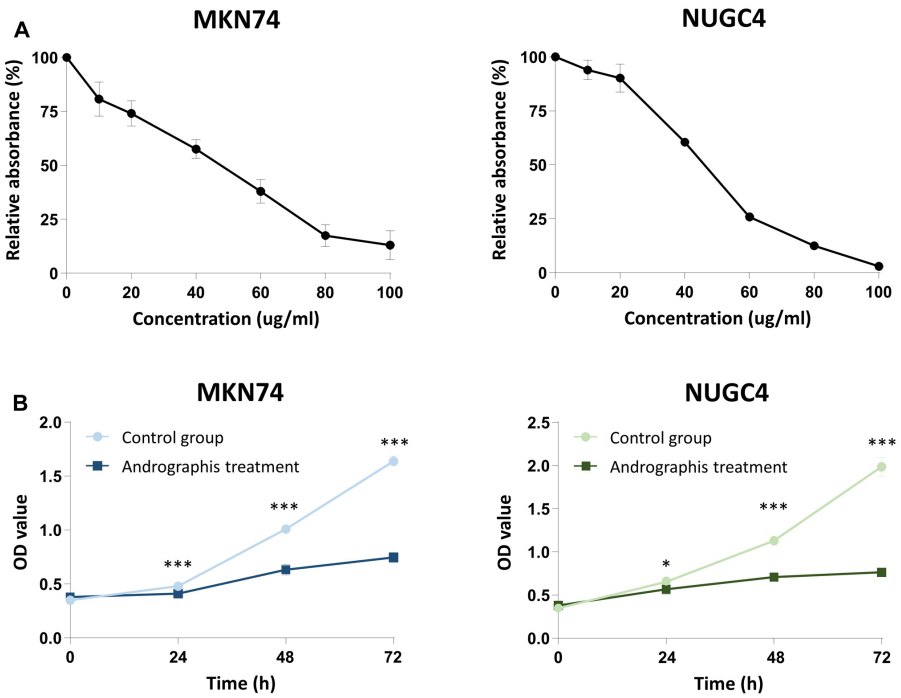

Figure 1. Antiproliferative effects of Andrographis in gastric cancer cells. (A) WST assay results revealing the dose-dependent effects of Andrographis on cell viability in MKN74 and NUGC4 cell lines. (B) WST assay results demonstrating the effects on cell viability following treatment with 40 μg/ml 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 ± 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±0.95% for MKN74 cells (P=0.001) and 6.18±1.6% for

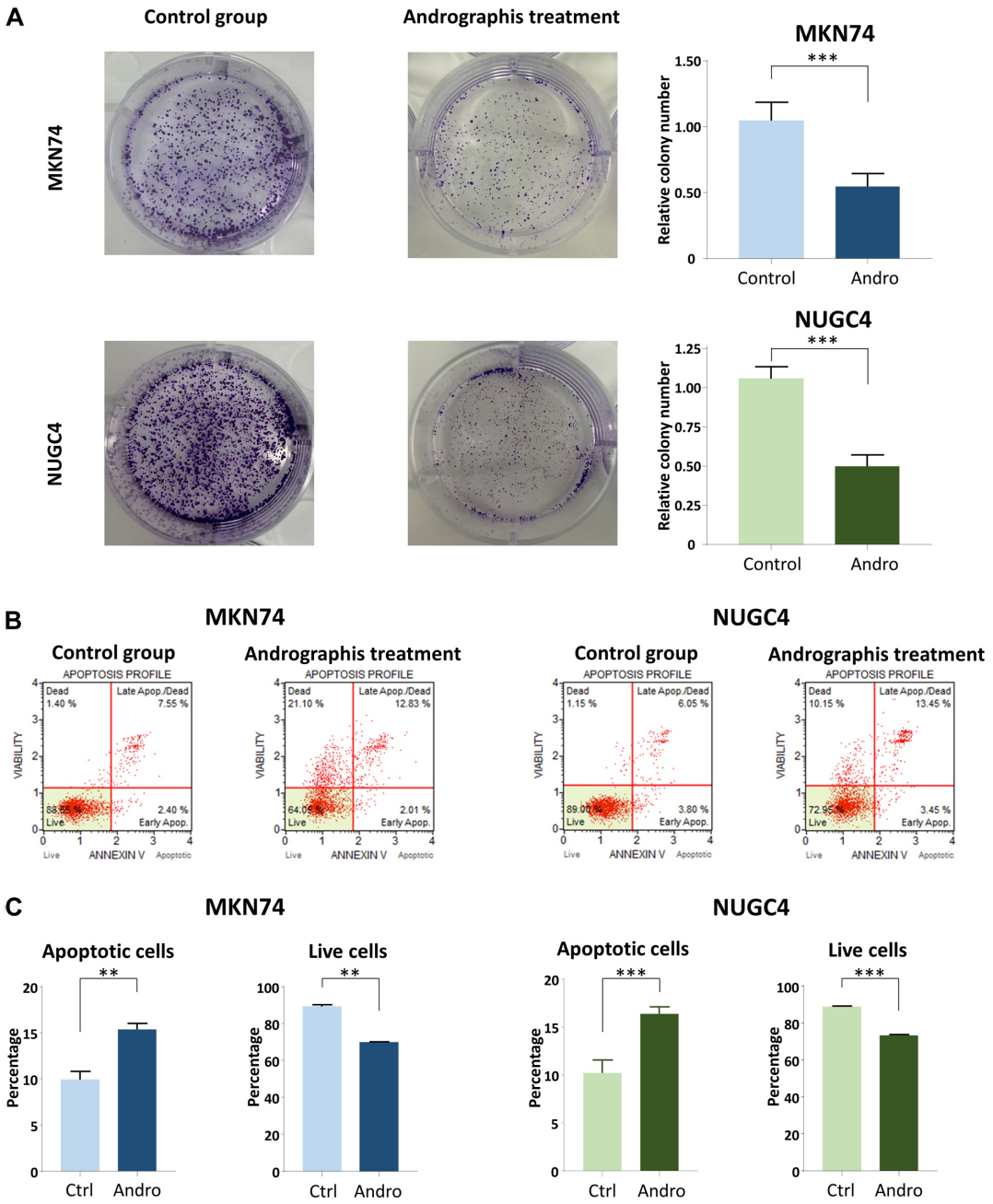

Figure 2. Inhibition of colony formation and enhancement of apoptotic activity induced by Andrographis in gastric cancer cells. (A) Colony formation assay to assess clonogenicity in MKN74 and NUGC4 cells following treatment with Andrographis. (B) Representative images illustrating the percentage of MKN74 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±1.13% for MKN74 cells (P=0.0001) and 15.42±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

