

Protective role of LRRC3B in preventing breast cancer metastasis and recurrence post-bupivacaine

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Abstract. The present study aimed to investigate the potential effect of leucine-rich repeat containing 3B (LRRC3B) with respect to the inhibition of breast cancer recurrence and metastasis post-anesthesia. The mRNA expression of LRRC3B in breast MDA-MB-231 and MCF-7 cell lines was detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The effect of bupivacaine on breast cancer cell invasion was analyzed using a Matrigel assay. LRRC3B specific small interfering (si)RNA was constructed and transfected into breast cancer cells using Lipofectamine[®] 2000 reagent. The influence of bupivacaine on LRRC3B expression was measured based on RT-qPCR. Additionally, the effect of LRRC3B silencing on the invasion of breast cancer cells treated with bupivacaine was analyzed. Compared with the control, LRRC3B expression significantly increased in MDA-MB-231 and in MCF-7 cells as the length of time increased ($P < 0.05$), but the expression of the gene significantly declined in 2 types of cancer cell when the cells were transfected with siRNA-LRRC3B plasma ($P < 0.05$). The administration of 50 $\mu\text{g/ml}$ bupivacaine promoted maximum breast cancer cell invasion, and suppressed LRRC3B mRNA expression in cells. However, when LRRC3B was silenced in cancer cells, 20 $\mu\text{g/ml}$ bupivacaine significantly promoted cancer cell invasion, indicating that bupivacaine suppresses the expression of LRRC3B and promotes cell invasion. The present study suggested that LRRC3B serves a protective role in preventing bupivacaine-induced breast cancer recurrence and metastasis.

Introduction

Breast cancer is one of the most common types of cancer among female malignancies, and has previously been

associated with high mortality worldwide (1). Primary breast cancer often occurs in the twinned mammary gland, which accounts for 2-11% of female breast tumors (2). Statistical data reveals that numerous types of factor function in the recurrence and metastasis of primary breast cancer subsequent to surgery (3). Primary breast tumor cells may metastasize to a number of positions in the human body such as the lung, bone and lymph node, a process involving a number of biological processes including tumor cell proliferation, migration and apoptosis (4,5). Surgery may be the best choice for patients with primary cancer, which results in the necessary administration of anesthesia. However, a possible association between anesthetic techniques and primary breast cancer has been reported (6).

Bupivacaine is a type of surgical local anesthetic, and studies have demonstrated that the excessive administration of bupivacaine results in central nervous system and cardiovascular system poisoning (7,8). Bupivacaine has been widely used in clinical surgeries such as tonsillectomy, neurolept-analgesia and tumor resections. A low dose of bupivacaine yielded satisfactory postoperative effects on female ovarian cancer resection, with little influence on hemodynamics and postoperative adverse reactions (9,10). Previous studies have demonstrated that anesthesia during primary breast cancer surgery may influence recurrence or metastasis (11). The administration of bupivacaine has been examined in breast cancer resection surgery. For example, the addition of 1 $\mu\text{g/kg}$ dexmedetomidine to 0.25% bupivacaine in paravertebral analgesia for patients undergoing modified radical mastectomy surgery, for the treatment of breast cancer, yielded no serious side effects (12). However, the mechanism of several types of anesthesia, including bupivacaine, on breast cancer recurrence and metastasis remain unclear.

The abnormal expression of leucine rich repeat containing 3B (LRRC3B) has been reported in numerous types of cancer such as breast, colon and ovarian (13,14). An increasing number of studies have mentioned the pivotal role of LRRC3B in tumor development and progression. For instance, Kim *et al* (15) reported that LRRC3B was a tumor suppressor for gastric cancer. Furthermore, LRRC3B was downregulated in a renal cell carcinoma line, which suggests that LRRC3B could be involved in the process of carcinogenesis as a tumor suppressor (13,16). Additionally, abnormal expression of LRRC3B may serve as a useful marker for diagnosis and prognosis in breast carcinoma (17). Despite numerous studies

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being focused on the effect of LRRC3B expression in tumor pathogenesis, the potential role of LRRC3B on breast cancer cell invasion post-anesthesia has not been studied.

