Levobupivacaine inhibits proliferation and promotes apoptosis of breast cancer cells through PI3K/Akt/mTOR signalling pathway.

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Research note

Keywords: levobupivacaine, breast cancer cells, PI3K/Akt/mTOR signalling pathway

DOI: https://doi.org/10.21203/rs.3.rs-38113/v1

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Abstract

Objective This study aimed to test the hypothesis that levobupivacaine has anti-tumour effects on breast cancer cells.

Results Colony formation and transwell assay were used to determine breast cancer cells proliferation, whereas flow Cytometry (annexin V and PI staining) was used to investigate breast cancer cells apoptosis. The effects of levobupivacaine on cellular signalling and molecular response were studied with Quantitative Polymerase Chain Reaction (qPCR) and western blot. Induction of apoptosis was confirmed by cell viability, morphological changes showed cell shrinkage, rounding, and detachments from plates. The results of the western blot and qPCR indicated activation of Caspase-3 and inhibition of FOXO1. The results of the flow Cytometry confirmed that levobupivacaine inhibited breast cancer cell proliferation and enhanced apoptosis of breast cancer cells. Quantitative Polymerase Chain Reaction and Western blot analysis showed increased p21 and decreased cyclin D. However, the qPCR and western blot analysis showed that levobupivacaine significantly increased Bax expression, accompanied by a significant decreased Bcl expression and inhibition of PI3K/Akt/mTOR signalling pathway. These findings suggested that levobupivacaine inhibits proliferation and promotes breast cancer cells apoptosis in vitro.

Introduction

Breast cancer is one of the most recorded cancer illness among women [1, 2]. More than 40,000 patients die from breast related-cancer illness yearly despite the advances in chemotherapy and targeted treatments [3].

Molecular signalling pathways that are involved in breast malignant transformation have become evident as promising therapeutic targets [4]. Reports indicate that inhibiting this signalling pathway could inhibit cancer cells proliferation and also stimulate them toward cell death [5, 6, 7, 8, 9, 10].

Growing evidence of local anaesthetics inhibiting cancer cell growth seems promising yet limited [11]. At the tissue level, administration of a certain amount of local anaesthetics either topical or local has shown to have a direct inhibitory effect on the action of epidermal growth factor receptor (EGFR), which is a potential target for anti-proliferation in cancer cells [12, 13]. Evidence also shows that ropivacaine and lidocaine impede cancer cells growth, invasion, migration and increase apoptosis of lung cancer cells [14–18]. The effect of levobupivacaine on breast cancer cells is yet to be determined. The present study aimed to investigate the anti-tumour effects of levobupivacaine on breast cancer cells.

Materials And Methods

Ethics statement
The ethical committee of the Dalian Medical University First Affiliated Hospital approved for this study to be carried out.

**Cell culture**

We acquired MCF-7 and MDA-MB231 breast cancer cells from the ATCC (Beijing Zhongyuan limited, China). We regularly maintained the MCF-7 and MDA-MB-231 cells with high-glucose DMEM or DMEM/F12 (Gibco, USA) medium. Also, 10% fetal bovine serum (FBS) (Gibco, USA), penicillin 100 units/ml and streptomycin 100 µg/ml (TransGen Biotech, China) were used to supplement the medium to maintain the cells. The MCF-7 and MDA-MB231 cells were kept in an incubator at 37 °C humidified air with 5% CO2 atmospheric condition. The cells were repeatedly subcultured afterwards.

**Antibodies and reagents**

#AP0304 Phospho-Akt1-T308Pab Antibody (ABclonal Technology), #A2845 Bcl2 Polyclonal Antibody (ABclonal Technology), #A11550 BAX Polyclonal Antibody(ABclonal Technology), #A0265 PIK3CA Polyclonal Antibody (ABclonal Technology), #A2934 FOXO1 Polyclonal Antibody (ABclonal Technology), #10176-2-AP Akt Rabbit Polyclonal antibody (Proteintech), Peroxidase-conjugated goat anti-rabbit IgG (Proteintech, China); PRAP antibodies (Proteintech, China), GAPDH antibodies (Proteintech, China) and #A11354 mTOR Polyclonal Antibody

**Cell viability assay and IC50**

Through CCK-8 assay, we determined the MCF-7 and MDA-MB 231 cells viability. Levobupivacaine at a concentration of 0, 1, 2 and 3 mM was used to treat MCF-7 and MDA-MB 231 cells which were plated in 96-well plates (1 × 10⁴ cells/well) and incubated for 12, 24, or 48 h respectively in an incubator at the atmospheric condition of 37 °C, with 5% CO₂. Procedure for the CCK-8 assay was the same as described elsewhere [19].

