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Sufentanil impairs autophagic degradation and inhibits cell migration in NCI-H460 *in vitro*

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Abstract. Metastasis, which involves the spread of cancer cells to distant tissues and organs, is a major cause of cancer-associated mortality. Although the use of anesthetics and analgesics may affect cancer cell metastasis, the underlying molecular mechanism remains unclear. Autophagy is a lysosome-based dynamic intracellular catabolic process that serves a crucial role in cancer cell metastasis. In order to investigate the role of autophagy in the migration of cancer cells treated with analgesics, immunofluorescence, western blotting, wound healing assay and cell invasion assay were performed in the present study. The results from immunofluorescence and western blotting demonstrated that the opioid analgesic sufentanil stimulated LC3 induction in NCI-H460 cells. Furthermore, sufentanil increased LC3 and Beclin1 protein levels, but inhibited the fusion of autophagosomes and lysosomes. In addition, sufentanil decreased cathepsin D protein level and increased p62 protein level. The addition of chloroquine (CQ) to sufentanil did not induce a further increase in LC3-II protein levels in NCI-H460 cells, suggesting the impairment of autophagic degradation. Furthermore, treatment with trehalose stimulated the migration of sufentanil-treated cells, whereas additional treatment with CQ did not further decrease the migration of sufentanil-treated cells. In addition, sufentanil co-treatment with trehalose significantly increased the invasion of lung cancer cells, whereas, additional treatment with CQ did not further reduce the invasion of sufentanil-treated cells. These results indicated that autophagy may be involved in the inhibition of NCI-H460 cell migration by sufentanil, and that sufentanil may be considered as a favorable analgesic for patients with lung cancer.

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Introduction

Although cancer is a disease with high mortality rate, the current treatments for patients remain limited due to the complexity of the different types of cancer and the high incidence of cancer cell metastasis (1). At present, surgical resection is the most commonly used method to treat cancer; however, tumor recurrence and metastasis remain the main cause of cancer-associated mortality in patients (2,3). Perioperative care and anesthesia management are recognized as important factors that may affect cancer recurrence, metastasis and patient survival (4). Previous retrospective studies reported that inhibition of immune function caused by postoperative pain could worsen the prognosis of patients with cancer, whereas intraspinal block, postoperative analgesia, and the use of non-steroidal anti-inflammatory analgesics can improve the prognosis of these patients (5,6). Previous studies demonstrated that local anesthetics can inhibit the proliferation of liver cancer cells (7) and further induce necrosis of prostate cancer and ovarian cancer cells (8). The general anesthetic propofol administered intravenously can decrease the malignant degree of prostate cancer cells by inhibiting N-methyl-D-aspartic acid receptors (9). Furthermore, midazolam can induce apoptosis in a variety of different types of cancer cell (10). However, certain studies reported that the general anesthetic isoflurane can increase the degree of malignancy of prostate and renal cancer cells (11,12). In addition, isoflurane can stimulate glioma stem cell viablity, thereby increasing the risk of cancer recurrence and metastasis (11,12). The selection and use of anesthetic drugs could therefore be associated with cancer cell proliferation and metastasis.

Tumors can be highly invasive. The infiltration of cancer cells and metastatic spread towards distant tissues and organs represent the most direct causes of cancer-associated mortality in patients (13); however, the underlying molecular mechanisms remain unclear. Metastasis comprises multiple discrete steps, as follows: i) Tumor cell invasion is initiated from the primary tumor site and is followed by intravasation into the vasculature or lymphatic circulation; ii) tumor cells lose their initial epithelial phenotype in order to survive in the circulation; iii) extravasation of individual tumor cells occurs at the target organ site; and iv) expansion and colonization of tumor cells at the secondary site is promoted (13). Therefore, inhibition of the aforementioned steps may inhibit cancer metastasis. However, studies on the effects of anesthetics on

tumor metastasis are currently rare, and most of them focus on the direct effects and underlying mechanisms of drugs on cancer cell proliferation and tumor growth.