The present study used 2 types of breast cancer cell lines to assess the effect and potential mechanism of LRRC3B silencing on breast cancer cell invasion post-anesthesia. A number of experimental methods were used to assess the effect of LRRC3B silencing and bupivacaine on breast cancer cell invasion. The present study aimed to investigate the potential role of LRRC3B on breast cancer cell invasion post-anesthesia and may provide a theoretical basis for the mechanism of LRRC3B in breast cancer cell invasion following the administration of anesthetic.

Materials and methods

Cell culture and drug treatment. Human breast cancer MDA-MB-231 and MCF-7 cell lines (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA) at 37°C with 5% CO₂.

Bupivacaine (Sigma-Aldrich; Merck KGaA) was diluted in dimethyl sulfoxide (DMSO) to 10 mg/ml and stored at room temperature with no exposure to light. Consequently, bupivacaine was diluted to concentrations of 1, 10, 20, 50 and 100 µg/ml for breast cancer cell treatment. The cells treated with DMSO only were considered as the control group.

Small interfering RNA (siRNA) transfection. The target sequence for LRRC3B specific siRNA was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). siRNA with no silencing sequence was transfected as control group. Cell transfection was conducted with Lipofectamine[®] 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturers protocol. G418 (Sigma-Aldrich; Merck KGaA) was used for the stable LRRC3B transfectants selection (18).

Cellular invasion assay. For the cellular invasion ability assay, the Matrigel method was used as previously described (19). The cells in each group previously cultured for 48 h were incubated in serum-free DMEM medium containing 10% FBS for 24 h at 37°C. The upper Transwell chamber (Corning Incorporated, Corning, NY, USA) was covered with serum-free DMEM supplemented with 50 mg/l Matrigel and air-dried at 4°C. Following the removal of the medium, 50 µl fresh serum-free medium containing 10 g/l bovine serum albumin (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) was added to the upper chamber, and the cells were cultured for 30 min at 37°C. Transwell inserts were subsequently added to the 24-well plates and the plates were cultured with DMEM mixed with 10% FBS. MDA-MB-231 and MCF-7 cells (5x10⁴ cells/well) in the Transwell inserts were then suspended in serum-free DMEM. Subsequent to 48 h, the Transwell inserts in each group were washed with PBS buffer to remove the upper cells on the microporous membrane, subsequent to fixing in ice-cold alcohol. Finally,

the Transwell inserts from each group were stained with 0.1% crystal violet for 30 min, and washed with 33% acetic acid. The absorbance of the eluents was observed at a wavelength of 570 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The Transwell chambers in the control group were treated without Matrigel.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from the breast cancer cells and cultured for 48 h at 4°C using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (20) and was treated with RNase-free Dnase I (Promega Corporation, Madison, WI, USA). The concentration and purity of the isolated RNA was measured with SMA 400 UV0VIS (Merinton Instrument, Ltd., Shanghai, China). Purified RNA at a density of 0.5 µg/µl with nuclease-free water was used for cDNA synthesis with the PrimerScript 1st Strand cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.). The expression level of LRRC3B in bupivacaine pre-treated MDA-MB-231 and MCF-7 cells were detected in an Eppendorf Mastercycler (Brinkmann Instruments Inc., Westbury, NY, USA) using the SYBR ExScript RT-qPCR kit (Takara Biotechnology Co., Ltd., Dalian, China). LRRC3B expression in cells without bupivacaine treatment served as negative controls. The experiment was repeated three times. The total reaction system of 20 µl volume was as follows: 1 µl cDNA, 10 µl SYBR Premix EX Taq, 1 µl each of the primers (10 µM), and 7 µl double-distilled H₂O. The PCR program was performed using Taq DNA polymerase (Roche Diagnostics, Basel, Switzerland) as follows: Denaturation at 50°C for 2 min; 95°C for 10 min; 45 cycles at 95°C for 10 sec; 60°C for 1 min. Melting curve analysis of the amplification products was performed at the end of each PCR to confirm that only 1 product was amplified and detected. Data were analyzed according to the 2^{-ΔΔC_q} method (21) and were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in each sample. Primers used for targets amplification were as follows: LRRC3B sense, 5'-GAGGTATTG GTTGGGTTTTG-3', and anti-sense, 5'-AACCCAACCTACC CACCTAA-3'; and GAPDH sense, 5'-TATGATGATATC AAGAGGGTAGT-3', and anti-sense, 5'-TGTATCCAACT CATTGTCATAC-3'.

