Natural bioactive gallic acid shows potential anticancer effects by inhibiting the proliferation and invasiveness behavior in human embryonic carcinoma cells

DONG YOUNG KANG[1*], SE WON BAE[2*] and KYOUNG-JIN JANG[3]

[1]Department of Immunology, School of Medicine, Institute of Biomedical Science and Technology, Konkuk University, Chungju. Chungcheong 27478, Republic of Korea; [2]Department of Chemistry and Cosmetics, Jeju National University, Jeju, Jejudo 63243, Republic of Korea; [3]Department of Integrative Biological Sciences and Industry, College of Life Science, Sejong University, Seoul 05006, Republic of Korea

Received October 11, 2024; Accepted February 6, 2025

DOI: 10.3892/mmr.2025.13516

Abstract. Embryonic cancer stem cells (CSCs), referred to as self-renewable cells, are commonly found in liquid and solid cancers and can also be attributed to tumor onset, resistance, expansion, recurrence and metastasis following treatment. Cancer therapy targeting CSCs using natural bioactive products is an optimal option for inhibiting cancer recurrence, thereby improving prognosis. Several natural compounds and extracts have been used to identify direct or indirect therapy effects that reduce the pathological activities of CSCs. Natural gallic acid (GA) is noted to have anticancer properties for oncogene expression, cycle arrest, apoptosis, angiogenesis, migration and metastasis in various cancers. The present study demonstrated that GA has various anticancer activities in NTERA-2 and NCCIT human embryonic carcinoma cells. In two types of embryonic CSCs, GA effectively induced cell death via late apoptosis. Furthermore, GA showed the G_0/G_1 cell cycle arrest activity in embryonic CSCs by inducing the increase of p21, p27 and p53 expression and the decrease of CDK4, cyclin E and cyclin D1 expression. The present study showed that GA inhibited the expression levels of mRNA and protein for stem cell markers, such as SOX2, NANOG and OCT4, in NTERA-2 and NCCIT cells. The induction of cellular and mitochondrial reactive oxygen species by GA also activated the cellular DNA damage response pathway by raising the phosphorylated-BRCA1, ATM, Chk1, Chk2 and histone. Finally, GA inhibited CSCs invasion and migration by inhibiting the expression of matrix metalloproteinase by the downregulation of EGFR/JAK2/STAT5 signaling pathway. Thus, it is hypothesized that GA could be a potential inhibitor of cancer emergence by suppressing CSC properties.

Correspondence to: Professor Kyoung-Jin Jang, Department of Integrative Biological Sciences and Industry, College of Life Science, Sejong University, 209 Neungdong-ro, Gwangjin, Seoul 05006, Republic of Korea
E-mail: jangkj@sejong.ac.kr

*Contributed equally

Key words: human embryonic carcinoma cells, gallic acid, cell cycle arrest, reactive oxygen species, DNA damage response, matrix metalloproteinase

Introduction

Globally, cancers are considered as one of the highest leading causes of mortality. The pathogenesis of cancer is initiated when normal cells acquire the ability to uncontrollably grow and eventually invade and damage the normal tissues of the body (1). Cancer development is comprised of multiple stages, that is, from precancerous changes to malignant tumors. Furthermore, some cancer cells may spread from their original location to other places in the body via the bloodstream or lymphatic system, referred to as metastasis (2). Embryonic cancer stem cells (CSCs) are known as a small subpopulation of whole cancer cells which exhibit high tumorigenic potential (3,4). In the past, CSCs were simply considered as tumor-initiating cells and also known to be the primary cause for tumorigenesis (5,6). CSCs are known to have additional features including a self-renewal capacity, differentiation into all types of cancers, invasion and metastasis into other tissues and drug resistance (7). These characteristics induce cancer aggressiveness and play a pivotal role in increasing cancer therapy difficulty. Additionally, CSCs have the fundamental properties of all types of cancers including high tumorigenicity, high metastasis potential, immune surveillance evasion, drug resistance and cancer relapse (3,5,6,8,9). Thus, several therapeutic attempts targeting these CSCs have been employed to improve the therapeutic effects and long-term clinical outcome, including efforts to overcome the unique carcinogenic properties of CSCs.

The transcription pluripotency factors, also known as stem cell markers, such as NANOG, SOX2 and OCT4, contribute in maintaining the phenotype of pluripotent embryonic stem (ES) cells in embryonic stem cells (ESCs) and enterochromaffin cells (10-12). Some studies suggest that the abnormal

ES cell self-renewal through the hyper-expression of these transcription pluripotency factors promotes carcinogenesis (13-15). Particularly, NANOG overexpression enhances poor prognosis in gastric adenocarcinoma indicating tumor progression (16). Reactive oxygen species (ROS) are known to be a subset of free radicals, which promote tumorigenesis, resulting in oxidative damages of DNA, protein or lipid in normal cells. In the prolonged stress state, cells activate DNA repair pathway, also known as a DNA damage response (DDR), for the repair of damaged DNA and maintaining genome fidelity (17-19). The DDR mechanism is initiated with the induction of phosphorylation in ATM, ATR and DNA-PKcs kinase (20,21). Moreover, Chk1/2 and BRCA1 are phosphorylated, thus activating p53-dependent pathway in the repair response to DNA damage, which leads to the induction of cell cycle arrests, DDR activation and apoptotic mechanism (22).

Cell cycle checkpoints are precisely controlled by both activators and inhibitors to evade normal cell carcinogenesis. Generally, the failed checkpoints for oncogenesis or tumorigenesis allow progression of uncontrolled cell cycle, induce failure of apoptosis and downregulate tumor suppressor factors. Cyclin-dependent kinases, such as CDK4, are considered to be significant cell cycle regulators through positively regulating multiple checkpoints (23,24). However, CDK inhibitors (CKIs) such as p21 ($p21^{Cip1/Waf1}$) and p53 are known as tumor suppressor factors that inhibit the abnormal cell cycle and carcinogenesis (25-27). Therefore, carcinogenesis in normal cells is related to abnormal operation of CDKs or CKIs, which indicates loss of the ability to induce DNA repair or apoptosis. These cell cycle checkpoints are being studied as target sites for anticancer treatment. Matrix metalloproteinases (MMPs), such as MMP-2, -3 or -9, are knowns as the family of metzincin proteases, which play a role in the degradation of various proteins in the extracellular matrix (ECM) (28,29). In tumor cells, it plays an essential role in a wide range of tumorigenesis, tumor growth, tumor invasion and metastasis (30-32). Additionally, MMPs are emerging as a key player in the entrance or exit of circulating cancer cells in the blood vessels (33). Hence, tumor-associated MMPs are good targets for developing MMP inhibitors for anticancer therapy.

Chemotherapy using various types of drugs has been used to effectively treat several types of cancer. Cancer cells are known to grow faster than normal cells, which indicates that it is easier for drugs to attack these fast-growing cells. However, similar to other cancer treatments, it often causes various side effects, including fatigue, nausea, vomiting, various pains or memory loss (34,35). Additionally, although chemotherapy is initiated depending on the cancer type, location, drugs and health condition of the patient, chronic drug exposure has been linked to serious side effects, including neutropenia, arrhythmia, neuropathy and cardiovascular breakdown. Thus, cancer treatment using natural ingredients could be a more optimal alternative as it has been known to alleviate side effects compared with chemotherapeutic drugs (36-40). Recently, anticancer chemotherapy using natural ingredients has been widely employed to extend the therapeutic domain in chemotherapeutic agents, decrease side effects or drug resistance (41-44).

Natural gallic acid (3,4,5-trihydroxybenzoic acid; GA) is a secondary metabolite, which is widely distributed in natural plants, vegetables, fruits and green tea (45-47). GA is considered to have numerous pharmacological properties, including antibacterial, anti-inflammatory, antioxidant, antiviral and antitumor activities (48-55). Furthermore, it has anticancer properties, including apoptosis induction and suppression of angiogenesis and metastasis. Some studies have reported that GA selectively activates the induction of cancer cell apoptosis in HeLa, HCT-15, SH-SY5Y and NSCLC cells (56-60). In addition, it suppresses cell proliferation and metastasis through the increase or decrease of fatty acid synthase in estrogen receptor (ER) alpha level in TSGH-8301 bladder cancer cells (60). A study suggests that GA decreases the progression of T24 bladder cancer cell via inducing mitochondrial dysfunction and suppressing the PI3K/Akt and NF-κB signaling pathway (61).

The present study revealed that natural GA showed multiple anticancer functions in two NTERA-2 and NCCIT human embryonic carcinoma cells. The anticancer properties of GA in terms of proliferation, apoptosis, cell cycle distribution, migration and invasion were assessed. It was demonstrated that GA effectively suppressed the cancer characteristics of CSC cells. Additionally, it was revealed that the reduction of MMPs by GA was associated with EGFR/JAK2/STAT5 signaling inhibition.

Materials and methods

Cell Culture. Embryonic carcinoma cells (NCCIT and NTERA-2 cells with biomarkers of NANOG, SOX2 and OCT4) were obtained from the Korea Cell Line Bank. NTERA-2 cells were maintained using Dulbecco's Modified Eagle's Medium (DMEM; cat. no. L0103; Biowest) and NCCIT cells were cultured using RPMI-1640 media (cat. no. L0498; Biowest) containing 10% fetal bovine serum (FBS; cat. no. A5670801; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (cat. no. 15140122; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2; $5x10^5$ cells/60 mm dish were cultured up to 70-80% confluence for 48 h and the adherent cells were harvested with trypsin-EDTA solution (cat. no. T4049; MilliporeSigma). The cells were either treated or left untreated with GA and were then further cultured for 24 h.

Reagents and antibodies. GA was bought from MilliporeSigma and dissolved in distilled water (stock concentration of 100 mM). MitoSOX and CM-H_2DCFDA were obtained from Invitrogen (Thermo Fisher Scientific, Inc.). The primary antibodies were purchased from Santa Cruz Biotechnology, Inc.: p21 (cat. no. sc-756), p53 (cat. no. sc-126), cyclin E (cat. no. sc-481), MMP9 (cat. no. sc-13520), STAT5b (cat. no. sc-1656), CDK4 (cat. no. sc-260) and β-actin (cat. no. sc-47778). Cell Signaling Technology, Inc. supplied: p27 Kip1 (cat. no. 3686), p-EGFR (cat. no. 3777), EGFR (cat. no. 4267), phosphorylated (p-)JAK2 (cat. no. 3776), JAK2 (cat. no. 3230), p-STAT5 (cat. no. 9351), pCHK1 (cat. no. 2348), p-CHK2 (cat. no. 2197), p-ATM (cat. no. 5883), p-Histone (cat. no. 9718) and p-BRCA1 (cat. no. 9009). MilliporeSigma supplied MMP3 (cat. no. AB2963), OCT4 (cat. no. MABD76), SOX2 (cat. no. MAB4423) and NANOG (cat. no. MABD24)

Abcam supplied Cyclin D1 (cat. no. ab6152) and EnoGene MMP2 (cat. no. E90317). Secondary antibodies were purchased from Cell Signaling Technology (cat. nos. 7074; anti-rabbit and 7076; anti-mouse). All antibodies were incubated at 4˚C for 12-16 h.

