Lidocaine Inhibits Breast Cancer Cell Proliferation and Induces Cell Apoptosis via Regulating MEK/ERK and PI3K/AKT Signalling Pathways

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Research Article

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Abstract

Background: Lidocaine is a commonly used local anesthetic in clinic, which is mainly used for anesthesia and analgesia. Lidocaine has been recently found to have an inhibitory effect on a variety of cancers.

Materials and Methods: We used MTT assay and cell proliferation assay to detect the inhibition of lidocaine on proliferation of MCF-7 and MDA-MB-231 breast cancer cells. Flow cytometric analysis was used to detect cell cycle and apoptosis. Western blot was used to detect protein levels of cyclin-dependent kinase 1(CDK1) ,cyclinB1, BCL2, BCL-XL, p62, LC3B, p-ERK,p-AKT, ERK and AKT.

Results: Lidocaine inhibited the proliferation of MCF-7 and MDA-MB-231 breast cancer cells, increased the percentage of G2 / M phase cells , apoptosis and autophagy by reducing the mRNA of CDK1 and cyclinB1, decreasing protein levels of CDK1,cyclinB1,BCL2, BCL-XL, p62, p-ERK and p-AKT protein, and increasing LC3B-II/LC3B-I protein levels.

Conclusion: Lidocaine may be a potential candidate for treatment of breast cancer.

Introduction

Breast cancer is the leading cause of cancer-related death among women worldwide, accounting for one-fourth of all cancers [1]. In the past few years, the incidence has been increasing and the age of onset has been decreasing [2]. Breast cancer is a highly heterogeneous tumor, which can be divided into luminal, HER-2-enriched and triple negative subtype [3]. These breast cancer subtypes showed different clinical outcomes: the luminal cancer responded well to therapy and had a good prognosis, while HER-2- enriched and triple negative breast cancer had a poor prognosis. Breast cancer tends to metastasize at an early stage [4]. Systemic adjuvant therapy is the standard treatment strategy for patients with breast cancer. However, the high toxicity of chemotherapeutic drugs and the accompanying antineoplastic drug resistance limit the treatment of breast cancer [5]. Therefore, there is an urgent need to develop new antineoplastic drugs to solve this situation.

It has been recently reported that cancer patients can reduce cancer recurrence and improve survival rate through the application of local anesthetic drugs [6,7]. Lidocaine is one of the most commonly used local anesthetics in clinic, which is mainly used for anesthesia and analgesia [8]. Therefore, growing attention has been paid to the study of the role of lidocaine in cancer. Up to date, it has been reported that lidocaine can inhibit lung cancer [9,10], hepatocellular carcinoma [11,12], cervical cancer [13], melanoma [14,15], colorectal cancer [16], glioma [17] and other cancers [18–20]. Although the potential antitumor effect of lidocaine has been reported, the role and mechanism of lidocaine in breast cancer are still unclear. The aim of this study was to investigate the effect of lidocaine on breast cancer cells and its mechanism.

Materials And Methods

Cell culture

The breast cancer cell lines MCF7 and MDA-MB-231 were derived from ATCC. Breast cancer cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen, CA, USA). All cells were cultured at 37 ℃ and 5% CO_2.

MTT assay

MTT method was used to determine cell viability of tumor cells after lidocaine treatment. In short, the cells ($5–6×10^3$) per well were seeded on a 96-well plate and cultured for 24 hours, and then exposed to lidocaine at 0mM, 3mM, 6mM for 12h, 24h, 48h. 20 μl 5mg/ml MTT was added to each well and incubated for another 4 h at 37°C. The supernatant was then removed.100μl DMSO was added to each well and gently rocked incubated for 10 minutes. The absorbance (OD) was measured at 490nm by SpectraMax iD3 (Molecular Devices).

Cell proliferation assay

Cells were seeded on 24-well plates at 3000 cells per well in 0.5 ml DMEM with 10% FBS. Normally, the culture medium was changed every two days. 24 hours before cell treatment, the medium was replaced with DMEM supplemented with lidocaine at indicated concentrations. At indicated time points, cells were fixed in 4% formaldehyde and stained with 2% crystal violet. Dye was extracted with 10% acetic acid and the relative proliferation was determined by the absorbance at 595nm(SpectraMax iD3,Molecular Devices).

