IMPACT OF ANESTHETICS ON CANCER BEHAVIOR AND OUTCOME

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PUBLISHED IN: Frontiers in Pharmacology and Frontiers in Oncology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88976-403-7
DOI 10.3389/978-2-88976-403-7

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IMPACT OF ANESTHETICS ON CANCER BEHAVIOR AND OUTCOME

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Citation: Lin, J.-A., Ma, D., Wu, S.-Y., eds. (2022). Impact of Anesthetics on Cancer Behavior and Outcome. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88976-403-7

Table of Contents

frontiers | Frontiers in Pharmacology

TYPE Editorial
PUBLISHED 04 August 2022
DOI 10.3389/fphar.2022.957070

OPEN ACCESS

EDITED AND REVIEWED BY
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SPECIALTY SECTION
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Pharmacology

RECEIVED 30 May 2022
ACCEPTED 04 July 2022
PUBLISHED 04 August 2022

CITATION
Lin J-A, Ma D and Wu S-Y (2022), Editorial: Impact of anesthetics on cancer behavior and outcome. *Front. Pharmacol.* 13:957070. doi: 10.3389/fphar.2022.957070

Editorial: Impact of anesthetics on cancer behavior and outcome

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KEYWORDS

local anesthetics, inhalation anesthetics, intravenous anesthetics, neoplasms, behavior, treatment outcome

Editorial on the Research Topic
Impact of anesthetics on cancer behavior and Outcome

In two review articles, the mechanisms of cancer inhibition by local anesthetics were reviewed in detail. Zhang et al. summarized the possible pathways in a schematic figure, which provided a framework for researchers to follow when one would like to investigate the impact of local anesthetics on cancer behaviors and associated mechanisms. Generally speaking, local anesthetics involve activating the death signaling and inhibiting survival pathways. According to the current results, their roles seem not independent, more likely to be modulating the chemotherapy during cancer treatment. Furthermore, Zhang et al. pointed out some of the solutions and research priorities, such as standardization of experimental methods, investigation of the stemness of the cancer cells, identification of the specific tumors or types of the cancer cells that may be particularly sensitive to certain local anesthetics, as well as the use of bioinformatics. From the viewpoint of residual cancer cells, Wall and Buggy specifically looked into the effect of lidocaine perioperatively, which has a well-established role either while being infused intravenously or in the provision of epidural anesthesia. Their review provided a state-of-the-art summary of recent advances regarding perioperative lidocaine and its in-depth biological effects from possible aspects. Based on a schematic overview of the pathophysiological mechanisms involved in perioperative metastasis formation, they explained potential tumor-related mechanisms of action of systemic lidocaine during surgery by a model of colonic tumor excision. Although published dosage guidelines may aid in ensuring safe practice, the

appropriateness of intravenous lidocaine has recently been questioned in terms of its impact on cancer behavior given as yet inconclusive benefits. Both review articles mentioned that the drug concentrations in most animal and cell experiments were significantly higher than in clinical use. Given the considerable degree of biological heterogeneity between different malignancies, it would be difficult to extrapolate the result of one cancer to another. Different anesthetics promote or inhibit metastasis, depending on the type of tumor cell as well as the type, dose, and regimen of anesthetics used.

This Research Topic also includes some specific analyses, including tumor-specific, anesthetic technique-specific, and anesthetic agent-specific publications to elucidate the association and possibly causal relationship. Most publications on this Research Topic involved female cancers. Liu et al. found that sevoflurane, though with increased migration of 4T1 breast cancer cells, has no difference compared with isoflurane and desflurane on the growth and lung metastasis in the mouse residual tumor model. Chamaraux-Tran et al. provided a prototypical model of combining metabolomics and onco-anesthesia to evaluate the impact of anesthetic on metabolites in triple-negative breast cancer cell lines. Especially for female cancers, ferroptosis might be an emerging target to assess the impact of anesthetics on cancer behavior. Both Zhao et al. and Sun et al. explored anesthetic-specific ferroptosis-related signaling pathways *in vitro* with or without chemotherapeutic agents. In the two retrospective propensity score matching study investigating ovarian cancer surgery Tseng et al. found propofol-based total intravenous anesthesia is associated with better survival compared with desflurane anesthesia, and Zhang et al. reported a positive effect of intravenous infusion of lidocaine on short-term outcomes and survival. However, robust prospective clinical evidence supporting the beneficial anti-cancer effect of intravenous lidocaine treatment is lacking. The scarcity of randomized controlled trials on this topic is also in line with the conclusion made by Luo et al. in this Research Topic. Using the bibliometric method Luo et al. found that the research hot spot in the influence of anesthesia on tumor prognosis mainly focused on retrospective studies over the past 20 years, and the direction is likely in a gradual transition from retrospective studies to prospective randomized controlled trials. Shi et al. conducted a prospective randomized controlled trial comparing propofol-based general anesthesia with local anesthesia in patients with hepatocellular carcinoma undergoing radiofrequency ablation. It would be interesting to know that even propofol-based general anesthesia would aggravate cancer behavior and precipitate pro-inflammatory cytokine secretion from the viewpoint of the patient's serum. Shi et al. speculated that physiological changes during general anesthesia or accompanying opioid usage during propofol-based anesthesia might be responsible for the results. However, another randomized trial directly evaluating early recurrence by Li et al. showed no difference between paravertebral-propofol-based regional anesthesia and sevoflurane-opioid-based general anesthesia for breast cancer surgery. A trend of reduced recurrence hazard under regional anesthesia in estrogen receptor-negative group, though not significant, warrants an even larger sample to clarify the influence in this high-risk group.

In many *in vitro* studies evaluating the effects of lidocaine or other local anesthetics, the drug was usually incubated with cultured cells for some time, but such a period of stable and direct drug exposure cannot be possible for an *in vivo* experiment when local anesthetics were locally injected unless it is given into the body compartment that would achieve a constant systemic concentration or where local anesthetic can directly expose themselves to the target tumor. Therefore, what we called clinically relevant concentrations would be serum concentration achieved by intravenous (<20 μM) or epidural lidocaine infusion (around 1 μM). Other situations with higher concentrations cannot be called clinically relevant concentrations unless they are administered into specific body cavities, such as intra-bladder or intra-abdominal injection for direct drug exposure. The difficulty in translating the higher concentration results to the clinical setting (such as local injection around the solid tumor) comes from unstable lidocaine exposure to the tumor since the highly soluble lidocaine hydrochloride can be cleared out *in vivo* quickly with rather limited duration. Otherwise, to retain lidocaine at the injection site much longer, lidocaine nanoparticles proposed by Yang et al. can significantly slow down the release rate of lidocaine and could be one of the solutions to help translate the results from higher concentrations studied in the web bench and make it more adequately compatible with the model of local injection around solid tumors. It would be even better if future publications could comment on the effects of lidocaine on cancer behaviors based on clinically relevant concentrations and stratified by body compartments administered, respectively, to help readers evaluate its true impact without being confused by describing the effects according to the results from mixed concentration scales (mM vs μM).

Although all of the above articles described the inhibitory effects of local anesthetics on cancer behaviors, a balanced report cannot be achieved without including the possible aggravating effects of intravenous lidocaine infusion on cancer behaviors. The biphasic effect, especially from drugs, is not uncommon in clinical practice from our previous experience (Lin et al., 2011). It might be premature to deny the possibility that clinically relevant concentrations of lidocaine (<20 μM) could exert opposite effects compared with high concentrations on the millimolar scale. Furthermore, the impact of lidocaine at concentrations corresponding to intravenous (<20 μM) or epidural infusion (around 1 μM) on cancer behavior has rarely been explored. Lidocaine has been proven to

consistently promote proliferation in cells derived from metastatic colon cancer (Siekmann et al., 2019). Nevertheless, previous studies have also reported that exposure to 8 μM lidocaine could reduce the barrier property of A549 lung cancer cells using electric cell-substrate impedance sensing (ECIS) technology (Chan et al., 2017). However, the effect of 8 μM lidocaine on cell migration is yet to be clarified. For lidocaine to be infused either intravenously or epidurally, the concentrations to which tumors are directly exposed must be below the toxic concentration (21 μM) to have significance in translational medicine. Contrary to previous reports, our preliminary data showed that lidocaine concentrations corresponding to intravenous infusion in clinical scenarios (1–20 μM) did not affect the growth and proliferation of lung cancer cells but promoted epithelial-mesenchymal transition, which further renders its clinical effects on lung cancer questionable. Including both sides (positive and negative impacts) in the review or discussion section of studies would help achieve a more robust study design in the future.

From describe above and meanwhile to meet the future directions pointed out by Zhang et al., experimental model wise, we call for further studies using clinically relevant concentration (<20 μM) to evaluate the impact of lidocaine on cancer behaviors to mimic stable circulatory lidocaine exposure to the tumors during intravenous or epidural infusion. Cancer stem cell and mechanisms associated with cancer stemness, such as epithelial-mesenchymal transition, would be the future research hotspot in this field. Network analysis using ingenuity pathway analysis corresponding to specific cancer settings might be a practical tool to systematically predict gene alteration according to the wet bench results following lidocaine exposure as a solution to meet the direction toward bioinformatics.

Author contributions

J-AL prepared the draft. DM and S-YWu revised the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 26 May 2021
doi: 10.3389/fphar.2021.681223

Lidocaine Promoted Ferroptosis by Targeting miR-382-5p /SLC7A11 Axis in Ovarian and Breast Cancer

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Ovarian and breast cancer are prevalent female malignancies with increasing occurrence incidence and metastasis, significantly affecting the health and life quality of women globally. Anesthetic lidocaine has presented anti-tumor activities in the experimental conditions. However, the effect of lidocaine on ovarian and breast cancer remains elusive. We identified the important function of lidocaine in enhancing ferroptosis and repressing progression of ovarian and breast cancer. Our data showed that lidocaine further repressed erastin-inhibited ovarian and breast cancer cell viabilities. The treatment of lidocaine induced accumulation of Fe^{2+}, iron and lipid reactive oxygen species (ROS) in ovarian and breast cancer cells. The ovarian and breast cancer cell proliferation was suppressed while cell apoptosis was induced by lidocaine *in vitro*. Lidocaine attenuated invasion and migration of ovarian and breast cancer cells as well. Regarding the mechanism, we found that lidocaine downregulated solute carrier family 7 member 11 (SLC7A11) expression by enhancing microRNA-382-5p (miR-382-5p) in the cells. The inhibition of miR-382-5p blocked lidocaine-induced ferroptosis of ovarian and breast cancer cells. MiR-382-5p/SLC7A11 axis was involved in lidocaine-mediated inhibition of ovarian and breast cancer cell proliferation *in vitro*. The miR-382-5p expression was down-regulated but SLC7A11 expression was up-regulated in clinical ovarian and breast cancer samples. Furthermore, the treatment of lidocaine repressed tumor growth of ovarian cancer cells *in vivo*, in which the miR-382-5p expression was increased while SLC7A11 expression was decreased. Consequently, we concluded that the lidocaine promoted ferroptosis by miR-382-5p/SLC7A11 axis in ovarian and breast cancer cells. The clinical value of lidocaine in the treatment of ovarian and breast cancer deserves to be proved in detail.

Keywords: ovarian cancer, breast cancer, lidocaine, ferroptosis, miR-382-5p, SLC7A11

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Pharmacology

Received: *16 March 2021*
Accepted: *10 May 2021*
Published: *26 May 2021*

Citation:
Sun D, Li Y-C and Zhang X-Y (2021) Lidocaine Promoted Ferroptosis by Targeting miR-382-5p /SLC7A11 Axis in Ovarian and Breast Cancer. Front. Pharmacol. 12:681223. doi: 10.3389/fphar.2021.681223

INTRODUCTION

Breast cancer and ovarian cancer are the most frequently occurred gynecological cancers globally, severely threatened the life quality of females (de Sousa Cunha et al., 2019; Siegel et al., 2020). Breast cancer has gain great attentions caused by its constantly increasing incidence despite of the improvement in treatment (Britt et al., 2020). Moreover, ovarian cancer represents a major clinical challenge due to the frequent late diagnosis at advanced tumor stage (Jayson et al., 2014). Ferroptosis is a novel form of cell death related to iron-caused accumulation of lipid

reactive oxygen species (ROS) (Dixon et al., 2012). Accumulating studies have indicated that targeting ferroptosis may be a feasible strategy for therapy of breast cancer and ovarian cancer (Li et al., 2020). It was proposed that triple negative breast cancer (TNBC) patients were more sensitive to ferroptosis than estrogen receptor (Wang et al., 2021)-positive patients, which provided a potential therapeutic strategy for TNBC patients (Doll et al., 2017). Ferroptosis also mediated platinum tolerance in ovarian cancer (Wang et al., 2021).

Lidocaine is a local anesthetic drug derived from cocaine, and widely used in clinical application such as anti-bacteria, anti-inflammation, local skin anesthesia and so on (Caracas et al., 2009; Adler et al., 2017; Cizmarikova et al., 2020). An increasing number of evidences have demonstrated that lidocaine could affect the development of multiple tumors including ovarian and breast cancer (Gao et al., 2018; Khan et al., 2019; Liu et al., 2021). For example, it was reported that intravenous infusion of lidocaine during operation was associated with improved overall survival of patients with pancreatic cancer (Zhang et al., 2020). Gao and colleagues suggested that the administration of lidocaine could enhance the apoptosis of breast cancer cells caused by cisplatin, as well as alleviating their metastasis *in vivo* (Gao et al., 2018). Moreover, lidocaine could also suppress the growth and metastasis of ovarian cancer (Liu et al., 2021). However, whether ferroptosis is involved in the function of lidocaine in breast and ovarian cancer is still unknown.

MicroRNA (miRNA) is a class of endogenous short-sequenced non-coding RNAs (Lee and Dutta, 2009). MiRNAs commonly function *via* interacting with the mRNA of targeted genes and impede their translation to functional proteins, which could lead to the regulation of various biological processes during carcinogenesis, such as growth, angiogenesis and metastasis (Lee and Dutta, 2009). For instance, miR-382-5p acted as a competitive endogenous RNAs (ceRNA) of circRNA-UBAP2 to promote proliferation and suppress apoptosis of ovarian cancer cells (Xu et al., 2020). Recent studies even revealed the involvement of miRNA in lidocaine-related suppression of cancer (Sui et al., 2019; Sun and Sun, 2019). Lidocaine regulated the expression of EGFR through miR-539, which further hindered the proliferation and metastasis of lung cancer cell (Sun and Sun, 2019). However, the detailed involvement of miR-382-5p in lidocaine-affected progression of ovarian cancer and breast cancer is not clear. SLC7A11 is the gate keeper for antiporter of cystine-glutamate, also involved in cancer development (Liu et al., 2019), Newest study have indicated that SLC7A11 mediated the radiotherapy and immunotherapy of cancers *via* ferroptosis, which implied the role of SLC7A11 in regulating ferroptosis of cancers (Lang et al., 2019).

In this work, we disclosed the inhibitory effect of lidocaine on growth and metastasis of breast cancer and ovarian cancer cells *via* affecting ferroptosis process. Mechanistically, lidocaine administration stimulated ferroptosis through upregulating miR-382-5p and the subsequent suppression of SLC7A11 level. Our work suggested a miR-382-5p/SLC7A11 regulatory axis mediating lidocaine-induced ferroptosis in breast and ovarian cancer, provided new basis for therapy of female malignancies.

MATERIALS AND METHODS

Cell Lines and Transfection

Ovarian cancer cell line SKOV-3 and breast cancer cell line T47D were purchased from the Shanghai Institute of Life Sciences at the Chinese Academy of Sciences (Shanghai, China), and cultured in the DMEM cell culturing medium (Hyclone, United States) containing 10% FBS (Gibco, United States) in a 37°C humidified atmosphere filled with 5% CO_2.