Western blot analysis. The two types of breast cancer cells were lysed by culturing for 48 h at 4°C in lysis buffer (Sigma-Aldrich; Merck KGaA). The cells were then centrifuged at 300 x g for 10 min at 4°C. The supernatant was collected to determine the protein concentration using a bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.). For western blotting (22), 30 µg cell lysate protein was separated by 10% SDS-PAGE, subsequently the gels were transferred onto a polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked in TBS and Tween-20 (TBST) containing 5% non-fat milk for 1 h. The membrane was then incubated with anti-LRRC3B antibody (catalog no. HPA015568) and anti-GAPDH antibody (1:100; catalog no. SAB2103104; Sigma-Aldrich; Merck KGaA), overnight at 4°C. The membrane was incubated with horseradish peroxidase labeled anti-rabbit secondary antibody (1:1,000; catalog no. 7054; Cell Signaling Technology,

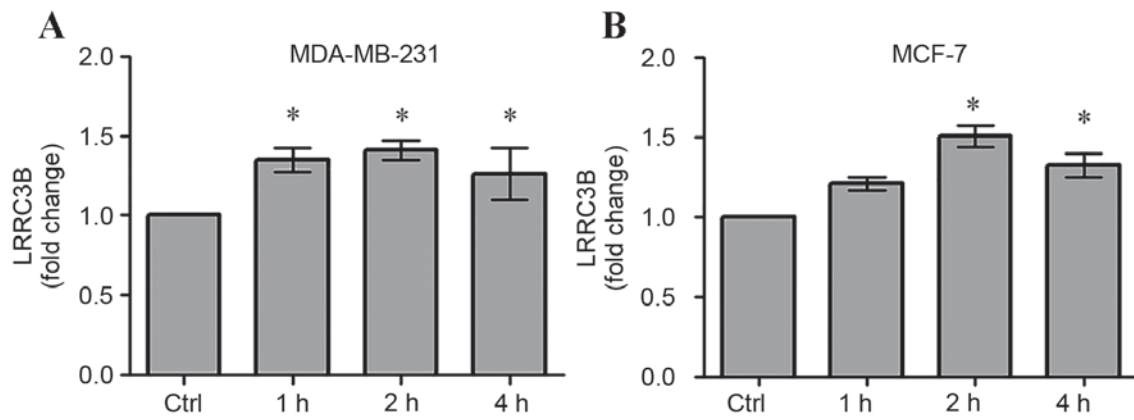


Figure 1. Expression of LRRC3B in breast cancer MDA-MB-231 and MCF-7 cell lines. (A) LRRC3B expression was significantly increased with time compared with the control group in MDA-MB-231 cells. (B) LRRC3B expression was significantly increased with time compared with the control group in MCF-7 cells. *P<0.05 compared with the control group. LRRC3B, leucine rich repeat containing 3B.