Protein Isolation And Western Blot

Protein isolation and Western blot
Protein extracts were prepared using NP-40 lysis buffer containing phosphatase and protease inhibitors. The cell lysates were then subjected to SDS-PAGE followed by immunoblot using indicated antibodies(CDK1, Proteintech, No.19532-1-AP, 1:2000;cyclinB1, Proteintech,No. 55004-1-AP,1:1000;BCL2, Proteintech,No. 12789-1-AP,1:2000;BCL-XL, Proteintech,No. 26967-1-AP,1:1000;p62, Proteintech, No. 18420-1-AP,1:2000;LC3B, Proteintech,No. 14600-1-AP,1:1000;p-ERK, Proteintech,No. 24390-1-AP,1:1000;ERK, Proteintech,No. 16443-1-AP, 1:2000; p-AKT, Proteintech,No. 66444-1-Ig,1:5000;AKT, Proteintech,No. 10176-2-AP,1:2000;β-actin, Proteintech,No. 66009-1-Ig,1:5000).

Flow cytometry for cell cycle analysis

Before treated with lidocaine, all breast cell lines were synchronized by serum starvation for 24 hours. Cells treated with lidocaine were digested with trypsin. After centrifugation, the supernatants were discarded, and the cell precipitations were resuspended in 5 ml PBS, then centrifuged again and resuspended in 0.5 ml PBS. The cells were then fixed on ice with 70% alcohol for at least 2 hours, and then centrifuged at 1500 RPM (Revolutions Per minute) for 10 minutes. The supernatants were discarded

and cell precipitations were then resuspended with PBS and centrifuged again. Cell precipitations were resuspended with 500 µl guava cell cycle reagent. After incubation at 37 ℃ for 30 min in the dark, cell cycle distribution was analyzed by the BD FACSMelody™ flow cytometer for counting 30000 cells.

Flow cytometry for apoptosis analysis

The lidocaine-treated cells were digested with trypsin, centrifuged at 300g for 5 minutes, the supernatants were discarded, the cells were collected, washed again with PBS, and then gently resuspended and counted. 5×10^5 cells were collected and centrifuged at 300g for 5 minutes, and the supernatants were discarded. The cells were resuspended with PBS again. After centrifugation, the supernatants were discarded. The cells were resuspended with 100 ul diluted 1×Annexin V Binding Buffer. 2.5 ul Annexin V-FITC and 2.5 ul propidium iodide (PI) staining were then added. The samples were incubated for 15 minutes in the dark at room temperature. 400ul diluted 1×Annexin V Binding Buffer were then added to mix the samples, apoptosis was analyzed by flow cytometer (BD FACSMelodyTM).

Quantitative RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen) and 1 µg total RNA was performed reverse transcription using PrimeScript RT reagent kit with gDNA eraser (TaKaRa), according to the manufacturer's instructions. Quantitative RT-PCR was performed with SYBR Green dye using (Applied Biosystems). The relative amount of cDNA was calculated by the comparative Ct method using GAPDH as a control. PCR reactions were performed in triplicate.The specific primer sequences were presented as follows:CDK1:F 5′-GGAAGGGGTTCCTAGTACTGC-3′,R 5′-CCATGTACTGACCAGGAGGG-3′;cyclinB1:F 5′-GCACTTCCTTCGGAGAGCAT-3′, R 5′-TGTTCTTGACAGTCCATTCACCA-3′; GAPDH:F 5'-GCACCGTCAAGGCTGAGAAC-3',R 5'-TGGTGAAGACGCCAGTGGA-3'.

Statistical analysis

The statistical analysis was performed with SPSS software, version 19.0(SPSS, INC., USA). All the experiments in vitro were performed a minimum of three times. Data were expressed as the mean ± standard deviation (SD). The results were demonstrated by charts using GraphPad Prism version 7.0(GraphPad Software, Inc., USA). The data were compared using a one-way ANOVA followed by a Dunnett post hoc test, as appropriate; When *variance* between groups was not homogeneous and they did not show normal distribution, data were compared using non-parametric Kruskal-Wallis rank sum test. Differences was considered statistically significant when $P < 0.05$.