Morphological analysis. NTERA-2 and NCCIT cells were cultured at a density of $1.5x10^5$ cells per well and treated with or without GA for 24 h, followed by washing with phosphate-buffered saline (PBS) twice and again incubated with DAPI staining solution for 1 h. A fluorescence microscope was used to capture the images (Olympus IX71/DP72; Olympus Corporation).

Cell viability assay. MTT (cat. no. M6494; Thermo Fisher Scientific, Inc.) is used to detect reductive metabolism in cells for viability, proliferation and cytotoxicity assays. Cells ($3x10^3$ cells per well) were cultured in a 96-well plate for 24 h. Thereafter, they were incubated using dimethyl sulfoxide (DMSO) as vehicle control or using different concentrations of GA (20-400 μM) at 37˚C for 24 h. The next day, treatment with 5 mg/ml of MTT reagent was performed and incubation for 4 h at 37˚C. After washing with 1X PBS, the formazan product was then dissolved in DMSO. At a 560-nm wavelength, the formazan product absorbance was measured with an Ultra Multifunctional Microplate Reader (Tecan Group, Ltd.).

Apoptosis analysis. An Apoptosis assay kit (cat. no. 556570; BD Biosciences) was used to measure apoptosis in NCCIT and NTERA-2 cells. First, the GA-treated or GA-untreated cells were washed with PBS and resuspended in a binding buffer at a concentration of $1x10^6$ cells. Thereafter, cells were stained with Annexin V-FITC and propidium iodide for 10 min in a dark box at room temperature. Finally, the percentage of apoptotic cells was measured using flow cytometry using a FACSCalibur flow cytometer (BD Biosciences) and dead cells are initially filtered through a 'singlets' gate that excludes aggregates, using the height vs. area signals of the same parameter (such as side scatter or forward scatter). Then, cells were gated for detecting apoptotic cells on Annexin V-FITC (FL1) vs. propidium iodide (PI) (FL3). FlowJo v10 software (FlowJo LLC) was used to perform the analysis. The apoptotic rates were calculated with apoptotic cells (Annexin V+/PI-), late apoptotic cells (Annexin V+/PI+), and necrotic cells (Annexin V-/PI+).

Cell cycle analysis. The DNA content from cells with or without GA was determined using a BD Cycletest Plus DNA Reagent Kit (BD Biosciences) according to the manufacturer's protocol. The samples ($5x10^5$ cells) were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Western blot analysis. All protein samples were obtained using a radioimmunoprecipitation assay lysis buffer (cat. no. 20-188; MilliporeSigma) on ice for 20 min and determined by the Bradford assay. The proteins isolated (10 μg/well) were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels (6-15%) and transferred to nitrocellulose western blotting membrane (cat. no. 10600002; Cytiva). Then 2% bovine serum albumin (BSA; cat. no. 5217; R&D systems) was used for blocking at 4˚C overnight. Target proteins were identified using immunoblotting assay at 4˚C overnight. The primary antibodies (1:1,000 dilution) and secondary antibodies (1:5,000-10,000) used are in the Reagents and antibodies section. The iBright™ CL1500 Imaging System (cat. no. A44114; Invitrogen; Thermo Fisher Scientific, Inc.) was used to capture images and analyze data from immunoblotting assay.

Reverse transcription-quantitative (RT-q) PCR. At between 70-80% confluence, cells were gently washed twice with PBS. Using TRIzol® (cat. no. 15596018; Thermo Fisher Scientific, Inc.), total RNAs were isolated and quantified by Nanodrop (ND-1000; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Subsequently, a thermal cycler was used to prepare cDNA from the total RNA using a first-strand cDNA synthesis kit (Bioneer Corporation) for RT-qPCR according to the manufacturer's instructions. The qPCR was performed using a LightCycler 480II (Roche Diagnostics). It was used for qPCR as follows: 2 μl diluted cDNA was mixed with 10 μl TB Green Advantage Premix (cat. no. 639676; Takara Bio, Inc.) and 1 μl (100 pM) each of forward and reverse primers according to the manufacturer's instructions. The cycling conditions were: 95˚C for 5 min for the initial denaturation, followed by 40 cycles of 95˚C for 40 sec, 58˚C for 40 sec, 72˚C for 40 sec and, finally, extension for 5 min at 72˚C. All reactions were conducted three times and normalized to GAPDH and quantifications were conducted using obtained Cp values. The sequences of all primers with GAPDH are in Table I.

Tumorsphere formation assay. Cells were cultured in DMEM/F-12-containing growth supplements, epidermal growth factor, basic fibroblast growth factor and B27 in low attachment six-well plates together with or without GA for 14 days. Cell status was checked on days 0, 7 or 14 using an optical microscope (BX-51; Olympus Corporation) at 100x and 200x magnification, and cells harvested for immunoblotting assay.

Mitochondrial and cellular ROS Analysis. $1x10^6$ cells were stained with 5 μM of MitoSOX to evaluate mROS or 5 μM of CM-H_2DCFDA to evaluate cellular ROS for 20 min at 37˚C. Then, the stained cells were used for ROS analysis using a FACSCalibur flow cytometer (BD Biosciences). FlowJo v10 software (FlowJo LLC) was used for the analysis.

Comet assay. Cellular DNA damage was measured using the comet assay kit (cat. no. 238544; Abcam) following the manufacturer's protocol. This assay is a single-cell gel electrophoresis method for a simple evaluation of cellular DNA damage. First, a base layer of comet agarose was created on a slide and then a layer of cells and agarose was added, followed by lysis. Next, under neutral conditions, electrophoresis was performed and cells were stained using a DNA dye. Finally, a fluorescence microscope was used to observe cell morphology (Olympus IX71/DP72; Olympus Corporation).

Invasion assay. Matrigel was used to pre-coat plates overnight at 4˚C. For the invasion assay, $5x10^4$ cells with or without GA were inserted into each invasion chamber (BD Biocoat; BD Biosciences) and further incubated at 37˚C for 24 h. Then,

Table I. Primer sequences for reverse transcription-quantitative PCR.

CDK4	Sense	5'-CCCGAAGTTCTTCTGCAGTC-3'
	Antisense	5'-CTGGTCGGCTTCAGAGTTTC-3'
Cyclin D1	Sense	5'-TGTTTGCAAGCAGGACTTTG-3'
	Antisense	5'-TCATCCTGGCAATGTGAGAA-3'
Cyclin E	Sense	5'-ATCCTCCAAAGTTGCACCAG-3'
	Antisense	5'-AGGGGACTTAAACGCCACTT-3'
p21	Sense	5'-ATGAAATTCACCCCCTTTCC-3'
	Antisense	5'-AGGTGAGGGGACTCCAAAGT-3'
p27	Sense	5'-CCGGCTAACTCTGAGGACAC-3'
	Antisense	5'-TTGCAGGTCGCTTCCTTATT-3'
SOX2	Sense	5'-CTGCAGTACAACTCCATGAC-3'
	Antisense	5'-GAGTGGGAGGAAGAGGTAAC-3'
Nanog	Sense	5'-ACCAGTCCCAAAGGCAAACA-3'
	Antisense	5'-TCTGCTGGAGGCTGAGGTAT-3'
Oct4	Sense	5'-CAAAGCAGAAACCCTCGTGC-3'
	Antisense	5'-AACCACACTCGGACCACATC-3'
MMP2	Sense	5'-TGATGGCATCGCTCAGATCC-3'
	Antisense	5'-GGCCTCGTATACCGCATCAA-3'
MMP3	Sense	5'-CACAGACCTGACTCGGTTCC-3'
	Antisense	5'-AGGTTCTGGAGGGACAGGTT-3'
MMP9	Sense	5'-GGACAAGCTCTTCGGCTTCT-3'
	Antisense	5'-TCGCTGGTACAGGTCGAGTA-3'
GAPDH	Sense	5'-CCCACTCCTCCACCTTTGAC-3'
	Antisense	5'-TCCTCTTGTGCTCTTGCTGG-3'

cells were stained with crystal violet at room temperate for 30 min and the invaded cells were observed using an optical microscope (BX-51; Olympus Corporation) at 100x and 200x magnification and counted.

Wound healing assay. NTERA-2 and NCCIT cells ($1x10^5$ per well) were seeded and incubated in six-well plates. After a confluent monolayer was attained, the cell layers were scratched using a 1,000 μl pipette tip and immediately incubated with GA (0 or 200 μM) for 24 h. Images were captured at different time intervals to assess the wound edges through an optical microscope (BX-51; Olympus Corporation) at 100x and 200x magnification and closure of the wound was quantified by ImageJ (v1.51; National Institutes of Health). All cells were cultured with culture medium without FBS.

Chromatin immunoprecipitation (ChIP) *assay*. A ChIP assay kit was purchased from MilliporeSigma (cat. no. 17-295) and the ChIP assay was performed according to the manufacturer's protocol. Cells ($1x10^6$) were fixed in 1% formaldehyde and quenched with 1.25 M glycine, washed with ice-cold PBS, suspended in a nuclear preparation and shearing buffer, and then sonicated (on/off 20 sec during 20 min on ice). The sheared DNA was centrifuged at 13,000 x g for 15 min at 4˚C, followed by protein/DNA immunoprecipitation from the cleared supernatant as follows. The clarified supernatant was diluted with buffer (1:1 ratio), and 5 μl aliquots of the diluted samples were used as internal controls. Next, the diluted supernatant was incubated with STAT5b anti-bodies in pre-coated wells for 90 min at room temperature. The controls were incubated with normal goat-IgG and anti-RNA polymerase II. Unbound DNA was removed and bound DNA collected using the cross-link reversal method with DNA release buffer containing proteinase K. The released DNA and internal control DNA were purified using the GenElute Binding Column G (cat. no. 17-295; MilliporeSigma). DNAs from each cell were quantified using a PCR instrument with the following primers for MMP2: Sense; 5'-TGATGGCAT CGCTCAGATCC-3' and Antisense; 5'-GGCCTCGTATAC CGCATCAA-3'.

Statistical analyses. All experiments were performed in triplicate. The values of three independent experiments conducted in triplicates (n=3) were represented as mean ± standard error of the mean. The controls were set to 100. One way analysis of variance (ANOVA) or Student's t-test were used for the statistical analyses. One-way ANOVA was also performed using Tukey's post hoc test. The analyses were performed using the SAS 9.3 software (SAS Institute, Inc.). The relative density of proteins by the effects of GA was compared with the GA non-treated control. $P<0.05$ was considered to indicate a statistically significant difference.