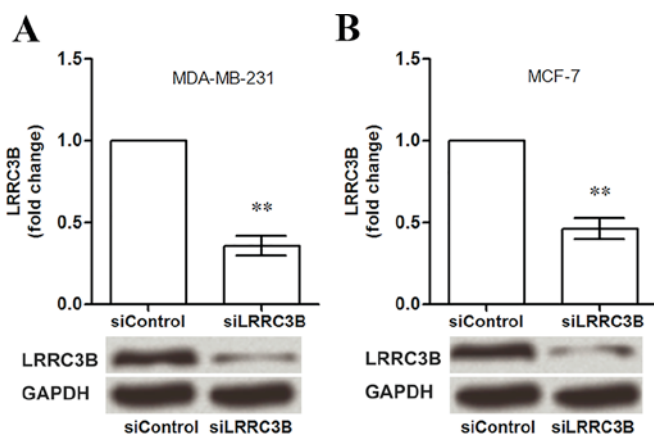


Figure 2. Expression of LRRC3B in breast cancer cells that transfected with siRNA-LRRC3B. (A) mRNA and protein expression of LRRC3B significantly decreased subsequent to the transfection of siRNA-LRRC3B in MDA-MB-231 cells. (B) Levels of mRNA and protein expression of LRRC3B significantly decreased subsequent to the transfection of siLRRC3B in MCF-7 cells. **P<0.01 compared with the control group. LRRC3B, leucine rich repeat containing 3B; siRNA, small interfering RNA.

Danvers, MA, USA) at room temperature for 1 h. The PVDF membrane was subsequently washed with 1X TBST buffer for 10 min 3 times. Detection was conducted with X-rays using the enhanced chemiluminescence (ECL) reagent (GE Healthcare, Chalfont, UK). GAPDH served as the internal control.

Statistical analysis. All experiments were conducted independently in triplicate. All experimental data is presented as the mean ± standard deviation and was performed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). A post hoc Tukey's test was used to calculate the differences between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of LRRC3B in breast cancer cell lines. The expression of LRRC3B in each type of breast cancer cell line was analyzed using RT-qPCR (Fig. 1). The results showed that the fold change of LRRC3B significantly increased as

time increased, up to 2 h, compared with the control in the MDA-MB-231 cell line, declining slightly at 4 h (P<0.05, Fig. 1A). The same tendency with respect to LRRC3B expression was exhibited in the MCF-7 cell line (P<0.05, Fig. 1B). The aforementioned results suggested that LRRC3B was overexpressed in the breast cancer cell lines.

In addition, when the breast cancer MDA-MB-231 and MCF-7 cell lines were transfected with siRNA-LRRC3B plasma, the expression of LRRC3B was detected with western blotting (Fig. 2). The results suggested that LRRC3B was downregulated in the MDA-MB-231 and MCF-7 cell lines.

Influence of siLRRC3B on protein expression in breast cancer cells. RT-qPCR analysis showed that when the breast cancer cells were transfected with a siLRRC3B vector, LRRC3B mRNA expression in MDA-MB-231 and MCF-7 cells significantly decreased compared with the controls (P<0.01; Fig. 2). A similar pattern of LRRC3B expression was observed in the western blotting, suggesting that LRRC3B silencing exerted an effect on LRRC3B expression in breast cancer cells.

Effect of bupivacaine on breast cancer cell invasion. Transwell assay was used to assess the effect of bupivacaine on breast cancer cell invasion (Fig. 3). Compared with the control DMSO group, the percentage migrated MCF-7 cells increased as bupivacaine concentration increased until 50 µg/ml and declined when bupivacaine concentration exceeded 50 µg/ml (Fig. 3A). However, the effect of bupivacaine at different concentrations on MDA-MB-231 cells was not the same as the effect in MCF-7 cells, with the results revealing that the concentration of bupivacaine that yielded the largest percentage migrated cells was 50 µg/ml (Fig. 3B). This data suggested that 50 µg/ml bupivacaine promoted breast cancer cell invasion in the 2 cell types.