Result

Lidocaine inhibits the proliferation of MCF-7 and MDA-MB-231 breast cancer cells.

To evaluate the effects of lidocaine on breast cancer cells proliferation, MTT assay was used to detect the effect of lidocaine at concentrations of 0,1′2′4′8 and 16mM on MCF-7 and MDA-MB-231 Breast

cancer cells for 12,24 and 48 h, respectively. As shown in Fig. 1A, the inhibitory effect of lidocaine on the proliferation of MCF-7 and MDA-MB-231 cells was dose- and time-dependent. In order to choose the best lidocaine concentration for subsequent experiments, The *IC50* values were determined by nonlinear regression fitting using GraphPad *Prism.*The semi-inhibitory concentrations (IC50) of MCF-7 cells treated with lidocaine at 12, 24 and 48h were 5.647 ± 0.684, 4.192 ± 0.509 and 3.527 ± 0.437 mM, respectively. The semi-inhibitory concentrations (IC50) of MDA-MB-231 cells treated with lidocaine at 12, 24 and 48h were 8.512 ± 1.362, 5.894 ± 0.746 and 3.425 ± 0.459 mM, respectively. When MCF7 and MDA-MB-231 cells were treated with 6mM lidocaine for 12h, cell proliferation was significantly reduced. Therefore, we used 3 and 6 mM lidocaine to treat MCF7 and MDA-MB-231 cells for 12 h in our subsequent experiments. We further verified the inhibitory effect of lidocaine on the growth of MCF7 and MDA-MB-231 breast cancer cells by growth experiment. (Fig. 1B).

Lidocaine induces cell cycle arrest at the G2/M phase in breast cancer cells

We used flow cytometry following PI staining to measure the effects of lidocaine at various doses of 0, 3, and 6mM on the cell cycle distribution of MCF7 and MDA-MB-231 breast cancer cell for 12h, respectively. As shown in Fig. 2A, after treatment with 3 and 6mM lidocaine for 12 h, the proportion of MCF7 cells in the G2/M phase was 12.6% and 19.9%, respectively, which was significantly higher than that of the control group (10.6%). Similarly, following treatment with 3 and 6 mM lidocaine for 12 h, the proportion of MDA-MB-231 cells in the G2/M phase was 20.3% and 25.2%, respectively, which was significantly higher than that of the control group (17.7%). In addition, we analyzed the mRNA and protein expression levels of CDK1 and cyclinB1 by qRT-PCR and Western blot, respectively. The results showed that the mRNA and protein expression of CDK1 and cyclinB1 decreased in a concentration-dependent manner(Fig. 2B and 2C). Therefore, lidocaine induced cell cycle arrest of MCF7 and MDA-MB-231 breast cancer cells in G2/M phase in a concentration-dependent manner.

Lidocaine Promoted Apoptosis In Breast Cancer Cells

After annexin V-FITC and PI staining, flow cytometry was used to determine the effect of lidocaine on apoptosis of MCF7 and MDA-MB-231 cells. As shown in Fig. 3A and 3B, lidocaine treatment significantly induced apoptosis of MCF7 and MDA-MB-231 breast cancer cells in a concentration-dependent manner. We further confirmed these results by Western blot, characterized by the decrease of BCL2 and BCL-XL(Fig. 4A).

Lidocaine caused autophagy in MCF7 and MDA-MB-231 breast cancer cells

Because of the crosstalk between autophagy and apoptosis [21], we investigated whether lidocaine can induce autophagy in MCF7 and MDA-MB-231 cells. We examined autophagy-related proteins by Western blot. As shown in Fig. 4B, LC3B-II/LC3B-I increased and p62 decreased, indicating that lidocaine can increase autophagy in MCF7 and MDA-MB-231 cells.

Lidocaine Inhibits The Activation Of Mek/erk And Pi3k/akt Pathways

PI3K/Akt and MEK/ERK pathways are the key pathways to maintain the normal growth and differentiation of cancer cells. Therefore, we tested whether lidocaine can affect PI3K / Akt, MEK / ERK pathway in breast cancer cells. Results from western blotting showed that p-ERK and p-Akt decreased in a concentration-dependent manner(Fig. 4C), while the expression of ERK and Akt did not change significantly. These data indicate that lidocaine can inhibit the activation of MEK/ERK and PI3K/Akt pathways in breast cancer cells.