The miR-382-5p mimics, miR-382-5p inhibitor, siSLC7A11, and the scramble controls (NC) were designed and purchased from RioBio (China). SKOV-3 and T47D cells were plated in 6-well plates and incubated 12 h before transfection. Next, miRNA or siRNA was mixed with lipofectamine 2,000 in the opti-MEM medium and added in each well. After a 24-hour transfection, cells were collected to perform subsequent experiments.

Patients and Tissue Samples

We included 38 ovarian cancer patients and 50 breast cancer patients, who received surgical operation in Cangzhou Central Hospital. Tumor tissues were collected during operation and subjected to real-time PCR to evaluate the levels of SLC7A11 and miR-382-5p. All experiments have acquired the consents of patients and were performed under approval of Cangzhou Central Hospital.

MTT

SKOV-3 and T47D cells were plated in 96-well plates at a density of 5×10^3 cell/well, and cultured for 12 h to form a monolayer. Subsequently, lidocaine (Sigma, United States) diluted by cell culture medium was added into each well and incubated for indicated time (24, 48, and 72 h). At the end time point, 10 μL MTT (5 mg/ml) were added into each well and the cells were cultured for another 4 h. Then the medium was discarded and 150 μL DMSO was added into each well to incubate for 10 min in dark. The absorbance values were detected at 490 nm.

Colony Formation

SKOV-3 and T47D cells were digested and suspended in DMEM as single cell. A total number of 1,000 cells were seeded into each well of 6-well plate, followed by administration of lidocaine 12 the next day. The medium was replaced with fresh DMEM containing lidocaine every three days. The cells were cultured for 15 days until visible clones formed. Subsequently, the clones were fixed, stained by violate crystal (Sigma) for 30 min at room temperature and captured in a microscope (Olympus, Japan).

Cell Apoptosis

The apoptotic cells were detected by an Apoptosis Assay Kit (Thermo, United States) under manufacturer's protocol. Briefly, SKOV-3, and T47D cells treated with lidocaine were collected, washed and double stained by FITC-Annexin V and PI reagents. The A flow cytometer (BD Biosciences, United States) was adopted to measure the cells undergoing early and late apoptosis.

Transwell

The transwell chambers (Corning, United States) covered with or without Matrigel (Corning) were used to check the invasion or migration ability of SKOV-3 and T47D cells. Cells were placed in the upper chamber with DMEM medium containing no FBS, while the lower chambers were filled with complete culturing medium with 10% FBS. After incubation for 48 h, the upper chambers were washed with PBS and stained with violate crystal.

Wound Healing

Wound healing assay was performed to determine cell migration. SKOV-3 and T47D cells were placed in 6-well plates at a density of 1×10^6 cell per well and cultured for 12 h to form a monolayer confluence. Then a 200 μL pipet was used to gently scratch a line on the monolayer. Then the medium was replaced by fresh FBS-free medium containing lidocaine. The pictures of scratches were taken at 0, 6, and 12 h.

Detection of Iron and ROS Level

Iron assay kit (Beyotime) and BODIPY C-11 (Sigma) staining were conducted to measure the intracellular Fe^{2+} level and the lipid ROS level in SKOV-3 and T47D cells under the manufacturers' instructions, separately.

Western Blotting

The proteins from SKOV-3 and T47D cells or tumor tissues were extracted by ice-cold RIPA lysis buffer (Solarbio, China) added with protease inhibitor cocktail (Thermo), and separated by 8–10% SDS-PAGE. The separated proteins were transferred to NC membranes. The blots were blocked and incubated with specific primary antibodies against GPX4 (1:1000, Proteintech, China), SLC7A11 (1:1000, Proteintech), Bcl-2 (1:1000, abcam, United States), Bax (1:1000, abcam, United States), caspase3 (1:1000, abcam, United States), cleaved-caspase3 (1:1000, abcam, United States), RIPK3 (1:1000, abcam, United States), and β-actin (1:1000, Proteintech) overnight at 4°C. Following washing with PBST, the blots were incubated at room temperature by peroxidase-conjugated secondary antibodies (1:1000, Proteintech). Proteins were visualized by using an ECL reagent (Millipore, United States) in an Image Lab Software (BIO RAD, United States).

RNA Quantification

Total RNA from tumor tissues and SKOV-3 and T47D cells were isolated using TRIzol® reagent (Sigma, United States). Quantitative real-time PCR (qPCR) for miR-382-5p and SLC7A11 mRNA was performed using a SYBR Green Mix

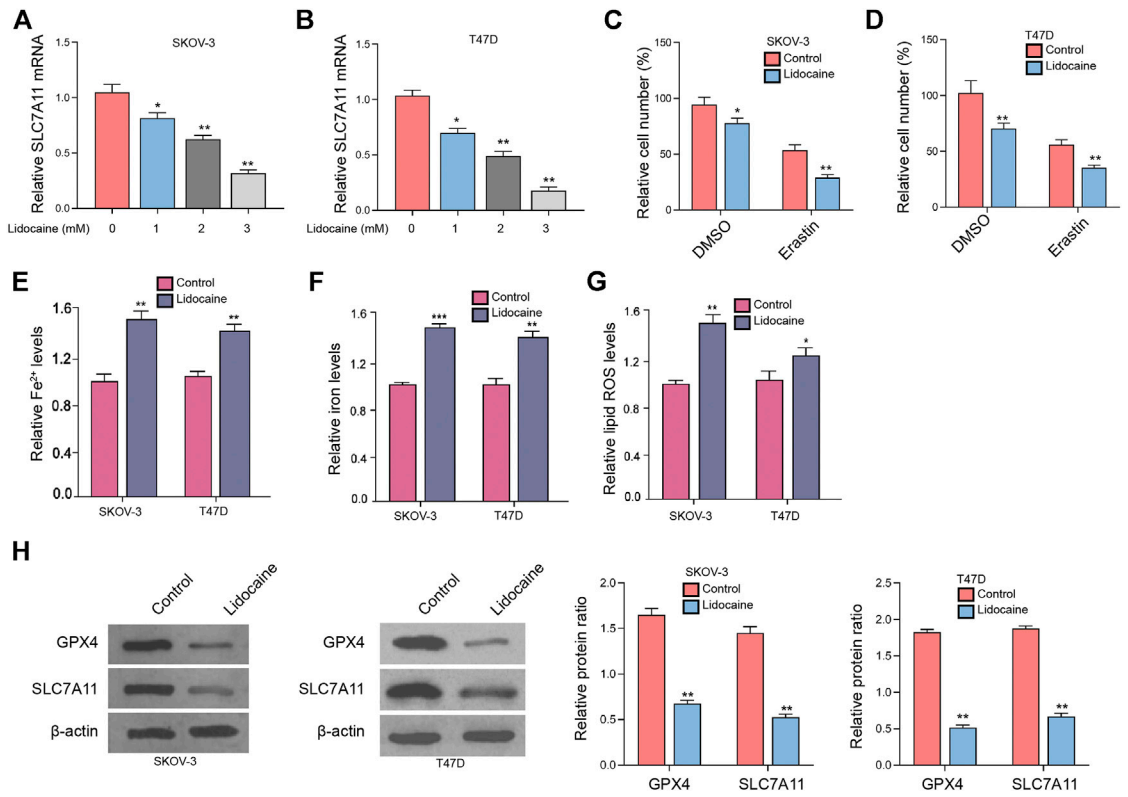

FIGURE 1 | Lidocaine induces ferroptosis of ovarian and breast cancer cells. **(A,B)** SKOV-3 and T47D cells were treated with lidocaine at the indicated concentrations. The mRNA expression of SLC7A11 was analyzed by qPCR. **(C,D)** SKOV-3 and T47D cells were co-treated with erastin (5 mmol/L) and lidocaine (3 mM). The cell viability was detected by MTT assays after 48 h of the treatment. **(E–H)** SKOV-3 and T47D cells were treated with lidocaine (3 mM). The Fe^{2+} **(E)**, iron (Tesfay et al., 2019), and lipid ROS levels **(G)** were detected. **(H)** The expression of GPX4 and SLC7A11 was measured by Western blot analysis. The results were quantified using ImageJ software. mean ± SD, $^{**}p < 0.05$, $^{**}p < 0.01$. The experiments were performed independently three times.

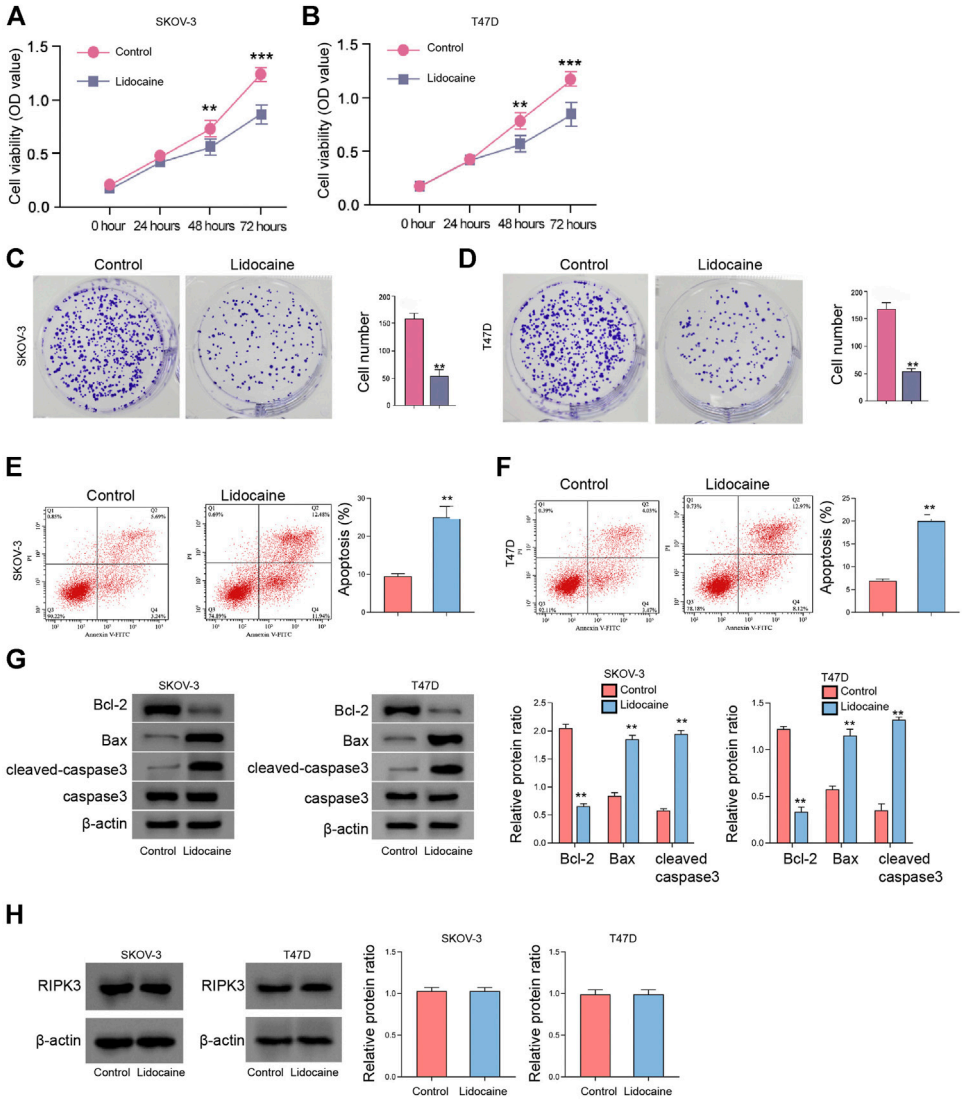

FIGURE 2 | Lidocaine reduces the proliferation of ovarian and breast cancer cells *in vitro*. **(A–H)** SKOV-3 and T47D cells were treated with lidocaine (3 mM). **(A,B)** The cell viability was detected by MTT assays. **(C,D)** The cell proliferation was measured by colony formation assays. **(E,F)** The apoptosis was analyzed by flow cytometry. **(G)** The expression of Bcl-2, Bax, caspase3, and cleaved-caspase3 was detected by Western blot. The results were quantified using ImageJ software. **(H)** The expression of RIPK3 was measured by Western blot. The results were quantified using ImageJ software. mean ± SD, $^{**}p < 0.01$. The experiments were performed independently three times.

(Thermo) and measured by a Bio-Rad CFX96 real-time PCR system (United States). Relative gene expression levels were calculated using a $2^{-\Delta\Delta Ct}$ method. The normalization was achieved by using U6 and GAPDH as the internal control of miR-382-5p and SLC7A11 mRNA. The primers are as follows: SLC7A11 sense: 5'- TCTCCAAAGGAGGTTACCTGC -3' and anti-sense: 5'- AGACTCCCCTCAGTAAAGTGAC -3'; miR-382-5p sense: 5'-ACACTCCAGCTGGGAAAGTGCTTCCC-3' and anti-sense: 5'-CTCAACTGGTGTCGTGGA-3'; GAPDH sense: 5'-TGGGTGTGAACCACGAGAA-3' and anti-sense: 5'-GGCATGGACTGTGGTCATGA-3'; U6, sense 5'-GCTTCGGCAGCA CATATAATAAAAT-3'; anti-sense 5'-CGCTTCACGAATTTG CGTGTCAT-3'.

Luciferase Reporter Gene

The predicted interaction and binding site between miR-382-5p and SLC7A11 was obtained by TargetScan website (http://www.targetscan.org/vert_72/). To clarify the interaction between miR-382-5p and SLC7A11, the luciferase reporter plasmids psiCHECK-2 (Promega, United States) inserted with wide type (WT) or mutated (Mut) 3'UTR of SLC7A11 were constructed. The T47D and SKOV-3 cells were transfected with SLC7A11-WT or SLC7A11-Mut together with miR-382-5p mimics or NC and pRL-TK as the internal control. The luciferase activity was measured in a microplate reader (PerkinElmer) by using a dual-luciferase assay system (Promega) under the manufacturer's instruction.

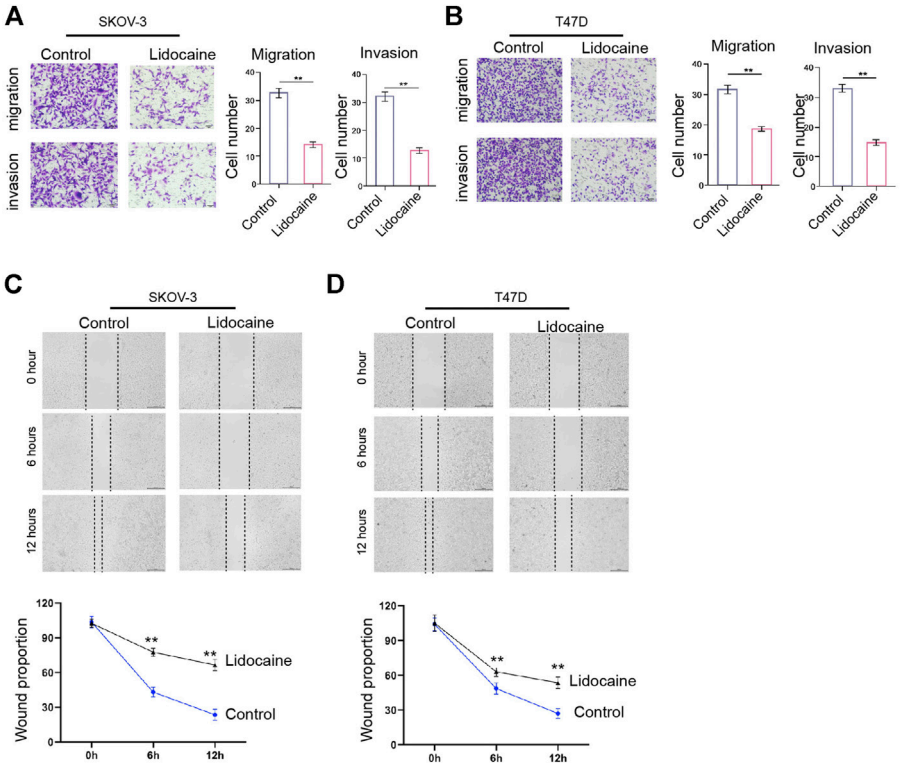

FIGURE 3 | Lidocaine suppresses invasion and migration of ovarian and breast cancer cells *in vitro*. **(A–D)** SKOV-3 and T47D cells were treated with lidocaine (3 mM). **(A,B)** The cell invasion and migration were measured by transwell analysis. **(C,D)** The cell migration was analyzed by wound healing analysis. mean ± SD, $^{**}p < 0.01$. The experiments were performed independently three times.