LRRC3B mRNA expression in breast cancer cells exposed to bupivacaine. The mRNA expression of LRRC3B in breast cancer cells post-anesthesia was measured using RT-qPCR analysis (Fig. 4). No significant difference was indicated for LRRC3B expression in MCF-7 or MDA-MB-231 cells at a variety of bupivacaine concentrations. However, the LRRC3B

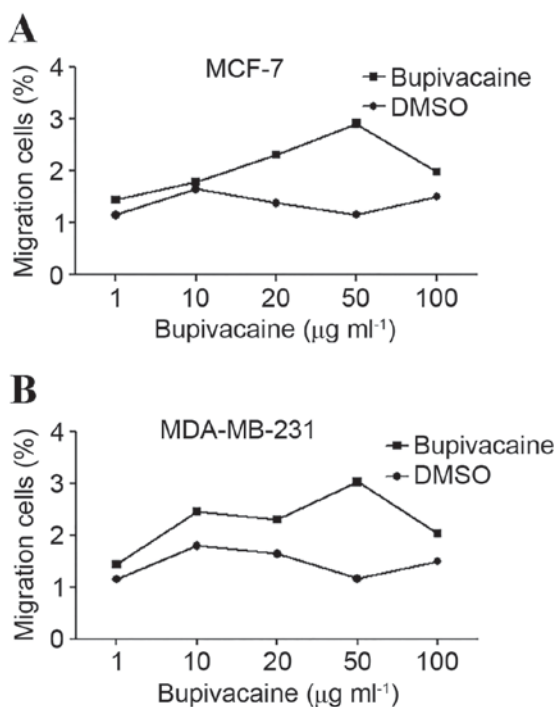


Figure 3. Effect of bupivacaine on breast cancer cell invasion. (A) The percentage invasion of MCF-7 cells increased as the concentration of bupivacaine increased. (B) The percentage invasion of MDA-MB-231 cells increased as the concentration of bupivacaine increased. DMSO, dimethyl sulfoxide.

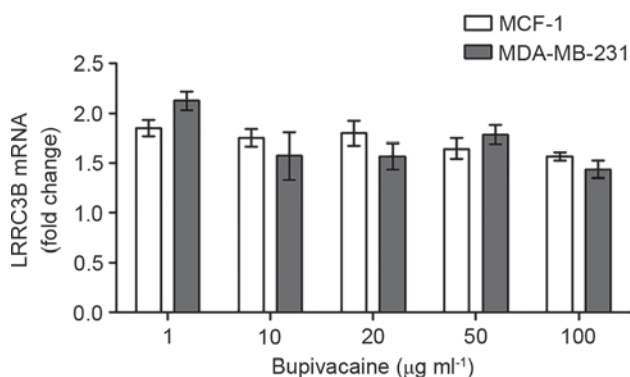


Figure 4. Bupivacaine suppressed LRRC3B mRNA expression in 2 types of breast cancer. LRRC3B, leucine rich repeat containing 3B.

mRNA expression reduced slightly at a high concentration (100 µg/ml) of bupivacaine.

Effects of LRRC3B silencing on breast cancer cells exposed to bupivacaine. When the breast cancer cells were transfected with siLRRC3B plasma, the percentage migrated cells was highest at 20 µg/ml bupivacaine in MDA-MB-231 and MCF-7 cells (Fig. 5), indicating that LRRC3B silencing suppresses bupivacaine-induced breast cancer cell invasion.

Discussion

Previous studies have reported that the administration of anesthetics may serve pivotal roles in cancer recurrence and metastasis during primary breast cancer surgery (6,23).