Discussion

A retrospective study of patients undergoing cancer surgery showed that the use of regional anaesthesia reduced the risk of tumour metastasis and recurrence [7,22,23]. Lidocaine is one of the most commonly used regional anaesthesia in clinic [8]. In addition to the most common anesthetic and analgesic effects, more and more attention has been paid to the research of lidocaine in anti-cancer. Lidocaine can inhibit the proliferation and metastasis of lung cancer cells by regulating GOLT1A or miR-539/EGFR axis [9,10]. It has been reported that lidocaine inhibited the proliferation and invasion of hepatocellular carcinoma by down regulating USP14 [24]. There are more and more studies on the effect of lidocaine on other cancers, including colorectal cancer, gastric cancer, melanoma, etc. Lidocaine has also been shown to induce apoptosis of breast tumor cells when its concentration is similar to that of clinical use [25]. However, the mechanism of lidocaine on breast cancer is rarely studied. We studied the effect of lidocaine on MCF7 and MDA-MB-231 breast cancer cells and found that lidocaine inhibited the proliferation of breast cancer cells in a concentration dependent and time-dependent manner. Flow cytometry showed that lidocaine promoted cell apoptosis in a concentration dependent manner. Lidocaine can reduce the mRNA and protein levels of CDK1 and cyclinB1 to inhibit cell cycle in G2/M phase. Meanwhile, lidocaine can promote autophagy and apoptosis by reducing BCL2, BCL-XL, p62 ,increasing Caspase-3, LC3B-II/LC3B-I and inhibiting MEK/ERK and PI3K/Akt pathways.

Ras/RAF/MEK/ERK signal cascade transmits upstream signals to downstream effector molecules and regulates physiological processes such as cell proliferation, differentiation, survival and death. The Raf / MEK / ERK cascade and RAF itself have different effects on the key molecules involved in preventing apoptosis. It has been reported that the Raf / MEK / ERK pathway can phosphorylate bad on S112, inactivate it and isolate it by 14-3-3 protein [26], which makes Bcl-2 form homodimer and produce anti apoptotic response. Therefore, lidocaine can promote apoptosis by inhibiting the activation of MEK/ERK pathway.

Autophagy and apoptosis are established processes of cell degradation, and they share many regulatory proteins [27]. Autophagy kills cells under certain conditions, this process is called autophagy death, which involves pathways and mediators different from apoptosis [28,29]. Therefore, autophagy may increase cell

death caused by apoptosis. Alternatively, it may induce cell death in a manner independent of apoptosis or necrosis. We found that both autophagy and apoptosis increased in a dose-dependent manner in MCF7 and MDA-MB-231 cells treated with lidocaine, indicating that autophagy and apoptosis are closely related, and the specific relationship and mechanism need to be further studied.

Conclusion

Our results implicate that lidocaine induces apoptosis, autophagy and G2/M phase cell cycle arrest via inhibiting the activation of MEK/ERK and PI3K/AKT pathways in human breast cancer cell. The anti-tumor effect of lidocaine provides a new opportunity for the treatment of breast cancer.

Declarations

Funding

This work was not supported by any funding.

Disclosure of interest

The authors report no conflict of interest.

Data Availability

The data used to support the founding of this study are available from the corresponding author upon request.

Author contributions

Ting Han designed the study and revised the manuscript. Xu Wang completed most of experiments and wrote the manuscript. Shi-Hang Xi, Qin Li made part of contributions in the experiment.

Ethics Approval and Consent to Participate

The proposed research project detailed above does not need Animal Researches Ethics Committee Approval.

Consent to Publish

All authors agree to publish article in this journal.

