

The anticancer effects of Levonorgestrel on human esophageal squamous carcinoma cells

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Abstract

Drug repositioning is a better way to cancer drug discovery. As a common used oral contraceptive, Levonorgestrel (LNG) was found to play important anticancer roles in several cancers, but its role in human esophageal squamous cell carcinoma (ESCC) was little known. Using ESCC cell line of Ec1 and human normal esophageal epithelial cell line of Het-1A, this study was aim to investigate the effect and molecular mechanism of LNG on the ESCC. The results showed that LNG inhibit the cell proliferation; LNG also induced the cell apoptosis of ESCC related to mitochondrial apoptotic pathway for its disruption of mitochondrial capacity and upregulation of cleaved-Caspase 3 and the declining of the ratio of Bcl-2/Bax; LNG can inhibit the cell migration of ESCC with the E-cadherin overexpression. The anticancer effect of LNG on ESCC mainly associated with Wnt/ β -catenin signaling pathway through up-regulation the phosphorylation level of β -catenin. At last, the study declared that LNG combined with cisplatin (CDDP) significantly suppressed the proliferation of Ec1. In conclusions, the LNG serves as efficient anticancer drug in ESCC cells and maybe used for drug repositioning to adjunctive therapy ESCC.

Introduction

Esophagus Cancer is one of alimentary tract malignancies with high mortality [1]. Esophageal squamous cell carcinoma (ESCC) is the most common pathological type and accounting for more than 90% of esophageal cancer in China, accounting for 53% of ESCC cases worldwide [2]. ESCC is generally only diagnosed at the later stages, and the 5-year survival rate is still poor (less than 15%) although surgery, chemoradiotherapy and other treatment methods continue to improve [1, 2]. Therefore, it is imperative to seek new drugs or/and alternative treatment methods to therapy ESCC.

It is well known that successfully bringing a new anticancer drug to clinic is a big challenge, mainly because of the need for large clinical trials, much of the costs and time. However, the advantages of drug repositioning are less time-consuming, less expensive and reduced risk of unexpected side effects, so the cancer researchers pay more intention to old drugs for their anticancer activity [3-5]. It is well known that Levonorgestrel (LNG) is usually used by women to prevent pregnancy as a short-term and long-term oral contraceptive, and sometimes it is implanted in body in from of a device, named the levonorgestrel intrauterine system (LNG-IUS), releasing certain portions of the active substance with time, and thus achieving the similar effect to regular oral intake [6]. Former studies showed that LNG or LNG-IUS was associated with endometrial, breast, ovarian, pancreatic, and lung cancers [7-9]. And it is amazing that many studies reported that LNG or LNG-IUS have different role in cancer occurrence, development and therapy, especially in breast and endometrial cancers [8-17]. However, little was known about the role of LNG on ESCC. In this study, a new strategy of LNG for the treatment of esophageal cancer was discussed by investigating the antitumor activity on the ESCC cells.

Materials And Methods

Cells and cell culture

Human ESCC cell line Ec1 and human esophageal epithelial cells Het-1A (from associate professor Zang Wenqiao, Department of Immunology, School of Basic Medical Sciences, Zhengzhou University). The cells were cultured under the condition of 37°C, 5% CO₂, in RPMI 1640 Medium (Corning) with 10% FBS (Gibco) with 100 units/ml streptomycin/penicillin (Solarbio).

Cell viability assay

Ec1 and Het-1A cells were seeded in the 96-well plate of 1×10^4 per well and treated with LNG (Peprotech, 0, 6.25, 12.5, 18.75, 25, 75, 250, 375, 500ng/ μ l), cisplatin (CDDP, MCE, 2.5, 5, 10, 20 and 25 μ M) for 24 h, then measured by Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan) according to the manufacturer's instruction. And the absorbance was measured at 450nm using an automated microplate spectrophotometer (ELx-800, BioTek, Vermont, USA). Then the cell viability rate of LNG and cisplatin on human Ec1 or/and Het-1A was calculated: Cell viability rate (%) = [(absorbance value of control group - absorbance value of drug administration group)/absorbance value of control group] \times 100%. At last, the optimal concentrations of LNG and CDDP were determined for subsequent experiments.

Cloning Formation Experiment

Ec1 were seeded into 6-well plates at a density of 1000 cells per well and treated with different concentrations of LNG (25, 50ng/ μ l), CDDP (10, 20 μ M), LNG (12.5, 25ng/ μ l) + CDDP (10, 20 μ M) for 10 days. The culture medium was discarded and rinsed with PBS for 2 times. And the plates were fixed with 4% paraformaldehyde solution (Beyotime Biotechnology, China) for 30 min at room temperature and then stained with 0.5% crystal violet (Beyotime Biotechnology, China) for 15 min. The number of colonies was counted and the data were obtained from three independent experiments.