Results

GA inhibits the propagation of embryonic CSCs and induces apoptosis. The structure of GA, also known as 3,4,5-trihydroxy benzoic acid, is shown by Fig. 1A. First, the present study performed the MTT assay for confirming the inhibition ability

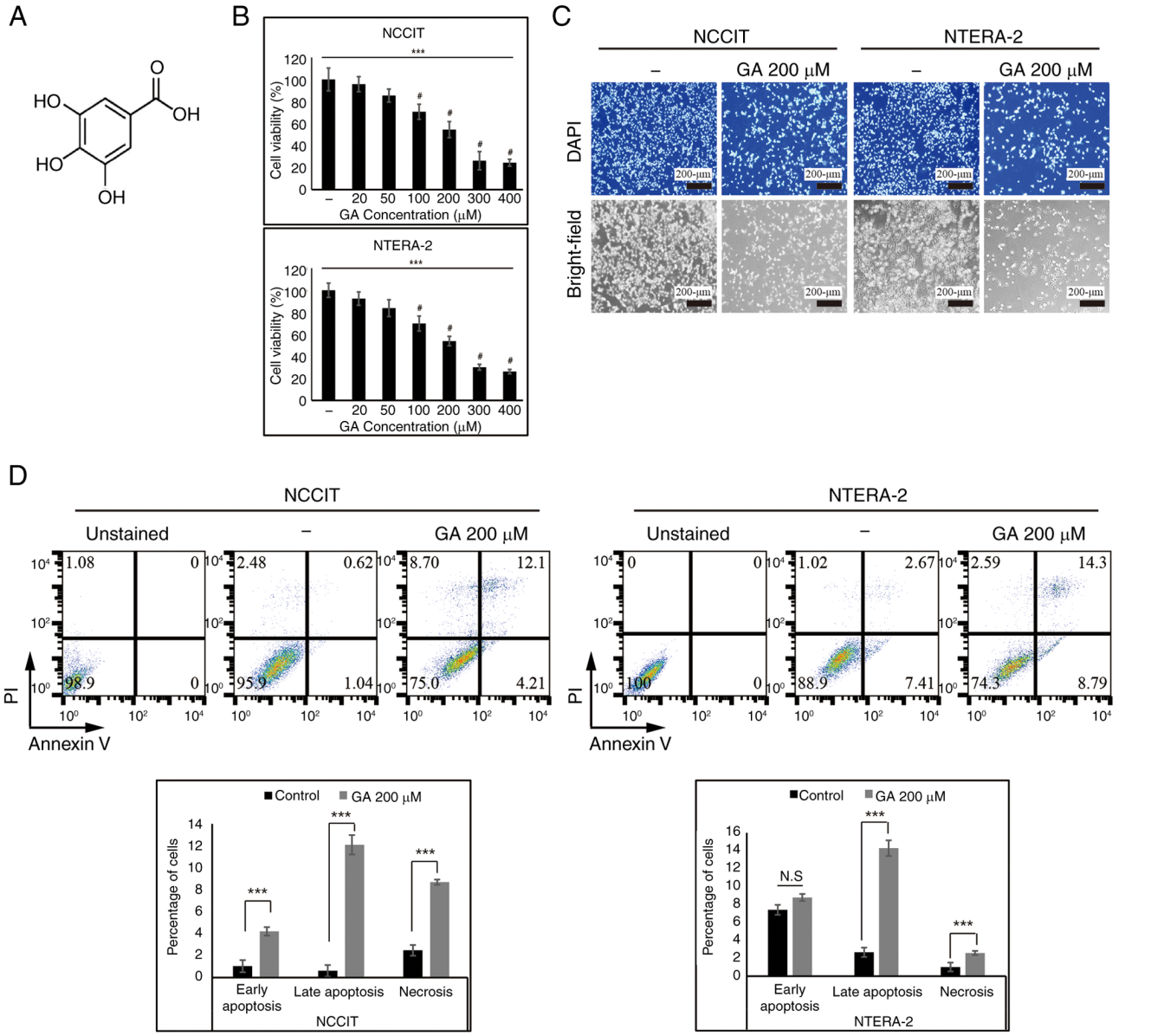

Figure 1. GA inhibits cell viability and induce apoptosis in CSCs. (A) Chemical structure of gallic acid. (B) The graphs display the cytotoxicity assay results in embryonic CSCs at 24 h. (C) GA induced nuclear abnormality in embryonic CSCs (scale bar, 200 μm). (D) The flow cytometry analysis results demonstrated induction of late apoptosis in embryonic CSCs with 0 or 200 μM GA, respectively, for 24 h. ***P<0.001 (ANOVA test) and $^{\#}$P<0.001 vs. control. GA, gallic acid; CSCs, cancer stem cells.

of cell proliferation by GA in embryonic CSCs. It confirmed a dose-dependent inhibition of cell viability through GA treatment in CSCs (Fig. 1B). The present study used 200 μM GA as the IC_{50} dose and 100- or 200 μM GA concentrations for dose-based studies. Then, the status of embryonic CSCs following 200 μM GA treatment was estimated using DAPI staining. This confirmed the characteristic features of apoptosis, such as the nuclear shrinkage and fragmentation as well as the chromatin condensation in CSCs treated with GA. A decrease in cell number following GA treatment was clearly observed compared with non-treated control (Fig. 1C). The inhibition of CSCs growth via GA treatment was verified with bright-field microscopic analysis. These results may provide evidence that GA can induce apoptosis. FACS-based Annexin V/PI staining assay was applied to assess the effects of GA on the apoptosis and necrosis of NTERA-2 and NCCIT cells. The assay may show that GA induced the increase in early-stage apoptosis (Annexin V+/PI-cells), late-stage apoptosis (Annexin V+/PI+ cells) and necrosis (Annexin V-/PI+ cells). Treatment with 200-μM GA into CSCs demonstrated the inhibitory effect of GA, suggesting that GA may induce apoptosis. In addition, increased cell death by 200-μM GA was observed and results revealed the induction of cell apoptosis by GA in both NTERA-2 and NCCIT cells (Fig. 1D).

GA induces cell cycle arrest in embryonic CSCs. The present study used 100- or 200-μM GA for further studies. Previous results suggested that GA may induce cell cycle arrest. The present study verified that cell cycle arrest in G_0/G_1 phase, which was increased through the administration of 200 μM GA, was found in CSCs through using a flow cytometry (Fig. 2A). To confirm the inhibitory induction of cell cycle

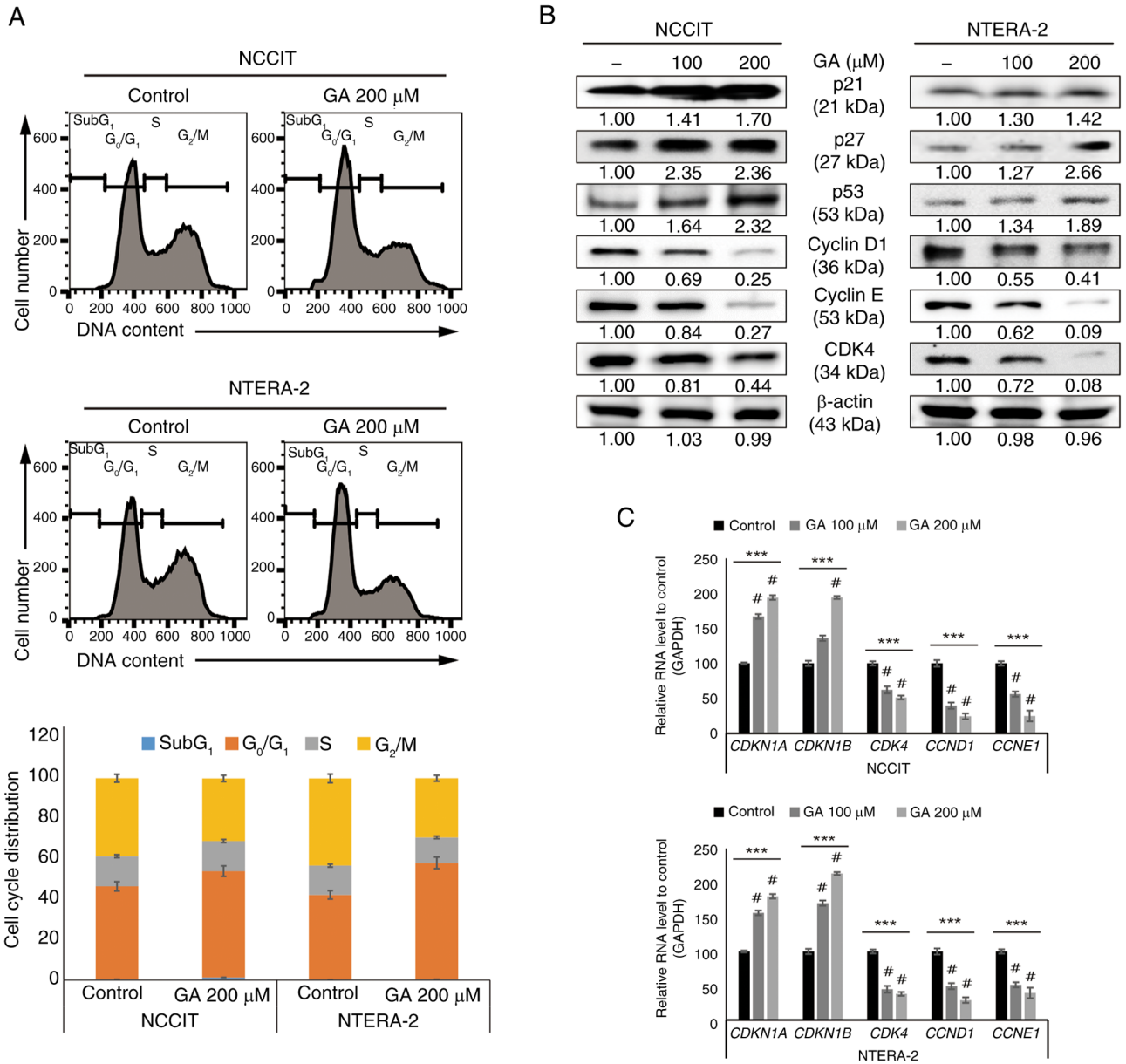

Figure 2. GA enhances the arrest of G_0/G_1 cell cycle in CSCs. (A) The flow cytometry results demonstrated the cell cycle distribution with 0 or 200 μM GA, respectively, for 24 h. Graphics display a G_0/G_1 arrest by GA in embryonic CSCs. (B) Immunoblotting analysis showed the expression patterns of p21, p27, p53, CDK4 or cyclin D1 and E with 0, 100, or 200 μM GA, respectively, for 24 h. All proteins were normalized to β-actin levels. (C) Reverse transcription-quantitative PCR represented the genetic expression of cell cycle factors, including *CDKN1A*, *CDKN1B*, *CDK4*, *CCND1* or *CCNE1* mRNA. ***P<0.001 (ANOVA test) and $^{\#}$P<0.001 vs. control. GA, gallic acid; CSCs, cancer stem cells.

by GA treatment, western blotting assay was performed to identify the lower-expression of cell cycle markers (CDK4 or cyclin D1 and E) and hyper-expression of tumor suppressor proteins (p21, p27 or p53) was confirmed (Fig. 2B). To confirm the RNA levels, qPCR was performed to assess for the expression of markers (p21, p27, CDK4 or cyclin D and E) and these results clearly demonstrated the inhibition of cell cycle arrest by GA (Fig. 2C). These results suggest anticancer activity of GA against CSCs.