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Figures

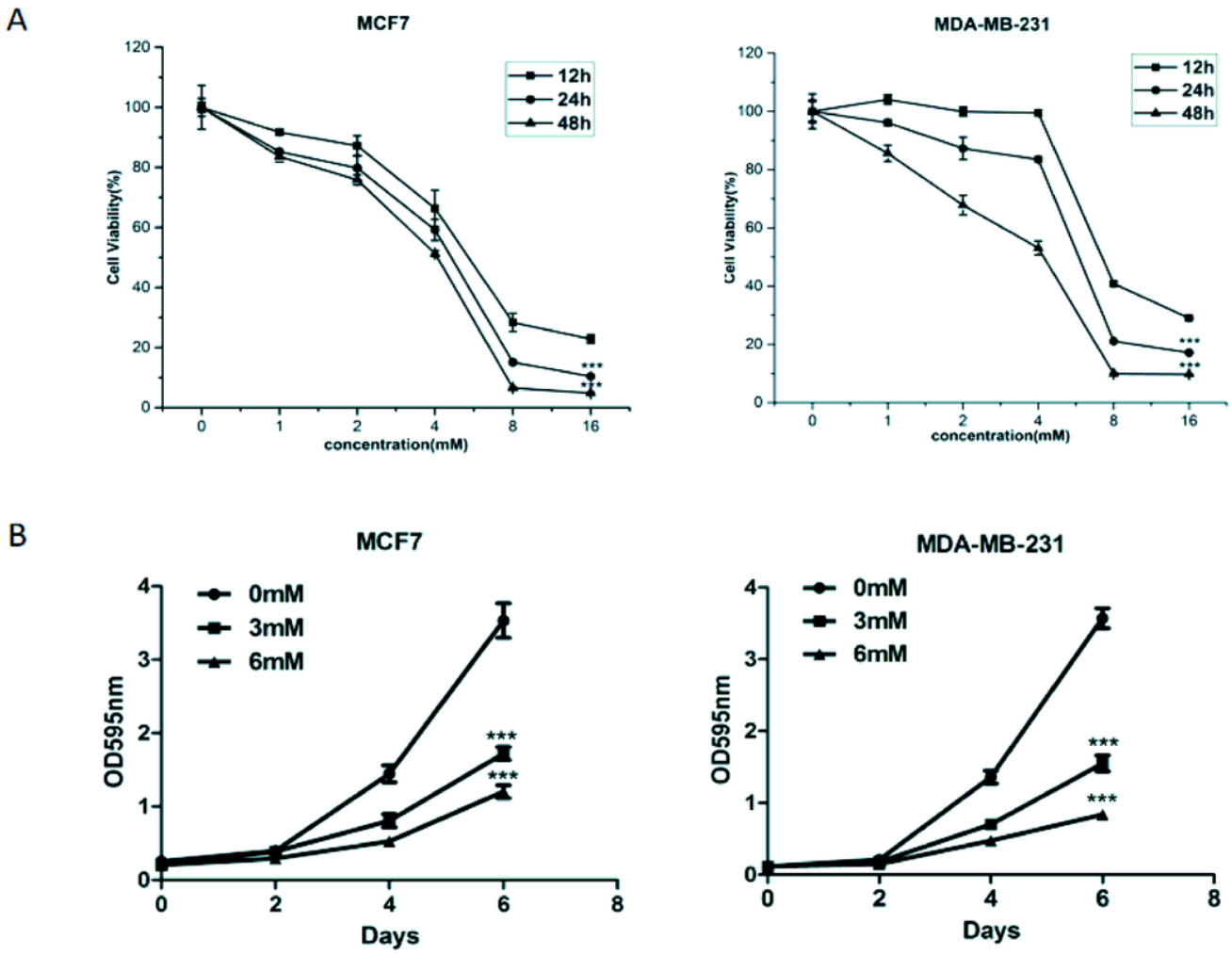

Figure 1

Lidocaine inhibits the proliferation of breast cancer cells. (A) Evaluation of the effect of various concentrations (0,1,2,4,8,16mM) of lidocaine on MCF-7(left) and MDA-MB-231(right) breast cancer cells at 12, 24 and 48 h by MTT assay. (Error bars are mean ± s.d, n=3 independent experiments, all P value were calculated by independent two-sample t-test, *** $p < 0.001$).(B) The effect of various concentrations (0,3,6mM) of lidocaine on MCF-7(left) and MDA-MB-231(right) breast cancer cells was detected by cell proliferation assay. (Error bars are mean ± s.d, n=3 independent experiments, all P value were calculated by independent two-sample t-test, *** $p < 0.001$).