Measurement of the mitochondrial membrane potential

Ec1 and Het-1A cells were treated with different concentrations of LNG (25, 50ng/ μ l) for 24 h. Then, cells were stained with Rhodamine123 (6 μ g/ml, Sigma) and Mito Tracker Green probes (5ug/ml, Yeason Biotechnology) respectively for 30 min at 37°C, and then rinsed twice with PBS, and checked by inverted fluorescence microscope (Nikon) and confocal microscopy (Olympus, CFV-2000), respectively. The averaged fluorescence of mitochondria was measured by the Image J software.

Scratch Wound Healing Assay

5×10^6 per well of Ec1 were seeded into 6-well plates and treated with LNG (25, 50ng/ μ l). When they were approximately 90% confluent, the monolayer cells were scratched with a tip 200 μ l aseptic pipette and rinsed with PBS to remove cellular debris. Subsequently, a fresh complete culture medium was added and cells were incubated at 37°C for 48h for migration into the cell-free area. The images of the migratory cells were captured at 0, 24 and 48 hours post wounding using a microscope (Nikon). Images were analyzed by the Tscratch software (CSE, Switzerland). Experiments were run in triple independent repeats.

Western-blotting analysis

The western blot (WB) protocol was according to our previous study with minor modified [18]: 5×10^6 per well of Ec1 and Het-1A were seeded into 6-well plates and treated with LNG (0, 12.5, 25, 37.5, 50ng/ μ l) for 24h. The primary antibodies are PCNA, Cleaved-Caspase3, Bcl2, Bax, E-cadherin, ERK1/2, p-ERK1/2, STAT3, p-STAT3, β -catenin, p- β -catenin and β -actin (ZEN BIO, 1:10000) and the secondary antibody was HRP-conjugated goat anti-rabbit IgG (Proteintech, 1:1000); The signals were detected using the ECL-Plus System (Genview). The optical density of each band was measured using the Image J software. The ratio between the optical density of target protein and β -actin of the same sample was calculated as relative content.

Statistical analysis

Data analysis was performed using GraphPad Prism 7.0. Data presented as mean \pm SD were representative of at least three independent experiments. Statistical analysis of intergroup differences was performed using the one-way analysis of variance. $P < 0.05$ was considered significant statistical analysis.

Results

LNG inhibits the proliferation of human ESCC cell of EC1

To evaluate the effect of LNG on cell viability, Ec1 and Het-1A cells were exposed to various concentrations (0, 6.25, 12.5, 18.75, 25, 75, 250, 375, 500ng/ μ l) of LNG for 24h. CCK-8 assay showed the IC_{50} concentration of LNG in human ESCC cells Ec1 and human normal esophageal epithelial cells Het-1A was 82.17ng/ μ l and 1427.28ng/ μ l, respectively, and the LNG inhibit the cell proliferation of ESCC cells Ec1 more effectively than that of human esophageal epithelial cells Het-1A (Fig.1A). Then we checked the colony formation in Ec1 treated with different concentration LNG (25, 50ng/ μ l) for 10 days. The results showed that the number of colony formation in LNG treated groups was significantly lower than that in control group, and total colony area and average colony size in 25ng/ μ l, 50ng/ μ l LNG treated groups were about 2.057, 5.543-fold smaller than that of control groups (Fig1B and 1C). WB analysis demonstrated that PCNA (Proliferating Cell Nuclear Antigen) expression was remarkably inhibited when treated with LNG not only in Ec1 but also in Het1A (Fig1D and 1E): the PCNA relative expression is about 0.79, 0.61, 0.62, 0.59-fold in Ec1, and 0.87, 0.67, 0.55, 0.52-fold in Het-1A treated with 12.5, 25, 37.5 and 50ng/ μ l LNG compared to control groups, respectively ($P < 0.05$).

LNG induce ESCC cell apoptosis associated with mitochondria

The change of mitochondrial potential checked by Rhodamine123 and Mito Tracker Green probes, respectively. The mitochondria shape of the Ec1 cells were regular, short rod or ball in control group; while the mitochondria of those cells showed pyknosis, smaller and deeper matrix and irregular morphology in LNG exposed groups (Fig.2A, left), the proportion of abnormal cells were about 2.98, 5.25-fold (treated with 25, 50ng/ μ l LNG for 24h, respectively) than control group (Fig.2C). And in Het-1A cells, the change of mitochondria is not obviously between control groups and LNG treated groups (Fig.2A, Right; Fig.2C).

Rhodamine 123 dye was used to evaluation of mitochondrial membrane potential (MMP) and indicated the cell apoptosis characteristics. The results showed that the cell numbers of apoptosis in LNG exposed groups were about 7.83, 25.60-fold (25, 50ng/ μ l, respectively) than control groups (Fig. 2B, left; Fig.2D); Small part of mitochondrial membrane ruptures were showed in some LNG treated Het-1A cells (Fig.2B, Right). In order to deeply explore the mechanism of the LNG in human ESCC, the cells of Ec1 and Het-1A were treated with different concentration LNG (12.5, 25, 37.5 and 50ng/ μ l) for 24h and WB analysis demonstrated that the protein expression level of cleaved-Caspase 3 increased (about 0.92, 0.90, 1.12, 1.81-fold than control groups) and the ratio of Bcl-2/Bax decreased (about 0.90, 0.89, 0.83, 0.70-fold than control groups) in Ec1 cells treated with the LNG (Fig.2E, 2F and 2G). It is noted that the change of those above protein expression level was not obviously in Het-1A (Fig.2E, 2F and 2G). Those data suggested that LNG induces a disruption of mitochondrial capacity, thereby promoting the cancer cells to undergo the mitochondrial apoptotic pathway in vitro.