Autophagy is a lysosome-based evolutionarily conserved and dynamic intracellular catabolic process (14) that serves a crucial role in cancer cell metastasis (13). The ability of autophagy to control necrosis and inflammation may limit the invasion and dissemination of tumor cells from a primary site, therefore inhibiting metastasis at an early step. However, autophagy may promote metastasis at later stages by protecting stressed tumor cells traveling through the vasculature and colonizing at distant sites (13). Autophagy can adapt to contextual demands and serve both prometastatic and antimetastatic roles (13). Regulating autophagy may, therefore, prevent tumor metastasis.

Autophagy is an important physiological process that can be triggered by the administration of anesthetics, including local anesthetics, inhaled general anesthetics, intravenous general anesthetics and analgesic drugs (15-17). Autophagy is involved in the effects of anesthetics on tumors. It has been demonstrated that Propofol induces protective autophagy and promotes renal fibroblast survival time under hypoxic conditions (18). Furthermore, the induction of autophagy protects glioma H4 cells from sevoflurane toxicity (19). In addition, it has been reported that fentanyl can trigger autophagy via the reactive oxygen species/MAPK signaling pathway and decrease lung cancer cell sensitivity to cisplatin (20). However, to the best of our knowledge, only a limited number of studies have examined the effect of anesthetics on the metastasis of lung cancer through regulating autophagy. The present study demonstrated that the analgesic sufentanil induced the accumulation of autophagosomes and impaired the autophagic degradation that may account from the inhibition of NCI-H460 cell migration. These findings indicated that sufentanil may be considered as a preferable analgesic that could be used in patients with lung cancer.

Materials and methods

Antibodies and agents. The antibody against LC3 (1:2,000; cat. no. NB100-2220) was purchased from Novus Biologicals, LLC. The antibody against sequestosome 1 (SQSTM1)/p62 (1:2,000; cat. no. ab56416) was purchased from Abcam. Antibodies against β-actin (1:1,000; cat. no. 60008-1-Ig), LAMP1 (1:100; cat. no. 21997-1-AP) and Beclin1 (1:1,000; cat. no. 11306-1-AP) were purchased from ProteinTech Group, Inc. Antibodies against cathepsin D (1:1,000; cat. no. sc-6486) and goat anti-rabbit immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC) (1:100; cat. no. SC2-12) were purchased from Santa Cruz Biotechnology, Inc. Horseradish peroxidase (HRP)-conjugated anti-rabbit (1:10,000; cat. no. W4011), HRP-conjugated anti-goat (1:10,000; cat. no. V805A) and HRP-conjugated anti-mouse (cat. no. W4021) antibodies were purchased from Promega Corporation. Enhanced chemiluminescence (ECL) kit was purchased from Biological Industries.

Cell culture. The present study used NCI-H460 as a human large cell lung carcinoma, cell line, 293 cells known as the Human Embryonic Kidney 293 cells and HepG2 as a liver cancer cell line. Cells were obtained from American Type Cell Culture (ATCC, Manassas, VA) and kindly provided by Professor Longping Wen from University of Science and Technology of China. NCI-H460 cells were cultured in RPMI 1640 (SH30809.01, Hyclone) medium, and 293 and HepG2 cells were cultured in or Dulbecco's Modified Eagle medium (Hyclone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (FBS; Biological Industries) at 37˚C in the presence of 5% CO_2. Cells were treated with 50 μM chloroquine (CQ; Sigma-Aldrich; Merck KGaA) or 100 mM trehalose (Tre; autophagy inducer; Sigma-Aldrich; Merck KGaA) for 24 h (21). Sufentanil was used at the concentration of 1 nM and incubated with cells for 24 h (22). Sufentanil was purchased from Yichang Hmanwell Pharmaceutical Co., Ltd.

Immunofluorescence. NCI-H460 cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 10 min and blocked with 1% FBS for 1 h at room temperature. For LC3 puncta and lysosomes staining, cells were incubated with antibodies against LC3 or LAMP1 antibody overnight at 4˚C, and with anti-rabbit or anti-mouse IgG-FITC antibody, respectively, at 37˚C for 1 h. Images were acquired using an Olympus IX71 fluorescence microscope at x400 magnification (Olympus Corporation). Data were analyzed using Image J software (version 1.35; National Institutes of Health).