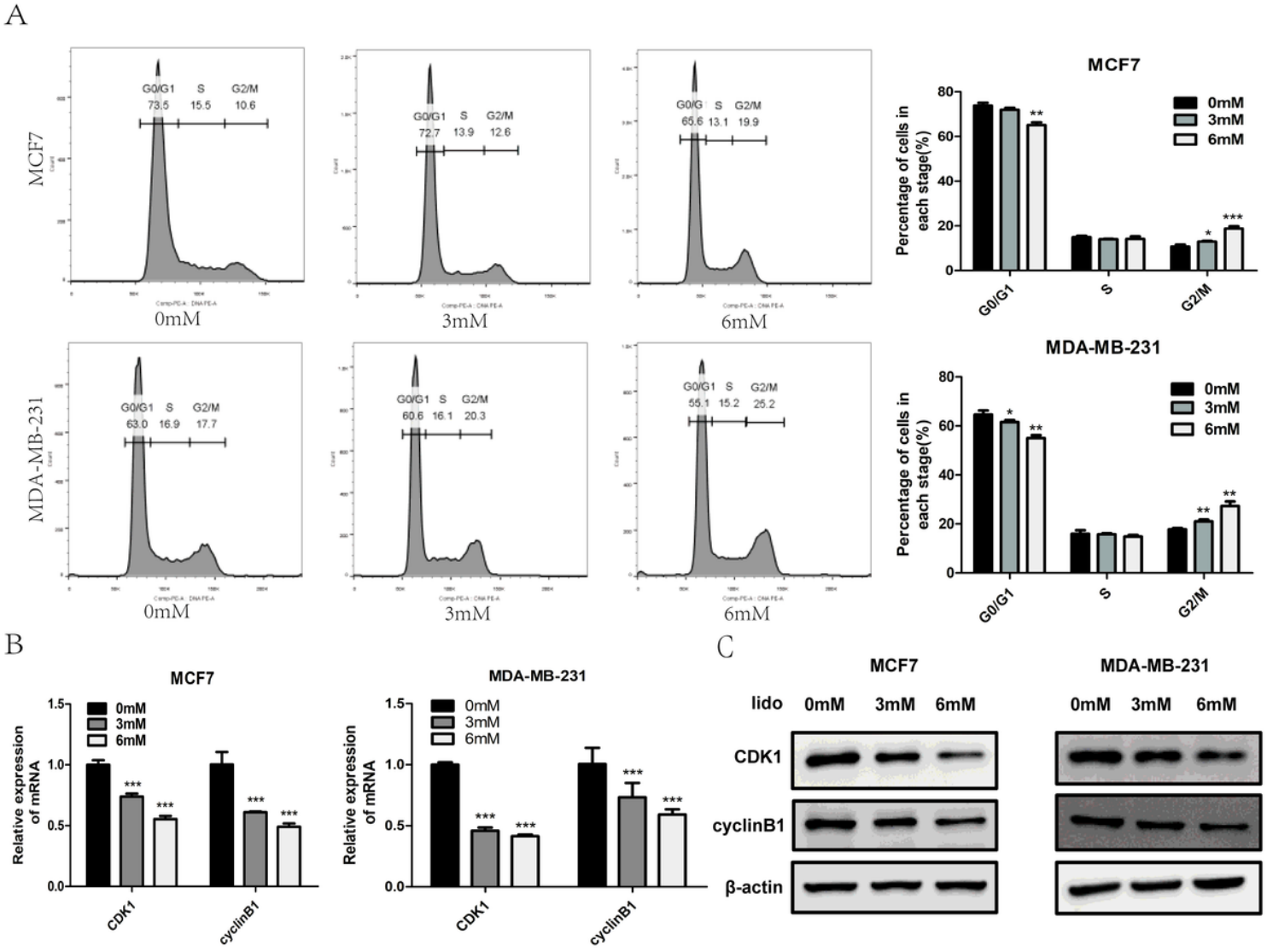

Figure 2

Lidocaine induces G2/M phase arrest in breast cancer cells. (A) The cell cycle distribution of breast cancer cells treated with various concentrations (0,3,6mM) of lidocaine was measured by flow cytometry. (Error bars are mean ± s.d, n=3 independent experiments, all P value were calculated by independent two-sample t-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) (B) The mRNA expression of CDK1 and cyclinD1 were measured by qRT-PCR. (Error bars are mean ± s.d, n=3 independent experiments, all P value were calculated by independent two-sample t-test, *** $p < 0.001$) (C) Western blot analysis of the related protein expression with indicated antibodies. All experiments were repeated 3 times independently, with similar results.