GA suppresses cancer stemness through the inhibition of tumorsphere formation in embryonic CSCs. For an improved replication of the conditions *in vivo*, tumorsphere formation assay was performed for 3D culture. It was determined whether GA could downregulate the expression of CSC markers. In the analysis of the CSC markers, NANOG, OCT4 or SOX2, in NTERA-2 and NCCIT cells upon GA treatment, a decrease in the expression level of CSC markers was clearly observed (Fig. 3A). Using RT-qPCR, CSC marker repression by GA was also confirmed at the level of mRNA transcripts (Fig. 3B). These results indicated inhibition of proliferation of CSCs by GA. A tumorsphere formation assay was further performed using NTERA-2 and NCCIT cells to evaluate the GA ability to inhibit the two CSCs. NTERA-2 and NCCIT cells were cultured in the tumorsphere medium using low attachment plates with GA for 14-days. Images were captured using a microscope, which indicated a significant reduction in tumorspheres following the treatment of 200 μM GA compared with untreated controls (Fig. 3C). To confirm CSCs suppression by GA on the protein level using these samples, western

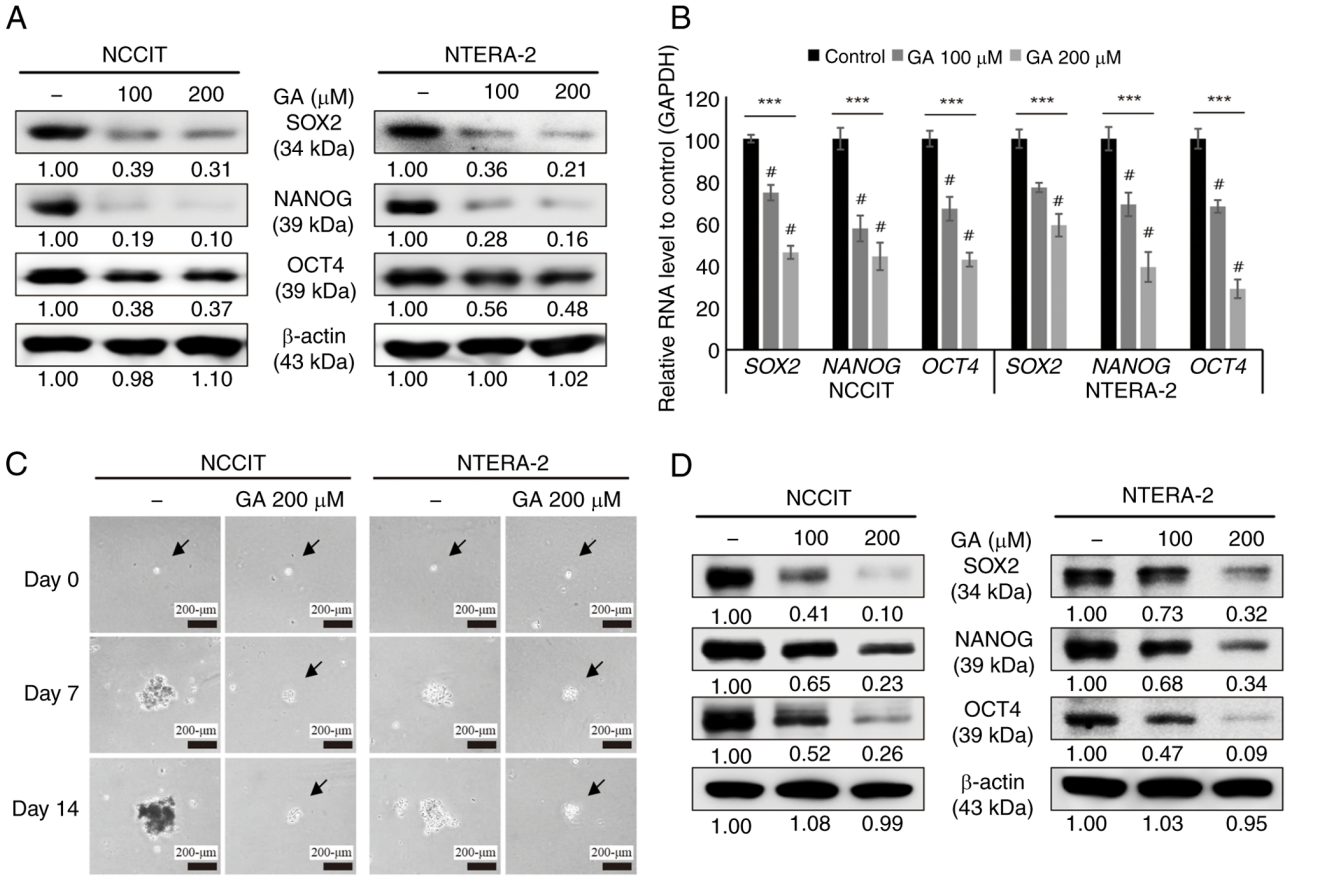

Figure 3. GA inhibits cancer stemness in CSCs. (A) The expression patterns of CSC markers, NANOG, SOX2 or OCT4, were incubated with 0 or 200 μM GA, respectively, for 24 h. (B) Reverse transcription-quantitative PCR of CSC marker mRNAs was performed and normalized to GAPDH mRNA. ***P<0.001 (ANOVA test) and $^{\#}$P<0.001 vs. control. (C) The tumorsphere formation assay of CSCs were performed with 0 or 200 μM GA, respectively, for 14 days (scale bar, 200 μm). (D) After 14 days, the CSC marker expression levels of NANOG, SOX2 or OCT4 were measured via western blotting. GA, gallic acid; CSCs, cancer stem cells.

blotting was used to identify the expression levels of specific CSC markers after sphere formation culture for 14 days. A significant downregulation of stem cell markers, NANOG, OCT4 or SOX2, by GA was observed (Fig. 3D). These results demonstrated the repression of target CSCs by GA.

GA activates ROS mediated-DNA damage in embryonic CSCs. The present study identified GA activity that induces cell cycle arrest and apoptosis in CSCs. Thus, it further confirmed whether ROS was also induced by GA, as this is crucial in anticancer study or application. MitoSOX and CM-H_2DCFDA reagents are widely used to identify mitochondrial and cellular ROS. MitoSOX and CM-H_2DCFDA reagents were used for detecting the mitochondrial and cellular ROS by GA. It was found that the treatment of 200-μM GA can show the considerable induction of cellular ROS levels in both NTERA-2 and NCCIT cells (Fig. 4A). As the mitochondria are known as the major source of cellular ROS in cells, an increase in cellular ROS levels by GA may imply ROS induction in mitochondria. The induction of higher mitochondrial ROS as in the case of cellular ROS by 200-μM GA in CSCs was also detected by flow cytometry analysis (Fig. 4B). Based on the hypothesis that the induction of cell death, cell cycle arrest or apoptosis was a result of DNA damage, presence of DNA damage was therefore confirmed. Excessive ROS product is known to cause DNA damage and induce the DDR pathway. To confirm this, the induction of DDR protein phosphorylation was investigated and confirmed upregulation in DDR proteins, p-BRCA1, ATM, CHK1, CHK2 or histone when treated with increased GA concentrations, focusing on NTERA-2 and NCCIT cells (Fig. 4C). Furthermore, the activity of GA to promote DDR mechanism was evaluated using a comet assay for detecting DNA fragmentation. A significant increase in comet-positive cells was also noted, including the length of comet in GA treated-cells compared with untreated-control cells (Fig. 4D). These results may validate the capability of GA to cause DNA damage in embryonic CSCs.

GA inhibits migration and invasion of embryonic CSCs. GA prevented CSCs from normally expanding and growing through DNA damage and inhibition of sphere culture growth. Hence, it was hypothesized that GA can inhibit tumor metastasis and invasion. The ability of GA to suppress CSCs migration and invasion was analyzed to confirm metastasis. First, the present study analyzed for molecular markers to confirm the capacity for invasion and the decrease of MMP proteins, MMP-2, -3 or -9, by GA was identified, suggesting the reduction of CSC progression and metastasis (Fig. 5A). These results were then validated via the analysis of gene expression patterns at the level of mRNA transcripts and