Western blotting. After treatment with drugs and reagents, NCI-H460, 293 and HepG2 cells were lysed with sample lysis buffer (Beyotime Institute of Biotechnology) at room temperature, and then boiled for 10 min. Protein concentration was determined using the BCA protein assay kit (cat. no. 23225; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Proteins (20-40 μg per lane) were separated via SDS-PAGE (15% gel) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat powdered milk for 1 h at 37˚C and incubated with the relevant primary antibodies at 4˚C overnight. The primary antibodies incubated membranes were washed 5 times with TBST (Beijing Solarbio Science & Technology Co., Ltd.) and then incubated with relevant secondary antibodies for 1 h at 37˚C. Eventually, bands were detected using enhanced chemiluminescence and visualized using a chemiluminescence imaging instrument (GE Image Quant LAS 4000; GE Healthcare Life Sciences).

Wound healing assay. NCI-H460, 293 or HepG2 cells ($1x10^6$ cells/3 ml) were seeded and cultured in FBS-supplemented medium in a 60 mm dish until they formed a confluent monolayer. The monolayer was wounded with a manual scratch using a 1 ml pipette tip. Cells were washed twice with PBS and were incubated with serum-free medium with or without sufentanil (1 nM) and CQ (50 μM) for 24 h. Images were subsequently captured using an Olympus IX71 microscope at x100 magnification. The wound healing closure was quantified using ImageJ 1.35 software (National Institutes of Health). The percentage of wound closure was calculated as follows: [(initial wound area-remaining wound area)/initial wound area].

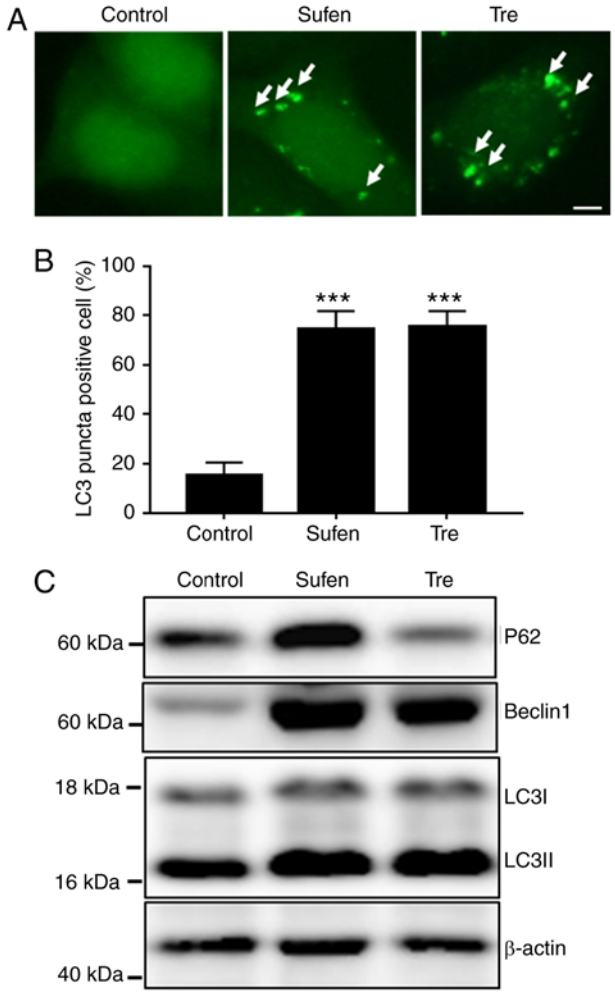

Figure 1. Sufentanil induced autophagosomes accumulation. (A) Immunofluorescence microscopy for LC3 labeling (green). (B) Quantification of LC3 positive cells from (A). (C) Western blotting analysis of LC3, Beclin1 p62 and β-actin levels. NCI-H460 cells were treated with sufentanil (1 nM) or trehalose (100 mM) for 24 h prior to the experiments. The arrows are pointing to autophagosomes.Scale bar, 5 μm. ***P<0.001 (comparison between any two means). Sufen, sufentanil; Tre, trehalose.

