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Regular paper

Chlorogenic acid regulates the proliferation and migration of high-grade serous ovarian cancer cells through modulating the miR199a5p/DDR1 axis

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This study aimed to demonstrate that chlorogenic acid (CGA) has anticancer effects against ovarian cancer. The MTT assay was used to assess the optimum concentrations of CGA on the ovarian cancer cell lines OVCA433 and SKOV3, followed by the rate of apoptosis using Annexin V-FITC/PI. The mitochondrial membrane potential of ovarian tumour cells treated with CGA was evaluated using mitochondrial staining kits followed by Western blot analysis, immunofluorescence, and RT-PCR assays. The Trans-well migration assay conducted the percentage of cell migration, followed by wound healing and colony formation assays. CGA induces activation of mitochondria-mediated intrinsic apoptotic pathways in ovarian cancer cells. The discovery that miR-199a-5p is inversely correlated to DDR1, a receptor tyrosine kinase involved in collagen synthesis, was the major consequence of examining the various mechanisms involved in the development of ovarian cancer. After treatment with CGA, cells derived from ovarian cancer cells were deregulated partially *via* the miR199a5p/DDR1 axis, significantly affecting tumour suppression. DDR1 has been identified as a direct target of miR199a5p in these ovarian cancer cells. We found that CGA-induced loss of DDR1 caused the inactivation of NF-κB signalling downstream in the MMP, migration, and EMT pathways. The study results showed that CGA is a promising drug candidate for treating ovarian cancer, particularly because it exhibits anti-invasive and migrastatic properties.

Keywords: chlorogenic acid, miR-199a-5p, ovarian cancer, collagen synthesis

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Abbreviations: CGA, chlorogenic acid; DDR1, Discoidin domain receptor 1; EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinase; TGF-β1, transforming growth factor β-1; LX-2, human hepatic stellate cell line; COL11A1, collagen type XI alpha 1 chain; PI3K, Phosphoinositide 3-kinase; Bcl-2, B-cell lymphoma 2; Apaf1, apoptosome complex protease 1; HES1, hes family b HLH transcription factor 1; MMP1, matrix metallopeptidase 1;ERK1, Extracellular signal-regulated kinase 1; HGSOC, high-grade serous ovarian cancer; MTP, mitochondrial transmembrane potential; PARP, poly-ADP ribose polymerase; Notch, Neurogenic locus notch homolog protein

INTRODUCTION

Globally, the burden of cancer is rising continuously, and the development of a drug that can be used as an effective anticancer therapy with the fewest adverse effects continues to be a challenge for drug discovery. Various cytotoxic drugs are available for treating cancer, which are effective against cancerous cells. Still, the drugs harm healthy cells, causing widespread and fatal side effects such as nerve ailments and cardiac and kidney toxicity (Nurgali *et al.*, 2018). There is evidence that compounds derived from plants have chemopreventive and anti-carcinogenic effects on the most common types of cancer or alleviate some of the toxic side effects associated with cancer. Additionally, coffee is consumed throughout the world and has various health benefits. It contains many polyphenols, including chlorogenic acid and its derivatives (Cano-Marquina *et al.*, 2013; Shimizu *et al.*, 1999). The average cup of coffee is estimated to contain between 30–600 mg of CGAs (Salomone *et al.,* 2017).

There have been several studies in both cell culture and animal models that have demonstrated that coffee consumption and CGA consumption inhibit the growth of cancer cells (Hou *et al.*, 2017; Miura *et al.*, 1997; Romualdo *et al.*, 2019; Shimizu *et al.*, 1999; Taha, Khalil *et al.*, 2020; Salzillo *et al.*, 2021; Sapio *et al.*, 2020). The study (Salomone *et al.*, 2017; Liu *et al.*, 2020) carefully examined the molecular mechanisms by which coffee and some of its components, such as CGA, suppress tumour growth in experimental models of liver cancer. These findings were based on an experiment (Miura *et al.*, 1997) that demonstrated coffee was effective in inhibiting the growth and invasion of hepatoma cells (AH109A) of the rat ascites in combination with rats' serum that were orally fed coffee, which also claimed to prevent proliferation and invasion of these cells. A study conducted (Hou *et al.*, 2017) showed that CGA inhibited the proliferation of HT29 and HCT116 cells (human colon cancer cells). CGA caused ROS generation in both cell types and prevented the ERK activation in both cell lines. This caused an inhibitory effect, i.e., it caused a cell cycle arrest at the S phase (Nwafor *et al.*, 2022). The study report (Romualdo *et al.*, 2019) examined the effects of coffee and chlorogenic acid on the development of oral and oesophagal cancers and colorectal cancer concerning malignant neoplasia. According to these authors, CGA's mechanisms of action are associated with ROS and other molecular pathways such as caspase-3, interleukin-IL-8, MMPs, miR-21, and Bax involved in cell lysis. A recent study (Taha *et al.*, 2020; Qin *et al.*, 2021) found that CGA and some of its derivatives showed strong interacting activity with several tumour-associated proteins, such as caspase-3, TP53 (tumour suppressor), and Akt1.

There have been a few studies that have investigated the possibility of miRNAs being affected by CGAs. For example, in a study conducted with LX2 cells and a fibrosis model induced by CCl4 in rats causing liver damage, CGA was shown to inhibit miR-21 expression, growth factors, Smad7, metalloproteinases, and TGF-β1, indicating that CGA may prevent fibrosis of liver damage by blocking miR-21-mediated Smad/TGF-β1 signalling mechanisms (Yang *et al.,* 2016). The study (Wang *et al*., 2017) reported similar results, demonstrating that CGA inhibited schistosomal infection-induced liver fibrosis by interfering with interleukin-13 (IL-13)/ Smad7/ miR-21 interconnected mechanisms in LX2 cells and mice infected with schistosomes. The presence of liver fibrosis is thought to be a major risk factor for HCC, suggesting CGA might have a beneficial role in preventing the disease.

On the other hand, CGA reduced the proliferative capability and migration and invasion potential of human Huh7 and H446 tumour cells by stabilizing the mRNA, resulting in a reduction in the level of the miR-20 family member miR-20a (Roehlen *et al*., 2020). These cells were used in xenograft experiments with similar results. In addition, it has been demonstrated that intraperitoneally administered 30–300 mg/kg/day of CGA to NOD/ SCID mice inhibits tumour growth, and it has also been demonstrated that administration of 25 mg/kg leads to a reduction in expression of members of the miR-17 family (Huang *et al*., 2019).