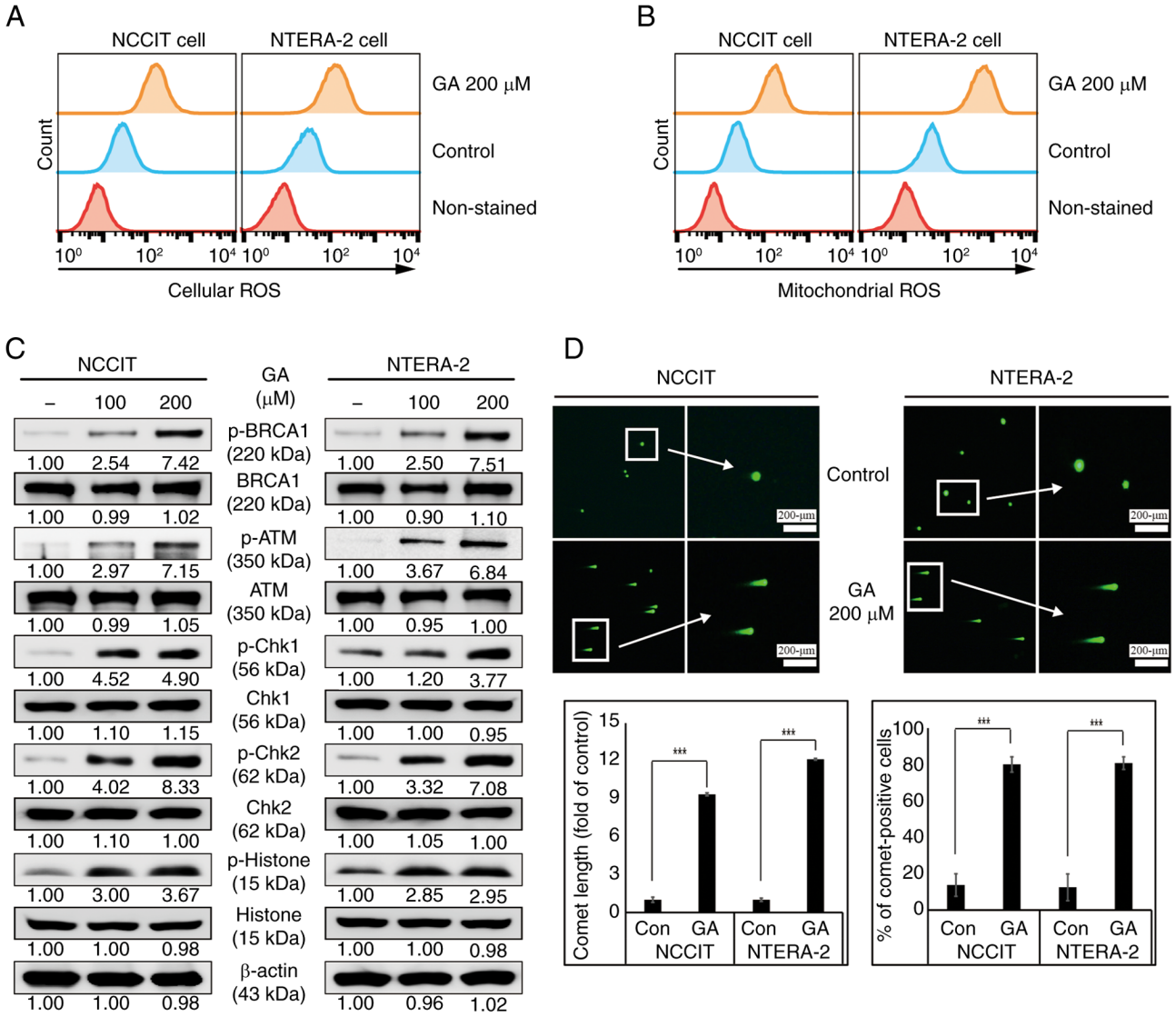

Figure 4. GA enhances ROS generation and DNA damage induction in CSCs. (A) The flow cytometry analysis for detecting cellular ROS with 0 or 200 μM GA, respectively, for 24 h. The image showed the embryonic CSCs with induction of cellular ROS. (B) The flow cytometry analysis for detecting mitochondrial ROS with 0 or 200 μM GA, respectively, for 24 h. The graphical image revealed embryonic CSCs with the induction of mitochondrial ROS. (C) The western blotting analysis results showed the induction of the DDR factors, p-BRCA1, p-ATM, p-Chk1, pChk2 and p-Histone with 0, 100 or 200 μM GA, respectively, for 24 h. (D) The comet assay results indicated the fragmented DNA migration from the nucleoid body and the percentage of comet-positive CSCs with 0 or 200 μM GA, respectively, for 24 h. ***P<0.001 (ANOVA test) vs. control. GA, gallic acid; ROS, reactive oxygen species; CSCs, cancer stem cells; p-, phosphorylated.

GA induced the downregulated mRNA levels of MMPs in two CSCs (Fig. 5B). An invasion assay was also performed to confirm whether GA can induce the inhibition of CSCs metastasis and the results indicated a significant suppression of invaded cells in GA treated-CSCs (Fig. 5C). Moreover, to determine whether CSCs can migrate after exposure to GA, migration-inhibiting effects by GA were confirmed through a wound-healing assay (Fig. 5D). Overall, these results indicated that GA effectively repressed cell migration and invasion through MMPs expression inhibition in embryonic CSCs.

GA inhibits the EGFR-STAT5 pathway and DNA-protein binding activity by STAT5. After confirmation that GA can inhibit migration and invasion, the mechanism of molecular signaling by GA was evaluated. The EGFR/JAK2/STAT5b pathway is known to be associated with tumor invasion and migration. To validate these results, the EGFR-STAT pathway was assessed through GA treatment and the suppression of EGFR phosphorylation blocked cellular signaling toward the downstream targets of EGFR (Fig. 6A). Additionally, to assess the relationship between STAT5 and MMP-2, GA and/or a specific STAT5 siRNA was used to confirm the STAT5-mediated MMP-2 expression (Fig. 6B). These results showed a significant inhibition of p-STAT5 and MMP-2 expression in GA-treated samples and its combination with STAT5 siRNA samples (Fig. 6B). To demonstrate the p-STAT5 binding onto the MMP-2 promoter region by GA inhibition, primers specific to the MMP-2 genome were designed and its binding was confirmed using ChIP assay. GA-treated samples indicated the considerable suppression of STAT5/MMP-2 binding (Fig. 6C). This result clearly

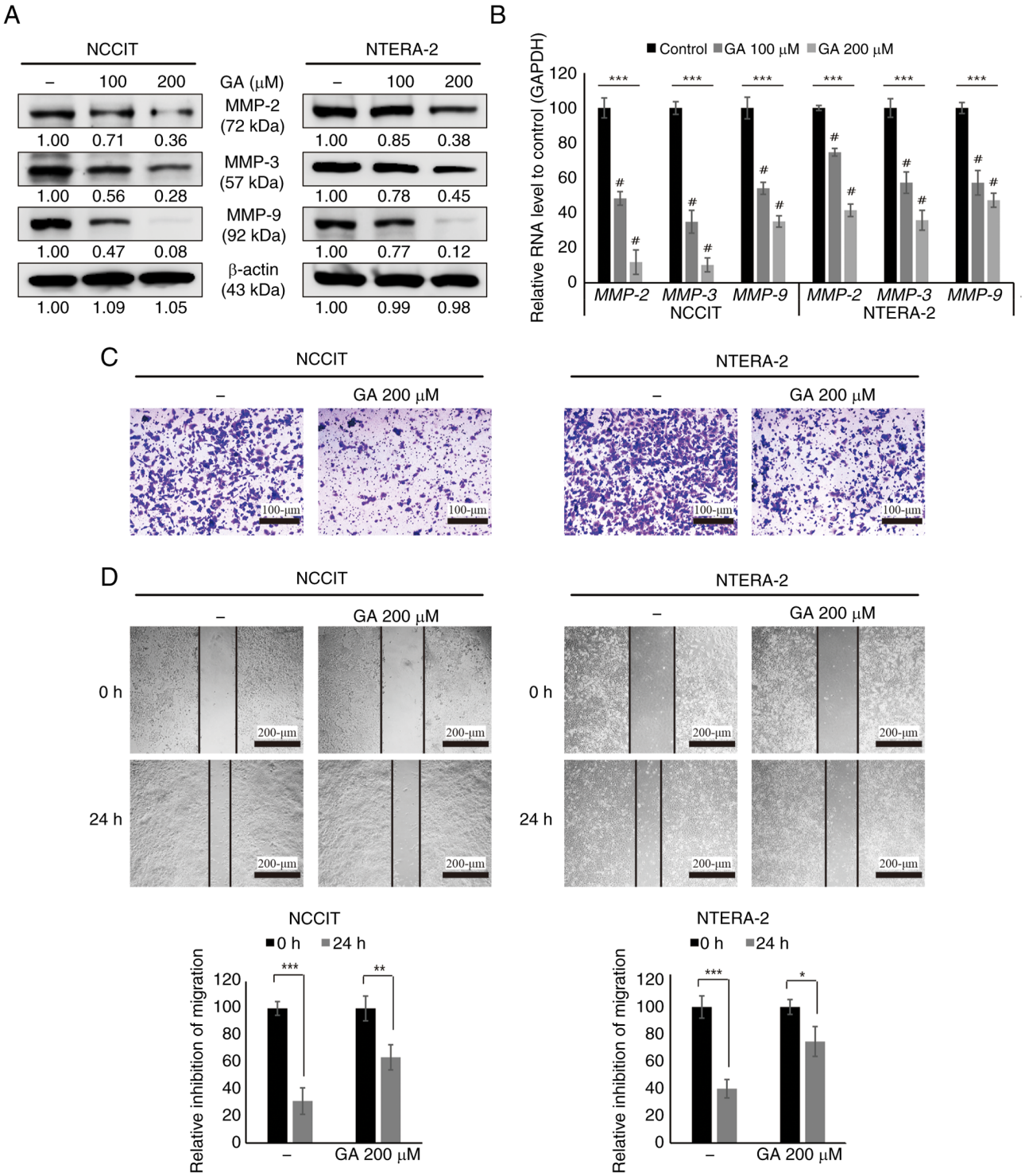

Figure 5. GA weakens migration and invasion in embryonic CSCs. (A) Immunoblotting analysis reveals the expression patterns of MMP-2, -3 or -9 protein with 0, 100 or 200 μM GA, respectively, for 24 h. (B) The analysis of RT-qPCR indicates the genetic expression of cell cycle factors including *MMP-2*, *-3* or -9 mRNA. (C) The results of a Matrigel invasion assay indicate the suppression of embryonic CSCs invasion with 0 or 200 μM GA, respectively, for 24 h (scale bar, 100 μm). (D) The wound healing assay shows the inhibitory migration of embryonic CSC with 0 or 200 μM GA, respectively, for 24 h (scale bar, 200 μm). ***P<0.001, **P<0.01 and *P<0.05 (ANOVA test); $^{\#}$P<0.001 vs. control. GA, gallic acid; CSCs, cancer stem cells; MMP, matrix metalloproteinase.

suggested the role of the EGFR/STAT5/MMP-2 signaling cascade in the anticancer ability of GA against embryonic CSCs. Altogether, GA can inhibit cancer hallmarks against NCCIT and NTERA-2 human embryonic carcinoma cells through EGFR-mediated JAK2/STAT5 signaling mechanisms (Fig. 7).