Xenograft Assays

All animal experiments were performed under the approvement of Animal Ethics Committee of the Cangzhou Central Hospital. SPF-level male nude mice aged 5–6 weeks and weighted around 20 g were purchased from Vitalriver (China). All mice were maintained in a 12-hour circadian rhythm, and had free access to water and food. Cancer cells were subcutaneously injected into the right flank of mice. Lidocaine was administrated to mice at a dose of 1.5 mg per kg injected through the vail tails. For control group, the mice were treated with saline. Tumor volume and mice body weight were monitored every 5 days. The tumor size was calculated *via* the following formula: length × width^2/2.

Statistics

Each experiment was performed at least three times. Date was shown as mean ± standard deviation (SD), and analyzed by SPSS software (version 19.0). The statistical comparison between groups were conducted *via* Student's t-tests or one-way analyses of variance (ANOVA). $p < 0.05$ was considered as statistically significant.

RESULTS

Lidocaine Induces Ferroptosis of Ovarian and Breast Cancer Cells

We were interested in the function of lidocaine in regulating ferroptosis of ovarian and breast cancer cells. Our data showed that lidocaine inhibited SLC7A11 mRNA expression in a dose-dependent manner, in which 3 mM lidocaine presented the highest effect and was selected in the subsequent analysis (**Figures 1A,B**). The co-treatment of lidocaine and erastin was able to enhance effect of erastin on the inhibition of SKOV-3 and T47D cells (**Figures 1C,D**). Meanwhile, total iron (iron) and ferrous iron (Fe^{2+}) were analyzed in the cells. The levels of Fe^{2+} and iron were induced by lidocaine in SKOV-3 and T47D cells (**Figures 1E,F**). In addition, lidocaine promoted lipid ROS accumulation in SKOV-3 and T47D cells (**Figure 1G**). The expression of SLC7A11 and GPX4 was repressed by lidocaine in SKOV-3 and T47D cells (**Figure 1H**). Taken together, these data suggest that lidocaine induces ferroptosis of ovarian and breast cancer cells.

Lidocaine Reduces the Proliferation of Ovarian and Breast Cancer Cells *In Vitro*

We then observed that the SKOV-3 and T47D cell viabilities were reduced by lidocaine (**Figures 2A,B**). Consistently, the colony formation numbers of SKOV-3 and T47D cells were suppressed by the treatment of lidocaine (**Figures 2C,D**). The apoptosis of SKOV-3 and T47D cells was induced by lidocaine (**Figures 2E,F**), indicating that lidocaine reduces the proliferation of ovarian and breast cancer cells *in vitro*. Moreover, the expression of apoptosis and necroptosis markers was detected. We found that Bcl-2

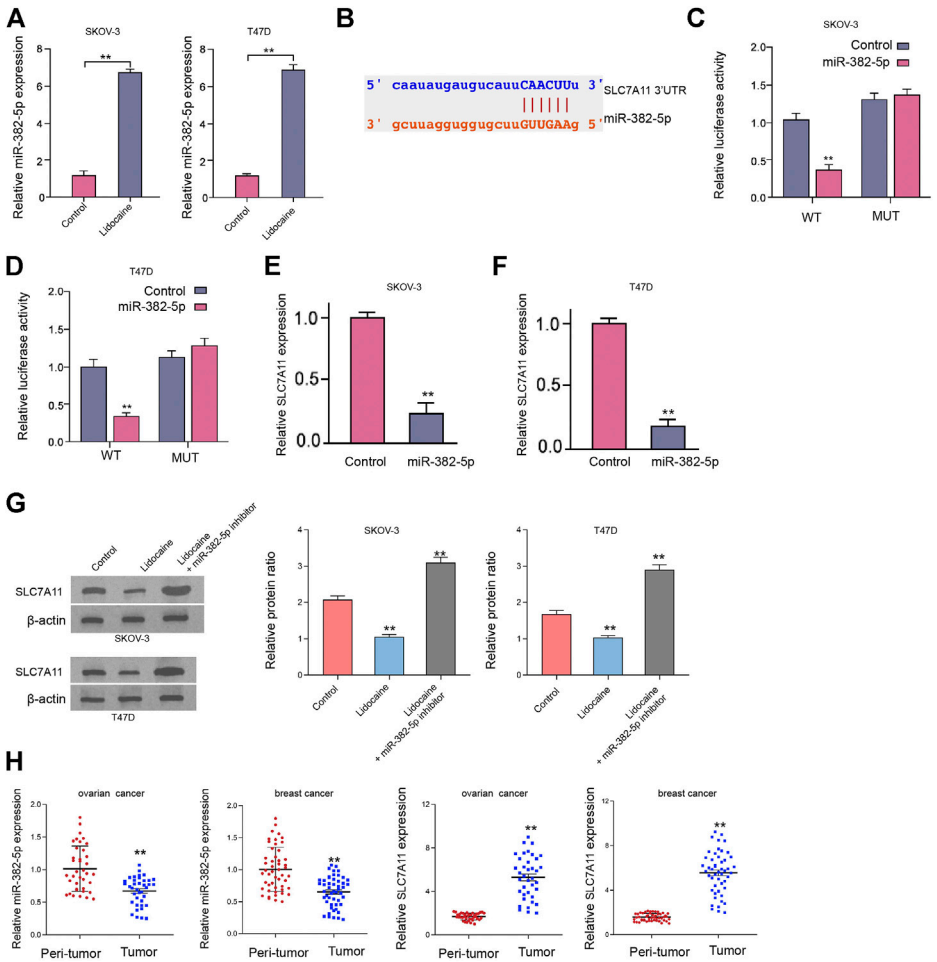

FIGURE 4 | Lidocaine inhibits SLC7A11 expression by upregulating miR-382-5p. **(A,B)** SKOV-3 and T47D cells were treated with lidocaine (3 mM). The expression of miR-382-5p was analyzed by qPCR assays. **(C–F)** SKOV-3 and T47D cells were treated with miR-382-5p mimic. **(C,D)** The luciferase activity of SLC7A11 mRNA 3'UTR was analyzed. **(E,F)** The mRNA levels of SLC7A11 were examined by qPCR. **(G)** The protein expression of SLC7A11 was detected by Western blot analysis in SKOV-3 and T47D cells co-treated with lidocaine and miR-382-5p inhibitor. **(H)** The expression of miR-382-5p and SLC7A11 was detected by qPCR in clinical ovarian cancer tissues (n = 38) and breast cancer tissues (n = 50) and the related adjacent tissues. mean ± SD, $^{**}p < 0.01$. The experiments were performed independently three times.

expression was repressed and Bax and cleaved-caspase3 expression was induced by lidocaine in SKOV-3 and T47D cells (**Figure 2G**). Meanwhile, the treatment of lidocaine failed to affect necroptosis marker RIPK3 expression in the cells (**Figure 2H**). Together these results indicate that lidocaine reduces the proliferation of ovarian and breast cancer cells *in vitro*.

Lidocaine Suppresses Invasion and Migration of Ovarian and Breast Cancer Cells *In Vitro*

Next, we also found that the treatment of lidocaine attenuated invasion and migration of SKOV-3 and T47D cells (**Figures 3A,B**). Similarly, the wound healing abilities of SKOV-3 and T47D cells were repressed by the treatment of lidocaine *in vitro* (**Figures 3C,D**). Together these results imply that lidocaine suppresses invasion and migration of ovarian and breast cancer cells *in vitro*.

Lidocaine Inhibits SLC7A11 Expression by Upregulating miR-382-5p

Next, our data demonstrated that the expression of miR-382-5p was up-regulated by lidocaine in SKOV-3 and T47D cells (**Figure 4A**). We predicted the binding site of miR-382-5p and SLC7A11 in a bioinformatic analysis (**Figure 4B**). The mRNA expression along with the luciferase activity of SLC7A11 were inhibited by miR-382-5p mimic in SKOV-3 and T47D cells (**Figures 4C–F**). The protein levels of SLC7A11 were repressed by the treatment of lidocaine while the inhibition of miR-382-5p blocked the effect of lidocaine in SKOV-3 and T47D cells (**Figure 4G**). The miR-382-5p expression was down-regulated but SLC7A11 expression was up-regulated in clinical ovarian cancer tissues (n = 38) and breast cancer (n = 50) tissues compared with the adjacent tissues (**Figure 4H**). Taken together, these data suggest that lidocaine inhibits SLC7A11 expression by upregulating miR-382-5p.

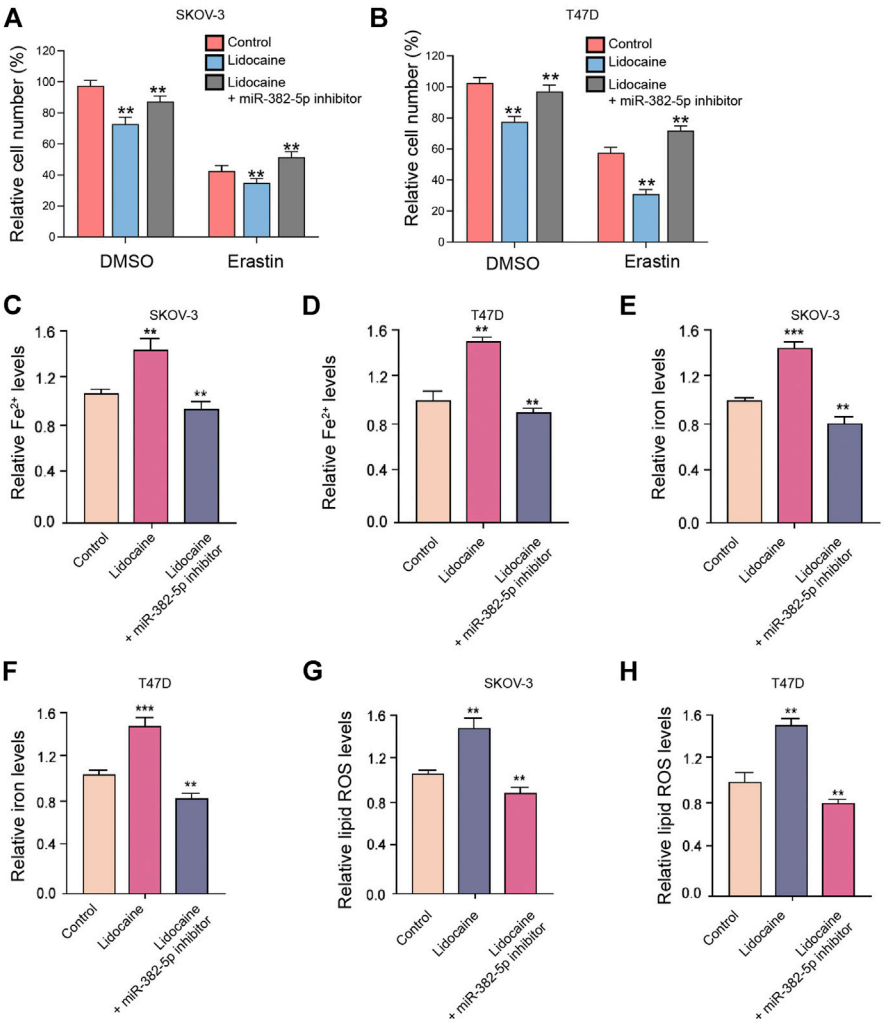

FIGURE 5 | The inhibition of miR-382-5p blocks lidocaine-induced ferroptosis of ovarian and breast cancer cells. **(A,B)** The erastin (5 mmol/L) -stimulated SKOV-3 and T47D cells were co-treated with miR-382-5p inhibitor and lidocaine (3 mM). The cell viability was detected by MTT assays after 48 h of the treatment. **(C–H)** SKOV-3 and T47D cells were co-treated with miR-382-5p inhibitor and lidocaine. The Fe^{2+} **(C,D)**, iron **(E,F)**, and lipid ROS levels **(G,H)** were detected. mean ± SD, $^{**}p < 0.01$. The experiments were performed independently three times.

Moreover, we identified that the SKOV-3 and T47D cell viabilities were reduced by miR-382-5p (**Supplementary Figures S1A,B**). The colony formation numbers of SKOV-3 and T47D cells were suppressed by the treatment of miR-382-5p mimic (**Supplementary Figures S1C,D**). The apoptosis of SKOV-3 and T47D cells was induced by miR-382-5p (**Supplementary Figures S1E,F**). The levels of Fe^{2+}, iron, lipid ROS were induced by miR-382-5p in SKOV-3 and T47D cells (**Supplementary Figures S1G–I**). Together these results indicate that miR-382-5p represses proliferation and induces ferroptosis of ovarian and breast cancer cells *in vitro*.

The Inhibition of miR-382-5p Blocks Lidocaine-Induced Ferroptosis of Ovarian and Breast Cancer Cells

Next, we found that miR-382-5p inhibition rescued cell viabilities repressed by lidocaine in erastin-stimulated SKOV-3 and T47D cells (**Figures 5A,B**). Moreover, the levels of Fe^{2+}, iron, and lipid ROS were enhanced by the treatment of lidocaine, in which the miR-382-5p inhibitor reversed these levels in SKOV-3 and T47D cells (**Figures 5C–H**).

We then showed that the treatment of lidocaine inhibited cell proliferation and stimulated cell apoptosis of SKOV-3 and T47D cells while miR-382-5p inhibitor or SLC7A11 overexpression was able to reverse the effect of lidocaine on SKOV-3 and T47D cell proliferation and apoptosis *in vitro* (**Figures 6A–D**). Taken together, these data suggest that inhibition of miR-382-5p blocks lidocaine-induced ferroptosis of ovarian and breast cancer cells.

Lidocaine Attenuates Proliferation of Ovarian Cancer Cells *In Vivo*

The tumorigenicity analysis further demonstrated that the tumor growth of SKOV-3 cells was attenuated by the

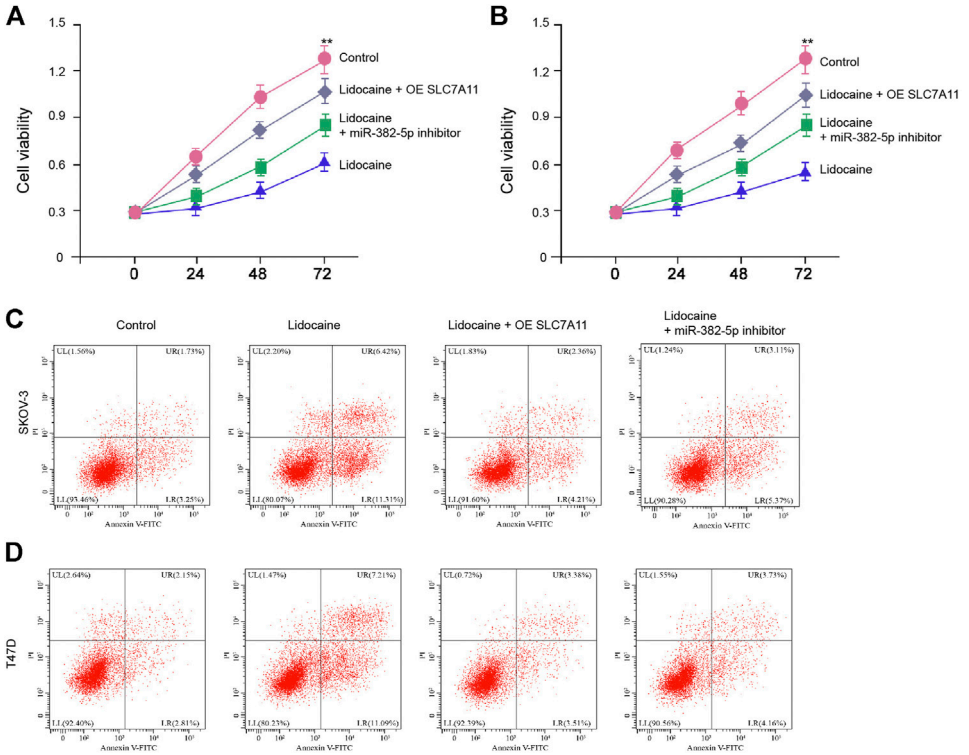

FIGURE 6 | Lidocaine/miR-382-5p axis reduces the proliferation of ovarian and breast cancer cells by targeting SLC7A11 *in vitro*. **(A–D)** SKOV-3 and T47D cells were co-treated with lidocaine (3 mM) and miR-382-5p inhibitor or SLC7A11 reconstitution vectors. **(A,B)** The cell viability was detected by MTT assays. **(C,D)** The apoptosis was analyzed by flow cytometry. mean ± SD, $^{**}p < 0.01$. The experiments were performed independently three times.