In recent years, it has been reported that the extracellular matrix plays a very critical role in the progression and metastasis of cancers (Walker *et al*., 2018). A key component of the ECM is collagen, one of the most abundant proteins in the body. Among the receptors for collagen are the DDR1 and DDR2 (Discoidin domain receptors), members of a subgroup of enzymes known as tyrosine kinases. Some evidence indicates that DDR1 signalling in tumours contributes to tumour growth, invasion, and chemotherapy resistance (Henriet *et al*., 2018). DDRs are therefore considered attractive therapeutic targets for treating cancer. For this reason, DDR inhibitors showed to suppress chemoresistance mediated by collagen synthesis, which is evident in resistant tumours (Gadiya & Chakraborty, 2018). It can be concluded that collagen IV mediated activation of DDR1 resulted in a feed-forward loop which increased MMP-9 and collagen IV, thus promoting myeloid leukaemia cell migration and adhesion to the bone marrow through activation of AKT pathways (Xu *et al*., 2019).

On the other hand, COL11A1 binding to DDR2 induced the activation of PI3K/AKT-NF-κB signalling mechanisms in ovarian cancer cells, which in turn caused apoptosis (Rada *et al*., 2018). It has been found that DDR1 expression is anti-correlated with miR-199a expression in acute myeloid leukaemia and hepatocellular carcinoma (Favreau *et al*., 2015). A growing body of evidence indicates that the aberrant expression of genes in cancer cells can be explained by deregulated microRNAs.

Several classes of small, non-coding RNAs play a crucial role in the transcriptional regulation of gene expression through their ability to attach to the 3'UTR of messenger RNA. There are two possible outcomes when miRNA binds to mRNA. Firstly, the bound mRNA can either be degraded (transcriptional block) or translated into a poorly processed protein (translational failure), which results in the absence of a protein (Lou *et al*., 2017). Several mechanisms are involved in cancer pathology and physiological processes that are affected by miRNA deregulation. Furthermore, the role of microRNAs in promoting tumour progression, angiogenic change, and tumour dissemination and their application to identify diagnostic and therapeutic targets have been extensively studied (Rupaimoole & Slack, 2017). In addition, miRNAs can interact with the UTRs of several genes and affect the expression of those genes. Several studies have investigated the impacts of miRNAs on mRNA expression by integrating miRNA levels and mRNA expression levels. A growing body of research relates to the cellular effects of drugs at the transcriptome level (Khatoon *et al*., 2014). To develop future therapeutics based on detecting the chemosensitivity, resistance, and toxicities of the drugs, we need to understand the changes that the drugs bring about by integrating miRNA and mRNA.

The objective of our study was to determine the sensitivity of CGA to the expression of miRNA and mRNA in ovarian cancer cells. The study was conducted to determine the sensitivity of CGA and decided to use two cell lines derived from ovarian cancer to perform this study to investigate the molecular mechanisms and mechanisms of cell death caused by CGA against ovarian cancer involving the OVCA433 and SKOV3 cell lines.

MATERIALS AND METHODS

Cell culture

This study utilized the human serous epithelial ovarian cancer cell lines OVCA433 and SKOV3 which were obtained from ATCC (American Type Culture Collection, Manassas, VA) for the purpose of this study. OVCA433 and SKOV3 cells were maintained in RPMI-1640 (Sigma-Aldrich, USA) and EMEM (Minimum Essential Medium Eagle, Sigma-Aldrich, USA) respectively, supplemented with 12% fetal bovine serum (Sigma-Aldrich, USA) and cultured in monolayers at room temperature with 5% CO_2 in a humidified incubator for 24 hours. The chlorogenic acid (CGA, Sigma-Aldrich, USA) was dispersed in phosphate buffer saline (PBS; pH 7.6) and reconstituted in hot water and cooled down. Additionally, different concentrations of the drug were prepared for different experiments. In the study, the control (distilled water) was used.

MTT assay

The cells (OVCA433 and SKOV3) were plated 3×10^4 cells per well of a 96-well plate for overnight incubation, exposing the cells to higher concentrations of CGA for 24–48 hours, then harvested. We treated the cells with MTT (MTT assay kit, Sigma-Aldrich, USA) at a concentration of 500 μg/ml after being incubated and allowed them to incubate until the formazan colour appeared. Firstly, the formazan crystals were solubilized by adding 40% DMSO solution (Sigma-Alrich, USA) and were kept for 3 hours at room temperature. Immediately after solubilization, absorbance measurements at 570 nm were made, and percentage cell viability was plotted to compare untreated control and treated samples.

Apoptosis assay

We evaluated the effects of the CGA drug on apoptosis using the Annexin V-FITC/PI kit (Invitrogen™, ThermoFisher Scientific USA). Following the instructions provided by the manufacturer, the assay was carried out for 14 days. In brief, both OVCA433 and SKOV3 cells

were treated with progressively higher concentrations of CGA for 48 hours, after which they were stained with Annexin V-FITC and PI using a flow cytometer (Becton Dickinson, Mississauga, ON, Canada) as the analysis tool. A fluorescence measurement of PI at 562 nm was obtained by using the Cell-Quest software (Becton Dickinson, CA, USA).

JC-1 assay

This study evaluated the mitochondrial membrane potential of ovarian tumour cells treated with CGA drugs using mitochondrial staining kits (Invitrogen™, ThermoFisher Scientific USA). After 48 hours of CGA treatment, OVCA433 and SKOV3 cells were isolated and stained with JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylimida carbocyanine iodide) for 30 minutes at room temperature. On the following day after the incubation, the fluorescence of the JC-1 antigen was determined by flow cytometry.

Western blot analysis

In brief, control and drug-treated cells and their lysates were isolated and lysed in a buffer (RIPA; Fisher Scientific, ON, Canada) comprising a protease inhibitor for total protein extraction. To lyse the cells, to extract nuclear proteins, 0.5% of NP-40 buffer (Thermo Scientific™ NP-40 lysis buffer, USA) was used and subjected to centrifugation for 15 minutes at 20 000 rpm at 4°C. The RIPA buffer was added to the pellet to further lyse the pellet obtained from the nuclear extract. To purify the cytoplasmic fractions from the supernatant, RIPA buffer was supplemented. The levels of proteins in the samples were assessed using the Bradford method, followed by the separation of 30–40 μg of the protein using SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes. We used the following antibody concentrations: BcL2, 1:2000 (Santa Cruz Biotechnology CA, USA); Apaf1, 1:2000 (Santa Cruz Biotechnology CA, USA); Cytochrome C, 1:800 (Cell Signaling Technology, MA, USA); Cleaved Caspase 9, 1:2000 (Cell Signaling Technology, MA, USA); Cleaved PARP, 1:1500 (Cell Signaling Technology, MA, USA); MMP1, 1:1500 (Cell Signaling Technology, MA, USA); MMP2, 1:1500 (Cell Signaling Technology, MA, USA); MMP9, 1:700 (Cell Signaling Technology, MA, USA); Notch, 1:800 (Cell Signaling Technology, MA, USA); Hes1, 1:1500 (Cell Signaling Technology, MA, USA); NFκB p65 (Santa Cruz Biotechnology CA, USA), 1:800; ERK1, 1:1500 (Santa Cruz Biotechnology CA, USA); GAPDH, 1:7000 (Santa Cruz Biotechnology CA, USA) and β-Tubulin (Santa Cruz Biotechnology CA, USA).