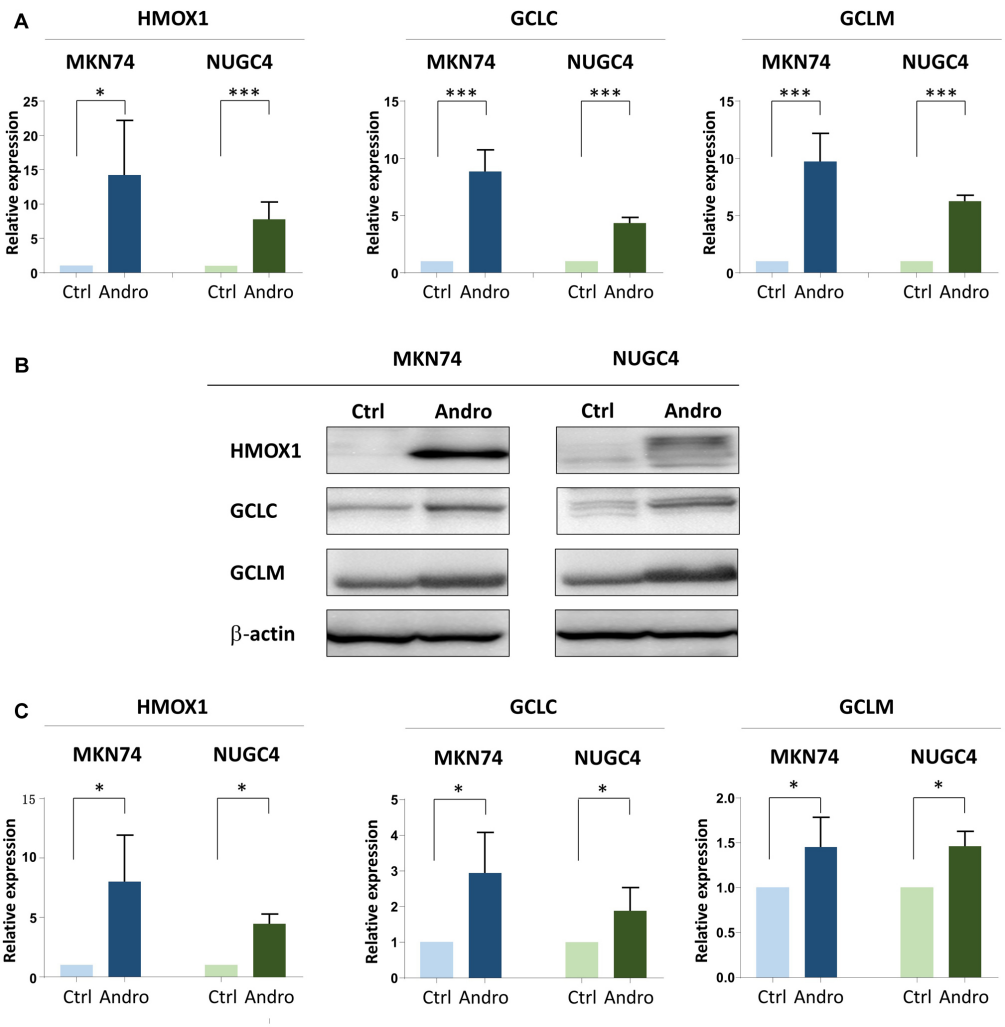

Figure 3. Altered mRNA and protein expression levels of the ferroptosis-associated targets *HMOX1*, *GCLC* and *GCLM* after Andrographis treatment in gastric cancer cells. (A) Changes in *HMOX1*, *GCLC* and *GCLM* mRNA expression after Andrographis treatment in MKN74 and NUGC4 cells. (B) Representative 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-kB/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.

Acknowledgements

The authors would like to acknowledge Ms. Yuki Orito (Department of Gastrointestinal and Pediatric Surgery, Institute of Life Sciences, Mie University Graduate School of Medicine) and Ms. Amphone Okada (Department of Gastrointestinal and Pediatric Surgery, Institute of Life Sciences, Mie University Graduate School of Medicine) for their experimental support, and Dr Priyanka Sharma (Department of Molecular Diagnostics and Experimental Therapeutics, Beckman Research Institute, City of Hope Comprehensive Cancer Center) for advice on the discussion of this article. Finally, the authors would like to thank Dr H. Nikki March and Dr Melissa Crawford, for editing a draft of this manuscript.

Funding

The present study was supported by a Grant-in-Aid for Scientific Research (grant nos. 18K08566 and 18K08700) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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, YOki, 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.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018. Erratum in: CA Cancer J Clin 70: 313, 2020.
2. Digklia A and Wagner AD: Advanced gastric cancer: Current treatment landscape and future perspectives. World J Gastroenterol 22: 2403-2414, 2016.
