Lidocaine inhibits the proliferation and migration of endometrial cancer cells, and promotes apoptosis by inducing autophagy

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Abstract. As a gynecological malignancy, endometrial cancer (EC) has a high incidence and mortality rate in women. The aim of the present study was to investigate the mechanism of EC and to identify novel effective treatment methods for this disease. The viability and proliferation of the RL95-2 human endometrial cancer cell line were assessed using Cell Counting Kit-8 assays. Colony formation, wound healing, Transwell, TUNEL and immunofluorescence assays were used to assess the effects of 5, 10 and 15 mM lidocaine on the colony formation, migration, invasiveness, apoptosis and Beclin 1 protein expression of RL95-2 cells, respectively. Furthermore, western blotting was used to analyze the protein expression levels of apoptosis- and autophagy-related proteins. The results demonstrated that lidocaine inhibited the viability, proliferation and migration of EC cells, and promoted apoptosis. Furthermore, lidocaine was demonstrated to induce autophagy and Beclin 1 protein expression in EC cells. In conclusion, lidocaine inhibited the proliferation and migration of EC cells, and promoted apoptosis via autophagy induction, which indicated that lidocaine may be a potential therapeutic drug for the treatment of EC.

Introduction

As a common gynecological cancer, the incidence rate of endometrial cancer (EC) has increased in recent years (1). It has been reported that 70% of EC cases occur in perimenopausal or postmenopausal women, while 4% of cases occur in women <40 years old (2). Moore and Brewer (3) hypothesized that the underlying causes of EC are obesity and hyperinsulinemia. Considering all EC stages, the overall 5-year survival rate for EC is ~80% (4). Currently, there are few effective detection strategies and treatments for EC. Therefore, the present study aimed to identify novel therapeutics for the prevention and treatment of EC.

Autophagy, which is also known as macroautophagy, is a controversial biological pathway in human cancer cells due its dual role as a self-protective and apoptotic mechanism (5). Autophagy induces apoptosis in tumor cells if the conditions in the cell mean that it is no longer viable, and this situation cannot be reversed (6). The role of autophagy has been investigated in EC, and the autophagy machinery may serve as a potential therapeutic target (5). Zhang *et al* (7) reported that four autophagy-related genes (cyclin dependent kinase inhibitor 2A, protein tyrosine kinase 6, erb-b2 receptor tyrosine kinase 2 and baculoviral IAP repeat containing 5) may potentially be independent predictive biomarkers and therapeutic targets for EC. Based on the results of the aforementioned studies, the present study investigated the underlying mechanism of autophagy in EC.

Lidocaine, one of the most commonly used local anesthetics, decreases the viability and migration of breast cancer cells (8). Chang *et al* (9) reported that lidocaine suppresses the viability of breast cancer cells via induction of apoptosis. Furthermore, lidocaine exhibits an inhibitory effect on the proliferation, epithelial-mesenchymal transition, migration and invasion of A2780 and SKOV3 ovarian cancer cells (10). Lidocaine has been investigated in numerous types of cancer; however, to the best of our knowledge, its effect in EC remains unclear. Furthermore, a recent study has reported the promotive effects of lidocaine on autophagy in different cell types. For example, in astrocytes, autophagy is activated by lidocaine treatment (11). However, the effects of lidocaine on autophagy in EC have not yet been reported. Therefore, the aim of the present study was to also determine the association of lidocaine and autophagy in EC.

Materials and methods

Cell culture and treatment. Human endometrial stromal cells (THESCs) and the human endometrial cancer cell line RL95-2 were provided by the Procell Life Science & Technology Co., Ltd. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin at 37˚C in a humidified incubator with 5% CO_2. Subsequently, lidocaine

(Beijing Solarbio Science & Technology Co., Ltd.) at different doss (5, 10 and 15 mM) was used to treat RL95-2 cells for 24, 48 and 72 h at 37˚C. To further explore the underlying mechanism of lidocaine, 1 mM 3-methyladenine (3-MA) (Beijing Solarbio Science & Technology Co., Ltd.), an autophagy inhibitor, was used to treat RL95-2 cells for 3 min at room temperature after lidocaine pre-treatment.

Cell Counting Kit-8 (CCK-8) assay. RL95-2 cells ($4x10^3$ cells/well) were inoculated into 96-well plates and incubated for 24 h at 37˚C. Subsequently, 10 μl CCK-8 reagent (Beyotime Institute of Biotechnology) was added to each well, and the cells were incubated for an additional 2 h. The absorbance was quantified at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Colony formation assay. RL95-2 cells ($3x10^5$ cells/well) were seeded into 6-well plates and maintained for 14 days to form colonies at 37˚C. After fixation and staining with 4% paraformaldehyde for 30 min at room temperature and 0.5% crystal violet solution for 10 min at 37˚C, respectively, the number of colonies (>50 cells were considered a colony) was counted under a Nikon Eclipse E600 microscope (Nikon Corporation; magnification, x100).