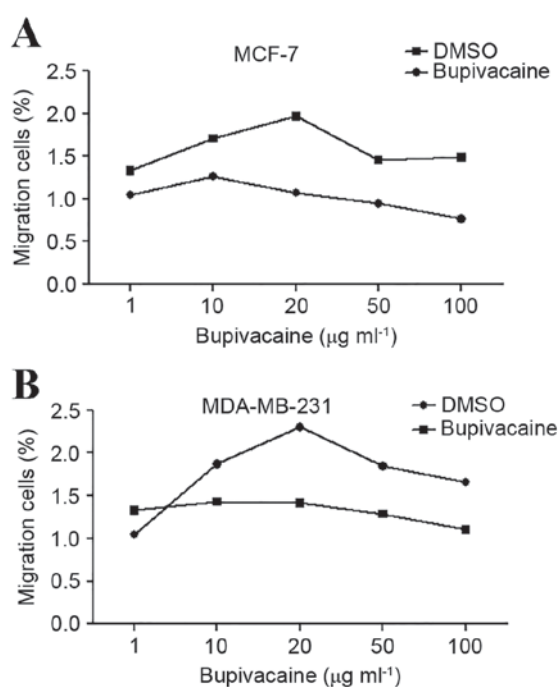


Figure 5. Influence of bupivacaine on breast cancer cell invasion was enhanced by siRNA-LRRC3B transfection compared with the control. (A) The percentage migrated MDA-MB-231 cells increased as bupivacaine concentration increased up to 20 µg/ml. (B) The percentage migrated MCF-7 cells increased as bupivacaine concentration increased up to 20 µg/ml compared with control. DMSO, dimethyl sulfoxide; LRRC3B, leucine rich repeat containing 3B; siRNA, small interfering RNA.

LRRC3B has been revealed to perform an important role in numerous types of cancer, however, there were previously no studies about the function of LRRC3B in breast cancer recurrence and metastasis post-anesthesia. The present study assessed the potential function of LRRC3B in breast cancer cell invasion subsequent to being treated with the anesthetic bupivacaine. The data showed that LRRC3B expression was inhibited by bupivacaine application. However, when LRRC3B was silenced, the effect of bupivacaine on breast cancer cell invasion was reversed, indicating the role of LRRC3B in the suppression of bupivacaine-induced breast cancer cell invasion.

The present study showed that LRRC3B expression increased significantly in breast cancer MDA-MB-231 and MCF-7 cell lines, but decreased significantly when the cells were transfected with siRNA-LRRC3B ($P < 0.05$). LRRC3B has been reported to be a tumor suppressor for gastric cancer (24), and Tian *et al* (25) revealed the tumor suppressor role of LRRC3B in colorectal cancer using a gene silencing method. The role of LRRC3B in breast cancer has not been fully discussed, even in primary breast cancer surgery. Based on the results of the present study, LRRC3B overexpression was speculated to be associated with breast cancer. However, the results showed that the application of 50 µg/ml bupivacaine yielded the maximum effect on breast cancer cell invasion, suggesting that bupivacaine administration may be associated with recurrence and metastasis in post-primary breast cancer surgery (26). Therefore, the present study investigated the association between bupivacaine and LRRC3B expression in breast cancer cells.

In the present study, LRRC3B expression levels decreased in breast cancer MDA-MB-231 and MCF-7 cells that were treated with different concentrations of bupivacaine, indicating that the administration of bupivacaine to breast cancer tumor cells suppressed the expression of LRRC3B. A number of studies have revealed that bupivacaine administration during cancer surgery serves a crucial role in cancer recurrence and metastasis by promoting cell migration (27,28). In line with previous studies, the data of the present study showed that 50 µg/ml bupivacaine yielded the maximum increase breast cell invasion. However, this effect was inhibited by LRRC3B silencing (Fig. 5). Jørgensen *et al* (29) demonstrated that the downregulation of LRRC3B inhibited the migration of breast cancer cells (29). The present study speculated, based on the aforementioned results, that LRRC3B silencing may perform a protective role in preventing breast cancer recurrence, post-bupivacaine, by suppressing cell invasion.

In conclusion, the data presented in the present study suggests that LRRC3B silencing may serve a protective role by preventing breast cancer recurrence and metastasis post-anesthesia *in vitro*. LRRC3B downregulation suppressed the breast cancer cell invasion caused by bupivacaine administration. The present study may provide a theoretical basis for the mechanism of action of LRRC3B and the clinical application of the gene in breast cancer. However, additional experimental studies are required to investigate the mechanism.

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