LNG Inhibit migration ability of ESCC cells

The role of LNG in ESCC cell motility was examined by scratch wound healing assay. As shown in Fig.3A and 3B, the healing rate of scratches in the LNG-treated group (about 6.38%, 6.76% for 24h; 12.13%, 9.78% for 48h treated with 25, 50ng/ μ l LNG, respectively) is significantly slower than that in the control group (about 23.56%, 31.58% for 24h and 48h, respectively). The WB analysis was used to check the E-cadherin expression levels for further investigating the mechanism of LNG on the migration of ESCC cells. The results showed that LNG (12.5, 25, 37.5 and 50ng/ μ l) could upregulate the E-cadherin expression, about 1.65, 2.30, 2.35, 2.8-folds compared to that in control groups (Fig.3C and 3D).

The potential regulated mechanism of LNG on ESCC cells

We further examined the potential regulated mechanism of LNG on ESCC cells by WB analysis (Fig.4A). Treated with 12.5, 25, 37.5 and 50ng/ μ l LNG, the relative protein expression ratios of pERK1/ERK1, pERK2/ERK2, pSTAT3/STAT3 and p β -catenin/ β -catenin are about 0.92, 0.90, 1.00, 0.92; 0.97, 1.05, 1.08, 1.01; 1, 1.02, 1.17, 1.03; 0.99, 0.95, 1.20, 1.46 in Ec1 cells; and the protein expression ratios of pERK1/ERK1, pERK2/ERK2, pSTAT3/STAT3 and p β -catenin/ β -catenin are about 0.77, 0.77, 0.81, 0.62; 0.97, 0.78, 0.69, 0.66; 0.97, 0.96, 0.96, 0.94; 1.09, 0.97, 1.02, 1.21 in Het-1A cells (Fig. 4B, 4C, 4D and 4E). The results suggested that the regulated role of LNG on Ec1 cells is mainly associated with the Wnt/ β -catenin signaling, while on Het-1A cells are mainly associated with the MAPK/ERK signaling.

The LNG promotes the inhibitory effect of CDDP on Ec1 proliferation

To investigate whether LNG modulates the inhibitory effect of CDDP on Ec1 cells proliferation, we treated the Ec1 cells with 25, 50ng/ μ l LNG and different concentration CDDP (2.5, 5, 10, 20, 25 μ M). CCK-8 assay indicated that the LNG can suppress the proliferation ability combined with CDDP (Fig.5A), and 10 and 20 μ M CDDP were selected for the colony formation assay. The results showed that LNG combined with CDDP significantly suppressed the proliferative ability of Ec1 cells compared with control groups and

CDDP treated groups, respectively ($P < 0.05$, Fig. 5B and 5C). These results indicate that The LNG promotes the inhibitory effect of CDDP on Ec1 proliferation in vitro.

Discussion

It is well-known that developing new anticancer drugs with traditional method is a long-term and expensive procedure. Then more and more researchers pay attentions to some known drugs used in non-oncological situations, such as diabetes, inflammation, alcoholism, and so on [3-5]. This method in drug development is called drug repositioning, also called drug rediscovery or drug repurposing. Recently, Steven M et al systematically analyzed more than 6000 already developed drug compounds and found nearly 50 that have previously unrecognized anti-cancer activity, and they also found that the targets of most of the non-oncology drugs that killed cancer cells in their study were previously unknown [19]. These findings further strengthens that drug repositioning is a better and attractive way to find new targets for anti-cancer and drug development.