Wound healing assay. RL95-2 cells ($3x10^5$ cells/well) were inoculated into 6-well plates and incubated at 37˚C until the cells reached 80-90% confluency. The cells were then incubated overnight at 37˚C with serum-free DMEM. Subsequently, a pipette tip was used to create a linear scratch in the cell monolayer. With the aim of removing cell debris, the cells were washed with PBS three times. Cells were then incubated at 37˚C with 5% CO_2, and were assessed at 0 and 24 h. The area occupied by the migrated cells was observed using a light microscope (magnification, x100) and quantified with Image J software (v1.52; National Institutes of Health).

Transwell assay. A Transwell assay was used to assess RL95-2 cell invasion. The upper chamber was pre-coated with Matrigel (Corning, Inc.) for 30 min at 37˚C. Subsequently, RL95-2 cells ($5x10^5$ cells/well) in serum-free DMEM were plated into the upper chamber with 8-μm pores at 37˚C with 5% CO_2, while DMEM supplemented with 10% FBS was added to the lower chamber. Following 24 h of incubation, the cells were fixed with 4% paraformaldehyde for 15 min at 37˚C and stained with 0.1% crystal violet for 10 min at 37˚C. Images were captured using a light microscope (magnification, x100) and quantified with Image J software (v1.52; National Institutes of Health).

TUNEL assay. A TUNEL assay was used to assess the effects of lidocaine on RL95-2 cell apoptosis. In brief, fixation and permeabilization of cells were performed using 4% paraformaldehyde at room temperature for 30 min and 0.25% Triton-X-100 at room temperature for 5 min, respectively. Subsequently, the cells that were rinsed with PBS were incubated with TUNEL reagent at 37˚C for 1 h in the dark. Next, 5 μg/ml DAPI was used to stain cell nuclei at 37˚C in the dark for 5 min and then 10 visual fields were randomly selected. The number of apoptotic cells was totaled using a fluorescence microscope (magnification, x100).

Western blotting. Total protein was extracted from RL95-2 cells using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). The protein concentration was quantified using a BCA kit (Thermo Fisher Scientific, Inc.). Protein samples (30 μg/lane) were separated using 10% SDS-PAGE. Subsequently, the separated proteins were transferred onto a PVDF membrane. The membranes were blocked with 5% skimmed milk at room temperature for 2 h, and then incubated with the primary antibodies against Bcl-2 (dilution, 1:1,000; cat. no. ab32124), Bax (dilution, 1:1,000; cat. no. ab32503), cleaved caspase-3 (dilution, 1:500; cat. no. ab32042), caspase-3 (dilution, 1:2,000; cat. no. ab184787), cleaved caspase-9 (dilution, 1:1,000; cat. no. ab2324), caspase-9 (dilution, 1:2,000; cat. no. ab202068), p62 (dilution, 1:10,000; cat. no. ab109012), LC3 (dilution, 1:2,000; cat. no. ab192890) and GAPDH (dilution, 1:2,500; cat. no. ab9485) (all from Abcam) at 4˚C overnight. Subsequently, the membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody (dilution, 1:2,000; cat. no. ab205718; Abcam) for 2 h at room temperature. Protein bands were visualized with an ECL kit (Thermo Fisher Scientific, Inc.) and analyzed using ImageJ (v1.51; National Institutes of Health).

Immunofluorescence staining. RL95-2 cells were fixed with 4% paraformaldehyde for 15 min at 4˚C and permeabilized with 0.1% Triton X-100 for 15 min at room temperature, following which blocking was performed with 10% goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at room temperature. Subsequently, the cells were incubated with primary antibody against Beclin 1 (dilution, 1:100; cat. no. ab62557; Abcam) at 4˚C overnight. On the next day, RL95-2 cells were incubated with FITC-conjugated goat anti-rabbit secondary antibody (dilution, 1:1,000; cat. no. ab6755; Abcam) for 1 h at room temperature. Next, the cells were counterstained with 5 μg/ml DAPI for 5 min at 37˚C, and the samples were imaged using a fluorescence microscope (Nikon Corporation).

Statistical analysis. All data are presented as the mean ± SD and were analyzed using GraphPad Prism 8.0 software (GraphPad Software, Inc.). One-way ANOVA and Tukey's post hoc test were used for statistical comparisons among different groups. Each experiment was repeated ≥3 times. P<0.05 was considered to indicate a statistically significant difference.