Discussion

CSCs are considered to be a subpopulation of tumor cells, commonly observed in a majority of liquid and solid cancers (3-6). CSCs feature specific characteristics, including self-renewal, tumorigenesis, multilineage differentiation

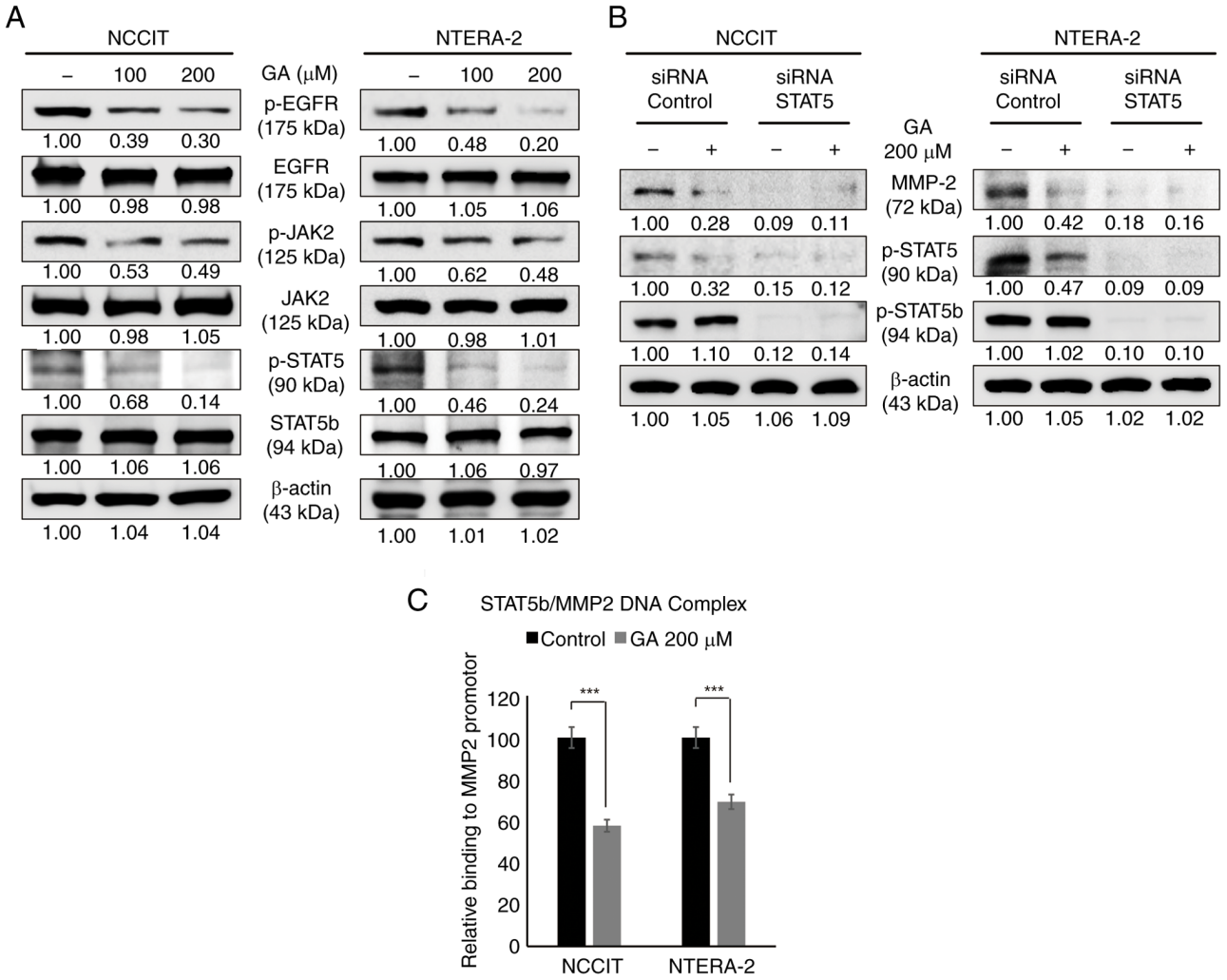

Figure 6. GA inhibits migration and invasion through the decrease of the EGFR-STAT5 pathway in embryonic CSCs. (A) The western blotting assay in embryonic CSCs with 0, 100 or 200 μM GA, respectively, for 24 h for p-EGFR, JAK2 or STAT5. (B) Immunoblotting analysis showed that treatment with 30 pM STAT5 siRNA or 200 μM GA for 24 h demonstrated the decrease of MMP-2, STAT5 or p-STAT5b levels. (C) The ChIP assay showed that GA inhibited the formation of the STAT5b/MMP2 complexes in embryonic CSCs with 200 μM GA, respectively, for 24 h. ***P<0.001 (Student's t-test). GA, gallic acid; CSCs, cancer stem cells; p-, phosphorylated; MMP, matrix metalloproteinase.

potential and tumorigenicity (7,62). Success in cancer treatment using drugs is mainly dependent not only on how it affects targeted cancer cells but also on its ability to prevent cancer recurrence of CSCs. Although several types of chemotherapeutic drugs successfully inhibit tumor progression, in a number of cases these fail to target CSCs which ultimately results in cancer recurrence (63,64). CSCs possess a formidable ability to evade or resist the killing effect of chemotherapeutic drugs through genetic instability, hypoxic stability and expression of drug efflux transports and anti-apoptotic proteins (65-68). It has been shown that various types of natural compounds have anticancer effects, indicating their potential as a drug (11,36,69). It has also been shown that a chemotherapeutic drug and a natural compound exhibit optimal efficacy when used in combination compared with using the drug alone (70). Therefore, chemotherapy using natural compounds may be an attractive method as it reduces side effects in both cancer cells and CSCs.

GA is a natural phenolic compound, well-recognized to have anticancer ability against diverse types of cancer cells *in vitro* and *in vivo* (48,56-58,71). Some studies have shown that GA can suppress cell proliferation by inducing cell cycle arrest and apoptosis in lung and ovarian cancer cells (72,73). The present study revealed that GA induced late apoptosis in both NTERA-2 and NCCIT cells compared with others (that is, early apoptosis and necrosis). Furthermore, GA induced the arrest of G_0/G_1 cell cycle through the downregulation of CDK4 or cyclin D1 and E and upregulation of p21, p27 or p53, suggesting its anticancer activity against CSCs.

CSC markers play a role in transcription factors, essential in maintaining the pluripotent ESC phenotype in CSCs (10). Furthermore, they are important factors in forming CSC tumorspheres. Thus, CSC markers are deemed good targets for drug development. In previous studies, a natural compound exhibited inhibition of sphere formation in CSCs (11) and suppressed the expression of Wnt/β-catenin-mediated CSC markers (74). The present study found that GA inhibited cancer stemness through the suppression of tumorsphere formation in CSCs. GA effectively downregulated CSC markers in mRNA and protein levels and inhibited CSC marker-mediated

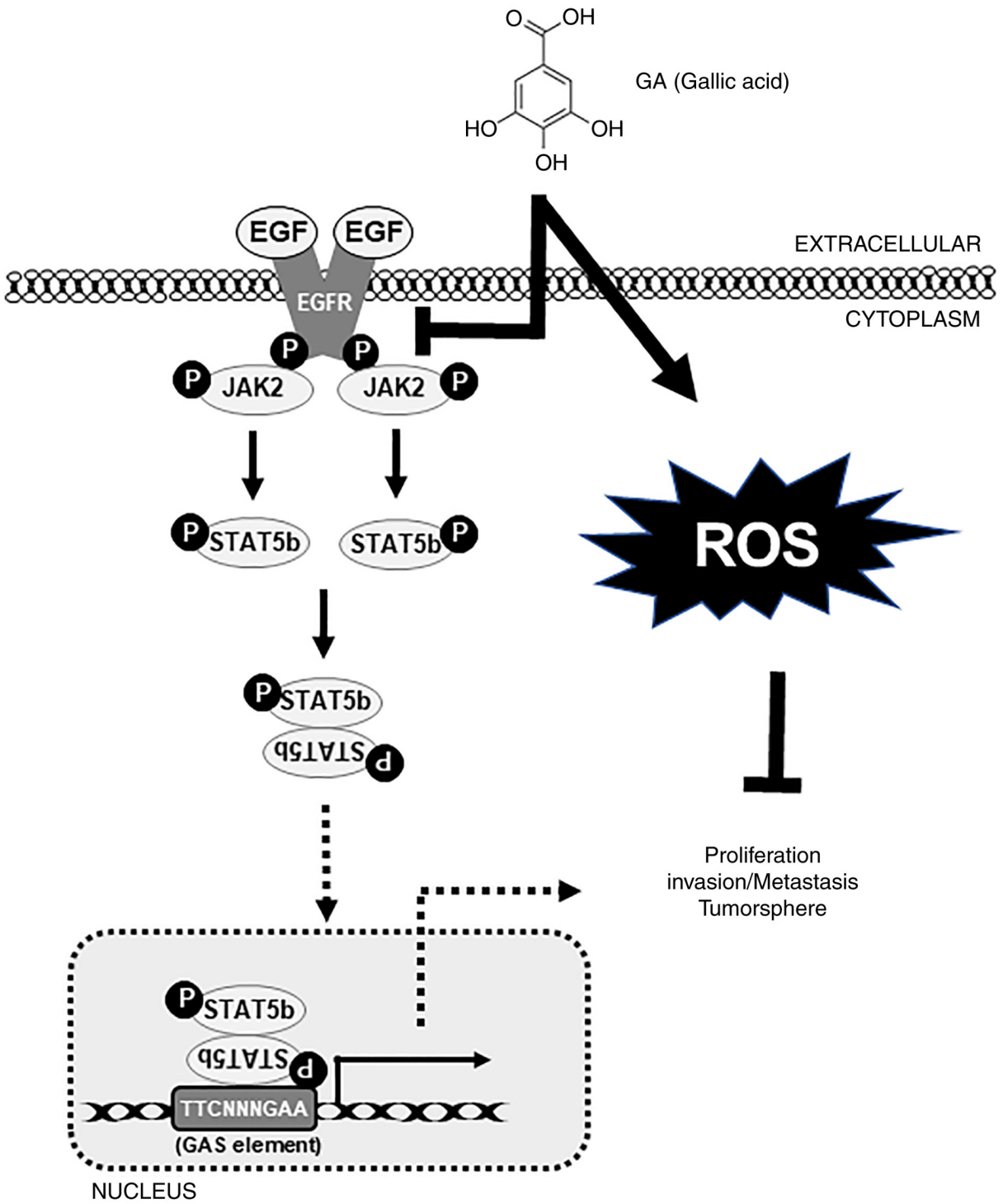

Figure 7. Molecular regulatory mechanism shows the anticancer activities of gallic acid in embryonic NCCIT and NTERA-2 cells. ROS, reactive oxygen species; p-, phosphorylization; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; JAK2, Janus Kinase 2; STAT5b, signal transducer and activator of transcription 5b.

tumorsphere formation. Generally, prolonged ROS generation is known to induce to DNA fragmentation, genomic instability and loss of heterozygosity. ROS play a pivotal role in tumorigenesis by inducing DNA damage that results in DDR mechanism. A number of natural compounds can activate the induction of DDR mechanism in cancer cells, finally leading to cell cycle arrest or apoptosis, suggesting that they can be possible candidates for further studies. Prior studies have demonstrated that GA prompts the induction of DNA damage and the inhibition of DNA repair-associated protein expression, resulting in DDR mechanism in some cancer cells (75-77). The present study observed that GA elevated cellular ROS and mitochondrial ROS in CSCs, suggesting an anticancer activity of GA. Furthermore, a high ROS generation by GA upregulated the DDR mechanism proteins, such as BRCA1, ATM or CHK1 kinase which are central sensors or regulator in DDR in cells. The results indicated that the increase in the expression of these kinases by GA enhanced the arrest of G_0/G_1 cell cycle and

apoptosis in CSCs. MMPs perform a key role in the decrease of various proteins in the ECM, resulting in tumorigenesis, tumor growth and tumor invasion and metastasis (30,31). Although the PI3K/AKT/mTOR pathway is related to tumor cell invasion and migration, the JAK/STAT pathway regulates the expression of MMPs, playing a crucial role in cancer progression via invasion or angiogenesis (33,78). Its regulation could be considered as one of the potential targets for halting tumor invasion or migration and thus cancer progression. Our previous study shows that a natural compound markedly inhibits cell invasion in compound-treated cancer cell (39). Thus, the present study demonstrated the decrease of these transmembrane signals that finally control MMPs in CSCs through GA. Eventually, GA decreased the EGFR/STAT5 pathway that enhanced MMP expression, leading to a significant inhibition of the STAT5/MMP2 binding in the nucleus. The present study confirmed that GA has anti-cell invasion and anti-migration properties by inhibiting the JAK/STAT pathway. However, it is necessary to additionally conform that GA suppresses invasion and migration of CSCs by inhibiting PI3K/AKT/mTOR pathway.