Levonorgestrel (LNG) is a synthetic progestogen and only the levonorgestrel isomer is active [20]. At present, levonorgestrel intrauterine system (LNG-IUS) a T-shaped intrauterine devices with a steroid reservoir containing levonorgestrel. The LNG, especially LNG-IUS, often used for contraception, control of menstrual disorders, treatment of endometriosis, and so on [11-14, 20-21]. Recently, more and more studies suggested that LNG or LNG-IUS play important roles in cancer, but the effect of LNG or LNG-IUS on cancers still has a dispute. Many researchers declared that LNG-IUS or LNG-IUS have a protective effect against endometrial malignant transformation [8, 11-14], but was associated with a higher than expected incidence of breast cancer [8-9, 23]; and they also found LNG-IUS was associated with a lower incidence of endometrial, ovarian, pancreatic, and lung cancers [8]. While some researchers reported that LNG or LNG-IUS associated with a decreased risk of ovarian and endometrial cancer, without increased risk of breast cancer [7, 10, and 15]. Scientists think that the different role of LNG or LNG-IUS in cancers may be associated with pathological process [12, 13], hormone levels [23], or the expression of molecules related hormone [24-25]. For example, Nikolopoulos et al and Westin et al found LNG-IUS offers an effective and safe treatment for early-stage, lowgrade endometrial cancer [12, 13]; Siegelmann-Danieli et al think that LNG-IUS was associated with a slightly increased risk for invasive breast cancer in the subgroup of women in their early 40's, while not associated with an increased total risk in perimenopausal women [23]. In this study, our results indicated that LNG exhibited anticancer effect on ESCC mainly associated with Wnt/ β -catenin signaling pathway and suggested LNG maybe used for drug repositioning to adjunctive therapy ESCC for its anticancer effect.

One of the important applications of drug repositioning is the combination of old drugs and existing drugs widely used in diseases [3-5, 19]. Studies found that LNG or LNG-IUS was safe, tolerable, and devoid of any noticeable pharmacokinetic interaction with some drugs associated with neurological diseases, pulmonary diseases, diabetes, and so on [26-29]. The combination of LNG-IUS and anticancer drug mainly focused on tamoxifen (for the treatment of advanced breast cancer and ovarian cancer), and the studies suggested that the LNG-IUS is benefit for the tamoxifen-treated breast cancer

patients [30-31]. In this study, our results suggested that the LNG promotes the inhibitory effect of cisplatin (a broad-spectrum anti-tumor drug) on Ec1 proliferation. And our former study declared that gold-LNG nanocluster can serve as efficient radiosensitizers for ESCC [16]. Those data suggested that LNG maybe have a new usage for anticancer.

Conclusion

In summary, the results showed that LNG exerts antitumor activity against ESCC related with Wnt/ β -catenin signaling pathway and can promotes the inhibitory effect of cisplatin on ESCC cells. Thus, LNG may be a potential novel therapeutic agent for the treatment of ESCC.

Declarations

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Conflicts of interest: The authors declare no conflicts of interest for this article.

Availability of data and material (data transparency): Not applicable.

Code availability (software application or custom code): Not applicable.

Authors' contributions: LY and ZW designed and participated the experiments and write original draft. HC, SC, YW and CZ performed the experiments and analyzed the data. WC and TJ gave intelligent advice. SM designed the study and the general supervision of the research group and made revision of this manuscript.

Ethics approval: This article does not contain any studies with human participants or animals performed.

Consent to participate: This article does not contain any studies with human participants.

Consent for publication: The Authors agrees to publish our report in the Journal of “Molecular and Cellular Biochemistry”. And all of the authors confirmed: the manuscript is approved by all authors for publication and has not been published or submitted elsewhere in whole or in part.