Results

Lidocaine inhibits the proliferation and migration of EC cells. RL95-2 cell viability was first detected using a CCK-8 assay. The results demonstrated that lidocaine markedly reduced the viability of RL95-2 cells and exhibited an inhibitory effect on cell viability in a dose-dependent manner. However, the viability of THESCs remained similar following lidocaine administration (Fig. 1A). Furthermore, lidocaine suppressed the proliferation of RL95-2 cells (Fig. 1B). When the lidocaine concentration was 5 mM, there was no statistically significant difference compared with the control at any of the three times measured, although cell proliferation was inhibited. By contrast, when the lidocaine concentration was 10 mM, the difference compared with the control was statistically significant in a time-dependent manner at 24 h (P<0.05),
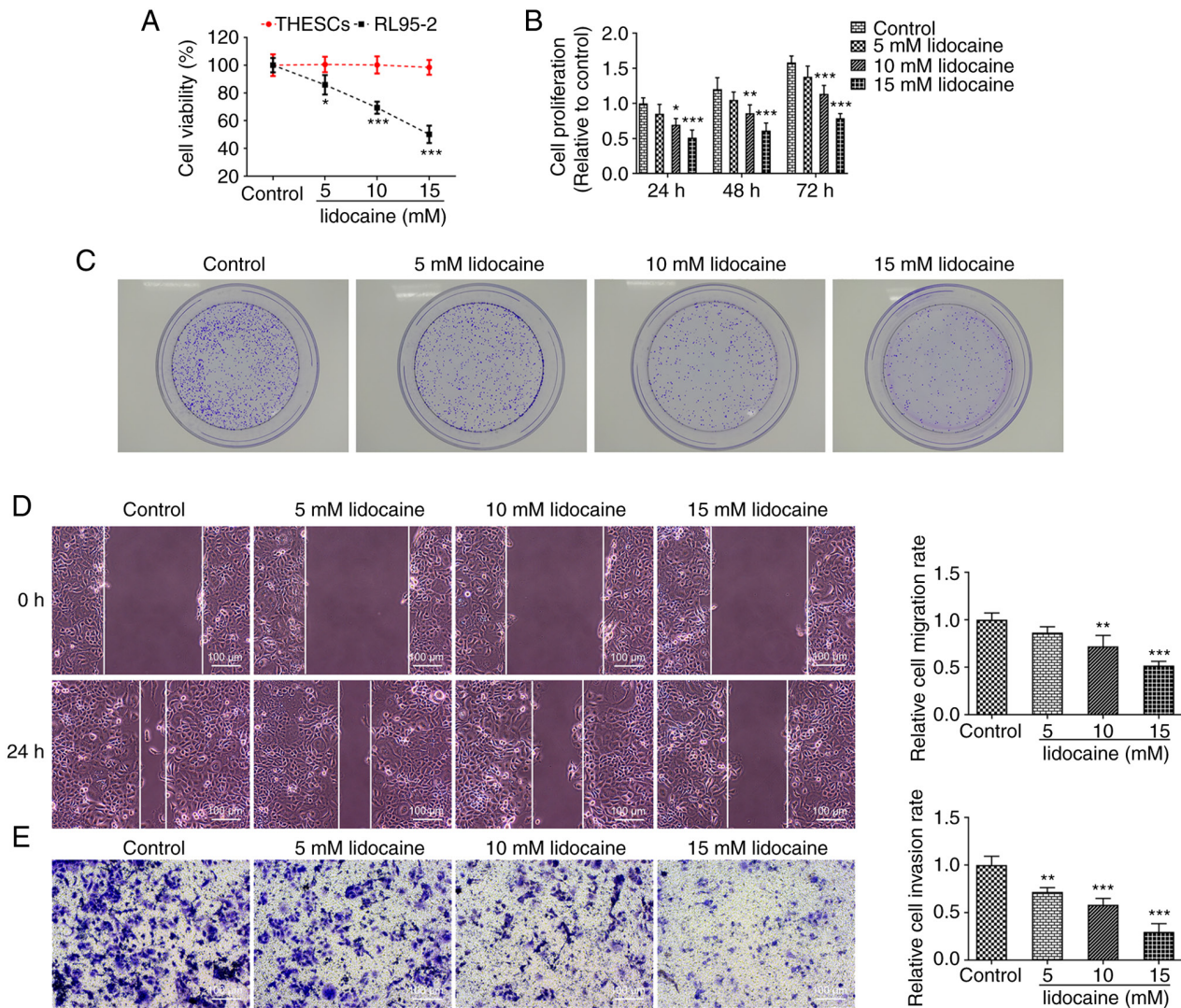
Figure 1. Lidocaine inhibits endometrial cancer cell proliferation and migration. (A) THESCs and RL95-2 cell viability was detected using a CCK-8 assay. (B) RL95-2 cell proliferation was detected using a CCK-8 assay (C) The colony formation of RL95-2 cells was evaluated using a colony formation assay. (D) RL95-2 cell migration was investigated using a wound healing assay. Scale bar, 100 μm. (E) RL95-2 cell invasion was detected using a Transwell assay. Scale bar, 100 μm. *P<0.05, **P<0.01 and ***P<0.001 vs. control. CCK-8, Cell Counting Kit-8.