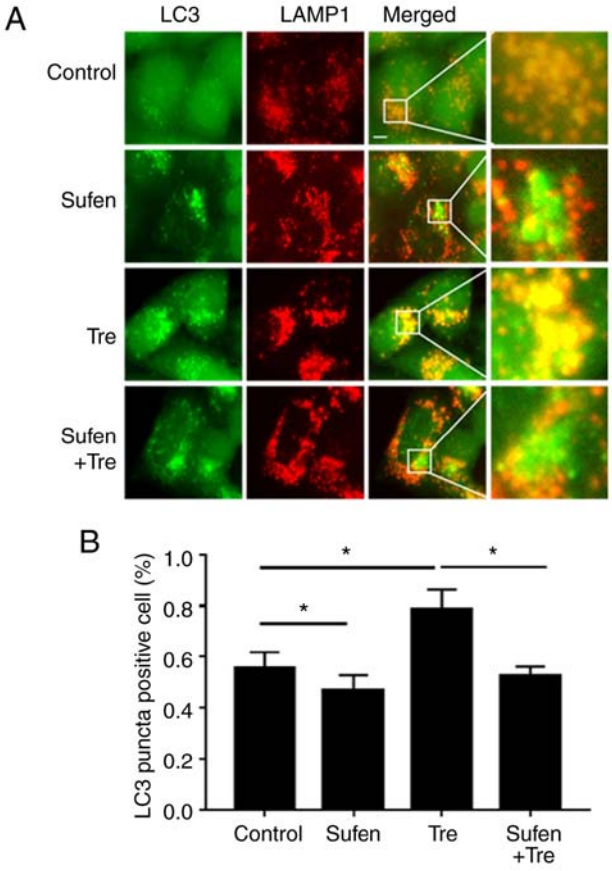

Figure 2. Sufentanil blocked the fusion of autophagosomes and lysosomes. (A) Immunofluorescence microscopy for LC3 (green) and LAMP1 (red) double staining. (B) Pearson's co-localization coefficient of cells from (A) NCI-H460 cells were treated with sufentanil (1 nM) or trehalose (100 mM) for 24 h prior to the experiments. Scale bar, 5 μm. *P<0.05 (comparison between any two means). LAMP1, lysosomal-associated membrane protein 1; Sufen, sufentanil; Tre, trehalose.

Cell invasion assay. Cell invasion was analyzed using Transwell chambers with 8 μm pore size (Corning Inc.). Prior to the invasion assay, Transwell were precoated with Matrigel (BD Biosciences) for 1 h at 37˚C. NCI-H460, 293 or HepG2 cells (5x10^5 cells/200 μl) were seeded into the top chamber and the bottom chamber was filled with 600 μl DMEM containing 10% FBS. Cells that migrated onto the bottom surface of the membrane were fixed in 100% methanol for 30 min and stained with 0.5% crystal violet for 20 min at room temperature after treatment with the specified drugs for 24 h. Images were subsequently captured using an Olympus IX71 fluorescence microscope at x100 magnification. Four randomly selected fields were counted for each experimental group per cell line.

Cell viability assay. MTT was used to assess the cell viability. Briefly, NCI-H460, 293 or HepG2 cells were grown in 96-well plates at a density of ~10,000 cells per well. After the different treatments, MTT (thiazoyl blue tetrazolium bromide; T0793-500MG, Bio Basic) was added to the growing cultures at a final concentration of 0.5 mg/ml, incubated for 4 h at 37˚C and dissolved in 100 μl dimethyl sulfoxide (D8370; Solarbio). The absorbance was measured at 570 nm with a spectrophotometer (Elx800, BioTek).

Statistical analysis. All data were expressed as the mean ± standard error of the mean. Differences among groups were analyzed by one- or two-way ANOVA followed by Tukey's Post-hoc test or the two-tailed Student's t-test. P<0.05 was considered to indicate a statistically significant difference. Pearson's co-localization coefficient of cells was calculated using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.).