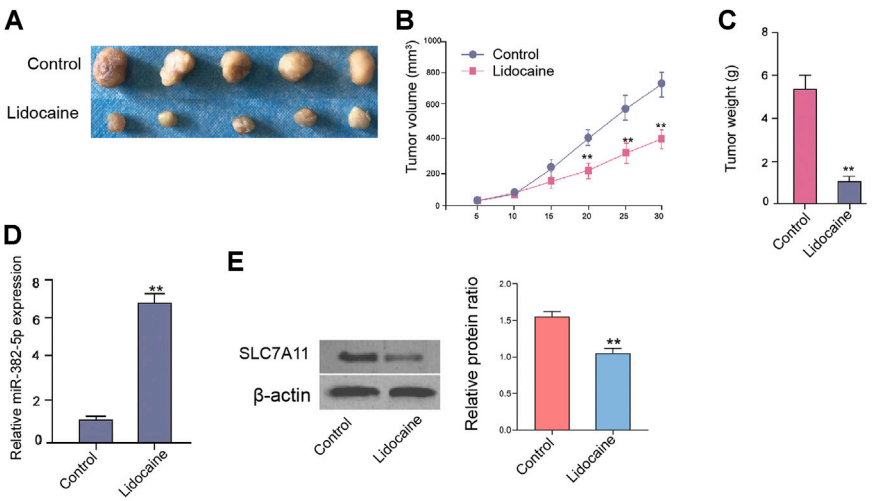

FIGURE 7 | Lidocaine attenuates proliferation of ovarian cancer cells *in vivo*. The nude mice were injected with SKOV-3 cells and treated with lidocaine (1.5 mg/kg). The tumor tissues **(A)**, tumor volume **(B)**, and tumor weight **(C)** were shown. **(D)** The expression of miR-382-5p was analyzed by qPCR assays. **(E)** The protein expression of SLC7A11 was detected by Western blot analysis. The results were quantified using ImageJ software. N = 5, mean ± SD, $^{**}p < 0.01$.

treatment of lidocaine in the nude mice (**Figures 7A–C**). As expected, the expression of miR-382-5p was enhanced and SLC7A11 expression was reduced in the tumor tissues of lidocaine-treated mice compared with that in control group (**Figures 7D,E**). Together these results indicate that lidocaine attenuates proliferation of ovarian cancer cells *in vivo*.

DISCUSSION

Ovarian and breast cancer are severe and common female malignancies with high recurrence and metastasis rates. Despite the anesthetic lidocaine has been identified to present potential anti-tumor effects, the functions of lidocaine in ovarian and breast cancer are unclear. In the present study, we reported a critical role of lidocaine in modulating ferroptosis of ovarian and breast cancer cells.

Multiple local anesthetics, such as bupivacaine and levobupivacaine, have presented the inhibitory effect on cancer development (Xuan et al., 2016; Li et al., 2019). The Regional anesthesia potentially benefits the clinical outcomes of cancer patients (Xuan et al., 2015; Wall et al., 2019). Lidocaine has demonstrated significant anti-cancer activities in the previous investigations. It has been reported that lidocaine represses cytotoxicity resistance by down-regulating miR-21 in DDP-resistant lung cancer cells (Yang et al., 2019). Lidocaine enhances apoptosis and reduces proliferation by inducing miR-520a-3p expression to inhibit EGFR in colorectal cancer cells (Qu et al., 2018). Lidocaine enhances apoptosis and reduces proliferation of cervical cancer cells by targeting the lncRNA MEG3/miR-421/BTG1 axis (Zhu and Han, 2019). Our data further found that lidocaine induced an inhibitory effect on ovarian and breast cancer cell proliferation *in vitro* and *in vivo*. It identifies an unreported function of lidocaine in attenuating the progression of female cancers, including ovarian and breast cancer. The clinical application of lidocaine in the treatment of ovarian and breast cancer are needed to prove in detail. Ferroptosis, as an emerging programmed cell death, plays critical functions in both of ovarian and breast cancer. These reports indicate that targeting ferroptosis may be the potential anti-tumor therapies in ovarian and breast cancer. Frizzled-7 regulates platinum-resistant ovarian cancer cells by modulating ferroptosis (Wang et al., 2021). Stearoyl-CoA desaturase 1 reduces ferroptosis of ovarian cancer cells (Tesfay et al., 2019). Ferroptosis is enhanced by the treatment of lapatinib and siramesine in breast cancer (Ma et al., 2016). The PI3K/AKT/mTOR signaling inhibits ferroptosis by SREBP-regulated lipogenesis in breast cancer (Yi et al., 2020). In this study, we found that lidocaine induced ferroptosis of both ovarian and breast cancer cells. It suggests that lidocaine may inhibit malignant progression of ovarian and breast cancer by stimulating ferroptosis, providing the valuable evidence of the relationship of lidocaine and ferroptosis. Moreover, there are some limitations of this study. For example, more direct evidence about the effect of lidocaine on ferroptosis and apoptosis need to explore in future investigations. In addition, the function of lidocaine in the modulation of ferroptosis and apoptosis should be validated in mouse model. Meanwhile, circRNA-UBAP2 represses apoptosis and contributes to the proliferation of ovarian cancer by miR-382-5p/PRPF8 axis (Xu et al., 2020). SNHG1 promotes invasion and proliferation of breast cancer by targeting miR-382 (Zheng et al., 2019). Furthermore, it has been identified that miR-382-5p can induces apoptosis by targeting PRPF8 and SPIN1 in ovarian cancer and lung cancer, respectively (Chen et al., 2020; Xu et al., 2020). Our data showed that lidocaine up-regulated the expression of miR-382-5p to reduce SLC7A11 expression. The inhibition of miR-382-5p blocked lidocaine-mediated ferroptosis in ovarian and breast cancer cells. Our finding indicates a new correlation of miR-382-5p with lidocaine in the regulation of ferroptosis during cancer development. MiR-382-5p/SLC7A11 axis may be just one of the downstream mechanisms underlying lidocaine-repressed cancer progression and more mechanisms are needed to be explored in future investigation.

Consequently, we concluded that the lidocaine promoted ferroptosis by miR-382-5p/SLC7A11 axis in ovarian and breast cancer cells. The clinical value of lidocaine in the treatment of ovarian and breast cancer deserves to be proved in detail.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Cangzhou Central Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Cangzhou Central Hospital.

AUTHOR CONTRIBUTIONS

DS designed the study; Y-CL performed experiments; X-YZ collected and analyzed data; Y-CL and X-YZ wrote the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.681223/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 02 August 2021
doi: 10.3389/fonc.2021.688896

Perioperative Intravenous Lidocaine and Metastatic Cancer Recurrence - A Narrative Review

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Cancer is a major global health problem and the second leading cause of death worldwide. When detected early, surgery provides a potentially curative intervention for many solid organ tumours. Unfortunately, cancer frequently recurs postoperatively. Evidence from laboratory and retrospective clinical studies suggests that the choice of anaesthetic and analgesic agents used perioperatively may influence the activity of residual cancer cells and thus affect subsequent recurrence risk. The amide local anaesthetic lidocaine has a well-established role in perioperative therapeutics, whether used systemically as an analgesic agent or in the provision of regional anaesthesia. Under laboratory conditions, lidocaine has been shown to inhibit cancer cell behaviour and exerts beneficial effects on components of the inflammatory and immune responses which are known to affect cancer biology. These findings raise the possibility that lidocaine administered perioperatively as a safe and inexpensive intravenous infusion may provide significant benefits in terms of long term cancer outcomes. However, despite the volume of promising laboratory data, robust prospective clinical evidence supporting beneficial anti-cancer effects of perioperative lidocaine treatment is lacking, although trials are planned to address this. This review provides a state of the art summary of the current knowledge base and recent advances regarding perioperative lidocaine therapy, its biological effects and influence on postoperative cancer outcomes.

Keywords: cancer, recurrence, anaesthesia, surgery, local anaesthetics, lidocaine

OPEN ACCESS

Edited by:
Daqing Ma,
Imperial College London,
United Kingdom

Reviewed by:
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

Received: *31 March 2021*
Accepted: *15 July 2021*
Published: *02 August 2021*

Citation:
Wall TP and Buggy DJ (2021) Perioperative Intravenous Lidocaine and Metastatic Cancer Recurrence - A Narrative Review. Front. Oncol. 11:688896. doi: 10.3389/fonc.2021.688896

INTRODUCTION

The burden of cancer as a global health issue is enormous – with an estimated 18.1 million new cases and 9.6 million related deaths in 2018, it is the second leading cause of death worldwide (1). Although the discovery of new chemotherapeutic agents and radiotherapy techniques continues to promise significant results, surgery is the mainstay of treatment for the majority of solid tumours that are detected prior to metastasis. Indeed, over 80% of all patients diagnosed with cancer will undergo a surgical procedure of some nature, for diagnostic or therapeutic purposes, with approximately 45 million surgical procedures estimated to be required per year by 2030 (2). Unfortunately, and despite optimal care, cancer often recurs following intended curative surgery in

the form of metastatic disease. Metastatic cancer is typically refractory to treatment and is the most common cause of death in cancer patients (3). Therefore the importance of minimising recurrence risk is paramount. The physiological stress response induced by surgery stimulates inflammation and angiogenesis, eventually leading to fibrosis and wound healing. Paradoxically, these pro-inflammatory and pro-angiogenic stimuli also facilitate the survival and proliferation of residual cancer cells (4, 5). In recent years other perioperative events and conditions have been suspected of modifying the risk of metastatic disease development. Factors such as hypothermia, blood transfusion, and use of open (rather than minimally-invasive) surgical techniques are hypothesised as having detrimental effects on recurrence risk (6–8). Among these modifiable factors is the choice of anaesthetic and analgesic agents used perioperatively (9). A large volume of laboratory research has identified numerous pro- and anti-neoplastic effects associated with commonly used anaesthetic agents (10). Some retrospective clinical evidence has also pointed to a beneficial effect on cancer outcomes associated with the choice of anaesthetic used (e.g. intravenous agents such as propofol versus inhalational agents such as sevoflurane) (11, 12). There are multiple biologic pathways through which these agents may exert such effects, with modulation of the immune and inflammatory responses, as well as direct effects on cancer cells among the most likely candidates (13). The following sections will outline the perioperative use of lidocaine and our current understanding of the pathophysiology underlying postoperative cancer recurrence, before summarising recent laboratory, preclinical and clinical studies as well as planned trials examining lidocaine's influence on cancer biology and outcomes.

METHODS

The keywords 'lidocaine cancer' were used to search the Medical Literature Analysis and Retrieval System (MEDLINE), Excerpta Medica database (EMBASE) and Web of Science databases. Studies from 1 January 2000 until 10 March 2021 were included as well as any referenced articles deemed significant irrespective of publication date. Randomised controlled trials, retrospective studies, meta-analyses and systematic reviews were included. Articles were assessed for relevance and data were qualitatively analysed.

PHYSICOCHEMICAL PROPERTIES OF LIDOCAINE AND CLINICAL USES

Lidocaine (or 2-diethylaminoaceto-2',6'xylidide, $C_{14}H_{22}N_{2O}$) is the prototype amide local anaesthetic (LA) and clinically used both as an anaesthetic and analgesic agent, as well as an anti-arrhythmic. Lidocaine principally acts by blocking voltage-gated sodium channels, preventing the rapid influx of sodium required to depolarise the cell and thereby blocking neural impulse conduction. Hence, the transmission of pain signals from peripheral tissue to the central nervous system (CNS) is blocked (14). Lidocaine also possesses activity at a wide range of other ion channels and cell receptors which potentially contributes to its observed analgesic effects (15). Compared to the other amide LAs (e.g. bupivacaine), lidocaine has a shorter half-life and is less toxic - as a result, it is the only amide LA compatible with intravenous administration. Lidocaine toxicity manifests as CNS involvement (tinnitus, altered consciousness, seizures, coma) followed by cardiac signs (arrhythmias potentially resulting in cardiac arrest). Toxicity is rare when plasma concentrations are maintained below 5 $\mu g.ml^{-1}$ (~22μM) (16). In the perioperative setting, lidocaine is typically administered either systemically (intravenously) or to provide regional anaesthesia. Systemic lidocaine is given as an infusion during surgery (and often continued post-operatively) primarily for analgesic purposes; intravenous use has also been associated with faster return of gastrointestinal motility following bowel surgery, although evidence remains uncertain (17). One suggested regime consists of a maximum loading dose of 1.5 $mg.kg^{-1}$ followed by a maximal infusion rate of 1.5 $mg.kg^{-1}.hr^{-1}$ for up to 24 hours (18), although 2 $mg.kg^{-1}.hr^{-1}$ may achieve better analgesic effects (19). Resultant plasma concentrations are in the range of 0.5 – 5 $\mu g.ml^{-1}$ (2 – 22μM) (20).

PATHOPHYSIOLOGIC BASIS OF POSTOPERATIVE CANCER RECURRENCE

Surgery, Circulating Tumour Cells and the Pre-Metastatic Niche

Metastasis begins when cancer cells are liberated from the primary tumour, enter the lymphatics or bloodstream (forming circulating tumour cells, CTCs) and subsequently seed distant tissues. Intraoperatively, CTCs may inadvertently be created when cancer cells are dislodged during tumour manipulation (**Figure 1**). Even after CTCs deposit in remote tissues, much adversity has to be overcome to successfully endure hostile immune surveillance and inadequate local homeostatic supports. Cancers, however, possess a remarkable ability to precondition distant organs to form pre-metastatic niches (PMNs) to aid the future survival and proliferation of arriving CTCs (5). PMNs are pre-programmed by extracellular vesicles (EVs) released by the primary tumour - these are cell-derived, membranous structures containing proteins, lipids, messenger RNAs and microRNAs (21, 22). MicroRNAs in particular are potent contributors to PMN formation, allowing malignancies to achieve remote 'epigenetic regulation' by altering gene expression in PMN cells to establish a pro-neoplastic milieu facilitating vascular permeability, angiogenesis and stromal degradation (5, 23, 24).