Transwell migration assay

The OVCA433 cells were pretreated with CGA drug and plated in the inner chamber of a 24-well plate with a density of $1x10^5$ cells/well in serum-free media. During incubation, a complete media containing serum was poured into the outer chamber of each transwell, and the plate was then incubated for 8 hours. After incubating the cells, the medium was separated from the cells. The inner chamber was dipped into phosphate buffer saline to segregate any remaining cells which do not adhere. The migrated cells were fixed in ethanol to stain, and staining was performed with crystal violet. Five different images characterized each transwell, and ImageJ software (Olympus, USA) calculated the percentage of migrated cells.

Wound healing assay

In a 24-well plate, we plated 1×10^5 OVCA433 cells per well and monitored their growth. A 48-hour exposure of the cells to a pipette tip resulted in the formation of wounds, which were then washed with PBS (phosphate buffer saline) after being removed from the scraped cells. Secondly, cells were further incubated in 3% serum media in the presence or absence of the CGA drug. We analyzed images taken at various time intervals. First, the wound area was manually measured employing the ImageJ image analysis software (Olympus, USA), and then the percentage of the closure area of the wound for each time point HG was plotted on a bar graph.

Colony formation assay

Wells were seeded with 2×10^3 cells/well overnight, stimulated with appropriate concentrations of CGA an hour before incubation, and allowed to incubate for 7 days. At the end of incubation, the medium (phosphate buffer saline) was discarded, the wells were washed with phosphate buffer saline, and the cells were stained with crystal violet (0.3%). ImageJ (Olympus, USA) was used to obtain the images and quantify the colony area using the images collected.

Immunofluorescence study

OVCA433 cells were seeded at an optimum density of 1×10^5 cells/well in a six-well plate, and then treated with CGA for 48 hours. After washing and fixing the cells in 5% paraformaldehyde, the cells were rehydrated and incubated for 20 minutes. Permeabilization was performed with 0.3% Triton-X100 in phosphate buffer saline followed by an hour's blocking in 5% BSA (Bovine Serum Albumin). Incubation was carried out overnight at 4°C with NF-κB p65 at a concentration of 1:400. Incubation was performed after washing with phosphate buffer saline and subsequently incubated with goat anti-rabbit biotinylated antibody (1:400; Cell Signaling Technology, MA, USA) for 40 minutes at 37°C following incubation with streptavidin-FITC conjugated antibody (1:300; (Invitrogen™, ThermoFisher Scientific USA) for 20 minutes at room temperature. A fluorescent microscope (Olympus, USA) was used to capture images.

Real-time PCR

RNAiso Plus (Takara Bio Inc, Nihonbashi, Chuo-ku, Tokyo) was used for the extraction of RNA from cells. PrimeScript RT Reagent Kit (Takara Bio Inc, Nihonbashi, Chuo-ku, Tokyo) was used to synthesize cDNA from 5 μg of RNA using the Prime-Script Real-Time Reagent Kit. We performed a quantitative real-time PCR (qRT-PCR) to determine miR199a5p levels. In this study, the reactions were carried out as follows: 80°C for 3 minutes, 42 cycles at 80°C for 50 seconds, 60°C for 25 seconds, and a dissociation stage. The primer sequences used for the miR199a5p PCR analysis were as follows: miR199a5p forward: 5'-GAAGTTAGTTACTACCAG-3' and reverse 5'-ATTAGTTAGCGGAATTGC-3'. In addition, U6 was included as an endogenous control.

The other primers sequences were as follows: miR-199a-5p mimic, 5'-AGUCCAUUGACUGGCUUAAGUAA-3'; miR-199a-5p mimic mutant 5'-AGGTUUGGCCUUGGTAGGUCCU-3'; DDR1, 5'-CCUGGGGAACCUUGAGGGAU-3'; scrambled control 5'-CCCAAGGAACCCUAAAGGAGU-3'. A miRNA inhibitor for miR-199a-5p and a negative control inhibitor

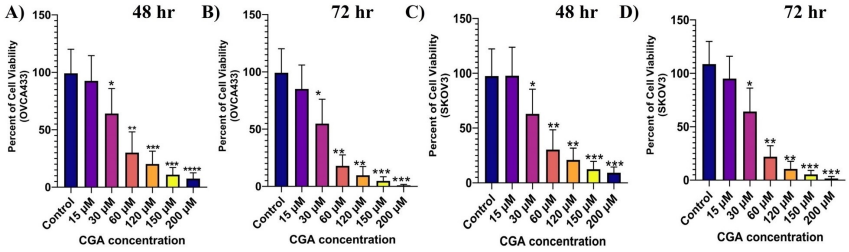

Figure 1a. Ovarian cancer cell lines were induced to undergo cytotoxicity and apoptosis by chlorogenic acid.
(**A–D**) OVCA433 and SKOV3 cells treated for 48 and 72 hours with increasing concentration of CGA using MTT assay. We plotted significance using p values and represented it as *(p<0.05), **(p<0.01), and ***(p<0.001). A p-value<0.001 was significant in post-test analysis for linear trend in both cell lines and for both time points for MTT assays.

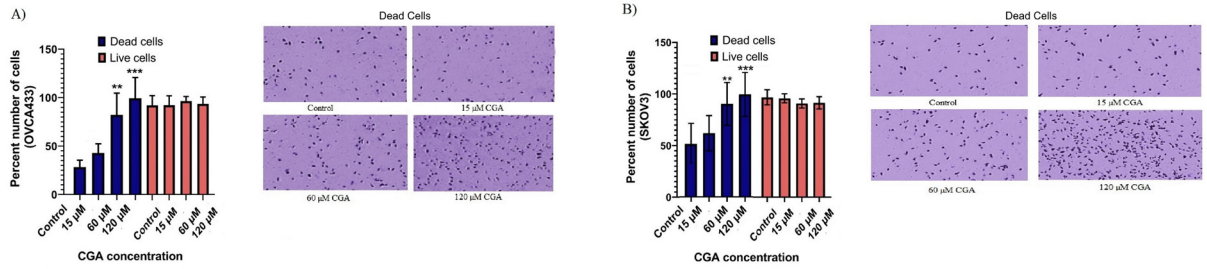

Figure 1b. Analysis of flow cytometry results of OVCA433 and SKOV3 cells treated with increasing concentrations of CGA for 48 hours.
The representative images of dead cells showing increase in the number of cell death with increase in the concentration of the CGA. Three independent flow cytometry experiments were used to calculate the percentage of each cell type. We plotted significance using p values and represented it as *(p<0.05), **(p<0.01), and ***(p<0.001).

was acquired from Exiqon (Denmark). Sequences were as follows: negative control inhibitor, 5'-ATTAGGCCCAATTGGACTAAC-3', miR-199a-5p inhibitor, 5'-AAGGCATCGTTACTGGCGT-3'.