The present study revealed that GA targets embryonic CSCs through the inhibition of cell proliferation. In addition, it was also evident that GA enhanced the induction of cellular and mitochondrial ROS, leading to DDR against to CSCs. In addition, it revealed that GA resulted in late apoptosis via the arrest of G_0/G_1 cell cycle. Finally, the significant suppression of MMPs, which are key factors for tumor invasion, was demonstrated by GA. Altogether, GA could be an attractive agent for preventing cancer recurrence by targeting CSCs.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) to KJ. (grant no. RS-2024-00450676). It was also supported by the Basic Science Research Program to Research Institute for Basic Sciences (RIBS) of Jeju National University through the National Research Foundation of Korea (NRF) funded by the Ministry of Education to SB. (grant no. 2019R1A6A1A10072987) and the Korea Basic Science Institute (National research Facilities and Equipment Center) grant funded by the Ministry of Education. (grant no. 2023R1A6C101A045). The present study was also supported by the faculty research fund of Sejong University in 2024.

Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

KJ designed the experiments and wrote the manuscript. DK and SB performed all the experiments and analyzed the data. DK, SB and KJ confirm the authenticity of all the raw data and helped to revise the manuscript. All authros read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Heron M and Anderson RN: Changes in the leading cause of death: Recent patterns in heart disease and cancer mortality. NCHS Data Brief 254: 1-8, 2016.
2. Steeg PS: Targeting metastasis. Nat Rev Cancer 16: 201-218, 2016.
3. Maiuthed A, Chantarawong W and Chanvorachote P: Lung cancer stem cells and cancer stem cell-targeting natural compounds. Anticancer Res 38: 3797-3809, 2018.
4. Pardal R, Clarke MF and Morrison SJ: Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 3: 895-902, 2003.
5. Koren E and Fuchs Y: The bad seed: Cancer stem cells in tumor development and resistance. Drug Resist Updat 28: 1-12, 2016.
6. Adorno-Cruz V, Kibria G, Liu X, Doherty M, Junk DJ, Guan D, Hubert C, Venere M, Mulkearns-Hubert E, Sinyuk M, *et al*: Cancer stem cells: Targeting the roots of cancer, seeds of metastasis, and sources of therapy resistance. Cancer Res 75: 924-929, 2015.
7. Nassar D and Blanpain C: Cancer stem cells: Basic concepts and therapeutic implications. Annu Rev Pathol 11: 47-76, 2016.
8. Andrews PW, Damjanov I, Berends J, Kumpf S, Zappavigna V, Mavilio F and Sampath K: Inhibition of proliferation and induction of differentiation of pluripotent human embryonal carcinoma cells by osteogenic protein-1 (or bone morphogenetic protein-7). Lab Invest 71: 243-251, 1994.
9. Donovan PJ and Gearhart J: The end of the beginning for pluripotent stem cells. Nature 414: 92-97, 2001.
10. Rodda DJ, Chew JL, Lim LH, Loh YH, Wang B and Ng HH, Robson P: Transcriptional regulation of nanog by Oct4 and Sox2. J Biol Chem 280: 24731-24737, 2005.
11. Sp N, Kang DY, Kim DH, Park JH, Lee HG, Kim HJ, Darvin P, Park YM and Yang YM: Nobiletin inhibits CD36-dependent tumor angiogenesis, migration, invasion, and sphere formation through the CD36/Stat3/NF-kB signaling axis. Nutrients 10: 772, 2018.
12. Pitrone M, Pizzolanti G, Tomasello L, Coppola A, Morini L, Pantuso G, Ficarella R, Guarnotta V, Perrini S, Giorgino F and Giordano C: NANOG plays a hierarchical role in the transcription network regulating the pluripotency and plasticity of adipose tissue-derived stem cells. Int J Mol Sci 18: 1107, 2017.
13. Zhang YS, Eades G, Yao Y, Li QL and Zhou Q: Estrogen receptor alpha signaling regulates breast tumor-initiating cells by down-regulating miR-140 which targets the transcription factor SOX2. J Biol Chem 287: 41514-41522, 2012.
14. Eini R, Stoop H, Gillis AJ, Biermann K, Dorssers LC and Looijenga LH: Role of SOX2 in the etiology of embryonal carcinoma, based on analysis of the NCCIT and NT2 cell lines. PLoS One 9: e83585, 2014.
15. Jeter CR, Yang T, Wang JC, Chao HP and Tang DG: Concise review: NANOG in cancer stem cells and tumor development: An update and outstanding questions. Stem Cells 33: 2381-2390, 2015.
16. Lin T, Ding YQ and Li JM: Overexpression of Nanog protein is associated with poor prognosis in gastric adenocarcinoma. Med Oncol 29: 878-885, 2012.
17. Yang J, Zhao XY, Tang M, Li L, Lei Y, Cheng P, Guo W, Zheng Y, Wang W, Luo N, *et al*: The role of ROS and subsequent DNA-damage response in PUMA-induced apoptosis of ovarian cancer cells. Oncotarget 8: 23492-23506, 2017.