Influence of Inflammation, Angiogenesis and the Surgical Stress Response on Cancer Progression

Surgery may not only disseminate tumour cells - it further promotes cancer development *via* the surgical stress response,

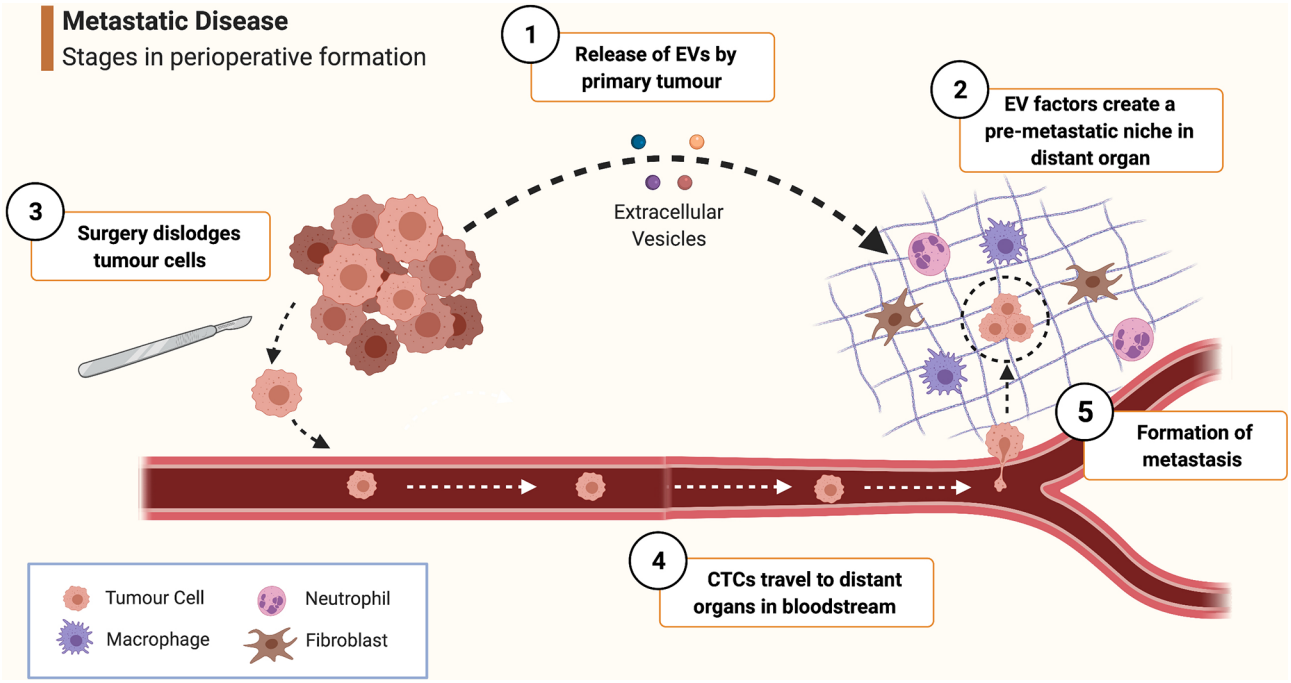

FIGURE 1 | Schematic overview of pathophysiological mechanisms involved in peri-operative metastasis formation. ① As it develops, the primary tumour releases extracellular vesicles (EVs) containing growth factors, miRNAs etc. ② EV-contained factors create a pre-metastatic niche in distant organs by stimulating local cells such as fibroblasts, macrophages and mesenchymal stem cells to promote pro-neoplastic processes such as angiogenesis, inflammation and stromal remodelling. ③ During surgery, malignant cells are dispersed from the primary tumour and are released into the bloodstream to form circulating tumour cells (CTCs). ④ CTC are borne in the circulation to distant tissue beds where they arrest and extravasate into a pre-metastatic niche. ⑤ Survival conditions for the tumour cell are rendered even more favourable by the effects of mediators of the surgical stress response and inflammation, furthering the processes of angiogenesis, immune evasion etc. thus enabling the cancer cell to survive and proliferate and eventually form a clinically significant metastasis. (Created with BioRender®).

inflammation and immunosuppression. Although vital for wound healing to occur, these physiological processes are strongly implicated in driving cancer progression; indeed, cancer has been called 'a wound that does not heal' (25). These processes may also cause previously formed micro-metastases to awake from dormancy and develop into significant metastatic disease. Thus excising cancerous tissue creates conditions which enhance the malignant potential of remaining cells (6).

Inflammation

Tissue injury creates an inflammatory state necessary to recruit and activate the cellular components responsible for wound healing (10). Macrophages and dendritic cells are activated and produce chemokines and pro-inflammatory cytokines including interleukins (such as IL-1, IL-1β, IL-6, IL-8, IL-12), tumour necrosis factor alpha (TNFα), and prostaglandins (26). Rapid increases in inflammatory mediators not only promotes local tissue healing but also stimulates cancer cell survival and proliferation (27). The immune system and the sensory nervous system (SNS) are tightly integrated: pro-inflammatory cytokines modulate pain transmission, causing peripheral and central pain sensitisation, increasing SNS and hypothalamic-pituitary-adrenal (HPA) axis outflow, in turn stimulating cytokine expression by immune cells (28). Expression of numerous signalling pathway elements are altered in the post-surgical inflammatory milieu, many of which are associated with cancer progression, including enzymes such as cyclo-oxygenase-2 (COX-2) and matrix-metalloproteinases (MMPs), and transcription factors such as nuclear factor kappa-beta (NF-κB) (29). Inflammatory cytokines impair endothelial integrity and endothelial function has been demonstrated to deteriorate for several days following surgery (30). Loss of endothelial function enables leucocyte transmigration and potentially facilitates the extravasation of CTCs into remote tissues (31). The tyrosine kinase enzyme Src contributes to this process *via* its actions as an important regulator of endothelial barrier integrity (32). Src is activated by inflammatory mediators, including TNFα, resulting in disruption of tight junctions between endothelial cells and eventual loss of endothelial function (32).

Angiogenesis

Surgical tissue injury causes localised tissue hypoxia, resulting in upregulation of hypoxia-inducible factor (HIF), in turn stimulating expression of vascular endothelial growth factor (VEGF). VEGF drives the synthesis of numerous tissue components involved in angiogenesis including integrins and extracellular matrix (33). Similarly, rapid growth of cancerous tissue creates a hypoxic cellular microenvironment, stimulating HIF and VEGF expression to create new blood vessels to supply the oxygen and nutrients necessary for further neoplastic

expansion. Overexpression of HIF and VEGF is associated with poorer prognosis in certain cancer types, including pancreatic and ovarian cancer (34, 35).

The Surgical Stress Response and Immunosuppression

The innate and adaptive components of the immune system act in unison to eliminate cancerous cells. Natural killer (NK) cells of the innate system, and T-cells (helper CD4+ Th1 cells and cytotoxic CD8+ T-cells) of the adaptive system provide cell-mediated immunity (CMI), the most important cellular anti-cancer immune response (36). This activity is influenced by post-operative pathophysiological changes - the initial inflammatory state is followed by a period of immunosuppression during which CMI is diminished (37). When the surgical stimulus activates the SNS and HPA axis, cortisol and catecholamines are released which inhibit the anti-tumour activity of NK cells and CD8+ T cells (6, 38). NK cytotoxicity is also reduced by increases in IL-6 and prostaglandin E2 (39). CMI is influenced by helper T-lymphocytes, which can be divided into two groups: Th1 cells favouring an anti-cancer CMI effect, and Th2 cells favouring antibody-mediated immunity (40). Post-operatively, Th2 proliferation increases, shifting the Th1/Th2 balance from a Th1-predominant CMI phenotype towards Th2 dominance, protecting cancer cells from immune attack (6).

Once considered relatively passive players, mounting evidence points to neutrophils having complex yet crucial roles in carcinogenesis (41). Circulating neutrophil counts are often increased by the post-operative inflammatory state, leading to an increased neutrophil-to-lymphocyte ratio (NLR) (42). NLR elevation is associated with poorer survival in some cancers – but whether this reflects causation or merely correlation is unclear (43). Circulating neutrophils can migrate into the tumour microenvironment where they adopt an anti- or pro-tumour phenotype, termed N1 and N2 respectively (44). N1 neutrophils phagocytose cancer cells whereas N2 neutrophils promote cancer in numerous ways, including reshaping stroma by expressing VEGF or MMP-9 (45). Neutrophils can also extrude decondensed chromatin to form web-like structures called neutrophil extracellular traps (NETs) (46). This process (termed NETosis) is implicated in neoplasia with elevated serum markers of NETosis associated with poorer prognosis in certain cancers, and poorer post-operative outcomes in metastatic colorectal cancer (47, 48). How NETs promote metastasis is unclear - NETs may sequester CTCs without killing them, facilitating their arrest and possibly shielding them from cytotoxic immune cells (49, 50).

EXPERIMENTAL EVIDENCE OF LIDOCAINE'S ANTINEOPLASTIC EFFECTS

Lidocaine's ability to inhibit cancer biology *in vitro* has been recognised for many years. Four decades ago, researchers identified that lidocaine exposure enhanced the cytotoxic effects of chemotherapeutic agents on cancer cells, with some authors attributing this phenomenon to inhibition of DNA damage repair (51). Since then, many cancers have been examined with numerous possible mechanisms of action proposed (52). To date, the accumulated evidence from many laboratory studies (**Table 1**) suggests that lidocaine possesses anti-neoplastic effects exerted *via* multiple biological pathways or components within cancer cells, and not just *via* voltage-gated sodium channels (31). In addition to direct effects on cancer cells, lidocaine also possesses anti-inflammatory properties which may modulate the pro-cancer effects of the stress response and preserve or enhance immune cell function (**Figure 2**) (82). Although *in vitro* studies are useful for establishing biological plausibility, their findings are not automatically transferrable to *in vivo* settings (83). Laboratory studies have often used human-toxic lidocaine concentrations, limiting the clinical applicability of their results. In addition, cancer in a host exists in a complex inter-relationship of cells, stroma, and cytokines, which is impossible to replicate *in vitro*. Lidocaine's effect on cancer *in vivo* has historically been infrequently examined; however, results from several recent animal studies have supported the largely beneficial effects of lidocaine observed *in vitro* (**Table 2**).

EFFECTS ON CANCER CELL BIOLOGY

Effects on Bax/Bak/Bcl-2 and Apoptosis

Whether a damaged or pre-cancerous cell undergoes programmed cell death or not depends on the intracellular balance between pro- and anti-apoptotic mediators. The pro-apoptotic proteins, Bax and Bak, induce the mitochondrial release of cytochrome c and other apoptosis-regulating factors (94). These in turn activate caspases (proteolytic enzymes) which degrade cellular components causing cell fragmentation and phagocytosis by macrophages (94, 95). Countering Bax and Bak is the protein Bcl-2 which exerts anti-apoptotic effects favouring cell survival (96). Aberrant regulation of these pathways is linked to carcinogenesis. Lidocaine has been shown to induce apoptosis in multiple cancer cell lines *in vitro* across numerous studies (**Table 1**). Ye et al. observed that lidocaine inhibited gastric cancer cell proliferation, migration and invasion as well as promoting apoptosis – a finding associated with decreased Bcl-2 and increased Bax expression (62). Similar lidocaine-induced alterations in the Bax/Bcl-2 ratio to favour apoptosis were also detected in lung cancer cells (76). Separate studies examining osteosarcoma, thyroid cancer and hepatocellular carcinoma cells found that lidocaine-associated apoptosis was accompanied not only by alteration of the Bax/Bcl-2 ratio but also activation of caspases (80, 81, 93).

Effects on EGFR and the MAPK Pathway

The epidermal growth factor receptor (EGFR) is a widespread transmembrane receptor activated by a number of extracellular ligands including the mitogens EGF and TGF-α. Binding of ligands activates complex downstream signalling cascades, including mitogen-activated protein kinase (MAPK) systems such as the extracellular signal-regulated kinase (ERK1/2) and p38 pathways (97). This results in DNA transcription and

TABLE 1 | Selected *in vitro* studies examining the effects of lidocaine treatment on cancer cell biology.

First author	Year	Cancer	Anti-neoplastic lidocaine effects detected	Proposed mechanism involved
D'Agostino (53)	2018	Breast	Inhibition of cancer cell migration	Inhibited CXCL12/CXCR4 signalling
Li (54)	2018	Breast	Only high concentration (over toxic concentrations) of lidocaine inhibited affected cell viability or migration	Cancer cells arrested in S phase of cell cycle
Zhu (55)	2019	Cervical	Inhibition of cancer cell viability and promotion of apoptosis	Modulation of lncRNA-MEG3/miR-421/BTG1 signalling
Zhang (56)	2019	Chorio-carcinoma	Lidocaine stimulates apoptosis in high concentrations, potentiation of the cytotoxicity of 5-FU	Reduction of ATP-binding cassette (ABC) transport protein expression
Qu (57)	2018	CRC	Inhibition of cancer cell proliferation and promotion of apoptosis	Suppression of EGFR expression by upregulation of microRNA miR-520a-3p
Siekmann (58)	2019	CRC	High concentration (1000μM) lidocaine reduced cell proliferation but low concentrations promote cell viability in metastatic cell lines	Not assessed
Tat (59)	2019	CRC	Reduced cell proliferation	Altered expression of caspase-8, HSP-27/60, IGF-II, IGF binding protein, p53, survivin
Bundscherer (60)	2017	CRC	Cell cycle arrest induced in two CRC cell lines by 1000μM lidocaine, but no change in cell proliferation noted	Cell cycle arrest
Zhu (61)	2020	Esophageal	Decreases cell growth, migration and survival	Causes mitochondrial dysfunction and oxidative damage, anti-migratory effects linked to decreased Rac1 activity
Ye (62)	2019	Gastric	Inhibition of cancer cell proliferation, migration, invasion and promotion of apoptosis	Decreased Bcl-2 expression, increased Bax expression, alteration of MAPK pathway
Sui (63)	2019	Gastric	Reduced cell viability, proliferation, migration and invasion, promoted apoptosis	Enhanced expression of miR-145, inactivation of MEK/ERK and NF-κB pathways, downregulated Bcl-2 expression, upregulated cleaved caspase-3/-7/-9 expression, decreased MMP-2/-9
Yang (64)	2018	Gastric	Inhibition of cancer cell proliferation and migration	Down-regulation of p-ERK1/2
Zhang (65)	2020	Gastric	Lidocaine inhibited cell migration and invasion, as well as reducing resistance to cisplatin	Inhibition of B-catenin and AKT/mTOR pathways by decreased expression of miR-10b
Izdebska (66)	2019	Glioma (rat)	Increased apoptosis and necrosis of cancer cells	Cytoskeletal reorganisation, possible induction of cytoprotective autophagy
Leng (67)	2017	Glioma (rat)	Lidocaine inhibits glioma cell proliferation	Inhibition of TRPM7 currents
Liu (68)	2018	HCC	Decreased HCC cell viability and colony formation	Upregulation of cytoplasmic polyadenylation element binding protein 3 (CPEB3)
Jurj (69)	2017	HCC	Inhibition of cell proliferation	Reduced expression level of p53
Le Gac (70)	2017	HCC	Lidocaine decreased viability and proliferation of HCC cell, increased apoptosis of HCC progenitor cells	Increased mRNA of APC, an antagonist of the Wnt/B-catenin pathway
Ni (71)	2018	Leukaemia stem cells	Lidocaine inhibited proliferation and colony formation of LSCs	Inhibition of Wnt/B-catenin signalling
Sun (72)	2019	Lung	Inhibited viability, migration, invasion; promotion of apoptosis	Increased expression of miR-539, inhibited EGFR signalling
Zhang (73)	2017	Lung	Reduced cancer cell proliferation	Downregulation of GOLT1A expression
Yang (74)	2019	Lung	Lidocaine reduced cancer cell viability, migration and invasion, as well as reducing resistance of lung cancer cells to cisplatin	Down-regulation of miR-21
Piegeler (75)	2015	Lung	Lidocaine reduced cancer cell invasion	Lidocaine reduced TNFα-induced activation of Akt, FAK, caveolin-1 and reduced MMP-9 secretion.
Dong (76)	2019	Lung	Lidocaine reduced viability of lung ca cells	Increased Bax/Bad expression, decreased Bcl-2 expression
Wang (77)	2016	Lung	Lidocaine decreases viability, invasion, migration and promotes apoptosis in NSCLC cells	Downregulation of $\Delta\Psi$m, provoked DNA damage, upregulated ROS production and activated MAPK pathways
Zheng (78)	2020	Melanoma	Lidocaine inhibited migration and proliferation of melanoma cells and increased apoptosis	Inhibition of small GTPases RhoA, Rac1 and Ras
Wang (79)	2017	Melanoma	Lidocaine sensitizes the cytotoxicity of 5-FU in melanoma cells	Upregulation of miR-493, potentially affecting SOX4-mediated pathways
Mirshahidi (80)	2020	Osteo-sarcoma	Lidocaine reduced viability of cancer cells, increased apoptosis	Bcl-2 and survivin expression decreased; Bax, cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase-1 were increased.
Chang (81)	2014	Thyroid	Decreases cell viability and colony formation, induces apoptosis	Activation of caspase 3/7, alters ratio of Bax/Bcl-2, attenuates ERK1/2 activity, activation of MAPK

promotion of processes leading to cell proliferation, migration and angiogenesis. MAPK pathways also play a role in apoptosis, where highly complex MAPK signalling may have either a pro- or anti-apoptotic effect depending on the cell type and stimulus involved (98). Defective EGFR signalling plays a major role in carcinogenesis, and many oncological therapies specifically target this signalling cascade (99). Lidocaine may also influence EGFR pathways resulting in antineoplastic effects. Researchers found lidocaine increased expression of miR-539 (an EGFR suppressor) in lung cancer cells treated *in vitro* resulting in