Results

Sufentanil induces autophagosome accumulation. The present study determined the induction of autophagy in sufentanil-treated NCI-H460 cells. During the autophagy process, the protein LC3 is cleaved from LC3-I into the lower molecular weight LC3-II and aggregates on autophagosome membranes (23). Beclin1 plays a central role in autophagy and is considered a marker protein for autophagy (24). At 1 nM, sufentanil exhibited a mild cytotoxicity in NCI-H460 cells

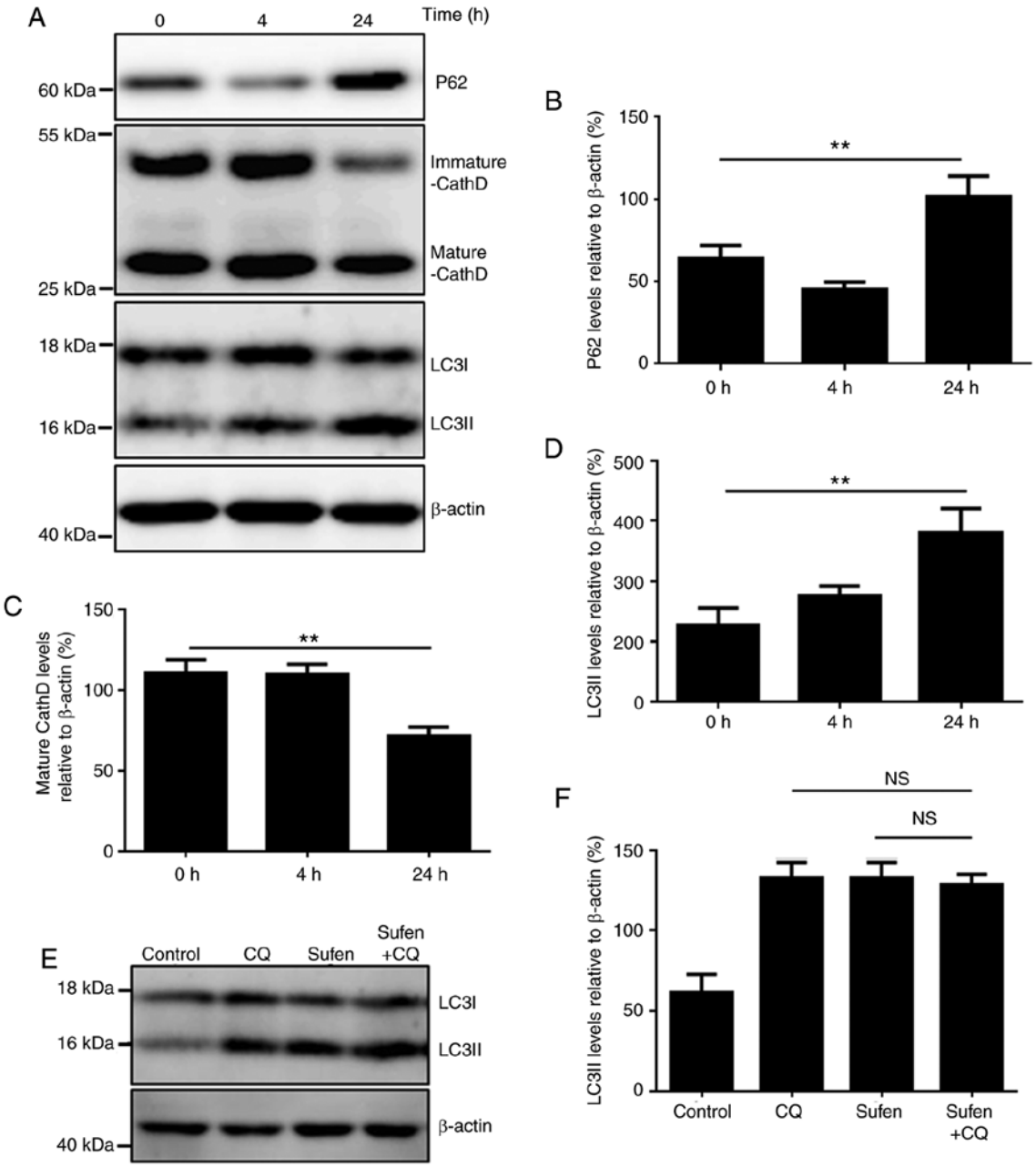

Figure 3. Sufentanil impaired autophagic degradation. (A) Western blot analysis of p62, LC3, CathD and β-actin levels. NCI-H460 cells were treated with 1 nM sufentanil for 24 h. (B-D) Quantification of p62, LC3II and CathD levels compared with β-actin levels. (E) Western blot analysis of LC3 and β-actin. Cells were treated with 1 nM sufentanil with or without CQ for 24 h prior to experiments. (F) Quantification of LC3II levels compared with β-actin levels. **P<0.01 (comparison between any two means); NS, not significant. Sufen, sufentanil. CathD, cathepsin D; CQ, chloroquine.