3. Smyth EC, Nilsson M, Grabsch HI, van Grieken NC and Lordick F: Gastric cancer. Lancet 396: 635-648, 2020.
4. Hironaka S, Sugimoto N, Yamaguchi K, Moriwaki T, Komatsu Y, Nishina T, Tsuji A, Nakajima TE, Gotoh M, Machida N, *et al*: S-1 plus leucovorin versus S-1 plus leucovorin and oxaliplatin versus S-1 plus cisplatin in patients with advanced gastric cancer: A randomised, multicentre, open-label, phase 2 trial. Lancet Oncol 17: 99-108, 2016.
5. Wagner AD, Syn NL, Moehler M, Grothe W, Yong WP, Tai BC, Ho J and Unverzagt S: Chemotherapy for advanced gastric cancer. Cochrane Database Syst Rev 8: CD004064, 2017.
6. Van Cutsem E, Sagaert X, Topal B, Haustermans K and Prenen H: Gastric cancer. Lancet 388: 2654-2664, 2016.
7. Muro K, Van Cutsem E, Narita Y, Pentheroudakis G, Baba E, Li J, Ryu MH, Zamaniah WIW, Yong WP, Yeh KH, *et al*: Pan-Asian adapted ESMO Clinical Practice Guidelines for the management of patients with metastatic gastric cancer: A JSMO-ESMO initiative endorsed by CSCO, KSMO, MOS, SSO and TOS. Ann Oncol 30: 19-33, 2019.
8. Hyodo I, Amano N, Eguchi K, Narabayashi M, Imanishi J, Hirai M, Nakano T and Takashima S: Nationwide survey on complementary and alternative medicine in cancer patients in Japan. J Clin Oncol 23: 2645-2654, 2005.
9. Chan A, Tan HL, Ching TH and Tan HC: Clinical outcomes for cancer patients using complementary and alternative medicine. Altern Ther Health Med 18: 12-17, 2012.
10. Liu TG, Xiong SQ, Yan Y, Zhu H and Yi C: Use of chinese herb medicine in cancer patients: a survey in southwestern china. Evid Based Complement Alternat Med 2012: 769042, 2012.
11. Newman DJ and Cragg GM: Natural products as sources of new drugs from 1981 to 2014. J Nat Prod 79: 629-661, 2016.
12. Singh S, Pandey P, Ghosh S and Banerjee S: Anti-cancer labdane diterpenoids from adventitious roots of *Andrographis paniculata*: Augmentation of production prospect endowed with pathway gene expression. Protoplasma 255: 1387-1400, 2018.
13. Islam MT, Ali ES, Uddin SJ, Islam MA, Shaw S, Khan IN, Saravi SSS, Ahmad S, Rehman S, Gupta VK, *et al*: Andrographolide, a diterpene lactone from *Andrographis paniculata* and its therapeutic promises in cancer. Cancer Lett 420: 129-145, 2018.
14. Liu G and Chu H: Andrographolide inhibits proliferation and induces cell cycle arrest and apoptosis in human melanoma cells. Oncol Lett 15: 5301-5305, 2018.
15. Suo XB, Zhang H and Wang YQ: HPLC determination of andrographolide in rat whole blood: Study on the pharmacokinetics of andrographolide incorporated in liposomes and tablets. Biomed Chromatogr 21: 730-734, 2007.
16. Lu WJ, Lee JJ, Chou DS, Jayakumar T, Fong TH, Hsiao G and Sheu JR: A novel role of andrographolide, an NF-kappa B inhibitor, on inhibition of platelet activation: The pivotal mechanisms of endothelial nitric oxide synthase/cyclic GMP. J Mol Med (Berl) 89: 1261-1273, 2011.
17. Jayakumar T, Hsieh CY, Lee JJ and Sheu JR: Experimental and clinical pharmacology of *Andrographis paniculata* and its major bioactive phytoconstituent andrographolide. Evid Based Complement Alternat Med 2013: 846740, 2013.