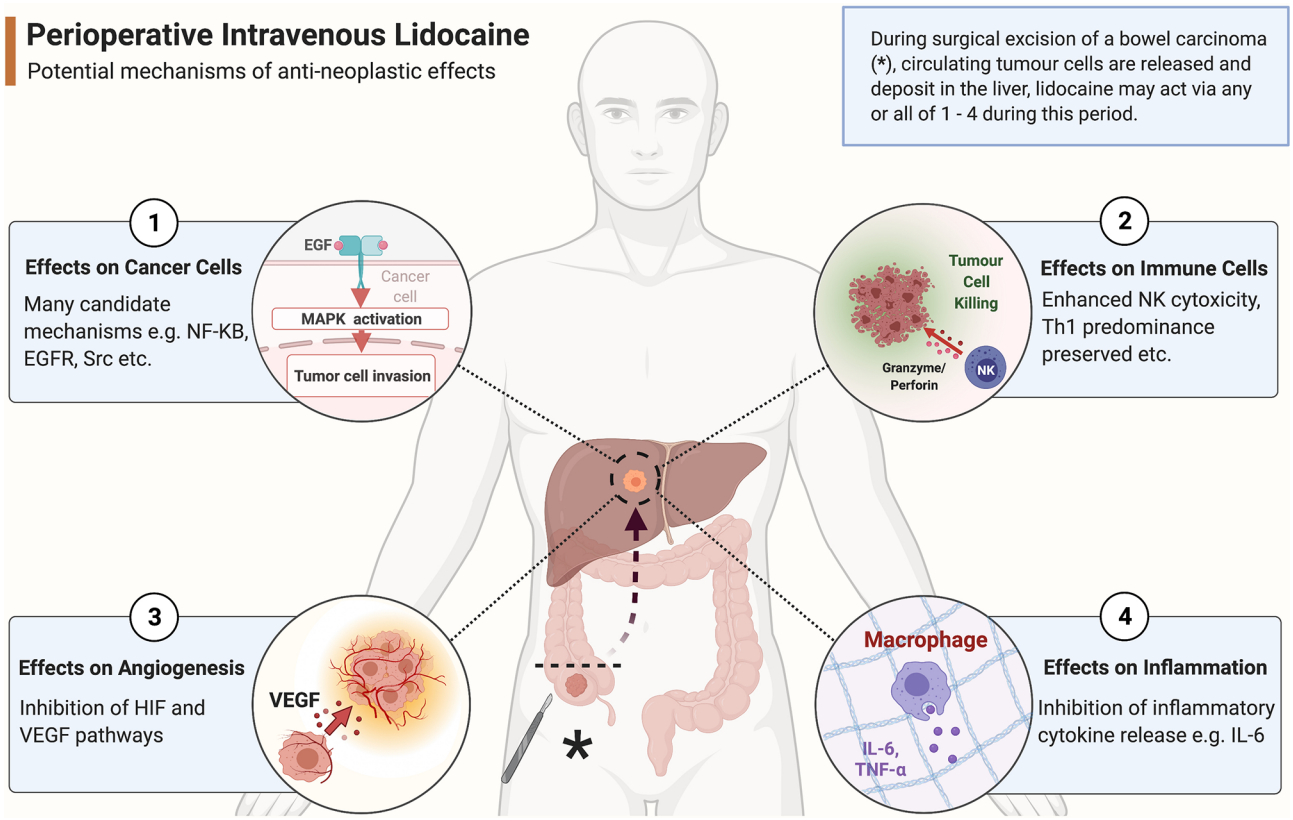

FIGURE 2 | Potential anti-neoplastic mechanisms of action of systemic lidocaine during surgery. As a colonic tumour is excised (marked with *), tumour cells are released into the circulation to form circulating tumour cells (CTCs). These CTCs arrest within liver parenchyma where the likelihood of forming future clinically significant metastatic disease depends on the balance of pro- and anti-neoplastic processes present in the tumour microenvironment. Perioperative systemic lidocaine bathes the tumour cells and their microenvironment during this sensitive period and potentially beneficially alters the odds of host survival *via an* effect on any of ① - ④ outlined in the figure. (Created with BioRender®).

EGFR inhibition and reduced viability, migration and invasion as well as apoptosis (72). To reinforce these findings, the anti-neoplastic effects of lidocaine were attenuated when miR-539 was silenced (72). Another miRNA-based effect on EGFR was detected when the mechanisms by which lidocaine inhibited proliferation and enhanced apoptosis in colorectal cancer cells were examined (57). In this instance miR-520a-3p directly targeted EGFR and lidocaine increased its expression (albeit at 500-1000μM). Similar lidocaine-induced alteration of the miR-520a-3p/EGFR relationship leading to anti-neoplastic effects was also noted in retinoblastoma cells (92).

Alteration of p38 and ERK1/2 pathways have been hypothesised as underlying lidocaine's anti-cancer effects in multiple *in vitro* experiments. In one study, lidocaine was noted to induce p38 phosphorylation in gastric cancer cells alongside an increase in apoptosis and decrease in proliferation, migration and invasion - the authors hypothesising that lidocaine-activated p38-MAPK signalling was the underlying mechanism (62). Other groups detected inactivation of both p38 and ERK1/2 pathways as well as activation of caspase 3 and alteration of the Bcl-2/BAX ratio when HCC cells were treated with lidocaine; in addition, viability was reduced and apoptosis increased in exposed cells (93). Further evidence linking anti-cancer effects of lidocaine to altered ERK signalling has been found in experiments examining gastric cancer, thyroid cancer and melanoma cells (64, 81, 90).

Effects on NF-κB

NF-κB is a protein transcription factor and regulator of numerous cellular processes occurring in response to tissue injury including immune response, inflammation, angiogenesis and apoptosis, in addition to playing a crucial role in cancer development (100). Cell stress signals (such as TNFα-receptor binding) are linked *via* intermediate steps to the translocation of the NF-κB complex into the cell nucleus whereupon transcription of potentially hundreds of target genes is activated or repressed (101). The exact nature of the resultant cellular response depends on complex, context-specific factors including cell type, cell health, and the nature of the stimulus. Adding to the complexity of NF-κB's functions, multiple points of crosstalk exist between the NF-κB pathway and disparate signalling pathways involving transcription factors, microRNAs and cytokines, amongst others (100).

TABLE 2 | Selected in *vivo* studies examining the effects of lidocaine treatment on cancer progression, metastasis or survival.

First Author	Year	Cancer	Study Type	Anti-neoplastic Lidocaine Effects Detected	Proposed Mechanism(s) Involved
Chamaraux-Tran (84)	2018	Breast	*In vitro* & in *vivo* (mouse)	Inhibition of cancer cell migration and viability; improved survival of mice with peritoneal carcinomatosis	Not assessed
Yang (85)	2018	Bladder	*in vitro* & in *vivo* (mouse)	Inhibition of cancer cell proliferation *in vitro*; *in vivo* intravesical lidocaine and mitomycin C combined prolonged survival and reduced bladder weight	Not assessed
Wall (86)	2019	Breast	*In vivo* (mouse)	Reduced post-surgical pulmonary metastasis count	Reduced MMP-2 expression
Johnson (87)	2018	Breast	*In vivo* (mouse)	Reduced post-surgical pulmonary metastasis count	Reduced pro-inflammatory and pro-angiogenic cytokine expression
Freeman (88)	2018	Breast	*In vivo* (mouse)	Decreased post-surgical pulmonary metastasis count when combined with cisplatin	No attributable change in cytokine expression detected
Freeman (89)	2018	Breast	*In vivo* (mouse)	Reduced post-surgical pulmonary metastasis count	No attributable change in cytokine expression detected
Chen (90)	2019	Melanoma	*In vitro* & in *vivo* (mouse)	Reduced cancer cell proliferation *in vitro*; in *vivo* lidocaine reduced tumour volume and weight	Cell cycle arrest in G1 phase, inhibited Ki-67 expression, inhibited ERK phosphorylation
Gao (91)	2019	Melanoma (*in vivo*)	*In vitro* (HUVEC) & in *vivo* (mouse)	In vitro lidocaine inhibited angiogenesis, *in vivo* lidocaine inhibited tumour angiogenesis and reduced tumour growth (mouse melanoma model)	Suppression of VEGF-activated phosphorylation of VEGF receptor 2 (VEGFR2), PLCγ-PKC-MAPK and FAK-paxillin
Xia (92)	2019	Retino-blastoma	*In vitro* & in *vivo* (mouse)	*In vitro* lidocaine inhibits proliferation and induces apoptosis; *in vivo* lidocaine reduces volume and weight of tumours	Increased expression of miR-520a-3p, decreased expression of EGFR
Xing (93)	2017	HCC	*In vitro* & in *vivo* (mouse)	Lidocaine inhibited HCC cell viability at higher concentrations (>0.5mM), apoptosis increased, cell arrest in G_0/G_1 phase; *in vitro* lidocaine inhibited tumour growth	Activation of caspase 3, decreased Bcl-2 and Bax expression, inactivation of ERK1/2 and p38 pathways

Alteration of NF-κB signalling by lidocaine has been demonstrated in a number of cancer types. Sui et al. detected inhibitory effects of lidocaine on gastric cancer cells, a finding attributed to upregulation of miR-145 resulting in inactivation of NF-κB and MEK/ERK pathways (63). miR-145 has been hypothesised as a potential gastric cancer suppressor and indeed Sui demonstrated that transfection with an miR-145 inhibitor reversed the anti-neoplastic effects of lidocaine on the cancer cells and the NF-κB and MEK/ERK pathways (63). Lidocaine has also been shown *in vivo* (in animal models of sepsis and sterile inflammation) to inhibit expression of the inflammatory mediator high mobility group box 1 (HMGB1), which in turn suppresses activation of NF-κB (102, 103). These findings are supported by a randomised control trial (RCT) which allocated patients undergoing radical hysterectomy to intraoperative i.v. lidocaine or placebo and found that lidocaine reduced serum HMGB1 and inhibited its expression by peripheral monocytes (104). Beneficial lidocaine-related NF-κB changes have also been detected in immune cells - Lahat et al. found that lidocaine reduced nuclear NF-κB in T-cells, inhibited T-cell proliferation *in vitro* and inhibited T-cell production of the pro-inflammatory cytokines IL-2, TNFα and IFN-γ (105).

Inhibition of the Wnt/β-catenin Pathway

Wnt pathways are complex signalling systems that direct cellular processes influencing organogenesis including cell fate determination, motility and stem cell renewal amongst others (106). β-catenin is a crucial component of the 'canonical' or Wnt/β-catenin pathway and acts as a transducer of this signalling mechanism which determines cell proliferation. Dysregulation of Wnt pathways is associated with development of numerous malignancies including colorectal cancer (107). The protein known as adenomatous polyposis coli (APC) contributes to the formation of the β-catenin destruction complex which degrades β-catenin leading to reduced Wnt/β-catenin signalling thereby inhibiting cell proliferation and migration (108). Recent experimental evidence has suggested that lidocaine's observed *in vitro* antineoplastic properties are potentially related to an effect on the Wnt/β-catenin pathway. One study identified that lidocaine increases the mRNA of the β-catenin antagonist APC ten-fold when applied to HCC cells, a finding associated with decreased cell viability and proliferation (70). Lidocaine repressed Wnt/β-catenin activity in two other *in vitro* studies examining gastric cancer and leukaemia cells respectively (65, 71). To the best of our knowledge, lidocaine's effect on this pathway has yet to be examined in an animal model.

Inhibition of Transient Receptor Potential Channels

Transient receptor potential (TRP) channels are a large family of widely expressed membrane ion channels, playing a role in cell growth, survival and proliferation (109). Several TRP family members (including TRPV1, TRPV6 and TRPM7) have been associated with oncogenesis, and increased TRP expression correlates negatively with tumour grade and patient survival in some cancers (110). Lidocaine can inhibit TRPM7 channel current, and TRMP7 suppression is associated with reduced proliferation, migration and invasion of glioma and breast cancer cells *in vitro* (67, 111–113). Similarly, lidocaine reduced TRPV6 expression, migration and invasion in TRPV6-positive breast, prostate and ovarian cancer cells (114). Lidocaine also increased apoptosis in glioma cells, an effect attributed to activation of the TRPV1 gene (115).

Effects on Src Signalling

Src is a non-receptor tyrosine kinase protein widespread in human cells and its encoding gene was the first proto-oncogene to be identified (116). Src is activated by various stimuli, such as TNFα binding to its receptor; activated Src phosphorylates a range of targets including the membrane protein caveolin-1. Src activation results in reduced endothelial barrier function and promotes cellular survival, proliferation, migration, invasion and angiogenesis (32, 117). Predictably then, activated Src in tumour cells is a potent oncogenic promoter and drives the pathogenesis of multiple cancers including colon, prostate and breast carcinomas (118, 119). Activated Src induces the expression of the enzymes MMP-2 and -9 which degrade basement membrane, thereby facilitating tumour cell migration and invasion (120). The effects of lidocaine on Src and associated signalling by-products have been studied both *in vitro* and *in vivo*. In separate experiments Piegeler et al. examined lung adenocarcinoma and lung endothelial cells *in vitro* and demonstrated that lidocaine not only inhibited TNFα-induced Src activation in both cell types, but also reduced cancer cell migration and endothelial cell permeability, as well as neutrophil adhesion (121, 122). In a subsequent study, the same group showed that lidocaine-related inhibition of TNFα-induced, Src-dependent signalling in lung adenocarcinoma cells resulted in reduced MMP-9 expression and reduced cancer cell invasion (75). Although Src inhibition by lidocaine has consistently demonstrated anti-tumour effects *in vitro*, this effect is yet to be confirmed *in vivo*. Our group examined whether an effect on Src underpinned lidocaine-related inhibition of pulmonary metastasis in a mouse model of breast cancer surgery by introducing an Src inhibitor alongside lidocaine. Although postoperative serum MMP-2 was reduced in lidocaine-treated animals, the results could not confirm an Src-dependent mechanism (86).