(Fig. S1). The results from immunofluorescence staining demonstrated that, similarly to trehalose-treated cells, 1 nM sufentanil induced the generation of a large number of LC3 puncta (Fig. 1A and B), which indicated the accumulation of autophagosomes. In addition, the levels of LC3-II, Beclin1 and p62 in sufentanil-treated NCI-H460 cells were increased (Fig. 1C), which further confirmed the accumulation of autophagosomes.

Sufentanil blocks the fusion of autophagosomes and lysosomes. It has been reported that autophagosomes can fuse with numerous lysosomes to form autolysosomes (23), and that the autophagic content found in the autolysosomes is degraded (23). LAMP1 is a lysosomal membrane protein frequently used as a lysosomal marker (25). Autolysosomes can therefore be identified by assessing the co-localization of LC3 and LAMP1 by fluorescence microscopy. Trehalose, which is a commonly used autophagy inducer, was used in the present study as a positive control. The results demonstrated that few LAMP1 and LC3 were co-localized in NCI-H460 cells following treatment with 1 nM sufentanil. However, the majority of LAMP1 signals were overlapped with LC3 in trehalose-treated cells (Fig. 2A). Statistical analysis of the co-localization rate between LAMP and LC3 was consistent with the results from fluorescence microscopy (P=0.04, sufen vs. control; P=0.03, tre vs. control; P=0.03, tre vs. tre+sufen; Fig. 2B).