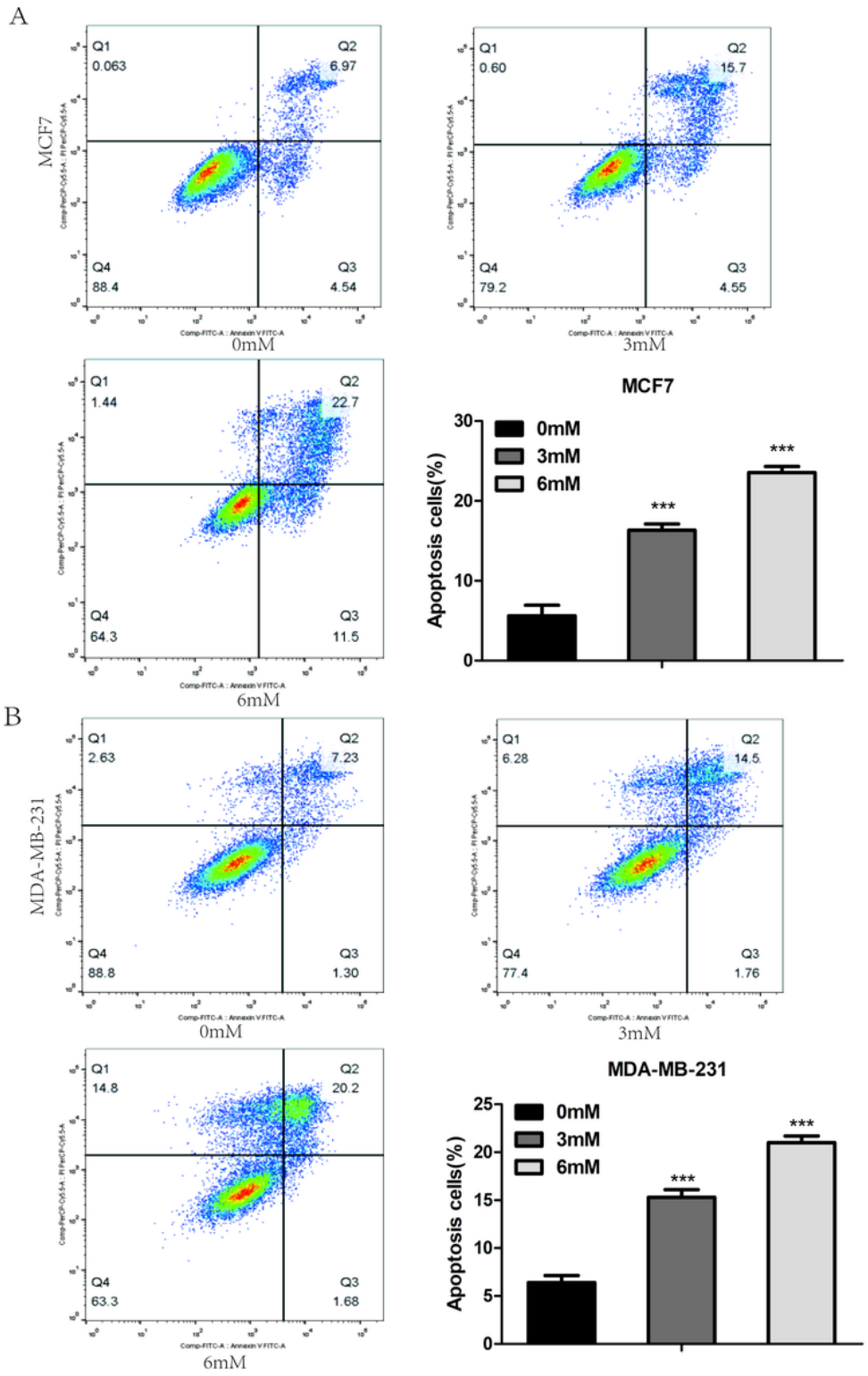

Figure 3

Lidocaine induced apoptosis in breast cancer cells. (A and B) MCF7 and MDA-MB-231 cells were treated with various concentrations (0,3,6mM) of lidocaine for 12 h (Error bars are mean ± s.d, n=3 independent experiments, all P value were calculated by independent two-sample t-test, *** $p < 0.001$).