In triplicate, three samples were assembled and run on a Step One Plus Real-Time PCR system.

Ovarian tumour samples

The samples from the high-grade serous ovarian cancer (HGSOC) tumour and normal ovarian tissues were obtained with the informed consent of the donors at the Department of Gynecology and Obstetrics. The medical ethics committee approved the experimental protocols of this hospital study, and all the methods carried out in line with the relevant guidelines and regulations were strictly adhered to.

Statistical analysis

The GraphPad Prism (Version 9.11) was used to perform the statistical analyses. The significance of the treatment groups concerning the control groups was determined using one-way ANOVA followed by Tukey's multiple comparisons. Results are presented as mean and standard deviation (S.D.). The significance of each subgroup was determined by p values, with p<0.05 considered significant. GraphPad Prism tool was used to calculate linear trend significance using one-way ANOVA.

RESULTS

Effect of CGA on MTT and Apoptosis Assays

It has been shown that CGA inhibits the proliferation of cells and causes apoptosis in cells derived from ovarian cancer. Therefore, the MTT procedure was conducted to determine whether the CGA has cytotoxic effects on the ovarian carcinoma cell lines (OVCA433 and SKOV3). We stimulated both cell lines with increasing CGA at various concentrations for 48 or 72 hours. There was a time- and dose-dependent effect (Fig. 1a A–D) in both cell lines at micromolar concentrations, indicating the drug's increased potency. According to the MTT assay, the IC_{50} was calculated for OVCA433 and SKOV3 at 38±1.8 µM and 45±1.3 µM, respectively.

To understand how CGA caused cell death, we performed the double staining assay of AnnexinV-FITC and PI. In both cell lines, CGA was administered at concentrations of 15 µM, 60 µM, and 120 µM for 48 hours, DAPI and AnnexinV-FITC/PI were used for staining, and flow cytometry was used for analysis. The study showed that at a concentration of 60 µM CGA, 80% of OVCA433 cells displayed apoptotic cells compared with 50% of SKOV3 cells (Fig. 1b A–B), showing OVCA433 to be more sensitive to CGA treatment. In addition, there were only 5–8% necrotic cells in OVCA433 and 41.4% in SKOV3 cells at 60 µM CGA concentration. This indicates that CGA induced apoptosis in these human ovarian tumour cell lines.

Effect of CGA on mitochondrial membrane potential by JC-1 assay

It has been found that CGA drugs alter mitochondrial membrane potential and activate a pathway intrinsic to apoptosis as soon as they are administered to ovarian cancer cells. The cytotoxic drugs cause apoptosis through a mechanism intrinsic to the process of apoptosis, which is mediated by the mitochondria. The first thing that must be determined to induce apoptosis must be the extent of mitochondrial dysfunction, which may presumably be determined by measuring the mitochondrial transmembrane potential (MTP). CGA treatment influences the mitochondria's function using JC-1, a cationic fluorescent dye. It was found that the cells from

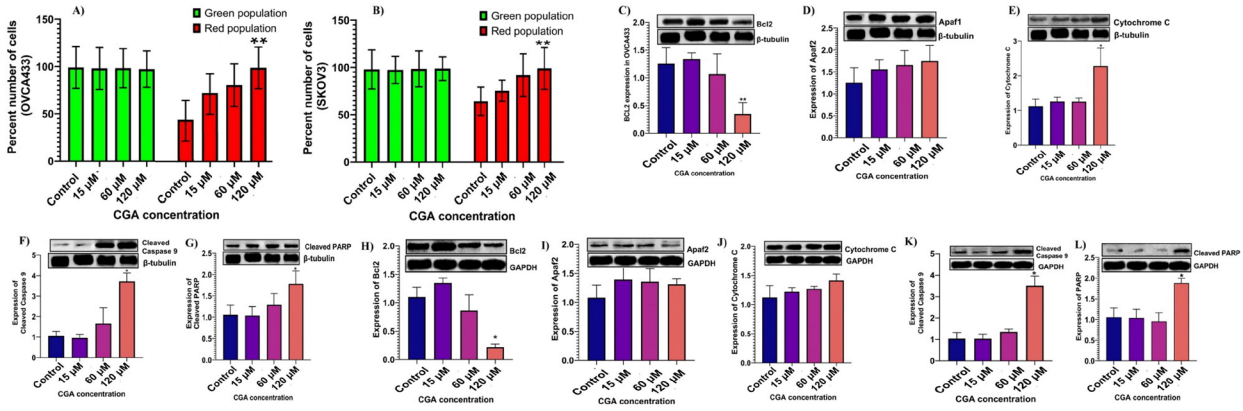

Figure 2. Ovarian cancer cell lines treated with CGA showed altered mitochondrial membrane potential and apoptotic proteins. (**A**) Flow cytometry analysis of the staining of JC1 in OVCA433 cells treated with CGA for 48 hours. (**B**) Flow cytometry analysis of JC1 staining of SKOV3 cells treated for 48 h with increasing concentrations of CGA. Three independent experiments were used to plot the median fluorescence intensity (MFI). (**C–G**) The expression of apoptosis-related proteins and the quantification of OVCA433 cells treated with increasing concentrations of CGA for 48 hours. (**H–L**) the expression of apoptosis-associated protein and quantification of SKOV3 cells treated with increasing levels of CGA for 48 hours. Statistical significance plotted using *p*-values, and represented as *($p<0.05$) and **($p<0.01$).

both cancer cell lines responded well to treatment for 48 hours with 15 μM, 60 μM, and 120 μM of CGA drug. These cells were stained with the JC-1 fluorescent dye and analyzed by flow cytometry. Increased CGA concentrations in either cell line (Fig. 2A–B) increased mitochondrial dysfunction. Interestingly, this study indicates that CGA induces cell death during mitochondrial-mediated apoptosis in both OVCA433 and SKOV3 cell lines of ovarian cancer.