**Flow cytometry**

Through Annexin V and propidium iodide (PI) staining assay, the apoptosis of MCF-7 and MDA-MB 231 cells was determined following the levobupivacaine treatment. After treating the cells for 24 h, 0.25% trypsin was used to harvest the treated cells and centrifugation at 1400 rcf for 10 min. The MCF-7 and MDA-MB 231 treated cells were again suspended with 1 × Binding Buffer, and then 5 µl of fluorochrome-conjugated annexin V (Sigma-Aldrich, Saint Louis, USA) was added into 100 µl of the cell suspension to stain intracellular phosphatidylserine (PS). In a dark, we carried out the cell’s incubation under room temperature. The cells were again suspended and we then added 5 µl propidium iodide staining solution (Sigma-Aldrich, Saint Louis, USA) into 100 µl of cell suspension. We detected the percentage of the apoptotic cells via FlowJo software (Treestar, Ashland, USA) through Flow cytometry (FACS Calibur, Becton Dickinson, and Sunnyvale, CA, USA).

**Quantitative polymerase chain reaction (qPCR)**
The procedure used for the qPCR was the same as previously described [19]. The primers sequences were: BAX: 5′-TGGCAGCTGACATGTTTTCTG-3′ (F), 5′-TCCGGAGGAAGTCCAATG-3′ (R). BCL2: 5′-ACGTTGGAGGAGCTCTTT-3′ (F), 5′-GCCGGTTTACAGCCTCAGTCATC-3′ (R). p21: 5′-GCGACTGTGATGCGCTAATG-3′ (F), 5′-GAAGGTAGAGCTTGGGCAGG-3′ (R). GAPDH: 5′-CATGTTCTCATGGGTGTGAA-3′ (F), 5′-GGCATGGACTGTGGGTGACTG-3′ (R).

**Western blot**

At the log phase of treated MCF-7 and MDA-MB 231 cells growth, we harvested the cells and then washed them twice with ice-cold PBS. The procedure used for the western blot was the same as described elsewhere [19].

**Colony formation assay**

The procedure used for the colony formation assay was the same as previously described [19].

**Transwell assay**

The MCF-7 and MDA-MBA-231 cells (5 × 10^4) that were pre-treated with different dose of Levobupivacaine (0, 1, 2 mM) for 24 h and resuspended in culture medium with the same concentrations of levobupivacaine were seeded onto the coated membrane in the upper chamber of the transwell (24-well millicell cell culture insert, 12 mm diameter, 8 µm pores; Merck KGaA, #P18P01250, China). The procedure used for the Transwell assay was the same as previously describe [19].

**Data analysis**

Values were expressed as the mean ± SD. Statistical analysis was performed with GraphPad Prism version 5.01(GraphPad Software, La Jolla, CA, US). One-way ANOVA was used to measure significance (p < 0.05). Dunnett’s post hoc tests were used to test the difference between groups.

**Results**

**Levobupivacaine inhibits breast cancer cell viability**

The MCF-7 and MDA-MBA-231 cell viability decreased as the concentration of levobupivacaine (0, 1, 2 and 3 mM) increased. For the MDA-MB-231 cells, more than 40% resulted in a cytotoxic effect, whereas the MCF-7 cells showed a similar responded cytotoxic effect of about 50% (Fig. 1A). Under a fluorescence microscope, cells treated with Levobupivacaine showed morphological changes after 24 h exposure. The features of the treated breast cancer cells showed morphological changes including cell rounding, cell shrinkage, and almost detachment from the plates (Fig. 1B). The change of cell viability was found to be significant as the viability of breast cancer cells decreased with an increase in dosage of levobupivacaine treatment for 24 h.