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Figures

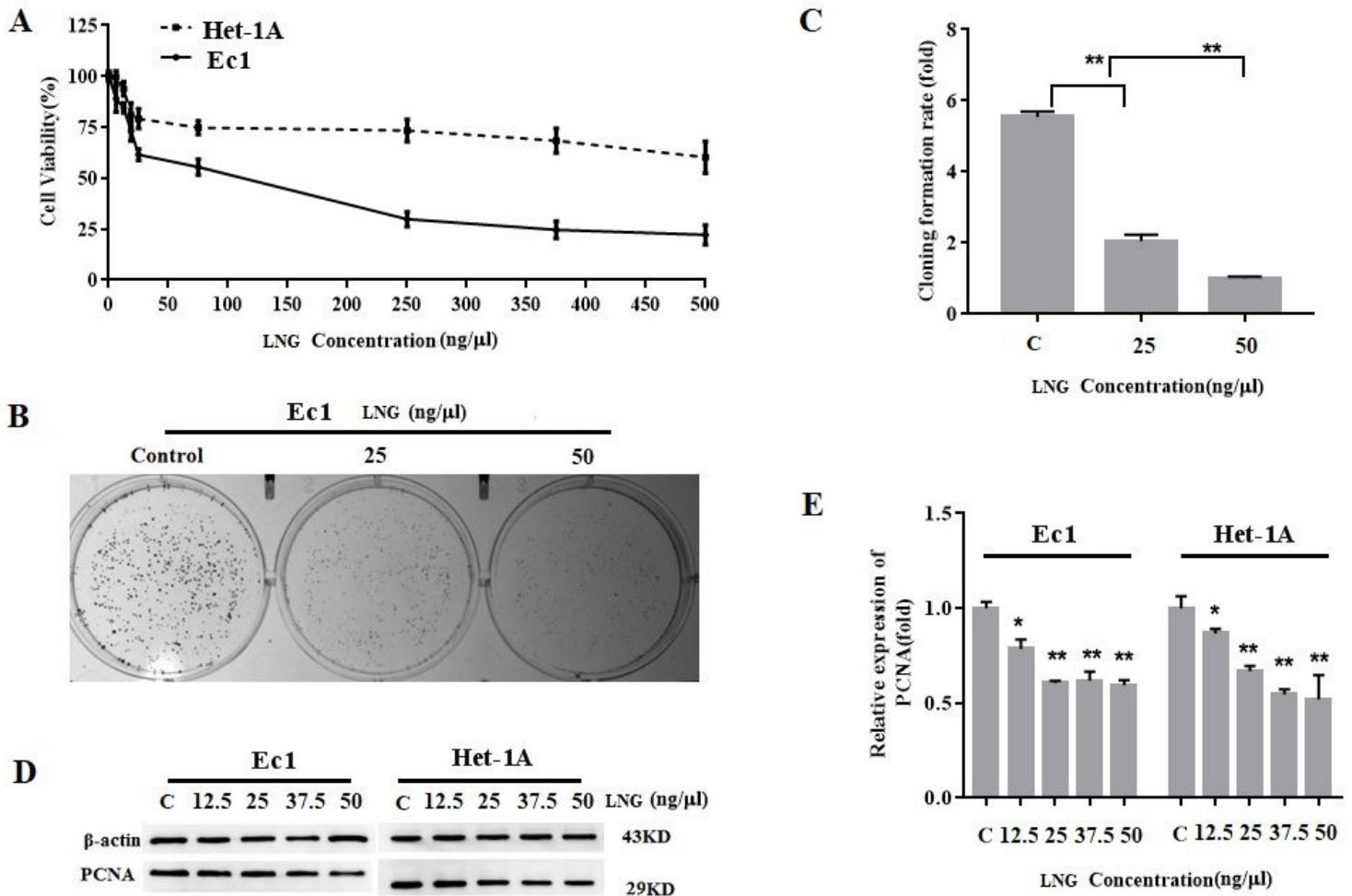


Figure 1

The inhibition role of LNG on cell proliferation in Ec1 and Het-1A (A) The effect of LNG on cell viability of Ec1 and Het-1A cells treated with various concentrations of LNG (0, 6.25, 12.5, 18.75, 25, 75, 250, 375, 500ng/μl) for 24 h. (B) and (C) The colony formation in Ec1 treated with different concentration LNG of 25, 50ng/μl for 10 days. (D) and (E) The effect of LNG (12.5, 25, 37.5 and 50ng/μl) on PCNA expression in Ec1 and Het-1A checked with WB. * P<0.05; **P<0.01