48 h (P<0.01) or 72 h (P<0.001). When the concentration of lidocaine was 15 mM, cell proliferation was significantly inhibited at 24 h (P<0.001), 48 h (P<0.001) and 72 h (P<0.001). The colony formation of RL95-2 cells was also decreased by lidocaine treatment in a dose-dependent manner (Fig. 1C).

To determine the effects of lidocaine on the migration and invasion of RL95-2 cells, wound healing and Transwell assays were performed. The results demonstrated that the migration and invasion of RL95-2 cells were inhibited following lidocaine treatment. Lidocaine at a concentration of 15 mM exhibited greater inhibitory effects (P<0.001) on the migration and invasion of RL95-2 cells compared with 5 or 10 mM (Fig. 1D and E).

Lidocaine promotes the apoptosis of EC cells. A TUNEL assay was performed to determine the effects of lidocaine on the apoptosis of RL95-2 cells. The results demonstrated that lidocaine treatment promoted the apoptosis of RL95-2 cells in a dose-dependent manner (Fig. 2A). Furthermore, lidocaine treatment downregulated Bcl-2 protein expression

but upregulated the protein levels of Bax, cleaved caspase-3 and cleaved caspase-9 (Fig. 2B). These results suggested that lidocaine may promote the apoptosis of EC cells.

Lidocaine induces autophagy in EC cells. The protein expression levels of Beclin 1 were markedly enhanced by lidocaine treatment in a dose-dependent manner (Fig. 3A). Lidocaine also regulated the protein expression levels of autophagy-related proteins, as demonstrated by the upregulated protein expression levels of LC3II/LC3I and downregulated p62 protein expression compared with those of the control group (Fig. 3B). These results indicated that lidocaine treatment may induce autophagy in EC cells.