Apoptosis is a controlled process in which apoptosis-inhibiting and apoptosis-promoting factors play a critical role in triggering cytochrome C production in mitochondria and the synthesis of apoptosomes, which in turn triggers the caspase cascade and ultimately leads to cell death. Subsequently, with the suppression of mitochondrial-mediated intrinsic apoptotic pathway expression upon the treatment of both cell lines of ovarian cancer with CGA, it became increasingly necessary to investigate the presence of particular proteins that regulate cell death, which are key components of the intrinsic apoptotic process that is mediated by mitochondria. Therefore, we treated ovarian cancer cell lines (OVCA433 and SKOV3) with 15, 60, and 120 μM of CGA drug for 48 hours.

Effect of CGA on western blot analysis of pro-apoptotic factors

We measured the expressions of survival-promoting factors, such as Apaf-1 (the apoptosome complex protease), Cytochrome *c*, Bcl-2, and the cleaved caspase-9. We also performed a genotyping analysis on the transcription of the residues that contain the catalytic fragment of the fragmented PARP enzyme. This is a marker for the impairment of DNA repair and a potential indicator of cell death (Henriet *et al.*, 2018). As a result, it was observed that both cell lines of ovarian cancer showed a dramatic reduction of pro-apoptotic Bcl-2 and a substantial increase in cytochrome c levels and cleaved caspase-9 activity (Fig. 2C–L), correlating with the observation that CGA is capable of causing intrinsic apoptosis in ovarian cancer cells.

Quantitative Real-Time PCR (qRT-PCR) was performed to detect DDR1 and miR199a5p expression levels

Several reports have indicated that DDR1 is widely expressed in various types of cancer (Lafitte *et al.*, 2020). To determine whether it is expressed in ovarian cancers and inversely correlated with miRNA199a5p, we evaluated their expression patterns in tumour samples collected from HGSOC patients. We then analyzed its expression in ovarian cancer. Six samples of stage-III HGSOC tissues and normal ovarian tissue samples were collected to perform this study. An analysis using qPCR of these samples confirmed that the DDR1 levels were high in ovarian cancers and the miR199a5p levels were low, thus establishing an anti-correlation of DDR1 and miR199a5p (Fig. 3A–B).

The graphs in Fig. 3C, D represent the expression levels and the inverse correlation between DDR1 and miR199a5p calculated using qPCR analysis of cells treated with CGA. These graphs showed the downregulation of DDR1 expression and the upregulation of miR199a5p expression. We investigated the expression of DDR1 in both OVCA433 cells and SKOV3 cells stimulated by the CGA series due to the upregulation of miR199a5p expressed in both cell lines. In addition, we found an inverse correlation between miR199a5p expression and DDR1 expression in ovarian tumour cells treated with CGA drugs. A significant reduction (i.e., dose-dependent) in DDR1 expression following CGA treatment (Fig. 3C–D). To investigate the functional implications of miR199a5p and its interaction with the DDR1, OVCA433 cells were transfected with anti-miR199a5p, a synthetic miRNA inhibitor. We demonstrated that anti-miR199a5p alone increased the proliferation of the cells, and our finding that the reduction in the proliferation of cells caused by CGA was reversible by anti-miR199a5p therapy (Fig. 3E). Proliferation was activated by administering 120 μM CGA at a higher dose. Furthermore, it was also found that the CGA-induced reduction of the amount of DDR1 protein could be restored when anti-miR199a5p was used, suggesting the endogenous binding of miR199a5p to DDR1 was inhibited (Fig. 3F).

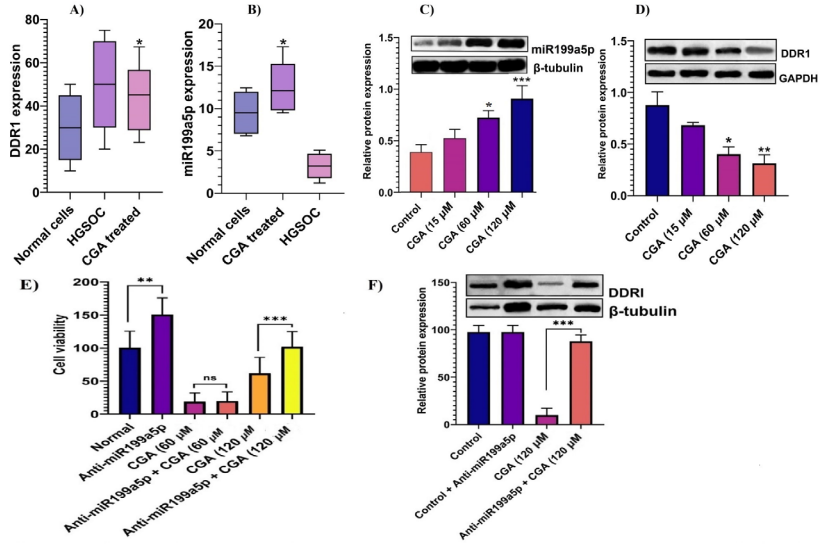

Figure 3. The correlation between DDR1 and miR-199a-5p in ovarian cancers is negative, and DDR1 is a direct target of miR-199a-5p. (**A**) The expression of DDR1 in Chinese HGSOC samples. (**B**) The expression of mir-199a-5p in Chinese HGSOC samples. The bar graph in (**C**) shows miR-199a-5p expression in OVCA433 cells treated with CGA. (**D**) shows the expression of DDR1 in OVCA433 cells treated with CGA. (**E**) MTT assays conducted on OVCA433 cells transfected with or without anti-miR-199a-5p (30 μM) and CGA. (**F**) Expression and quantification of the DDR1 protein in OVCA433 cells transfected with or without anti-miR-199a-5p and 120 μm CGA. A bar graph is plotted as the mean and standard deviations from three independent experiments. We plotted the significance based on the p value, and it is represented by *(p<0.05), **(p<0.01), and ***(p<0.001).

Effects of CGA on activities of MMPs and wound closure

It has been shown that the down-regulation of DDR1 when caused by CGA, inhibits the activity of MMPs, which reduces migration, colony-forming processes, and wound closure in OVCA433 cells. A large number of studies have linked DDR1 to tumour progression, and it is believed that it controls the adhesion and migration of cells by modulating the production of matrix metalloproteinases (MMPs) such as MMP-1, MMP-2, and MMP-9 (Reel *et al.*, 2015). The MMPs are a class of regulating proteins that play a role in the degradation of the extracellular matrix (ECM), which aids the progression of cancer, invasion, and metastasis. Therefore, we investigated the level of MMP expression in both cell lines