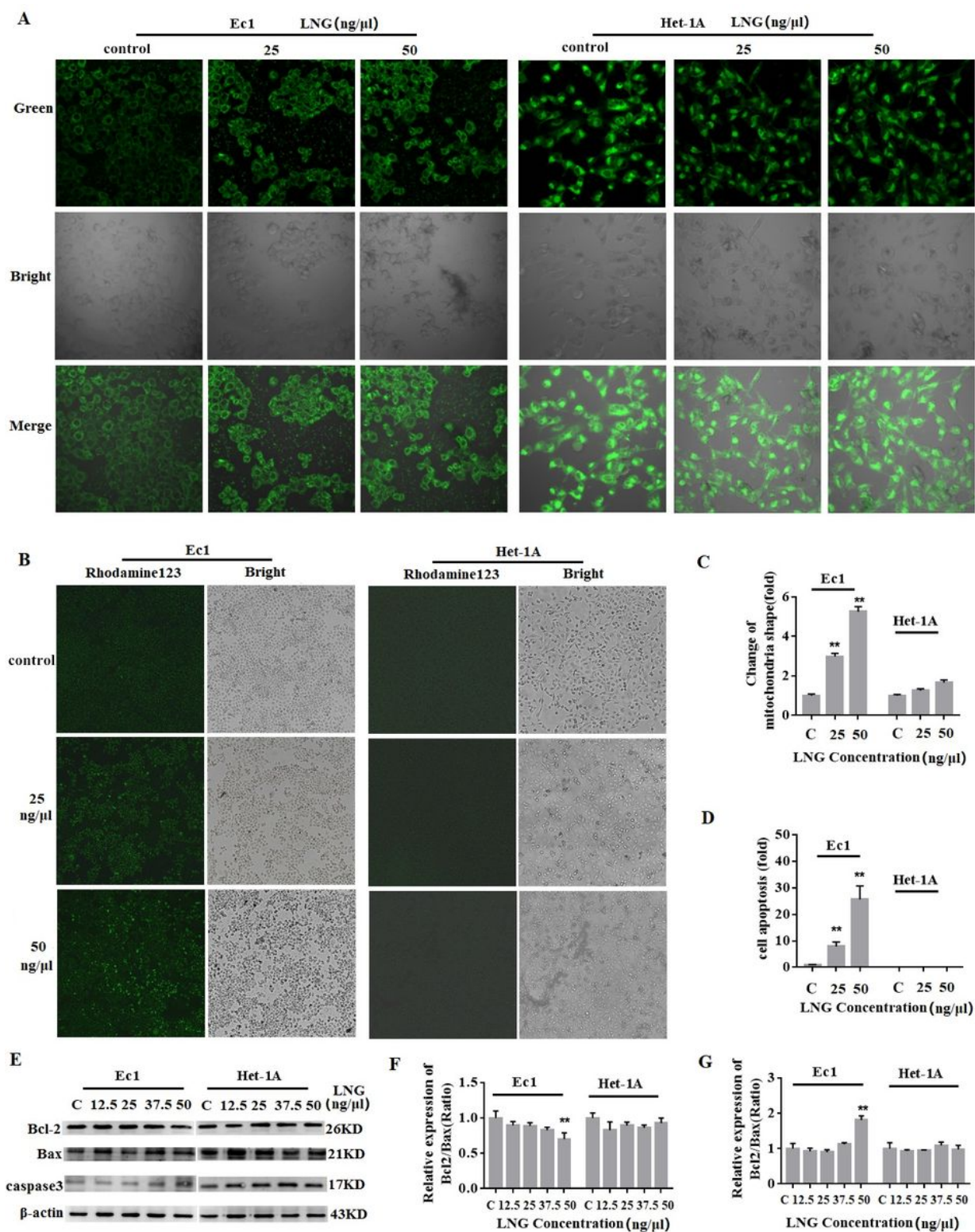


Figure 2

The effect of LNG on cell apoptosis in Ec1 and Het-1A. The change of mitochondrial potential checked by Mito Tracker Green probes (A, C) and Rhodamine123 (B, D) treated with 25, 50ng/μl LNG in Ec1 and Het-1A; the effect of LNG (12.5, 25, 37.5 and 50ng/μl) on cleaved-Caspase 3, Bcl-2 and Bax expression in Ec1 and Het-1A checked with WB analysis (E, F and G). **P<0.01