18. Poolsup N, Suthisisang C, Prathanturarug S, Asawamekin A and Chanchareon U: *Andrographis paniculata* in the symptomatic treatment of uncomplicated upper respiratory tract infection: Systematic review of randomized controlled trials. J Clin Pharm Ther 29: 37-45, 2004.
19. Dai Y, Chen SR, Chai L, Zhao J, Wang Y and Wang Y: Overview of pharmacological activities of *Andrographis paniculata* and its major compound andrographolide. Crit Rev Food Sci Nutr 59 (Suppl 1): S17-S29, 2019.
20. Kumar D, Das B, Sen R, Kundu P, Manna A, Sarkar A, Chowdhury C, Chatterjee M and Das P: Andrographolide analogue induces apoptosis and autophagy mediated cell death in U937 cells by inhibition of PI3K/Akt/mTOR pathway. PLoS One 10: e0139657, 2015.
21. Li L, Yue GG, Lee JK, Wong EC, Fung KP, Yu J, Lau CB and Chiu PW: The adjuvant value of *Andrographis paniculata* in metastatic esophageal cancer treatment - from preclinical perspectives. Sci Rep 7: 854, 2017.
22. Banerjee M, Chattopadhyay S, Choudhuri T, Bera R, Kumar S, Chakraborty B and Mukherjee SK: Cytotoxicity and cell cycle arrest induced by andrographolide lead to programmed cell death of MDA-MB-231 breast cancer cell line. J Biomed Sci 23: 40, 2016.
23. Lai YH, Yu SL, Chen HY, Wang CC, Chen HW and Chen JJ: The HLJ1-targeting drug screening identified Chinese herb andrographolide that can suppress tumour growth and invasion in non-small-cell lung cancer. Carcinogenesis 34: 1069-1080, 2013.
24. Li Y, Zhang P, Qiu F, Chen L, Miao C, Li J, Xiao W and Ma E: Inactivation of PI3K/Akt signaling mediates proliferation inhibition and G2/M phase arrest induced by andrographolide in human glioblastoma cells. Life Sci 90: 962-967, 2012.
25. Yuan M, Meng W, Liao W and Lian S: Andrographolide antagonizes TNF-α-induced IL-8 via inhibition of NADPH oxidase/ROS/NF-κB and Src/MAPKs/AP-1 axis in human colorectal cancer HCT116 cells. J Agric Food Chem 66: 5139-5148, 2018.
26. Zhang R, Zhao J, Xu J, Jiao DX, Wang J, Gong ZQ and Jia JH: Andrographolide suppresses proliferation of human colon cancer SW620 cells through the TLR4/NF-κB/MMP-9 signaling pathway. Oncol Lett 14: 4305-4310, 2017.
27. Deng Y, Bi R, Guo H, Yang J, Du Y, Wang C and Wei W: Andrographolide enhances TRAIL-induced apoptosis via p53-mediated death receptors up-regulation and suppression of the NF-κB pathway in bladder cancer cells. Int J Biol Sci 15: 688-700, 2019.
28. Bao GQ, Shen BY, Pan CP, Zhang YJ, Shi MM and Peng CH: Andrographolide causes apoptosis via inactivation of STAT3 and Akt and potentiates antitumor activity of gemcitabine in pancreatic cancer. Toxicol Lett 222: 23-35, 2013.
29. Yang W, Zhao J, Wang Y, Xu H, Wu Z, Hu Y, Jiang K, Shen P, Ma C, Guan Z, *et al*: In vivo inhibitory activity of andrographolide derivative ADN-9 against liver cancer and its mechanisms involved in inhibition of tumor angiogenesis. Toxicol Appl Pharmacol 327: 1-12, 2017.
30. Lim JC, Chan TK, Ng DS, Sagineedu SR, Stanslas J and Wong WS: Andrographolide and its analogues: Versatile bioactive molecules for combating inflammation and cancer. Clin Exp Pharmacol Physiol 39: 300-310, 2012.