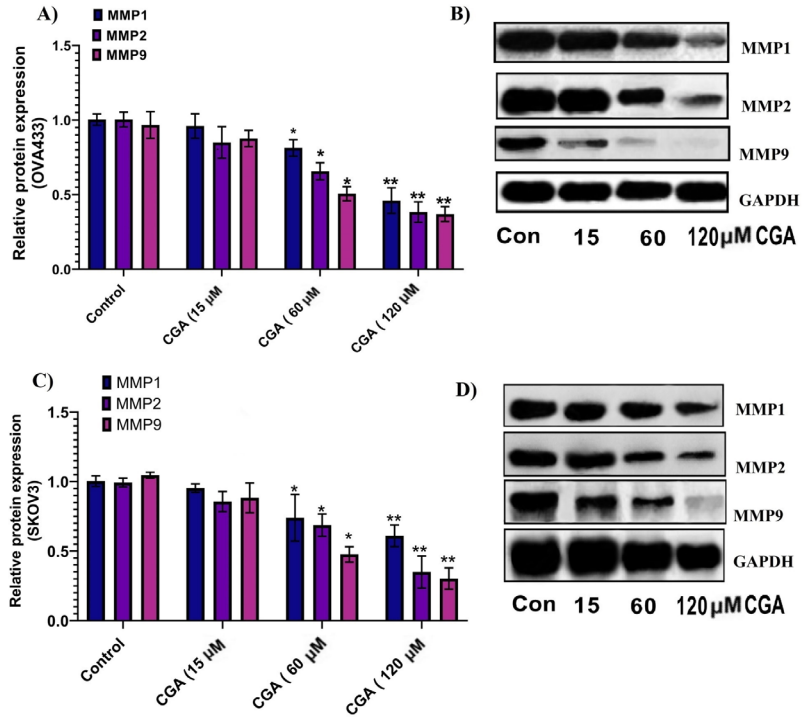

Figure 4. MMPs and downstream targets of DDR1 are downregulated by CGA in both OVCA433 and SKOV3 cells.
(**A**) MMP expression in OVCA433 cells and (**B**) Corresponding quantification of the MMP following treatment for 48 hours with increasing concentrations of CGA. (**C**) Expression of MMP proteins, and (**D**) Measurements of MMP for SKOV3 cells treated with increasing concentrations of CGA for 48 h. The bar graphs are plotted as the mean ± S.D. of three independent experiments. *p*-values were used to calculate significance, and the results were represented as *(p<0.05) and **(p<0.01) respectively.

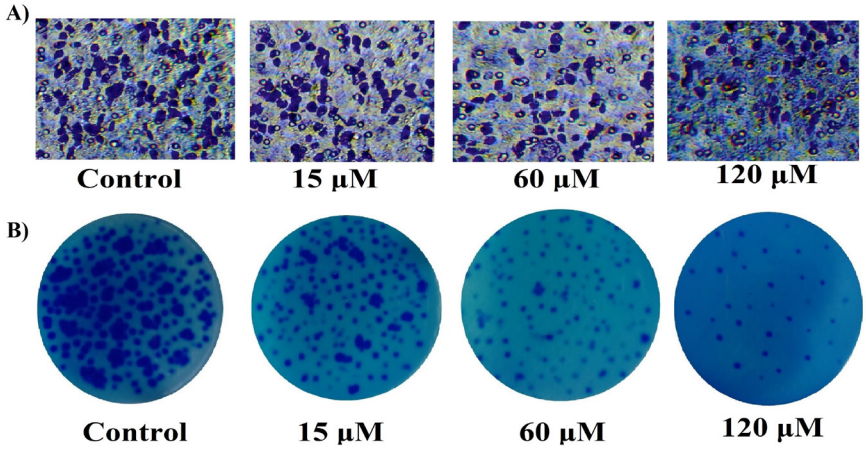

Figure 5. (A) Migration assay showing inhibition of migration (B) Clonogenic assay showing colony-forming properties of CGA-treated OVCA433 cells. Scale bar represents 200 μm

used in the study as a direct result of the downregulation of DDR1 after CGA treatment. There was a remarkable reduction in the expression levels of MMP-1, MMP-2, and MMP-9 when CGA treatment was administered to OVCA433 cells (Fig. 4A–B) and SKOV3 cells (Fig. 4C–D), suggesting that DDR1 signalling may be responsible for the change in MMP expression.

The expression of MMP is downregulated in response to CGA, making it essential to examine whether these cells have the capacity for migration. It is a significant indicator of the ability of cancer cells to invade and spread. In our study, we examined the migration of individual cells *via* transwell migration and the migration of collective cells via wound healing assays. The tests were performed using OVCA433 ovarian cancer cells as a model system. As shown in Fig. 5, in the Transwell Migration Study, an experiment where a serum-containing medium was used as a chemoattractant led to a substantial decline in the migration ability of OVCA433 cells with a 40% decrease at 120 μM CGA therapy (Fig. 5A). A clonogenic assay was performed to quantify the effects of the CGA drug on OVCA433 cells during their ability to self-renew, survive, and grow clonally. Notably, the number of colonies decreased in a dose-dependent manner after CGA treatment (Fig. 5B) in the assay. These studies demonstrate that CGA has migrastatic properties when incubated with ovarian cancer cell lines.

Moreover, wound healing assays showed that, compared to the control group, where the wounds healed within 48 hours after treatment with CGA, the cells showed no wound closing and increased wound size in a dose-dependent manner (Fig. 6).

Effects of CGA on ERK, NOTCH and NF-κB signaling pathways

The CGA targets downstream DDR1 pathways, known for their antimetastatic and anti-invasive prop-