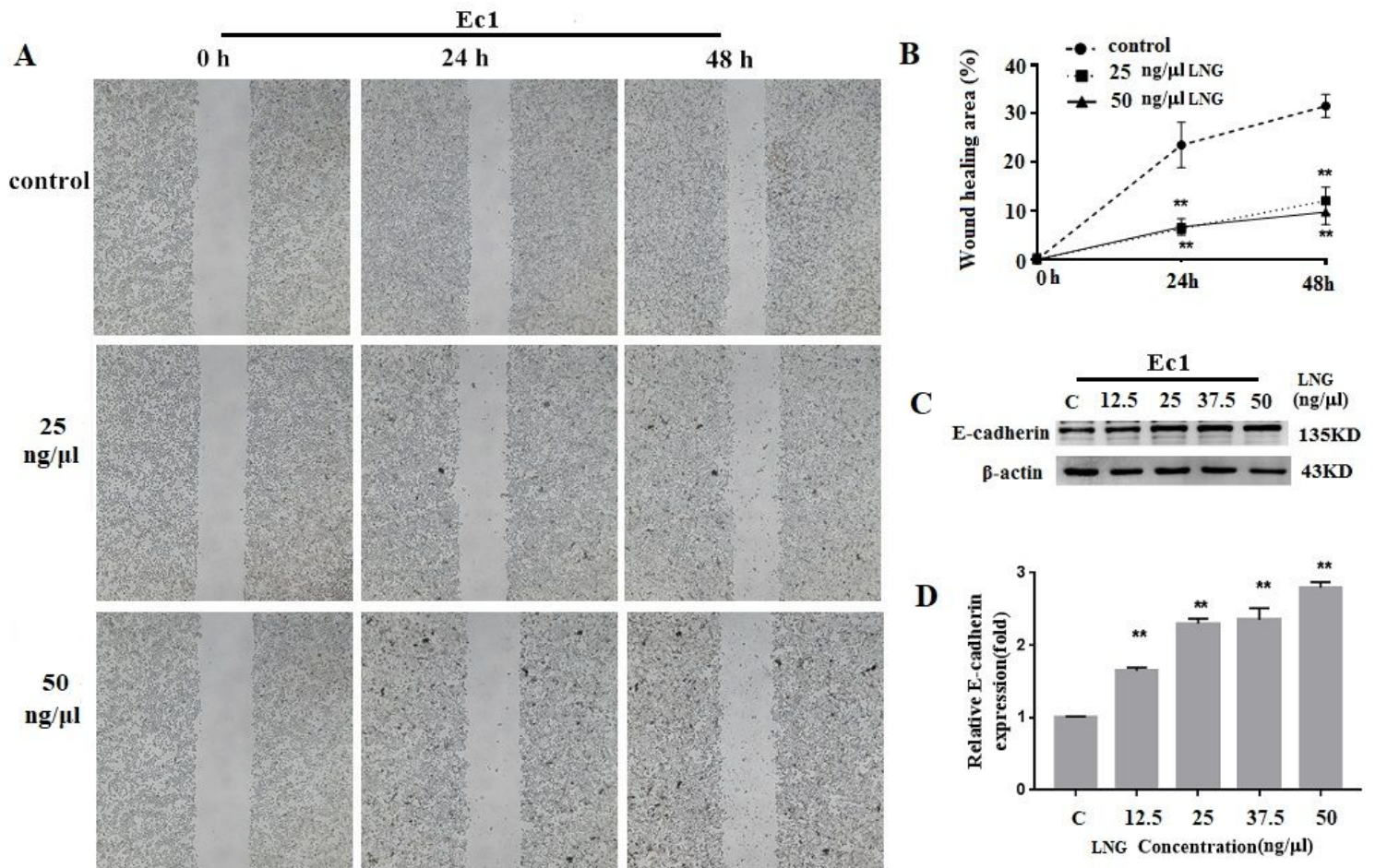


Figure 3

The role of LNG on cell motility in Ec1 cells Scratch wound healing assay was used to examine Ec1 cell motility when treated with 0, 25, 50ng/μl LNG (A and B); the effect of LNG (12.5, 25, 37.5 and 50ng/μl) on E-cadherin expression in Ec1 was checked with WB (C and D). **P<0.01

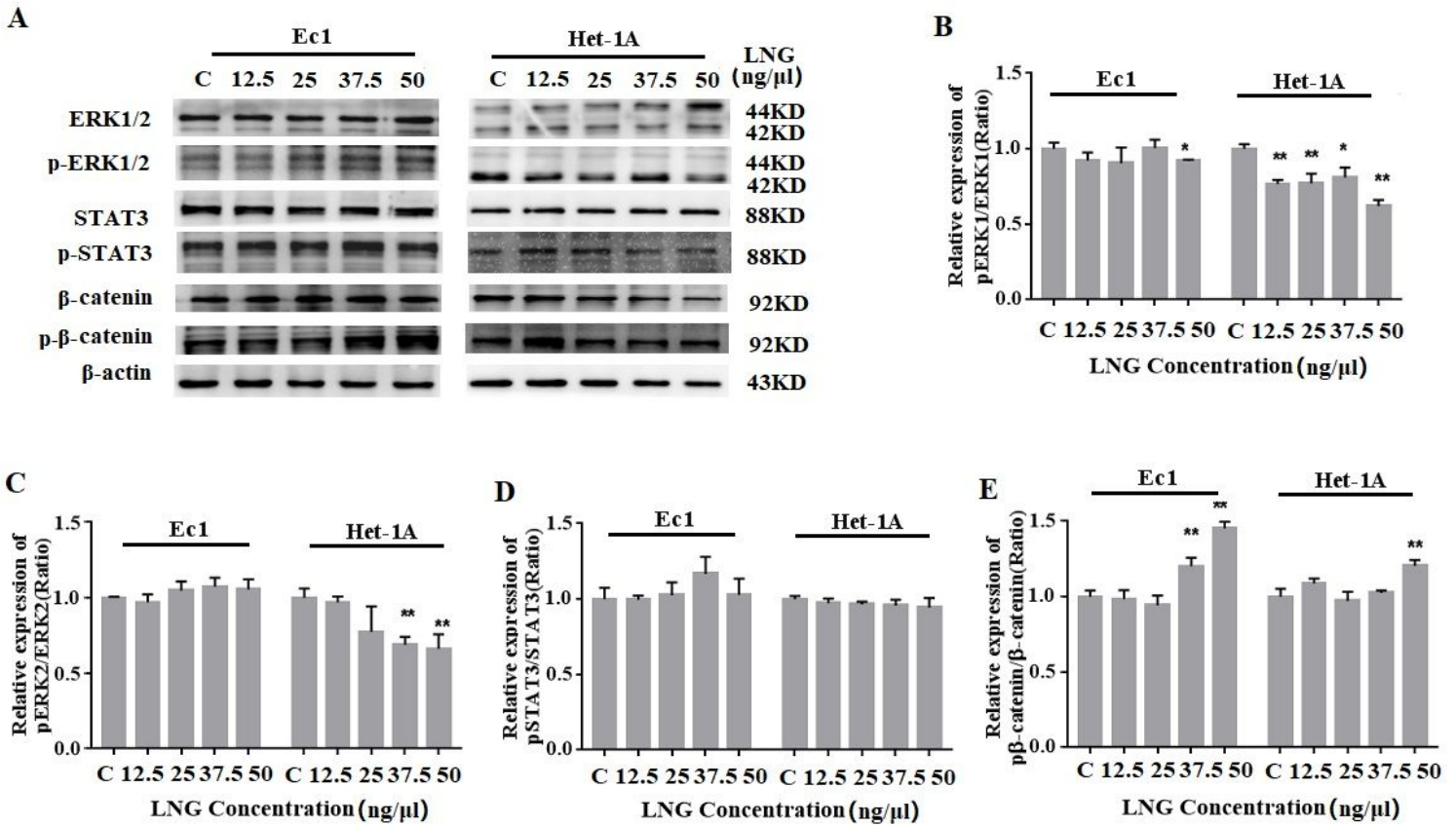


Figure 4

The potential regulated molecular mechanism of LNG on ESCC cells (A) The key proteins related MAPK/ERK, Wnt/β-catenin and JAK/STAT3 signaling pathway checked by WB analysis in Ec1 and Het-1A cells treated with 12.5, 25, 37.5 and 50ng/μl LNG , (B, C, D and E) The relative protein expression ratios of pERK1/ERK1, pERK2/ERK2, pSTAT3/STAT3 and pβ-catenin/β-catenin in Ec1 and Het-1A cells. * P<0.05; **P<0.01