Lidocaine inhibits the proliferation, invasion and migration of EC cells by inducing autophagy. To investigate the mechanism of the effect of lidocaine treatment on the proliferation, invasion and migration of EC cells, 3-MA, an autophagy inhibitor, was used to treat RL95-2 cells. Compared with that of cells in the control group, the proliferation of RL95-2 cells was
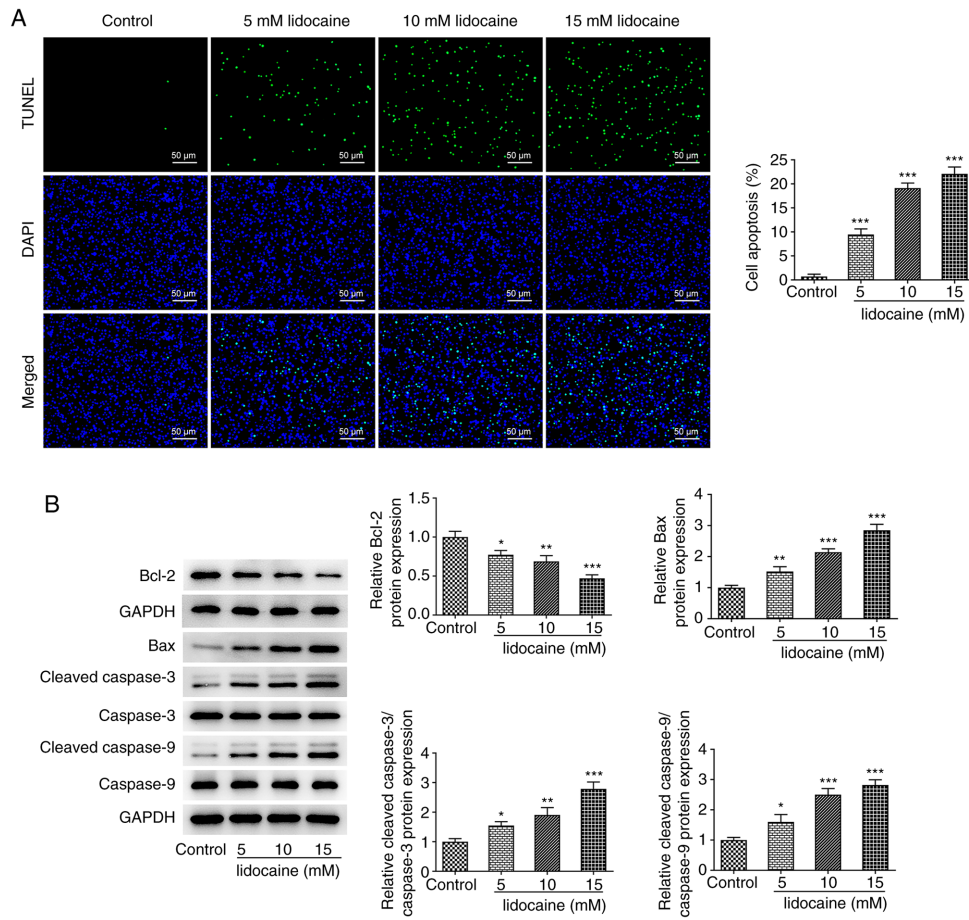
Figure 2. Lidocaine promotes the apoptosis of endometrial cancer cells. (A) RL95-2 cell apoptosis was detected using a TUNEL assay. Scale bar, 50 μm. (B) Protein expression levels of apoptosis-related proteins were detected by western blotting. *P<0.05, **P<0.01 and ***P<0.001 vs. control.
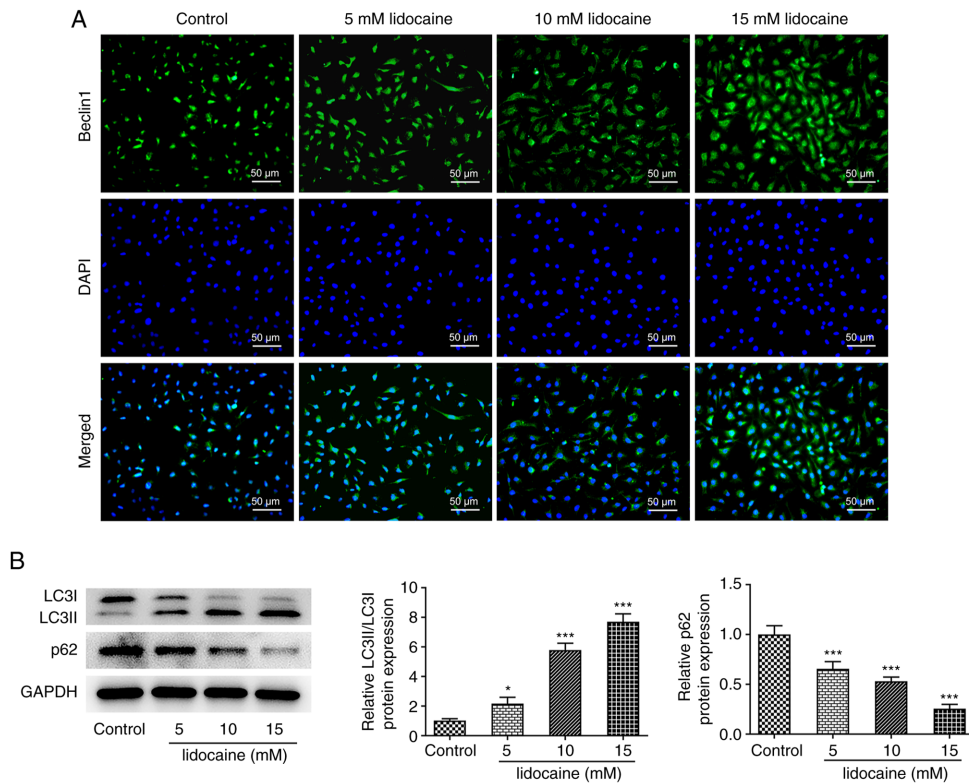