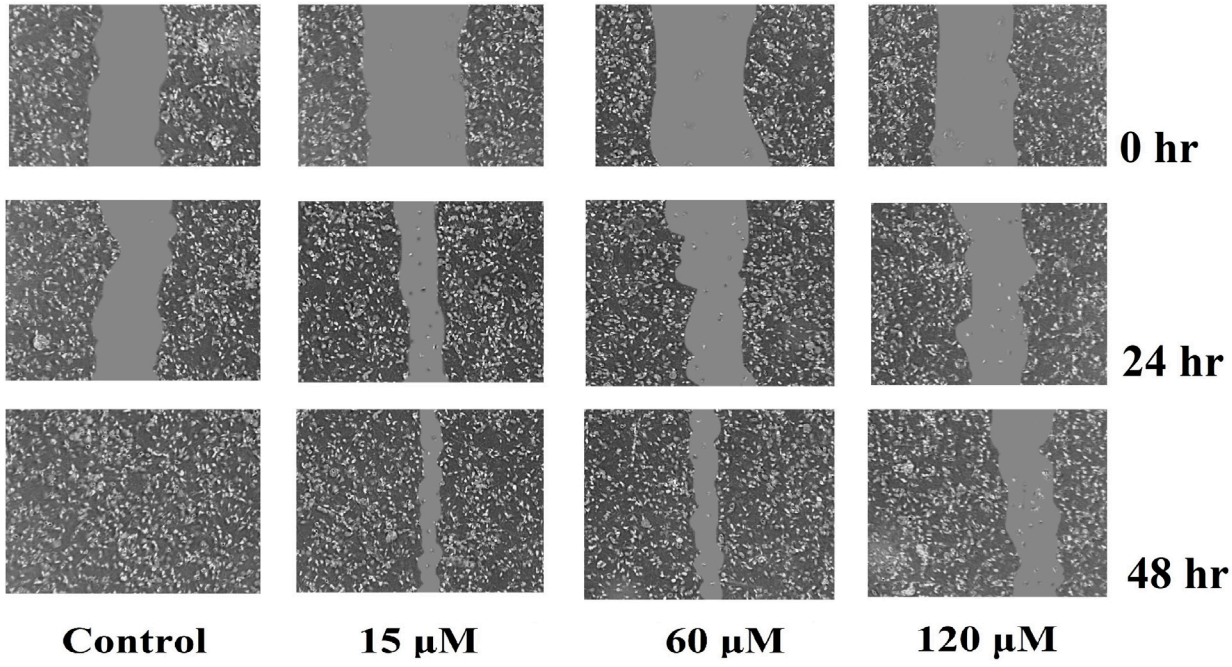

Figure 6. Wound healing assay showing wound closure at 0, 24 and 48 h time points of OVCA433 cells treated with increasing concentrations of CGA. Scale bar represents 200 μm.

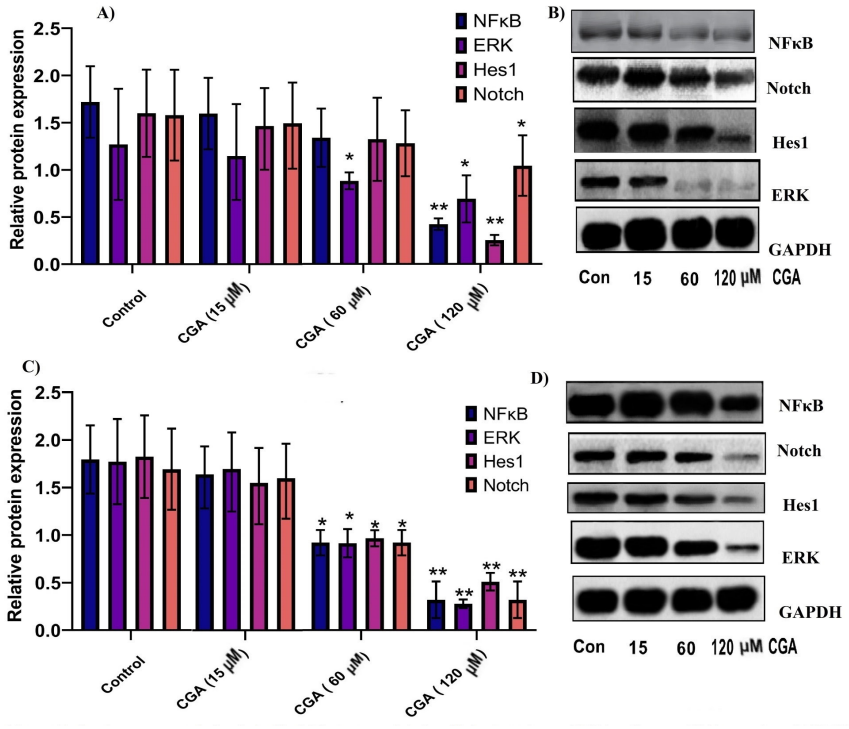

Figure 7. Ovarian cancer cells treated with CGA downregulated multiple downstream DDR1 pathways.
(**A**) Expression of NOTCH, HES1, ERK and NF-κB. (**B**) Quantification of those proteins in OVCA433 cells exposed to increasing concentrations of CGA for 48 hours. (**C**) Protein expression of NOTCH, Hes1, ERK, and NF-κB, and (**D**) corresponding protein quantifications in SKOV3 cells treated with increasing concentrations of CGA for 48 hours. The bar graphs are depicted as means ± standard deviations from three independent experiments. The significance of the results was determined based on the p-values, and was represented as follows: *(p<0.05) and **(p<0.01) respectively.

erties in ovarian cancer cells, to exert their effects. In addition, DDR1 has been shown to act as an activator of a variety of intracellular signalling pathways that are critical for cell growth, proliferation, differentiation, and invasiveness, such as the pathway of ERK and NOTCH signalling, as well as NF-ϰB signalling pathways by its effect on receptor kinase activity (Gadiya & Chakraborty, 2018) (Yeh *et al.*, 2019). Likewise, it has been reported that DDR1 is also involved in epithelial-to-mesenchymal transition (EMT) and is required for cell motility and invasion. In addition, several pathways contribute to cancer progression by causing the development of tumours with metastatic and invasive features. As a result, to gain a deeper understanding of the effects of CGA on subsequent pathways involving DDR1, we analyzed the degree of expression of markers of these pathways in two different cell lines of ovarian cancer (OVCA433 and SKOV3) that were exposed to increasingly high concentrations of CGA for 48 hours.

On the other hand, we found a dose-dependent reduction in the protein levels of the transcription factor ERK, NOTCH, and its negative regulator HES1 (Fig. 7A–D). Figures 7A, D, and E show that the transcript levels of NF-ϰB and its downstream target COX2 were also significantly reduced. Based on the immunofluorescence of OVCA433 cells treated with CGA and protein expression in nuclear extracts, expression patterns of nuclear factor NF-ϰB are decreased, corresponding to a decreased nuclear translocation. According to these results, the downregulation of DDR1 induced by OVCA433 in conjunction with downstream targets can inhibit the invasion and metastasis of ovarian cancer cells (OVCA433 and SKOV3). On the other hand, Fig. 6 depicts the overall effects of CGA on ovarian cancer cells in terms of its ability to induce apoptosis and migrateostasis.

DISCUSSION

It is estimated that as much as 70 percent of the chemotherapeutic drugs used in treating cancer are synthetically produced derivatives of natural substances (Liu *et al.*, 2018). It has been demonstrated that the natural phytochemical CGA, which is composed of high levels of antioxidants, may have tremendous therapeutic potential in treating a wide variety of diseases other than just cancer due to its multimodality and the fact that it targets multiple cells in the body, which result in a variety of biological effects in the body (Yang *et al.*, 2016; Nwafor *et al.*, 2022).

The results show that CGA causes cytotoxic effects in ovarian cancer cell lines, which causes apoptosis, as demonstrated by AnnexinV-FITC and PI staining. Furthermore, recent research has demonstrated that the mitochondria facilitate apoptosis through membrane depolarization of the mitochondrial membrane and release anti-apoptotic molecules such as cytochrome *c*, activation of caspases, and decrease pro-apoptotic proteins such as Bcl2. Overall, it was observed that the CGA drug had a significant effect (dose-dependent) on both of the studied ovarian cancer cell lines. It is noteworthy that the cell line showed a substantial improvement over the SKOV3 ovarian cancer cell line due to its significant impact.