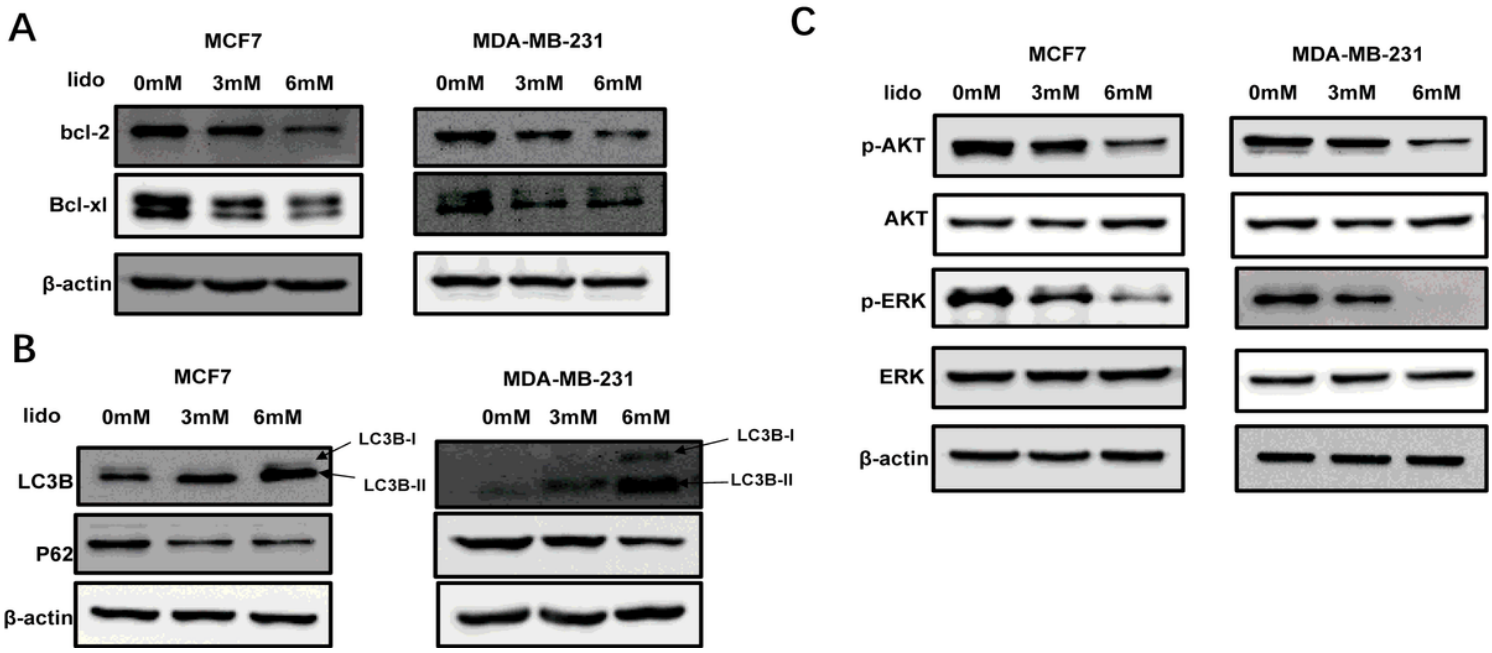

Figure 4

Cell lysates were prepared and analyzed by western blotting using indicated antibodies. (A) Apoptosis-related proteins were detected by Western blot. β-actin was used as a loading control. (B) Autophagy-associated proteins were detected by Western blot. β-actin was used as a loading control. (C) MEK/ERK and PI3K/AKT pathways were analyzed by Western blot. β-actin was used as a loading control.All experiments were repeated 3 times independently, with similar results.