**Levobupivacaine inhibits proliferation in breast cancer cells**
Transwell assay indicated a significant decreased in the invasion ability of MCF-7 and MDA-MB-231 cells as the concentration of the levobupivacaine increased compared with the control (Fig. 2A). The results showed a significant decreased in the number of clones of the treated cells compared with the control (Fig. 2B). The data showed that the mRNA level of p21 significantly increased following levobupivacaine treatment (Fig. 2C). Western blot analysis showed a similar increased in p21 and decreased in FOXO1 and cyclin D1 expressions (Fig. 2D, E).

**Levobupivacaine promote apoptosis in breast cancer cells**

Levobupivacaine significantly reduced the number of cells showing nuclear staining compared with untreated cells (Fig. 3A). The qPCR data showed a decreased in BCL and an increased in Bax expressions in MCF-7 and MDA-MB-231 cells compared with the control (Fig. 3B, C). Consistently, Western blot analysis showed a similar decreased in BCL and increased expressions of Caspase 3 and Bax as the concentration of the levobupivacaine increased compared with control (Fig. 3D, E).

**Levobupivacaine inhibits proliferation and promotes apoptosis in breast cancer through PI3K/Akt/mTOR signalling pathway**

The results showed a significant decreased in the expression of the nuclear localization of p-PI3K, p-Akt, and p-MTOR compared with the control using western blot (Supplementary Fig. 1A, B).

**Discussion**

Several retrospective studies have demonstrated that regional anaesthesia is associated with a decreased risk of recurrence or metastasis of multiple carcinomas, including breast, prostate and cervical cancers [20–22]. Recent growing evidence demonstrates that local anaesthetics have an antitumour effect and may suppress the motility of cellular function and invasiveness more likely via voltage-gated sodium channel inhibition. The cellular modification of treated cells is likely dependent on the duration of exposure and the dose of local anaesthetic [23–37]. In this study, we used MCF-7 and MDA-MB-231 cells as models and found that levobupivacaine could effectively inhibit breast cancer cell proliferation and promotes apoptosis in vitro. The anti-proliferation and apoptosis effects observed in this study suggested that levobupivacaine may have the potential therapeutic effects against breast cancer.

PI3K/Akt/mTOR signalling pathway may play a vital role in cell proliferation, survival, development, metabolism, motility and also, regulation of the immune response. Breast cancer cell resistance to therapies can result from the activation of PI3K/Akt/mTOR signalling pathway [38–41]. Hence, making it an important object of study for comprehending the development and progression of breast cancer. Consequently, in patients with breast cancer, PI3K/Akt/mTOR signalling pathway can be a target for diagnostic, treatment, and also for a prognostic purpose [2, 42–47]. In this study, the role of levobupivacaine on the expression of PI3K, Akt, and mTOR was investigated to illustrate the potential
molecular mechanism. We observed a significantly decreased expression of p-Akt, p-PI3K, p-mTOR and subsequent decreased expression of FOXO, Cyclin D1 and Bcl-2 following levobupivacaine treatment which correlated with decreased breast cancer cells proliferation and increased apoptosis. The findings of this study suggested that levobupivacaine inhibits proliferation and promotes apoptosis through the PI3K/Akt/mTOR signalling pathway which demonstrated possible antitumour effects of levobupivacaine on breast cancer.

Conclusion

levobupivacaine has the potency of reducing breast cancer cell viability, proliferation and causes cell death through the PI3K/Akt/mTOR signalling pathway. These pieces of evidence could lead to clinical studies which will seek to examine the anti-cancer effects of levobupivacaine which may increase the benefits in cancer patient as well as improve patient care.

Limitations

Numerous studies have reported on the antitumour effects of local anaesthetics on various cancer cells [48-50]. However, our work is not without limitations. Further, in vivo and clinical studies on the anti-tumour effects of levobupivacaine are needed.