31. Yue GG, Li L, Lee JK, Kwok HF, Wong EC, Li M, Fung KP, Yu J, Chan AW, Chiu PW and Lau CB: Multiple modulatory activities of *Andrographis paniculata* on immune responses and xenograft growth in esophageal cancer preclinical models. Phytomedicine 60: 152886, 2019.
32. Lim SC, Jeon HJ, Kee KH, Lee MJ, Hong R and Han SI: Andrographolide induces apoptotic and non-apoptotic death and enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in gastric cancer cells. Oncol Lett 13: 3837-3844, 2017.
33. Stewart MJ and Watson ID: Standard units for expressing drug concentrations in biological fluids. Br J Clin Pharmacol 16: 3-7, 1983.
34. Takahashi M, Sung B, Shen Y, Hur K, Link A, Boland CR, Aggarwal BB and Goel A: Boswellic acid exerts antitumor effects in colorectal cancer cells by modulating expression of the let-7 and miR-200 microRNA family. Carcinogenesis 33: 2441-2449, 2012.

35. Schneider CA, Rasband WS and Eliceiri KW: NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9: 671-675, 2012.
36. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
37. Li SG, Wang YY, Ye ZY, Shao QS, Tao HQ, Shu LS, Zhao YF, Yang YJ, Yang J, Peng T, *et al*: Proliferative and apoptotic effects of andrographolide on the BGC-823 human gastric cancer cell line. Chin Med J (Engl) 126: 3739-3744, 2013.
38. Dai L, Wang G and Pan W: Andrographolide inhibits proliferation and metastasis of SGC7901 gastric cancer cells. BioMed Res Int 2017: 6242103, 2017.
39. Yu AL, Lu CY, Wang TS, Tsai CW, Liu KL, Cheng YP, Chang HC, Lii CK and Chen HW: Induction of heme oxygenase 1 and inhibition of tumor necrosis factor alpha-induced intercellular adhesion molecule expression by andrographolide in EA.hy926 cells. J Agric Food Chem 58: 7641-7648, 2010.
40. Seo JY, Pyo E, An JP, Kim J, Sung SH and Oh WK: Andrographolide activates Keap1/Nrf2/ARE/HO-1 pathway in HT22 cells and suppresses microglial activation by Aβ42 through Nrf2-related inflammatory response. Mediators Inflamm 2017: 5906189, 2017.
41. Lu CY, Yang YC, Li CC, Liu KL, Lii CK and Chen HW: Andrographolide inhibits TNFα-induced ICAM-1 expression via suppression of NADPH oxidase activation and induction of HO-1 and GCLM expression through the PI3K/Akt/Nrf2 and PI3K/Akt/AP-1 pathways in human endothelial cells. Biochem Pharmacol 91: 40-50, 2014.
42. Lee JC, Tseng CK, Young KC, Sun HY, Wang SW, Chen WC, Lin CK and Wu YH: Andrographolide exerts anti-hepatitis C virus activity by up-regulating haeme oxygenase-1 via the p38 MAPK/Nrf2 pathway in human hepatoma cells. Br J Pharmacol 171: 237-252, 2014.
43. Guan SP, Tee W, Ng DS, Chan TK, Peh HY, Ho WE, Cheng C, Mak JC and Wong WS: Andrographolide protects against cigarette smoke-induced oxidative lung injury via augmentation of Nrf2 activity. Br J Pharmacol 168: 1707-1718, 2013.
44. Chao CY, Lii CK, Hsu YT, Lu CY, Liu KL, Li CC and Chen HW: Induction of heme oxygenase-1 and inhibition of TPA-induced matrix metalloproteinase-9 expression by andrographolide in MCF-7 human breast cancer cells. Carcinogenesis 34: 1843-1851, 2013.