Figure 3. Lidocaine induces autophagy in endometrial cancer cells. (A) Protein expression levels of Beclin 1 were determined using immunofluorescence. Scale bar, 50 μm. (B) Protein expression levels of autophagy-related proteins were detected via western blotting. *P<0.05 and ***P<0.001 vs. control.
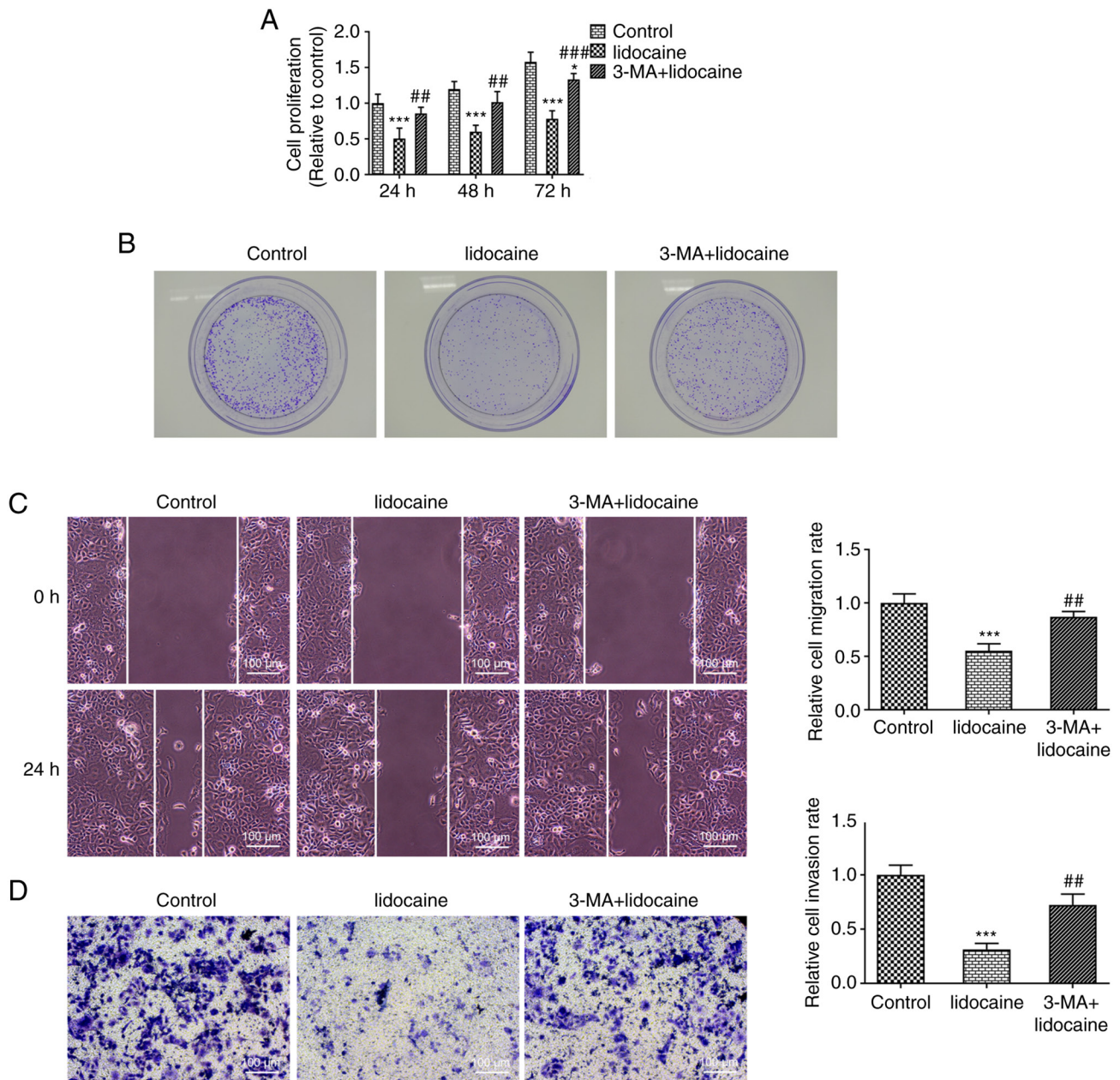
Figure 4. Lidocaine inhibits the proliferation, invasion and migration of endometrial cancer cells by inducing autophagy. (A) RL95-2 cell proliferation was detected using a Cell Counting Kit-8 assay. (B) RL95-2 cell colony formation was detected using a colony formation assay. (C) RL95-2 cell migration was detected using a wound healing assay. Scale bar, 100 μm. (D) RL95-2 cell invasion was detected using a Transwell assay. Scale bar, 100 μm. *P<0.05 and ***P<0.001 vs. control; $^{##}$P<0.01 and $^{###}$P<0.001 vs. lidocaine. 3-MA, 3-methyladenine.

significantly decreased following lidocaine treatment, whereas 3-MA partially reversed the inhibitory effects of lidocaine, as evidenced by the increased cell proliferation of the 3-MA + lidocaine group (Fig. 4A). The decreased colony formation observed in the lidocaine-treated group was also increased following treatment with 3-MA (Fig. 4B). Furthermore, lidocaine reduced the migration and invasion of RL95-2 cells compared with those of the control. This effect was reversed following 3-MA treatment (Fig. 4C and D).