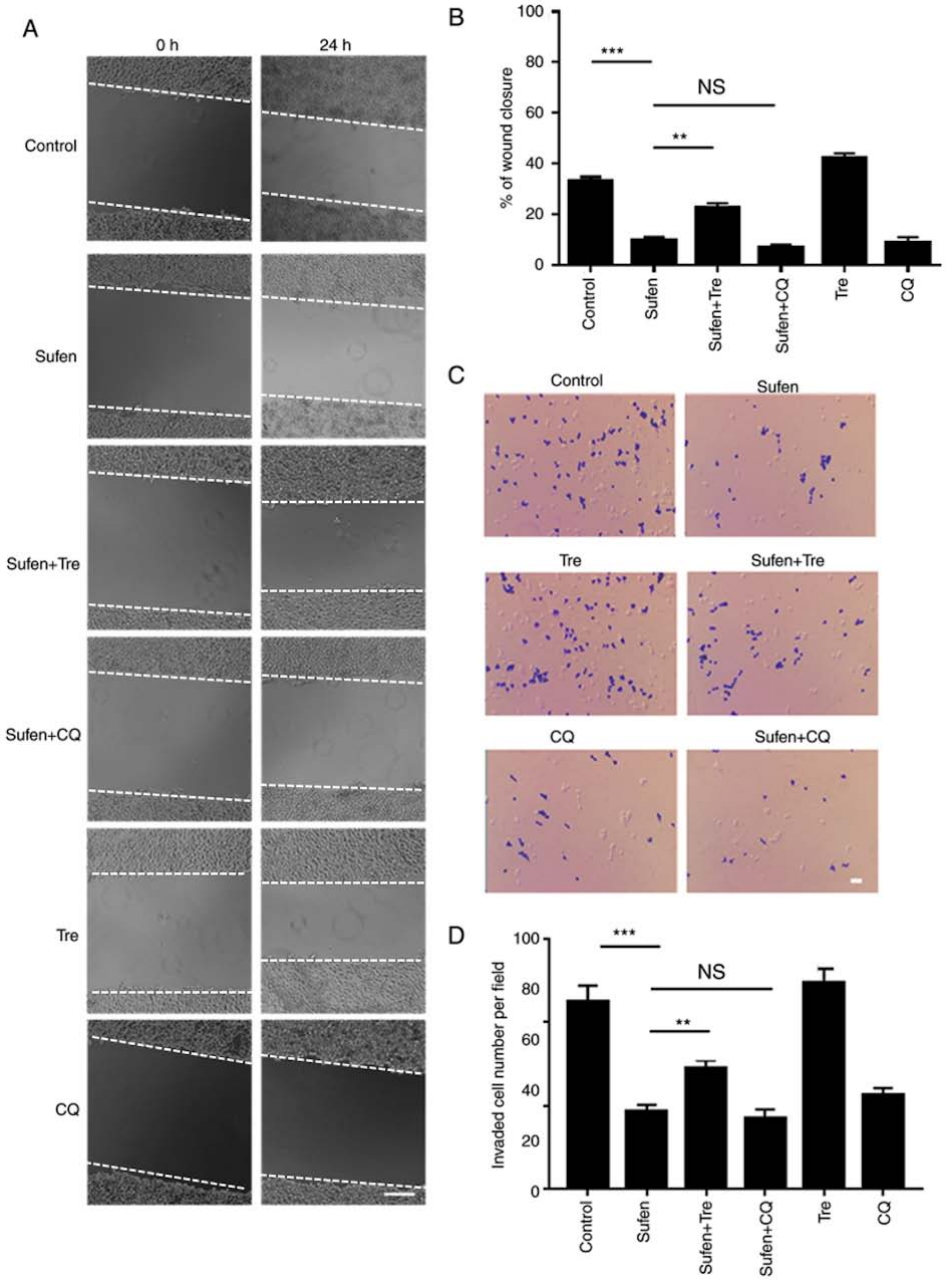

Figure 4. Autophagy was involved in the inhibition of migration by sufentanil. (A) Sufentanil suppressed wound closure. Scratched NCI-H460 cells were treated with 1 nM sufentanil, PBS, 1 nM sufentanil + 50 μM CQ or 1 nM sufentanil + 100 mM trehalose for 24 h. Scale bar, 1 mm. (B) Wound closure quantification from (A). (C) Cell invasion images. Scale bar, 50 μm. (D) Quantification of the invaded cells per field from (C). **P<0.01 and ***P<0.001 (comparison between any two means). CQ, chloroquine; NS, not significant; Sufen, sufentanil; Tre, trehalose.

Sufentanil gradually impairs autophagic degradation. Autophagy is a lysosomal-based intracellular degradation process (14). In the present study, the ability of autophagosomes to induce degradation was detected in sufentanil-treated NCI-H460 cells. SQSTM1/p62 is a protein substrate that is selectively incorporated into the autophagosomes and degraded during autophagy (26). The results from the present study demonstrated that the p62 level was slightly decreased after 4 h treatment, which indicated that sufentanil may induce a complete autophagy process at early stage of sufentanil treatment. Over time, sufentanil gradually inhibited autophagy, and after 24 h, p62 level was significantly increased (P<0.01), which suggested that autophagy may have been blocked (Fig. 3A and B) (27,28). Furthermore, the protein level of the autophagic marker LC3-II was significantly increased over time (Fig. 3A and C). The protein level of the lysosomal protease cathepsin D, which reflects the lysosomal function, was also determined (29). The results demonstrated that the level of the mature form of cathepsin D after 24 h treatment with sufentanil was significantly decreased (P<0.01), which suggested that sufentanil may have disturbed autophagic degradation in NCI-H460 cells (Fig. 3A and D). In addition, p62 and LC3-II levels in 293 and HepG2 cells were increased following 24 h treatment with sufentanil (Fig. S2), suggesting that autophagy may have been impaired.

The former malaria drug CQ is now widely used as an inhibitor of autophagy in cell culture and *in vivo* assays (23,28). CQ is a lysosomotropic weak base, which diffuses into the lysosome in its monoprotonated form. This compound is then entrapped in the lysosome and becomes diprotonated. Protonated CQ can alter the lysosomal pH, thereby inhibiting the autophagic degradation in the lysosome (28). Similar to CQ treatment, treatment with 1 nM sufentanil increased the levels of LC3-II, Beclin1 and p62, and decreased the level of mature cathepsin D in NCI-H460, 293 and HepG2 cells (Figs. 3E, S3 and S4). No significant difference in LC3-II level was observed between sufentanil- and CQ-treated cells. Furthermore, additional treatment with CQ did not further increase LC3-II level in sufentanil-treated cells (Fig. 3E and F), which suggested that sufentanil may disrupt autophagic degradation.