It was shown that CGA reversed the OVCA433 and SKOV3 cells' proliferation and induced EMT genes, suggesting CGA may play an influential role in causing a dramatic change in the pro-oncogenic and anti-oncogenic signalling pathways. There is growing importance in can-

cer biology for short, non-coding RNAs (microRNAs) that regulate genes post-transcriptionally, which can play crucial roles in cancer. MicroRNAs represent a marker of disease progression, invasion, metastasis, and even susceptibility to drugs (Peng & Croce, 2016). Based on qPCR analysis of the cell lines from ovarian cancer after drug treatment with CGA, miR199a5p was identified as a dominantly expressed miRNA. This was in addition to an aberrantly expressed miRNA. The miR199a5p has previously been identified as an anti-tumour factor in colorectal, glioblastomas, breast, cervix carcinoma, bladder, and liver cancer. Still, it has also been associated with an oncomir function in melanoma and stomach cancer (Wang *et al*., 2019). Furthermore, it has been demonstrated that miRNAs have a bidirectional function in cancer (Si *et al*., 2019) as a tissue-specific function.

Furthermore, based on the analysis of miRNA-mRNA interactions, it was revealed that DDR1, which was a non-integrin receptor for collagen, was the target of miR199a5p after the compound CGA was administered. CGA significantly decreased the levels of DDR1 protein in SKOV3 cells, as well as the levels of miR199a5p, a signalling molecule up-regulated by CGA. In the present study, anti-miR199a5p treatment abolished the downregulation of DDR1 induced by CGA, suggesting the inactivation of miR199a5p, an endogenous miRNA produced in cells of ovarian cancer after treatment with CGA. This study confirms that DDR1 is one of the direct targets of miR199a5p. Although transcript levels were not in agreement with the levels of protein expression in SKOV3 cells following CGA treatment, further investigation indicated that several miRNAs targeting DDR1 were expressed at higher levels in the same cell type based on the nucleotide sequence analysis of miRNA, indicating that specific interactions are likely to occur. Nevertheless, the interaction between miR199a5p and DDR1 in OVCA433 cells co-transfected with miR199a5p mimic was further validated. It has been previously reported that miR199a5p and DDR1 inverse correlations have also been shown to be associated with other types of cancer, such as colon, liver, and breast cancers, but not in ovarian cancer (Hu *et al*., 2014; Matà *et al*., 2016; Shen *et al*., 2010).

It has been found that this combination of factors is associated with an increased risk of tumour invasion in patients with these types of cancer (Gadiya & Chakraborty, 2018; Jing *et al*., 2018). In addition, it is associated with poor prognoses. It has also been confirmed that there is an inverse correlation between miR199a5p and DDR1 in stage-III samples of HGSOC tumours compared to the normal ovary. There has been an increase in DDR1 with miR199a5p loss. We found that miR199a5p regulates DDR1 expression upon CGA treatment, which provides the mechanistic basis for regulating DDR1 by miR199a5p.

It has been reported that DDR1 is related to tumour progression, invasion, and drug resistance (Gadiya & Chakraborty, 2018; Jing *et al*., 2018). It has been proposed that high levels of DDR1 in patients with high-grade or advanced ovarian cancer have been associated with a poor prognosis (Quan *et al*., 2011). It was found that knocking down the DDR1 gene significantly increased the sensitivity of ovarian cancer cells to cisplatin (Deng *et al*., 2017). It should also be noted that DDR1-depleted breast cancer cells displayed an increase in sensitivity to etoposide (Das *et al*., 2006). On the other hand, DDR1 has also been proposed as an effective cancer treatment against metastatic malignancies such as colon, breast, gastric, pancreatic, and lung cancer (Lafitte *et al*., 2020). A collagen receptor is attached to a receptor tyrosine kinase (RTK) responsible for several distinct signalling pathways, including cell proliferation, migration, and wound healing. It has been suggested that DDR1 may activate MMPs and the migration of certain cancers (Castro-Sanchez *et al*., 2011; Reel *et al*., 2015).

The present study showed a marked reduction of MMP-1, MMP-2, and MMP-9 in both CGA-treated cell lines of ovarian cancer with loss of DDR1. Furthermore, it was found that both OVCA433 cells treated with CGA showed dramatic reductions in their ability to migrate individually and collectively. Clonogenic assays assessed the survival capability of OVCA433 cells and also revealed the cytotoxicity of CGA. Studies have demonstrated that CGA induces migrastatic properties via downstream DDR1 pathways, possibly involved in the process.

It has been shown that DDR1 can regulate the ERK, NOTCH, and EMT pathways, which are associated with tumour invasion and the spread of metastatic disease (Liu *et al*., 2018; Zhao *et al*., 2021). The concept of EMT is regarded as one of the critical processes in the progression of metastatic disease and the activation of migration. It is also noteworthy that CGA loss of DDR1 was associated with a decrease in ERK and NOTCH proteins, suggesting that CGA can inhibit these pathways. On the contrary, it has been reported that DDR1 regulates chemoresistance by activating the NF-κB pro-survival pathway mediated by COX2. On the contrary, it was also found that NF-κB was a specific target of microRNA199a5p and demonstrated an inverse correlation to changes occurring in ovarian cancer cells (Das *et al*., 2006).

CGA treatment inhibited NF-κB expression and COX2 transcript levels in ovarian cancer lines, indicating the presence of the miR199a5p: DDR1 axis. Additionally, we observe that nuclear NF-κB expression is significantly diminished in a dose-dependent manner concerning nuclear NF-κB expression. A further noteworthy observation is that transcriptome analysis did not show NF-kB levels in either down- or up-regulated categories. Based on this finding, miRNA repression appears to be mediated by translation rather than by mRNA degradation.

CONCLUSION

Our findings showed that CGA induced cytotoxicity in the cell lines OVCA433 and SKOV3 through activating intrinsic mitochondrial apoptotic pathways. Based on the analysis of the miRNA-transcriptome profiles after treatment with CGA, the miR199a5p/DDR1 axis (miRNA loci) was found to play a role in tumour-suppressing activities. Our findings established that the treatment with CGA showed down-regulation of the tyrosine kinase DDR1 receptor and its signalling pathways (NF-κB, ERK), MMP activation, the migration of cells, and EMT. Our results also demonstrated that the CGA acted through an anti-survival pathway, suggesting it is a chemosensitive drug, possibly containing migration-suppressing characteristics, and is, therefore, a promising candidate for the treatment of ovarian cancer.

Declarations

Acknowledgements. Not applicable.

Conflicts of Interest. Authors declare no conflicts of interest in the publication of this research article.

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