Lidocaine promotes EC cell apoptosis by inducing autophagy. The increase in cell apoptosis following lidocaine treatment was decreased by 3-MA treatment, which indicated that 3-MA inhibited apoptosis in lidocaine-treated RL95-2 cells

(Fig. 5A). Furthermore, lidocaine downregulated Bcl-2 protein expression but upregulated the protein levels of Bax, cleaved caspase-3 and cleaved caspase-9. These effects were reversed when the cells were treated with 3-MA (Fig. 5B). Overall, the present results suggested that lidocaine may promote the apoptosis of EC cells by inducing autophagy.

Discussion

As one of the most prevalent neoplasms in developed countries, EC poses a serious threat to human health (12). To investigate the mechanism of EC, EC cells were treated with lidocaine in the present study. CCK-8, colony formation, wound healing and Transwell assays were performed to detect
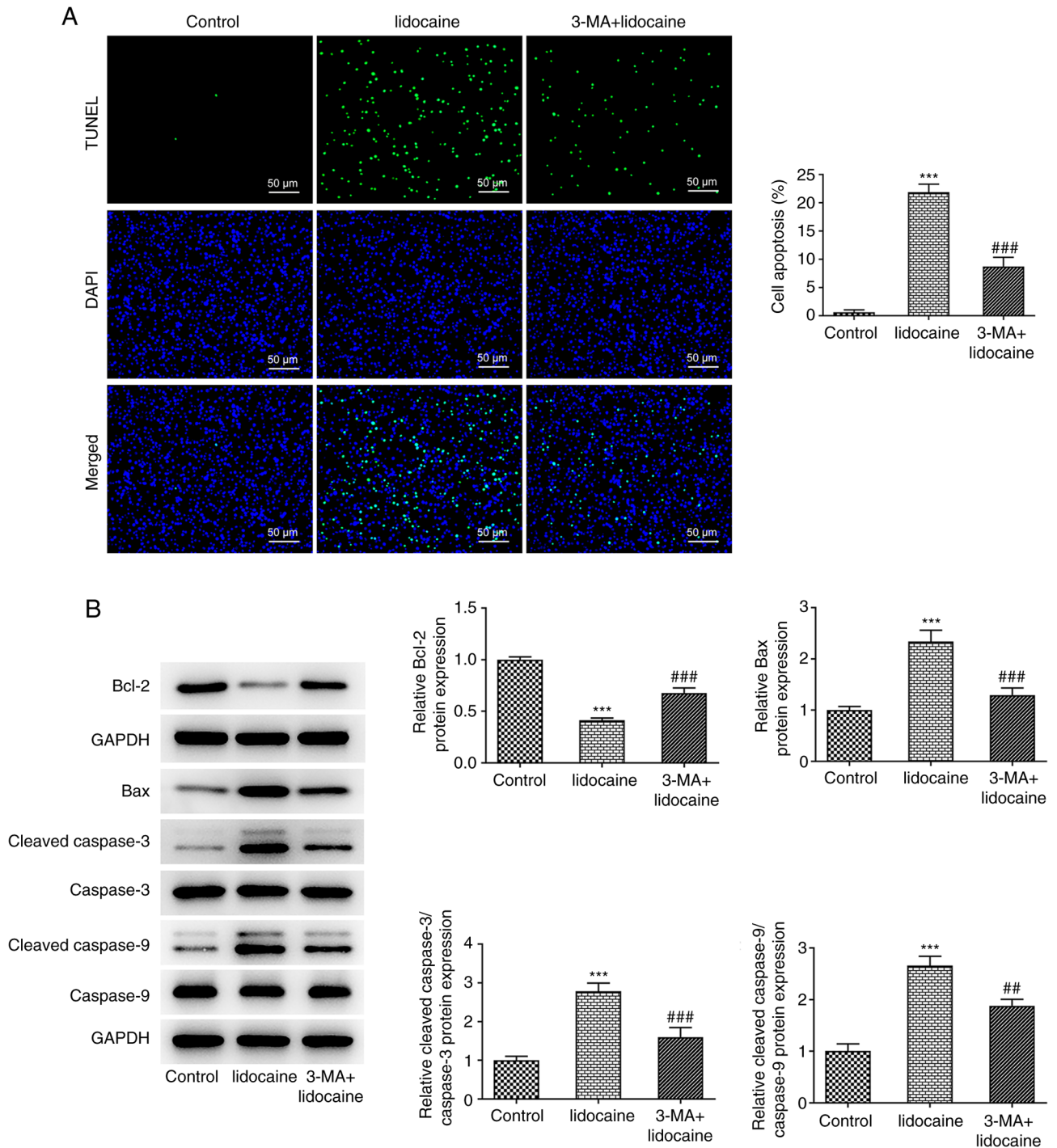
Figure 5. Lidocaine promotes endometrial cancer cell apoptosis by inducing autophagy. (A) RL95-2 cell apoptosis was detected using a TUNEL assay. Scale bar, 50 μm. (B) Protein expression levels of apoptosis-related proteins were detected via western blotting. ***P<0.001 vs. control; ##P<0.01 and ###P<0.001 vs. lidocaine. 3-MA, 3-methyladenine.