Impaired autophagic degradation is involved in sufentanil-inhibited cell metastasis in vitro. A wound healing assay was used to investigate the effect of sufentanil on the migratory capability of NCI-H460 cells. Following 24 h of treatment with 1 nM sufentanil, cell migration was significantly decreased compared with the control group (Fig. 4A and B). Furthermore, additional treatment with CQ did not further decrease the migration of sufentanil-treated cells. However, the increase in the level of autophagy (Fig. 1C) following trehalose treatment significantly increased the wound closure compared with sufentanil-treated cells. In addition, a lower number of invasive cells was observed following 1 nM sufentanil treatment compared with the control group (Fig. 4C and D). Additional treatment with CQ did not further decrease the invasive capability of sufentanil-treated cells (Fig. 4C and D). Cell treatment with trehalose significantly increased the invasive capacity of NCI-H460 cells compared with sufentanil-treated cells (Fig. 4C and D). These results demonstrated that impaired autophagic degradation may be involved in the inhibited migration of NCI-H460 cell induced by sufentanil. Similar results on the migratory and invasive capacities of 293 and HepG2 cell lines following treatment with the aforementioned drugs were observed (Fig. S5).

Discussion

The results from the present study demonstrated that cell treatment with sufentanil could inhibit the autophagosome-lysosome fusion and the disruption of the autophagic degradation. These findings may explain the inhibition of NCI-H460 cell migratory capacity, and indicated that sufentanil may be considered as a potential analgesic compound for the treatment of patients with lung cancer.

Opioids are the most commonly used type of analgesic for perioperative analgesia (30); however, whether opioids may favor the prevention of metastasis and recurrence following cancer surgery remains unclear (30). For example, morphine has been reported to promote the invasive and migratory capacities of breast and lung cancer cells via the upregulation of matrix metalloproteinases (MMPs) (31). However, a previous study demonstrated that morphine can significantly decrease the adhesion, invasion and metastasis capabilities of colon cancer cells via the downregulation of MMPs (31). The present study demonstrated that sufentanil inhibited the migration of NCI-H460 cells, which was consistent with previous studies (31,32). However, additional in-depth and extensive analyses are required in order to determine the impact of opioids on tumor metastasis.

Numerous retrospective studies reported a lower incidence of cancer recurrence following post-surgery regional anesthesia with low doses of opioids in patients with breast cancer, prostate cancer, colon cancer and melanoma (33,34). It has been demonstrated that pain can activate the stress response and suppress the immune system in both animals and humans (35,36), suggesting that pain could promote tumor recurrence and metastasis. Analgesics may therefore have the potential to alter tumor recurrence and metastasis via pain reduction (35). Furthermore, it has been demonstrated that opioids have extensive immunomodulatory activities, both in the cellular and humoral immune responses, and are able to modulate inflammatory cytokine production (37,38), which suggests that tumor metastasis may be inhibited by opioid analgesics. The present study aimed to evaluate the effect and molecular mechanism underlying anesthetics and analgesics on tumor cells in a simple and economical way, in order to provide some recommendations for the choice of analgesics in clinical surgery.

The results from the present study demonstrated that autophagy may be involved in the inhibited migration of NCI-H460 cell induced by sufentanil. The ability of autophagy to restrict necrosis and inflammation may limit the invasion and dissemination of tumor cells from the primary site, inhibiting therefore metastasis at an early stage (13). However, autophagy could promote metastasis at later stages by protecting stressed tumor cells as they travel through the vasculature and colonize at distant sites (13). Further investigations are required in order to determine how the impairment of autophagy and cell migration are associated in sufentanil-treated cells. The present study demonstrated that inhibition of autophagy was involved in sufentanil-mediated inhibition of tumor cell migration. Sufentanil may, therefore, have a beneficial anti-tumor effect in the late stages of lung cancer, and may be considered as an optimal choice for surgical analgesia in patients with advanced lung cancer.

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Availability of data and materials

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

Authors' contributions

HJ, CW and HW designed the present study, and analyzed and interpreted the data, HJ and HW wrote the manuscript. WZ, YH and CC performed the experiments study. CW critically revised the 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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