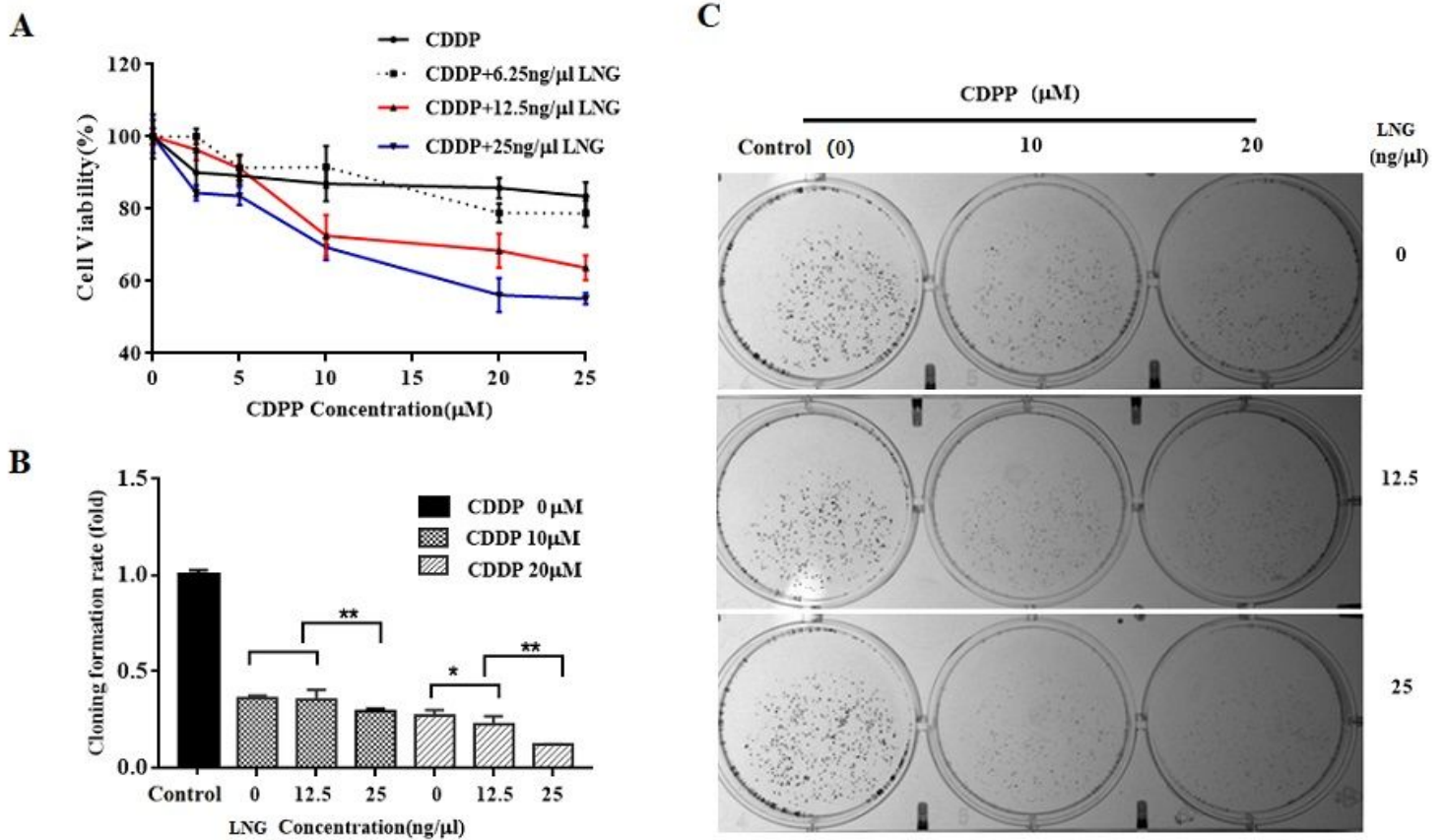


Figure 5

The LNG promotes the inhibitory effect of CDDP on Ec1 proliferation in vitro (A) CCK-8 assay and the colony formation assay (B and C) were used to check the role on the proliferation ability of Ec1 combined with CDDP. * $P < 0.05$; ** $P < 0.01$