18. Srinivas US, Tan BWQ, Vellayappan BA and Jeyasekharan AD: ROS and the DNA damage response in cancer. Redox Biol 25: 101084, 2019.
19. Ye Z, Shi Y, Lees-Miller SP and Tainer JA: Function and molecular mechanism of the DNA damage response in immunity and cancer immunotherapy. Front Immunol 12: 797880, 2021.
20. Maréchal A and Zou L: DNA damage sensing by the ATM and ATR kinases. Cold Spring Harb Perspect Biol 5: a012716, 2013.
21. Zhou BB and Elledge SJ: The DNA damage response: Putting checkpoints in perspective. Nature 408: 433-439, 2000.
22. Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y and Ziv Y: Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science 281: 1674-1677, 1998.
23. Visconti R, Della Monica R and Grieco D: Cell cycle checkpoint in cancer: A therapeutically targetable double-edged sword. J Exp Clin Cancer Res 35: 153, 2016.
24. Malumbres M: Cyclin-dependent kinases. Genome Biol 15: 122, 2014.
25. Deng C, Zhang P, Harper JW, Elledge SJ and Leder P: Mice lacking P21Cip1/WAF1 undergo normal development, but are defective in G1 checkpoint CONTROL. Cell 82: 675-684, 1995.
26. Abbas T and Dutta A: p21 in cancer: Intricate networks and multiple activities. Nat Rev Cancer 9: 400-414, 2009.
27. Hong B, van den Heuvel AP, Prabhu VV, Zhang S and El-Deiry WS: Targeting tumor suppressor p53 for cancer therapy: Strategies, challenges and opportunities. Curr Drug Targets 15: 80-89, 2014.
28. Stöcker W, Grams F, Baumann U, Reinemer P, Gomis-Rüth FX, McKay DB and Bode W: The metzincins-topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases. Protein Sci 4: 823-840, 1995.
29. Lohi J, Wilson CL, Roby JD and Parks WC: Epilysin, a novel human matrix metalloproteinase (MMP-28) expressed in testis and keratinocytes and in response to injury. J Biol Chem 276: 10134-10144, 2001.
30. Egeblad M and Werb Z: New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2: 161-174, 2002.
31. Hadler-Olsen E, Winberg JO and Uhlin-Hansen L: Matrix metalloproteinases in cancer: Their value as diagnostic and prognostic markers and therapeutic targets. Tumour Biol 34: 2041-2051, 2013.
32. Wu JS, Sheng SR, Liang XH and Tang YL: The role of tumor microenvironment in collective tumor cell invasion. Future Oncol 13: 991-1002, 2017.
33. Chambers AF and Matrisian LM: Changing views of the role of matrix metalloproteinases in metastasis. J Natl Cancer Inst 89: 1260-1270, 1997.
34. Russo S, Cinausero M, Gerratana L, Bozza C, Iacono D, Driol P, Deroma L, Sottile R, Fasola G and Puglisi F: Factors affecting patient's perception of anticancer treatments side-effects: An observational study. Expert Opin Drug Saf 13: 139-150, 2014.
35. Wamukaya JW and Philis PB: Outcome of supportive management in the prevention of chemotherapy induced nausea and vomiting in a resource limited set up-nurse experience. Asia-Pac J Clin Oncol 10: 194-196, 2014.
36. Sp N, Kang DY, Joung YH, Park JH, Kim WS, Lee HK, Song KD, Park YM and Yang YM: Nobiletin inhibits angiogenesis by regulating Src/FAK/STAT3-mediated signaling through PXN in ER+ breast cancer cells. Int J Mol Sci 18: 935, 2017.
37. Sp N, Kang DY, Lee JM, Bae SW and Jang KJ: Potential antitumor effects of 6-gingerol in p53-dependent mitochondrial apoptosis and inhibition of tumor sphere formation in breast cancer cells. Int J Mol Sci 22: 4660, 2021.
38. Lin SR, Fu YS, Tsai MJ, Cheng H and Weng CF: Natural compounds from herbs that can potentially execute as autophagy inducers for cancer therapy. Int J Mol Sci 18: 1412, 2017.
39. Rugamba A, Kang DY, Sp N, Jo ES, Lee JM, Bae SW and Jang KJ: Silibinin regulates tumor progression and tumorsphere formation by suppressing PD-L1 expression in non-small cell lung cancer (NSCLC) cells. Cells 10: 1632, 2021.
40. Sp N, Kang DY, Jo ES, Lee JM, Bae SW and Jang KJ: Pivotal role of iron homeostasis in the induction of mitochondrial apoptosis by 6-gingerol through pten regulated PD-L1 expression in embryonic cancer cells. Front Oncol 11: 781720, 2021.
41. Ouyang L, Luo Y, Tian M, Zhang SY, Lu R, Wang JH, Kasimu R and Li X: Plant natural products: From traditional compounds to new emerging drugs in cancer therapy. Cell Prolif 47: 506-515, 2014.
42. Huang M, Lu JJ and Ding J: Natural products in cancer therapy: Past, present and future. Nat Prod Bioprospect 11: 5-13, 2021.
43. Ali Abdalla YO, Subramaniam B, Nyamathulla S, Shamsuddin N, Arshad NM, Mun KS, Awang K and Nagoor NH: Natural products for cancer therapy: A review of their mechanism of actions and toxicity in the past decade. J Trop Med 2022: 5794350, 2022.
44. Talib WH, Alsalahat I, Daoud S, Abutayeh RF and Mahmod AI: Plant-derived natural products in cancer research: Extraction, mechanism of action, and drug formulation. Molecules 25: 5319, 2020.
45. Shahrzad S, Aoyagi K, Winter A, Koyama A and Bitsch I: Pharmacokinetics of gallic acid and its relative bioavailability from tea in healthy humans. J Nutr 131: 1207-1210, 2001.
46. Nabavi SF, Habtemariam S, Di Lorenzo A, Sureda A, Khanjani S, Nabavi SM and Daglia M: Post-stroke depression modulation and in vivo antioxidant activity of gallic acid and its synthetic derivatives in a murine model system. Nutrients 8: 248, 2016.
47. Abdelwahed A, Bouhlel I, Skandrani I, Valenti K, Kadri M, Guiraud P, Steiman R, Mariotte AM, Ghedira K, Laporte F, *et al*: Study of antimutagenic and antioxidant activities of gallic acid and 1,2,3,4,6-pentagalloylglucose from Pistacia Lentiscus. Confirmation by microarray expression profiling. Chem Biol Interact 165: 1-13, 2007.
48. Velderrain-Rodríguez GR, Torres-Moreno H, Villegas-Ochoa MA, Ayala-Zavala JF, Robles-Zepeda RE, Wall-Medrano A and González-Aguilar GA: Gallic acid content and an antioxidant mechanism are responsible for the antiproliferative activity of 'Ataulfo' mango peel on LS180 cells. Molecules 23: 695, 2018.
49. Kim SW, Han YW, Lee ST, Jeong HJ, Kim SH, Kim IH, Lee SO, Kim DG, Kim SH, Kim SZ and Park WH: A superoxide anion generator, pyrogallol, inhibits the growth of HeLa cells via cell cycle arrest and apoptosis. Mol Carcinog 47: 114-125, 2008.
50. Sorrentino E, Succi M, Tipaldi L, Pannella G, Maiuro L, Sturchio M, Coppola R and Tremonte P: Antimicrobial activity of gallic acid against food-related pseudomonas strains and its use as biocontrol tool to improve the shelf life of fresh black truffles. Int J Food Microbiol 266: 183-189, 2018.
51. Couto AG, Kassuya CAL, Calixto JB and Petrovick PR: Anti-inflammatory, antiallodynic effects and quantitative analysis of gallic acid in spray dried powders from Phyllanthus Niruri leaves, stems, roots and whole plant. Rev Bras Farmacogn 23: 124-131, 2013.
52. Lee JH, Oh M, Seok JH, Kim S, Lee DB, Bae G, Bae HI, Bae SY, Hong YM, Kwon SO, *et al*: Antiviral effects of black raspberry (Rubus coreanus) seed and its gallic acid against influenza virus infection. Viruses 8: 157, 2016.
53. Rasooly R, Choi HY, Do P, Morroni G, Brescini L, Cirioni O, Giacometti A and Apostolidis E: whISOBAX™ inhibits bacterial pathogenesis and enhances the effect of antibiotics. Antibiotics (Basel) 9: 264, 2020.
54. Schimites PI, Segat HJ, Teixeira LG, Martins LR, Mangini LT, Baccin PS, Rosa HZ, Milanesi LH, Burger ME and Soares AV: Gallic acid prevents ketamine-induced oxidative damages in brain regions and liver of rats. Neurosci Lett 714: 134560, 2020.
55. Zhang TX, Ma LJ, Wu PF, Li W, Li T, Gu R, Dan X, Li Z, Fan X and Xiao Z: Gallic acid has anticancer activity and enhances the anticancer effects of cisplatin in non-small cell lung cancer A549 cells via the JAK/STAT3 signaling pathway. Oncol Rep 41: 1779-1788, 2019.
56. You BR, Moon HJ, Han YH and Park WH: Gallic acid inhibits the growth of HeLa cervical cancer cells via apoptosis and/or necrosis. Food Chem Toxicol 48: 1334-1340, 2010.
57. Subramanian AP, Jaganathan SK, Mandal M, Supriyanto E and Muhamad II: Gallic acid induced apoptotic events in HCT-15 colon cancer cells. World J Gastroenterol 22: 3952-3961, 2016.
58. Tang HM and Cheung PCK: Gallic acid triggers iron-dependent cell death with apoptotic, ferroptotic, and necroptotic features. Toxins (Basel) 11: 492, 2019.
59. Phan AN, Hua TN, Kim MK, Vo VT, Choi JW, Kim HW, Rho JK, Kim KW and Jeong Y: Gallic acid inhibition of Src-Stat3 signaling overcomes acquired resistance to EGF receptor tyrosine kinase inhibitors in advanced non-small cell lung cancer. Oncotarget 7: 54702-54713, 2016.
60. Liao CC, Chen SC, Huang HP and Wang CJ: Gallic acid inhibits bladder cancer cell proliferation and migration via regulating fatty acid synthase (FAS). J Food Drug Anal 26: 620-627, 2018.

61. Zeng M, Su Y, Li K, Jin D, Li Q, Li Y and Zhou B: Gallic acid inhibits bladder cancer T24 cell progression through mitochondrial dysfunction and PI3K/Akt/NF-κB signaling suppression. Front Pharmacol 11: 1222, 2020.
62. Li Z, Bao S, Wu Q, Wang H, Eyler C, Sathornsumetee S, Shi Q, Cao Y, Lathia J, McLendon RE, *et al*: Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells. Cancer Cell 15: 501-513, 2009.
63. Dawood S, Austin L and Cristofanilli M: Cancer stem cells: Implications for cancer therapy. Oncology (Williston Park) 28: 1101-1107, 1110, 2014.
64. Brehmer B, Kauffmann C, Blank C, Heidenreich A and Bex A: Resection of metastasis and local recurrences of renal cell carcinoma after presurgical targeted therapy: Probability of complete local control and outcome. World J Urol 34: 1061-1066, 2016.
65. Ewald B, Sampath D and Plunkett W: Nucleoside analogs: Molecular mechanisms signaling cell death. Oncogene 27: 6522-6537, 2008.
66. Wilson TR, Johnston PG and Longley DB: Anti-apoptotic mechanisms of drug resistance in cancer. Curr Cancer Drug Targets 9: 307-319, 2009.
67. Rochat B: Importance of influx and efflux systems and xenobiotic metabolizing enzymes in intratumoral disposition of anticancer agents. Curr Cancer Drug Targets 9: 652-674, 2009.
68. Ho MM, Ng AV, Lam S and Hung JY: Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. Cancer Res 67: 4827-4833, 2007.
69. Kang DY, Darvin P, Yoo YB, Joung YH, Sp N, Byun HJ and Yang YM: Methylsulfonylmethane inhibits HER2 expression through STAT5b in breast cancer cells. Int J Oncol 48: 836-842, 2016.
70. Sp N, Darvin P, Yoo YB, Joung YH, Kang DY, Kim DN, Hwang TS, Kim SY, Kim WS, Lee HK, *et al*: The combination of methylsulfonylmethane and tamoxifen inhibits the Jak2/STAT5b pathway and synergistically inhibits tumor growth and metastasis in ER-positive breast cancer xenografts. BMC Cancer 15: 474, 2015.
71. Kang DY, Sp N, Jo ES, Rugamba A, Hong DY, Lee HG, Yoo JS, Liu Q, Jang KJ and Yang YM: The inhibitory mechanisms of tumor PD-L1 expression by natural bioactive gallic acid in non-small-cell lung cancer (NSCLC) cells. Cancers (Basel) 12: 727, 2020.
72. Ko EB, Jang YG, Kim CW, Go RE, Lee HK and Choi KC: Gallic acid hindered lung cancer progression by inducing cell cycle arrest and apoptosis in A549 lung cancer cells via PI3K/Akt pathway. Biomol Ther (Seoul) 30: 151-161, 2022.
73. He Z, Liu X, Wu F, Wu S, Rankin GO, Martinez I, Rojanasakul Y and Chen YC: Gallic acid induces S and G2 phase arrest and apoptosis in human ovarian cancer cells in vitro. Appl Sci (Basel) 11: 3807, 2021.
74. Sp N, Kang DY, Jo ES, Lee JM and Jang KJ: Iron metabolism as a potential mechanism for inducing TRAIL-mediated extrinsic apoptosis using methylsulfonylmethane in embryonic cancer stem cells. Cells 10: 2847, 2021.
75. Weng SW, Hsu SC, Liu HC, Ji BC, Lien JC, Yu FS, Liu KC, Lai KC, Lin JP and Chung JG: Gallic acid induces DNA damage and inhibits DNA repair-associated protein expression in human oral cancer SCC-4 cells. Anticancer Res 35: 2077-2084, 2015.
76. Liu KC, Ho HC, Huang AC, Ji BC, Lin HY, Chueh FS, Yang JS, Lu CC, Chiang JH, Meng M, *et al*: Gallic acid provokes DNA damage and suppresses DNA repair gene expression in human prostate cancer PC-3 cells. Environ Toxicol 28: 579-587, 2013.
77. Setayesh T, Nersesyan A, Mišík M, Noorizadeh R, Haslinger E, Javaheri T, Lang E, Grusch M, Huber W, Haslberger A and Knasmüller S: Gallic acid, a common dietary phenolic protects against high fat diet induced dna damage. Eur J Nutr 58: 2315-2326, 2019.
78. Lockhart AC, Braun RD, Yu D, Ross JR, Dewhirst MW, Humphrey JS, Thompson S, Williams KM, Klitzman B, Yuan F, *et al*: Reduction of wound angiogenesis in patients treated with BMS-275291, a broad spectrum matrix metalloproteinase inhibitor. Clin Cancer Res 9: 586-593, 2003.