45. Kwon MY, Park E, Lee SJ and Chung SW: Heme oxygenase-1 accelerates erastin-induced ferroptotic cell death. Oncotarget 6: 24393-24403, 2015.
46. Sharma P, Shimura T, Banwait JK and Goel A: Andrographis-mediated chemosensitization through activation of ferroptosis and suppression of β-catenin/Wnt-signaling pathways in colorectal cancer. Carcinogenesis 41: 1385-1394, 2020.
47. Yang G, Li X, Li X, Wang L, Li J, Song X, Chen J, Guo Y, Sun X, Wang S, *et al*: Traditional chinese medicine in cancer care: a review of case series published in the chinese literature. Evid Based Complement Alternat Med 2012: 751046, 2012.
48. Zhou J, Ong CN, Hur GM and Shen HM: Inhibition of the JAK-STAT3 pathway by andrographolide enhances chemosensitivity of cancer cells to doxorubicin. Biochem Pharmacol 79: 1242-1250, 2010.
49. Liang C, Zhang X, Yang M and Dong X: Recent progress in ferroptosis inducers for cancer therapy. Adv Mater 31: e1904197, 2019.
50. Ye Z, Liu W, Zhuo Q, Hu Q, Liu M, Sun Q, Zhang Z, Fan G, Xu W, Ji S, *et al*: Ferroptosis: Final destination for cancer? Cell Prolif 53: e12761, 2020.
51. Hassannia B, Vandenabeele P and Vanden Berghe T: Targeting Ferroptosis to Iron Out Cancer. Cancer Cell 35: 830-849, 2019.
52. Yu Y, Xie Y, Cao L, Yang L, Yang M, Lotze MT, Zeh HJ, Kang R and Tang D: The ferroptosis inducer erastin enhances sensitivity of acute myeloid leukemia cells to chemotherapeutic agents. Mol Cell Oncol 2: e1054549, 2015.
53. Yang S, Evens AM, Prachand S, Singh AT, Bhalla S, David K and Gordon LI: Mitochondrial-mediated apoptosis in lymphoma cells by the diterpenoid lactone andrographolide, the active component of *Andrographis paniculata*. Clin Cancer Res 16: 4755-4768, 2010.
54. Xia X, Fan X, Zhao M and Zhu P: The relationship between ferroptosis and tumors: a novel landscape for therapeutic approach. Curr Gene Ther 19: 117-124, 2019.
55. Chen Y, Fan Z, Yang Y and Gu C: Iron metabolism and its contribution to cancer (Review). Int J Oncol 54: 1143-1154, 2019.
56. Roh JL, Kim EH, Jang HJ, Park JY and Shin D: Induction of ferroptotic cell death for overcoming cisplatin resistance of head and neck cancer. Cancer Lett 381: 96-103, 2016.
57. Louandre C, Ezzoukhry Z, Godin C, Barbare JC, Mazière JC, Chauffert B and Galmiche A: Iron-dependent cell death of hepatocellular carcinoma cells exposed to sorafenib. Int J Cancer 133: 1732-1742, 2013.
58. Puntawee S, Theerasilp M, Reabroi S, Saeeng R, Piyachaturawat P, Chairoungdua A and Nasongkla N: Solubility enhancement and in vitro evaluation of PEG-b-PLA micelles as nanocarrier of semi-synthetic andrographolide analogue for cholangiocarcinoma chemotherapy. Pharm Dev Technol 21: 437-444, 2016.
59. Basuli D, Tesfay L, Deng Z, Paul B, Yamamoto Y, Ning G, Xian W, McKeon F, Lynch M, Crum CP, *et al*: Iron addiction: A novel therapeutic target in ovarian cancer. Oncogene 36: 4089-4099, 2017.
60. Eling N, Reuter L, Hazin J, Hamacher-Brady A and Brady NR: Identification of artesunate as a specific activator of ferroptosis in pancreatic cancer cells. Oncoscience 2: 517-532, 2015.