EFFECTS ON INFLAMMATORY CYTOKINE PRODUCTION

Lidocaine has long been known to possess anti-inflammatory properties (82). What has been more difficult to determine is the mechanism(s) by which inflammation is suppressed and the resulting clinical significance, if any. In vitro evidence from a number of studies has demonstrated that lidocaine inhibits release of leukotrienes, histamine and IL-1α from leukocytes - all potent inflammatory mediators (123, 124). Lidocaine may also inhibit the 'priming' or potentiation of neutrophil response to certain triggers of inflammation and thus reduce cytokine expression (125). In addition, lidocaine experimentally inhibits immune cell adhesion, migration and proliferation within areas of tissue injury (126). This may result from a protective effect of lidocaine on endothelium, preventing inflammatory mediator-induced injury and thus preserving endothelial barrier integrity (127). Conceptually then, perioperative lidocaine may inhibit immune cell infiltration into the pre-metastatic niche and prevent such cells releasing pro-metastatic inflammatory cytokines into this nascent tumour microenvironment, so reducing the risk of future metastasis development.

A number of small RCTs have examined the effect of i.v. lidocaine on post-operative cytokine expression (**Table 3**). Ortiz et al. randomised laparoscopic cholecystectomy patients (n=44)

TABLE 3 | Selected RCTs comparing the effects of systemic lidocaine versus saline placebo on serum cytokine concentration.

First Author	Year	Surgery Type & No. Recruited	I.V. Lidocaine Dosing	Effects on Postoperative Serum Inflammatory Marker Concentrations
Ortiz (128)	2016	Laparoscopic cholecystectomy (n=44, 22 per group)	1.5 $mg.kg^{-1}$ bolus then 3 $mg.kg^{-1}.h^{-1}$ until 1 hour post-surgery	IL-1, IL-6, IFN-γ, and TNFα reduced in i.v. lidocaine group and IL-10 increased compared with placebo group
Song (129)	2017	Laparoscopic cholecystectomy (n=80, 40 per group)	1.5 $mg.kg^{-1}$ bolus then 2 $mg.kg^{-1}.h^{-1}$ until end of surgery	IL-6 and IL-8 reduced in i.v. lidocaine group and no effect on IL-1ra compared with saline placebo
Kuo (130)	2006	Colon cancer surgery (n=60, 30 per group)	2 $mg.kg^{-1}$ bolus then 3 $mg.kg^{-1}.h^{-1}$ until end of surgery	IL-6, IL-8 and IL-1ra reduced by both i.v. and epidural lidocaine compared with saline placebo
Herroeder (131)	2007	Colorectal surgery (n=60, 30 per group)	1.5 $mg.kg^{-1}$ bolus then 2 $mg.kg^{-1}.h^{-1}$ until 4 hours post-surgery	Lidocaine attenuated increase of IL-6 and IL-8, no effect on IL-1β and TNF-α
Yardeni (132)	2009	Open hysterectomy (n=65, 32/33 in each group)	2 $mg.kg^{-1}$ bolus then 1.5 $mg.kg^{-1}.h^{-1}$ until end of surgery	Lidocaine attenuated the increase of IL-6 and IL-1ra produced by lipopolysaccharide–stimulated peripheral blood mononuclear cells
Sridhar (133)	2015	Open abdominal surgery (n=134, 67 per group)	2 $mg.kg^{-1}$ bolus then 1.5 $mg.kg^{-1}.h^{-1}$ until 1 hour post-surgery	Lidocaine attenuated IL-6 compared to saline placebo
Dewinter (134)	2017	Spinal surgery (n=70, 35 per group)	1.5 $mg.kg^{-1}$ bolus then 1.5 $mg.kg^{-1}.h^{-1}$ until 6 hours post-surgery	No significant differences between IL-6 and IL-1ra between the lidocaine and placebo groups
van den Heuvel (135)	2020	Breast cancer surgery (n=48, 24 received lidocaine)	1.5 $mg.kg^{-1}$ bolus then 2 $mg.kg^{-1}.h^{-1}$ until 1 hour post-surgery	No effect attributed to lidocaine on serum IL-1β, IL-6, IL-10, IL-1ra
de Oliveira (136)	2015	Open hysterectomy (n=40, 20 per group)	No bolus, 2 $mg.kg^{-1}.h^{-1}$ infusion during surgery	No difference in serum IL-6 detected
Xu (137)	2021	Laparoscopic hysterectomy (n=160, 4 groups of 40)	1.5 $mg.kg^{-1}$ bolus then 1.5 $mg.kg^{-1}.h^{-1}$ until 30 mins before end of surgery	No difference in serum IL-1, IL-6 and TNF-α between control group receiving saline and group receiving lidocaine

to receive either i.v. lidocaine (1.5 $mg.kg^{-1}$ bolus at surgical start then 3 $mg.kg^{-1}.h^{-1}$ until 1 hour after surgery) or i.v. saline as placebo (128). At 24 hours post-surgery compared to those receiving saline, i.v. lidocaine recipients had significantly reduced serum levels of pro-inflammatory cytokines (IL-1, IL-6, TNFα, IFN-γ) and an increase in the anti-inflammatory cytokine IL-10 suggestive of an overall anti-inflammatory effect. 5 other RCTs have detected that lidocaine has an inhibitory effect on serum cytokine concentrations following abdominal surgery, with IL-6 expression the most consistently suppressed; effects on clinical cancer outcomes were not assessed (129–133).

Not every RCT published to date has demonstrated lidocaine-related anti-inflammatory effects. Similar studies examining breast surgery, spinal surgery and hysterectomy patients found no difference in post-operative serum cytokines in their lidocaine treatment arms (134, 135, 137). There may be a number of reasons underlying the variable results observed in these trials. The enrolled numbers in the RCTs performed were small and most were powered to detect clinical outcomes (such as pain) as the primary outcome rather than cytokine changes. Significant heterogeneity existed not only in the dose and duration of infusion administered, but also in the time points at which cytokines were measured. Notably, all the RCTs reporting lidocaine-related cytokine reductions examined abdominal surgery, and indeed lidocaine's clinical benefits in terms of analgesic effects, hastening return of bowel function and decreasing hospital stay appear most pronounced in this cohort (16).

EFFECTS ON ANGIOGENESIS

Given that inflammation and angiogenesis are often inextricably linked, it is difficult experimentally to isolate purely angiogenic pathways from inflammatory ones (138). There is significant overlap between the intracellular signalling pathways activated by both hypoxia and inflammation – hypoxia inducible factors (HIFs) increase transcription of NF-κB in the same way that inflammatory stimuli do, leading to amplification of inflammatory mediator production, as well as increasing expression of pro-angiogenic VEGF (138). The effects of lidocaine on HIF or VEGF specifically has infrequently been studied in laboratory or preclinical experiments. Gao et al. examined endothelial cells *in vitro* and found that VEGF-stimulated cell migration and proliferation was inhibited by lidocaine (50μM), as well as suppression of VEGF/VEGF receptor 2 (VEGFR2) signalling at 100μM (91). Using a mouse melanoma model, the same group found that intraperitoneal lidocaine treatment resulted in smaller tumours with reduced blood vessel formation. Separately, Suzuki et al. detected similar lidocaine-associated anti-angiogenic effects on endothelial cells *in vitro* although at lower concentrations (4 - 44μM), with similar suppression of VEGF/VEGFR2 signalling noted (139). In contrast, Nishi et al. reported that lidocaine (lowest concentration 30μM) did not affect hypoxia-induced HIF activation or alter expression of hypoxia-induced genes (140). Although choice of anaesthetic technique can alter post-operative serum VEGF in certain cohorts of cancer patients, the clinical significance of any such change is unknown and no definite effect on cancer outcomes has been proven (141, 142). Only one RCT has examined the effect of systemic lidocaine on serum VEGF (though not as the primary outcome): breast cancer surgery patients (n=120) were randomised to anaesthesia using propofol or sevoflurane, with or without i.v. lidocaine – post-operative serum VEGF concentrations were unaffected by any treatment (143).

EFFECTS ON IMMUNE CELLS

The ability of some anaesthetic and analgesic agents to modify immune cell numbers and function is supported by laboratory evidence, although definitive clinical evidence of effects on patient outcomes is not confirmed (13, 38). Similarly, experimental evidence has accumulated indicating that lidocaine may modulate various cellular components of the immune system (31). As immune function and inflammation are closely associated, this effect may result from lidocaine's anti-inflammatory properties as outlined previously. Or it may result from a direct action of lidocaine on immune cells, or indirectly *via* effects on SNS or HPA axis activity, or from some combination of these. Systemic lidocaine reduced circulating cortisol levels in parturients undergoing caesarean section in one trial, and post-operative urinary catecholamines in cholecystectomy patients in another (144, 145). Conversely, this effect was not observed in studies examining cortisol and/or catecholamine levels in colectomy or hysterectomy patients (146, 147). Based on this admittedly small body of evidence, SNS/HPA suppression cannot convincingly be identified as the primary means by which lidocaine influences immune cells.

Dendritic cells and macrophages treated with lidocaine *in vitro* express reduced amounts of inflammatory cytokines, a potentially beneficial anti-inflammatory and anti-cancer effect (148, 149). Conversely, lidocaine-related suppression of Th1 differentiation was detected both *in vitro* and in a mouse model, a potentially detrimental effect as Th1 cells contribute to cell mediated immunity (CMI). Clinically achievable concentrations of lidocaine may also benefit CMI by enhancing the cytotoxic effects of NK cells - *in vitro* NK cytotoxicity against leukaemia cells was promoted by lidocaine treatment (at 0.01μM to 50μM), an effect attributed experimentally to enhanced lytic granule release (150). Similar NK cytotoxicity enhancement was identified in a study which isolated NK cells from healthy donors and cancer patients (both pre- and post-operatively) - NK cells treated *in vitro* with lidocaine had greater cytotoxic effects against cancer cells (151).

A small RCT randomised 30 patients undergoing radical hysterectomy to i.v. lidocaine (1.5 $mg.kg^{-1}$ bolus then 1.5 $mg.kg^{-1}.h^{-1}$ during surgery) or saline (152). Lidocaine treatment preserved post-operative lymphocyte proliferation and inhibited apoptosis. Another RCT, again involving

hysterectomy patients (n=65), randomised subjects to i.v. lidocaine (2 $mg.kg^{-1}$ bolus then 2 $mg.kg^{-1}.h^{-1}$ during surgery) or saline (132). Again an immune protective effect was detected - lidocaine attenuated suppression of the lymphocyte proliferative response, in addition to inhibiting expression of IL-6 and IL-1ra. Not all clinical studies have demonstrated beneficial immune effects - i.v. lidocaine (1 $mg.kg^{-1}$ bolus) in patients with herpes zoster-related pain did not affect NK numbers or activity (153).

Lidocaine inhibits neutrophil adhesion and migration *in vitro*, with effects on the integrin member CD11b-CD18 or the Nav1.3 voltage-gated sodium channel among the mechanisms postulated (154, 155). Evidence of lidocaine's effects on neutrophils has also been established by several animal and human studies. One *in vivo* study found that lidocaine (administered intra-peritoneally in a mouse peritonitis model) inhibited neutrophil apoptosis and macrophage clearance and delayed the resolution of the inflammatory response and return to normal homeostasis (156). Systemic lidocaine also inhibited leukocyte accumulation in animal models of peritonitis and myocardial ischaemia (157, 158). Clinical evidence is limited – in an RCT conducted by Berger et al., intravenous lidocaine administered to septic patients reduced chemokine-induced adhesion and transmigration of neutrophils through endothelium without affecting expression of adhesion molecules (159).

Lidocaine has long been recognised as affecting neutrophil phagocytic function (160), although accumulated evidence appears contradictory as to whether function is enhanced or impaired. Kawasaki et al. treated human neutrophils with lidocaine (at supraclinical 400μM) *in vitro* and found respiratory burst and phagocytic ability were impaired (161). Similar effects on respiratory burst have also been reported in neutrophils isolated from umbilical cord blood from newborns and treated *in vitro* with high concentration lidocaine (4mM), whereas low concentrations (2μM) appeared to increase reactive oxygen species production (162). However, other groups did not detect any lidocaine-related effect on *in vitro* neutrophil function or reactive oxygen species production when clinically achievable concentrations were tested (163–165). One clinical study examining neutrophils taken from lidocaine-treated patients detected significantly reduced superoxide anion release compared to patients who didn't receive lidocaine (166). Contrary to this finding, a clinical trial studying bolus lidocaine (1.5 $mg.kg^{-1}$) administered at induction of anaesthesia found that lidocaine actually preserved neutrophil respiratory burst compared to neutrophils from control patients who received saline (167).

The choice of anaesthetic technique can modulate the neutrophil-to-lymphocyte ratio (NLR) post-operatively, however significant effects on clinical outcomes are not proven (168, 169). Evidence from one small RCT also suggests beneficial effects of lidocaine on post-operative NLR following breast cancer surgery although, again, clinical outcomes were not assessed (170). Unsurprisingly given the relatively recent discovery of the phenomenon of NETosis, it has infrequently been studied in the context of cancer surgery. However, one RCT found that i.v. lidocaine reduced serum biomarkers of NETosis (namely neutrophil myeloperoxidase and citrullinated histone H3) after breast cancer surgery (143).

PRECLINICAL STUDIES OF CANCER OUTCOMES

Although to date far fewer preclinical studies have been conducted than laboratory experiments, a number of animal studies have identified beneficial effects of lidocaine on *in vivo* cancer growth and outcomes (**Table 2**). Chamaraux-Tran et al. injected immunodeficient mice intraperitoneally with human breast cancer cells, randomised the animals to weekly intra-peritoneal lidocaine or saline treatment, and demonstrated that lidocaine treatment significantly improved survival and reduced tumour growth (84). Similarly, lidocaine treatment has been proven to reduce tumour size and improve survival when administered intravesically alongside mitomycin C in a mouse model of bladder cancer (85). Lidocaine also decreased tumour size when administered intraperitoneally in mouse models of melanoma and hepatocellular carcinoma, and intravenously in models of melanoma and retinoblastoma (90–93). We previously established a syngeneic mouse breast cancer model to mimic the effects of anaesthesia and surgery on postoperative metastatic progression (87). In this model, animals that received an intravenous lidocaine infusion alongside sevoflurane anaesthesia during resection of primary breast tumours had consistently fewer pulmonary metastases when measured two weeks postoperatively (86, 88, 89).

CLINICAL STUDIES OF CANCER OUTCOMES

Following reports from retrospective analyses suggesting decreased cancer recurrence rates associated with regional anaesthetic techniques in breast and prostate cancer surgery, there has been an increased focus on establishing which anaesthetic technique, if any, provides the greatest outcome benefit following surgery (171, 172). Evidence accumulated from laboratory and retrospective clinical studies suggests that intravenous (i.e. propofol-based) and regional anaesthesia are potentially beneficial in terms of effects on cancer outcomes compared to volatile anaesthesia and opioids (10). However, the first large RCT examining this topic, which randomised breast cancer surgery patients to a propofol-regional anaesthesia technique versus a volatile-opioid technique, found no difference in recurrence rates between the two groups (173). Given the huge degree of biological heterogeneity between different malignancies, it is difficult to determine how applicable these findings may be to other cancer surgery types e.g. colorectal cancer surgery. Other trials currently underway, assessing anaesthetic technique and cancer outcomes across a range of different cancer types, will go some way towards addressing this uncertainty.

Although numerous studies have examined the effects of intravenous lidocaine on biochemical or haematological markers of inflammation, angiogenesis and immune function, to the best of our knowledge, only one study to date has reported on clinical outcomes. Zhang et al. in a recent retrospective study of 2239 patients undergoing resection of pancreatic carcinomas found that those who received perioperative i.v. lidocaine (1.5 $mg.kg^{-1}$ bolus followed by 2 $mg.kg^{-1}.hr^{-1}$) had significantly better overall survival at 1 and 3 years (68.0% *vs* 62.6%, p<0.001; 34.1% *vs* 27.2%, p=0.011), although disease-free survival was unaffected (174).