the proliferation, colony formation, migration and invasion of EC cells. In addition, 3-MA, an inhibitor of autophagy, was used to treat EC cells to explore the effects of lidocaine on autophagy. Overall, the current results demonstrated that lidocaine exerted desirable anticancer effects on the proliferation, migration, invasion and apoptosis of EC cells via the induction of autophagy.

Lidocaine, a local anesthetic that is the first choice of treatment for ventricular tachycardia and tremors, exerts its efficacy within 1-3 min after administration, and its effects

last for 1-3 h (13,14). Clinically, lidocaine was initially shown to have remarkable analgesic effects in laparoscopic and open surgery, but it is now applied in different clinical settings, including the perioperative period for spinal, breast, nose and throat surgery (15-17).

Lidocaine has been widely investigated in numerous cancer types. For example, lidocaine was demonstrated to exert an antitumor effect in human gastric cancer, as evidenced by inhibited cell proliferation, migration and invasion (18). Yang *et al* (19) reported that lidocaine may be a novel therapy

for the treatment of bladder cancer. Furthermore, it has been demonstrated that lidocaine inhibits the malignant development of cervical cancer by suppressing cell proliferation and inducing apoptosis (20). In the present study, the effects of lidocaine on RL95-2 cell proliferation, migration, invasion and apoptosis were explored. It was demonstrated that lidocaine markedly suppressed the viability, proliferation and colony formation of RL95-2 cells in a dose-dependent manner. Furthermore, lidocaine exhibited suppressive effects on the migration and invasion of RL95-2 cells.

Autophagy, a conserved self-degradation system that is critical for maintaining cellular homeostasis during stress conditions, is associated with the progression of numerous diseases (21). Autophagy serves an important role in cell survival and maintenance by degrading organelles, proteins and macromolecules, and by recycling degradation products (22). In addition, it serves a dichotomous role in cancer by suppressing the growth of benign tumors but supporting the growth of advanced cancer (23,24). Autophagy has been identified as a potential therapeutic target for cancer (23,25). Izdebska *et al* (26) reported that lidocaine induced protective autophagy in the rat C6 glioma cell line. To understand the effects of lidocaine on autophagy in EC cells, 3-MA was used to treat EC cells in the present study. The results revealed that the protein expression levels of Beclin 1 were markedly increased in lidocaine-treated RL95-2 cells compared with in the control cells. Furthermore, lidocaine treatment upregulated the protein expression levels of LC3II/LC3I, but downregulated p62 protein expression compared with those of the control cells. Therefore, these results suggested that lidocaine may induce autophagy. The effects of lidocaine on the proliferation and apoptosis in EC cells were reversed by 3-MA treatment, which indicated that lidocaine may inhibit cell proliferation and promote apoptosis in EC via autophagy induction.

Apoptosis, a type of programmed cell death, serves an indispensable role in a number of physiological and pathological processes (27). Apoptosis induction has been considered a target for cancer treatment (28-30). Previous studies have reported that lidocaine could induce apoptosis (31,32). Therefore, the present study also investigated the effects of lidocaine on apoptosis in EC. It was demonstrated that lidocaine treatment promoted the apoptosis of EC cells in a dose-dependent manner, which was inhibited by 3-MA treatment. Furthermore, the downregulated Bcl-2 protein expression, and the upregulated protein expression levels of Bax, cleaved caspase-3 and cleaved caspase-9 caused by lidocaine treatment were reversed following 3-MA treatment.

In conclusion, lidocaine inhibited the proliferation and migration of EC cells, and promoted apoptosis by inducing autophagy. However, the absence of an animal model, the use of only one type of EC cell line and the absence of deeper analysis of signaling pathways are limitations of the present study. In future studies, the current results will be validated in different EC cells and the signaling pathways involved will be thoroughly investigated. In conclusion, lidocaine may be a potential therapeutic drug for the prevention and treatment of EC.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DL, YC, LQ and YD conceived and designed the study, and acquired and interpreted the data. DL was a major contributor in writing the manuscript. DL and YD confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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