61. Liu Q and Wang K: The induction of ferroptosis by impairing STAT3/Nrf2/GPx4 signaling enhances the sensitivity of osteosarcoma cells to cisplatin. Cell Biol Int 43: 1245-1256, 2019.
62. Li Y, Yan H, Xu X, Liu H, Wu C and Zhao L: Erastin/sorafenib induces cisplatin-resistant non-small cell lung cancer cell ferroptosis through inhibition of the Nrf2/xCT pathway. Oncol Lett 19: 323-333, 2020.
63. Sugiyama A, Ohta T, Obata M, Takahashi K, Seino M and Nagase S: xCT inhibitor sulfasalazine depletes paclitaxel-resistant tumor cells through ferroptosis in uterine serous carcinoma. Oncol Lett 20: 2689-2700, 2020.
64. Bussolati B, Ahmed A, Pemberton H, Landis RC, Di Carlo F, Haskard DO and Mason JC: Bifunctional role for VEGF-induced heme oxygenase-1 in vivo: Induction of angiogenesis and inhibition of leukocytic infiltration. Blood 103: 761-766, 2004.
65. Farombi EO and Surh YJ: Heme oxygenase-1 as a potential therapeutic target for hepatoprotection. J Biochem Mol Biol 39: 479-491, 2006.
66. Jozkowicz A, Was H and Dulak J: Heme oxygenase-1 in tumors: Is it a false friend? Antioxid Redox Signal 9: 2099-2117, 2007.
67. Chiang SK, Chen SE and Chang LC: A Dual Role of Heme Oxygenase-1 in Cancer Cells. Int J Mol Sci 20: 20, 2018.
68. Trachootham D, Alexandre J and Huang P: Targeting cancer cells by ROS-mediated mechanisms: A radical therapeutic approach? Nat Rev Drug Discov 8: 579-591, 2009.
69. Suttner DM and Dennery PA: Reversal of HO-1 related cytoprotection with increased expression is due to reactive iron. FASEB J 13: 1800-1809, 1999.
70. Chang LC, Chiang SK, Chen SE, Yu YL, Chou RH and Chang WC: Heme oxygenase-1 mediates BAY 11-7085 induced ferroptosis. Cancer Lett 416: 124-137, 2018.
71. Chau LY: Heme oxygenase-1: Emerging target of cancer therapy. J Biomed Sci 22: 22, 2015.
72. Hill M, Pereira V, Chauveau C, Zagani R, Remy S, Tesson L, Mazal D, Ubillos L, Brion R, Asghar K, *et al*: Heme oxygenase-1 inhibits rat and human breast cancer cell proliferation: Mutual cross inhibition with indoleamine 2,3-dioxygenase. FASEB J 19: 1957-1968, 2005.
73. Ferrando M, Gueron G, Elguero B, Giudice J, Salles A, Leskow FC, Jares-Erijman EA, Colombo L, Meiss R, Navone N, *et al*: Heme oxygenase 1 (HO-1) challenges the angiogenic switch in prostate cancer. Angiogenesis 14: 467-479, 2011.
74. Nishizawa H, Matsumoto M, Shindo T, Saigusa D, Kato H, Suzuki K, Sato M, Ishii Y, Shimokawa H and Igarashi K: Ferroptosis is controlled by the coordinated transcriptional regulation of glutathione and labile iron metabolism by the transcription factor BACH1. J Biol Chem 295: 69-82, 2020.
75. Lu SC: Glutathione synthesis. Biochim Biophys Acta 1830: 3143-3153, 2013.
76. Huang CS, Lii CK, Lin AH, Yeh YW, Yao HT, Li CC, Wang TS and Chen HW: Protection by chrysin, apigenin, and luteolin against oxidative stress is mediated by the Nrf2-dependent up-regulation of heme oxygenase 1 and glutamate cysteine ligase in rat primary hepatocytes. Arch Toxicol 87: 167-178, 2013.