List Of Abbreviations

qPCR - Quantitative Polymerase Chain Reaction
EGFR- epidermal growth factor receptor
PI - Propidium iodide
PS – Phosphatidylserine
NC - nitrocellulose

Declarations

Ethics approval and consent to participate: The ethical committee of the First Affiliated Hospital of Dalian Medical University approved the study protocol, and because this study used breast cancer cells, consent to participate was not applicable for the study.

Consent to publish: Not applicable

Availability of data and materials: The data used and/or analysed in this study are available from the corresponding author upon reasonable request.
Competing interests: Authors declare that they have no competing interests

Funding: This study was supported by the Key Laboratory of Liaoning Provincial Education Department (Grant NO: LZ2016002) and Liaoning Natural Science Foundation (Grant NO: 20170540290).

Authors’ contributions: AKK, SK, QY and QPW conceived and designed the study. QPW and QY were responsible for the supervision and coordination of this study. AKK, SK, JL, MNR, QY, and QPW conducted the data collection. SK led the data analysis with inputs from AKK, QY, and QPW. AKK wrote the first draft of the manuscript, and then JL, MNR, SAR, AAF, JA, and EAN contributed to revising and reviewing the manuscript. All authors read and approved the final manuscript before submission.

Acknowledgements: We thank the First Affiliated Hospital and The Department of Biochemistry of Dalian Medical University for making available all the necessary materials needed for this work. We also thank the Key Laboratory of Liaoning Provincial Education Department (Grant NO: LZ2016002) and Liaoning Natural Science Foundation (Grant NO: 20170540290) of China for supporting this work. Our thanks also go to the China Scholarship Council and the Government of the Republic of Ghana for giving financial aid to some of the authors to study at Dalian Medical University.

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Signal Transduction Pathways in Breast Cancer: The Important Role of PI3K/Akt/mTOR. *Journal of Oncology* 2020.

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**Figures**
Figure 1

Effects of levobupivacaine on breast cancer cells. (A) Cell viability was measured by CCK-8 assay. IC50 results of levobupivacaine on MCF-7 and MDA-MB 231 cells. The breast cancer cells were treated with different concentrations of levobupivacaine for 24 h. (B) Effect of levobupivacaine on the morphology of MCF-7 and MDA-MB 231 cells. Treatment with levobupivacaine induced breast cancer cells morphological change after 24 h exposure. The data was statistically significant and indicates P < 0.05; P <0.01; and P < 0.001 compared with control. The data represent the mean ± SD of three independent experiments.
Figure 2

Levobupivacaine treatment inhibited proliferation in breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with different concentrations of levobupivacaine. (A) The invasion potentials of the cells were examined by invasion assay (Transwell assay). Levobupivacaine inhibits cell invasion in MCF-7 and MDA-MB-231 cells. (B) Colony formation of MCF-7 and MDA-MB 231 cells treated with various concentrations of Levobupivacaine with staining by crystal violet. (C) The mRNA expression levels of p21, Caspase 3 and GAPDH were analysed by qPCR. (D, E) Protein expression assessment of MCF-7 and MDA-MB-231 cells by western blot against antibodies FOXO1, p21, Cyclin D1 were used and GAPDH as control. The data was statistically significant at * indicates P< 0.05; ** indicates P< 0.01; *** indicates P< 0.001 compared with control. This data corresponds to the mean ± SEM of three independent experiments.
Figure 3

Effects of levobupivacaine on cell cycle and apoptosis in breast cancer cells. (A) MCF-7 and MDA-MB 231 cells were treated with concentrations of levobupivacaine for 24 h. The cells were later on stained with fluorescein-conjugated annexin V and PI and analysed by flow cytometry. Error bars represent standard error of the mean. P< 0.05 versus the control. (B, C) Relative gene expression of Bax and BCL\textsuperscript{2} following levobupivacaine treatment of breast cancer cells with different concentrations for 24 h were analysed by qPCR. (D, E) MCF-7 and MDA-MB 231 cells were treated with levobupivacaine for 24 h and the activities of Bax, BCL\textsuperscript{2}, and Caspase 3 were examined by Western blot analysis using specific antibodies. GAPDH was used as internal controls. The data was statistically significant at * indicates P< 0.05; ** indicates P< 0.01 compared with control. The data correspond to the mean ± SEM of three independent experiments.
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