FUTURE RCTs - ESTABLISHING SYSTEMIC LIDOCAINE'S EFFECT ON CANCER OUTCOMES

The question of whether perioperative systemic lidocaine has any influence on postoperative cancer outcomes can only be answered by the completion of a suitably powered RCT. No such trial has ever been completed, which is understandable considering the cost, patient number and length of follow up required. However, this question will be addressed for a subset of cancers by the Volatile Anaesthesia and Perioperative Outcomes Related to Cancer trial (VAPOR-C, NCT04316013) which is planned to complete in 2025. VAPOR-C will recruit 5736 colorectal and lung cancer patients and in a 2x2 factorial study randomise them to either sevoflurane or propofol anaesthesia, plus lidocaine infusion (1.5 $mg.kg^{-1}$ bolus followed by 2 $mg.kg^{-1}.hr^{-1}$ for 4 hours then 1.5 $mg.kg^{-1}.hr^{-1}$ thereafter) or saline placebo (175). The primary outcome measure will be disease-free survival, with overall survival as a secondary endpoint.

The ALLEGRO RCT (ISRCTN52352431), which is currently ongoing and aims to recruit 562 patients, is examining the effect of systemic lidocaine (1.5 $mg.kg^{-1}$ bolus followed by 1.5 $mg.kg^{-1}.hr^{-1}$ for 6 or 12 hours) during colorectal surgery on post-operative bowel function. Cancer outcomes will be also be studied up to 10 years post enrolment, although these are tertiary endpoints so will likely be underpowered but will potentially be a useful addition to the knowledge base (176). Other small trials are examining perioperative systemic lidocaine and cancer outcomes in colorectal surgery (NCT02786329) and pancreatic surgery (NCT04449289).

LICENCING AND SAFETY CONCERNS

The appropriateness of intravenous use of lidocaine given the potential risks and as yet inconclusive benefits has recently been questioned (177). Lidocaine remains unlicensed for intravenous use for analgesic purposes, although many drugs used routinely in anaesthesia are similarly used in an 'off-label' manner. The likelihood of encountering toxicity appears very small when carefully dosed and under continuous monitoring, with one surgical unit reporting over 2200 patients treated with perioperative i.v. lidocaine with no reported adverse effects (20). Despite this, the potential for toxicity can never be completely excluded and therefore the potential risks and benefits of systemic lidocaine should be carefully considered by the practitioner for each patient prior to commencing treatment. Recently published dosage guidelines may aid in ensuring safe practice, with dosages reduced accordingly (or usage avoided) in the presence of conditions known to enhance toxicity (18). In addition, as recently proposed, adoption of institutional guidelines regarding administration, monitoring, detection and treatment of systemic toxicity appears prudent wherever i.v. lidocaine is administered, and training of all involved staff should be mandatory (178). Perhaps, as recently suggested by Pandit and McGuire, use of intravenous lidocaine is currently best confined to subjects participating in clinical trials (including VAPOR-C) under rigorous safety conditions and where the results of usage can contribute to establishing definitive evidence of clinical benefits or otherwise (179).

CONCLUSION

The cancer patient's perioperative course is increasingly recognised as a period during which future malignant progression may be influenced for better or worse. Cancer progression appears dependent on the development of a harmful imbalance between pro- and anti-neoplastic humoral and cellular effects, in favour of the malignancy. Circulating tumour cells released by dissection, which under normal conditions would be eradicated by the immune surveillance system, may instead establish themselves in pre-metastatic niches in distant organs, where their survival is facilitated by the pathophysiological effects generated by the surgical insult. Or pre-established micro-metastatic deposits may be woken from their dormancy in the tumour microenvironment by the same processes. Any intervention made during this critical time which can rebalance these systems in favour of host survival holds tremendous promise for improving patient outcomes. Lidocaine has been shown experimentally to possess numerous beneficial effects, potentially affecting multiple biological pathways to act as an anti-inflammatory, immune cell modulator and/or direct inhibitor of cancer cells. An intravenous infusion of lidocaine administered perioperatively may act as a simple, inexpensive and effective chemotherapeutic agent in addition to its potential analgesic properties. Only evidence from adequately powered, randomised, controlled clinical trials will confirm lidocaine's efficacy in improving cancer outcomes - the planned VAPOR-C trial should go some way towards establishing this.

AUTHOR CONTRIBUTIONS

Conceptualization: TW and DB. Writing – Original Draft Preparation: TW and DB. Writing – Review & Editing: TW and DB. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 12 August 2021
doi: 10.3389/fonc.2021.683232

A 20-Year Research Trend Analysis of the Influence of Anesthesia on Tumor Prognosis Using Bibliometric Methods

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OPEN ACCESS

Edited by:
Daqing Ma,
Imperial College London,
United Kingdom

Reviewed by:
Hongmei Cui,
Lanzhou University, China
Wankun Chen,
Fudan University, China

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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

Received: *20 March 2021*
Accepted: *26 July 2021*
Published: *12 August 2021*

Citation:
Luo J, Shi Y, Wang X, Zhang R, Chen S, Yu W, Su D and Tian J (2021) A 20-Year Research Trend Analysis of the Influence of Anesthesia on Tumor Prognosis Using Bibliometric Methods. Front. Oncol. 11:683232. doi: 10.3389/fonc.2021.683232

Background: Bibliometric analysis is used to gain a systematic understanding of developments in the field of *the influence of anesthesia on tumor prognosis* and changes in research hot spots over the past 20 years.

Methods: Relevant publications from the Web of Science Core Collection (WoSCC) were downloaded on May 5, 2021. Acquired data were then analyzed using the Online Analysis Platform of Literature Metrology (http://biblimetric.com) and the CiteSpace software was used to analyze and predict trends and hot spots in this field.

Results: 1,521 publications on the influence of anesthesia on tumor prognosis were identified and 1494 qualifying records were included in the final analysis. The leading country in this field was the United States of America (USA). The University of Texas MD Anderson Cancer Center (Houston, TX, USA) and Pennsylvania State University (State College, PA, USA) featured the highest number of publications among all institutions. Co-citation cluster labels revealed characteristics of ten main clusters: total intravenous anesthesia, opioid growth factor receptor, gastric cancer cell, opioid receptor, murine model, natural killer cell activity, health-related quality, glioma cell, opioid switching and mu-type opioid receptor. Keyword burst detection indicated that randomized controlled trials (RCTs), volatile anesthetics, and ropivacaine were the newly emerging research hot spots.

Conclusions: This study compiled 1494 publications covering *anesthesia and tumor prognosis* research and showed that the direction of these studies is likely in transition from opioids and their receptors to other anesthetics, and from retrospective studies to prospective randomized controlled trials. It provides guidance for further research and clinical applications on choosing anesthetic methods and drugs.

Keywords: anesthetic methods, tumor recurrence, bibliometric analysis, hot spots, CiteSpace

INTRODUCTION

Cancer has become a major disease threatening the length and quality of people's lives in modern times. Consequently, Bray et al. predicted that the incidence of all cancer cases may rise to 22.2 million by 2030 (1). Although cancer diagnosis and treatment methods have improved, surgery remains the first-line treatment of solid tumor therapy. However, some researchers raised concerns that surgery-induced stress and inflammatory responses, together with anesthesia, could extend long past the time of surgery, which may affect long-term patient survival (2).

Therefore, an increasing number of researchers have investigated whether anesthetic technique and anesthetics used during cancer resection surgery can influence long-term tumor recurrence or metastasis (2–4). However, research on *anesthesia and cancer* involves many types of cancer and various anesthetic drugs and methods. Thus, it is challenging to grasp a general direction of this body of research and launch investigations in this field if equipped with little or no prior knowledge. While a plethora of experimental and observational clinical data have been published over the past 20 years, systematic summaries of these studies are insufficient. Thus, it is useful to collect data from relevant publications to assist investigators in reading vast amounts of literature on this subject.

Bibliometric analysis is a method used to analyze large amounts of heterogeneous literature; it is based on mathematics and statistics. Combining visualizing processing tools, like CiteSpace, helps gather data on contributions to certain fields from multiple perspectives, including different countries/regions, institutions, journals, authors, co-cited networks, and detailed research trends or hot spots (5).

The aim of this study was to provide a comprehensive understanding of developments in the research field of anesthesia and tumor prognosis by analyzing historic achievements over the past 20 years. Interpreting and summarizing these articles can help predict possible trends and provide a reference for future researchers, especially for those who have an interest, but are new to this field.

MATERIALS AND METHODS

Data Sources and Search Strategies

A literature search was conducted using the Web of Science Core Collection (WoSCC) database on May 5, 2021, to reduce bias incurred by database updating. The search strategy employed was as follows: TI=(an*esthesia or an*esthetic or narcotic or Propofol or etomidate or Opioid or *fentanyl or morphi* or Dexmedetomidine or midazolam or *caine or *flurane or ketamine or naltrexone or naloxone) AND TS=(tum*r or neoplasm or cancer or carcinoma) NOT TS=(non-cancer or "chronic pain") AND TS=(prognos*s or outcome or recurrence or "overall survival" or "recurrence free survival" or "relapse-free survival" or proliferation or invasion or metastas*s) NOT TI=(guideline or recommendation or consensus or "case report" or meta or review) AND Language=English, and the "Document Type" was set to include "Articles" only from 2001 to 2020. After the primary data search, two researchers (Jiamei Luo and Yumiao Shi) screened all manuscripts individually to ensure they were relevant to the subject of this study.

Bibliometric Online Platform Analysis

Web of Science (https://wcs.webofknowledge.com) was used to analyze retrieval results and extract the histogram showing the publication trend. For analysis of different countries' publication trends, the WoSCC data was converted to UTF-8 format and imported into the Online Analysis Platform of Bibliometrics (http://bibliometric.com/) choosing the "total literature analysis" option. For intercountry/regional analysis, we chose the "partnership analysis" option.

Citespace Software Analysis

Full records and cited references of these publications were downloaded from the WoSCC database and saved as.txt format, and then imported into the Citespace software V5.6R5 SE, 64 bits (Drexel University, Philadelphia, PA, USA), using the following settings: Time slicing from January 2001 to December 2020, years per slice choosing 1. The selection used a modified g-index in each slice: $g2 \leq k \Sigma_{i \leq g} c_i, k \in Z^+, k = 25$. For inter-institutional analysis, "Institution" was chosen in the Node Types parameter area, and the rest of the settings were left as default values. For Co-authorship network analysis, "Author" was chosen in the Node Types after importing data into CiteSpace. For document co-citation, the following related parameters were chosen: "References" as the Node Type, "Cosine" to calculate relationship strength, and as the Pruning parameters area "Pathfinder" and "Pruning the merged network" were chosen to simplify the network and highlight its important structural features (6). For keywords burst detection, "Keywords" was chosen as the Node Type. Again, "Cosine" was used to calculate the burst strength. After removing keywords with little real significance (like cells, mice, etc), the top 20 keywords with the strongest burst strength were identified and are displayed in Microsoft Excel 2016.

RESULTS

Quantity and Trend Analysis of Published Papers

1521 publications met the inclusion criteria when using our search strategy. After removing duplicate entries, 1482 Articles, 1 Book Chapter, 12 Early Access, 24 Proceedings Papers and 2 Retracted Publications were identified, among which 1494 qualified records (1482 Articles + 12 Early Access) were included in the final analysis. Results showed that research on *anesthesia and cancer* can be roughly divided into two time periods (**Figure 1A**). The early stage (2000-2010) saw fluctuations in the number of publications at a level < 50. However, a trend of increased publications in this field was seen in the 10 years that followed, indicating that the *anesthesia and cancer* field was becoming a

Abbreviation: WoSCC, Web of Science Core Collection; USA, United States of America; PA, Pennsylvania; TX, Texas; OGF, Opioid growth factor; RCTs, Random control trials; TI, Title; TS, Topical subject; IF, Impact factors.

research hot spot. Moreover, we used Microsoft Excel 2016 to build a growth trend model as follows: $f(x)=ax^3+bx^2+cx+d$, which indicated that nearly 600 articles will be published by 2025 (**Supplementary Figure 1**).

In order to find out which countries/regions were leading in research in this field, further analysis of publications in different countries and regions was conducted using the Online Analysis Platform of Bibliometrics (http://bibliometric.com/). The bar chart shows the total number of published articles of the top 10 countries/regions over the past 20 years. We found that the United States was a pioneer in this field, and the number of publications has increased steadily. Even though China was initially lagging behind, its annual publication output in this field grew rapidly, outpacing the USA from 2015 onward (**Figure 1B**). Notably, **Figures 1A, B** are from two different websites. **Figure 1A** calculates the number of articles actually published each year, while **Figure 1B** shows the number of articles published online each year. Therefore, **Figure 1B** includes the number of articles published in 2021.

Analysis of Intercountry/Regional and Inter-Institutional Cooperation

Next, we analyzed cooperation efforts among different countries using the bibliometrics online analysis platform (**Figure 2A**). Results of intercountry/regional cooperation suggested that 65 countries worked in partnerships, especially the USA and China. However, China showed less international cooperation compared to the USA.

In order to find out about research institutional and interinstitutional cooperation efforts in the *anesthesia and cancer* field, we next imported TXT format files into the CiteSpace software. Results of the collaborative relationship between different institutions showed 537 nodes and 523 links (**Figure 2B**). The top two of the most prolific institutions, the University of Texas MD Anderson Cancer Center and Pennsylvania State University, were both located in the USA, followed by a Chinese institution, Sun Yat-sen University, also indicating that contributions from the USA and China cannot be ignored in this field (**Figure 2B**).

The names of the top 10 most productive institutions were labeled. Since five institutions had the same number of publications and shared the tenth place, there are a total of 14 institutions displayed in **Figure 2B**. The size of the concentric circles represents the number of publications, therefore, institutions with more published articles are represented with larger concentric circles. Some institutions are too small to be identified. Links between two institutions represent collaboratively published articles. Line thickness indicates the

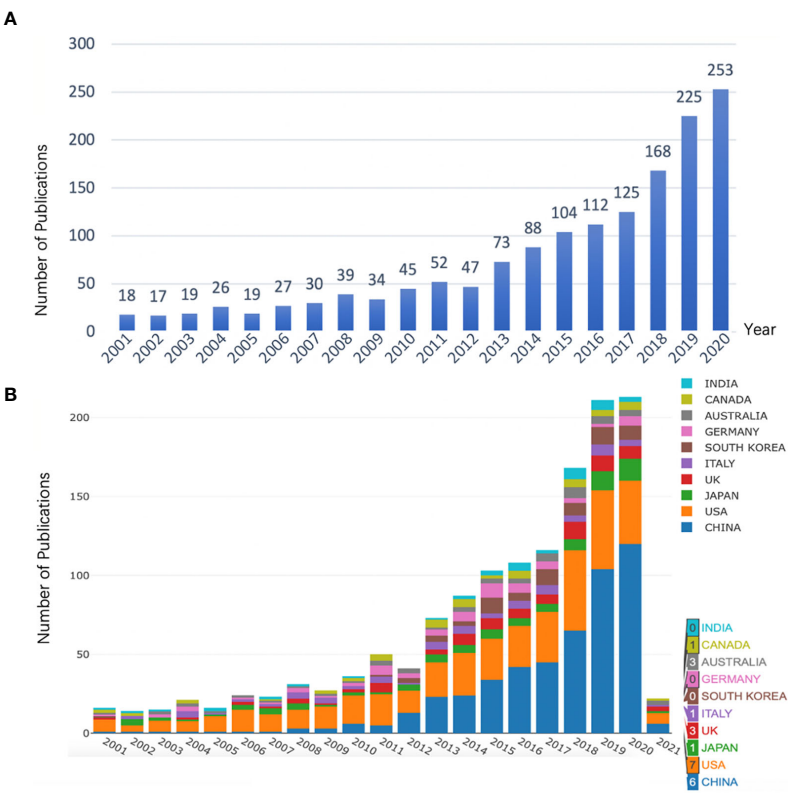

FIGURE 1 | **(A)** Number of annual research publications and growth trends on the topic of anesthesia and tumor prognosis from 2001 to 2020, export of results from Web of Sciences (https://wcs.webofknowledge.com). **(B)** Number of annual publications and growth trends of the top 10 countries/regions on research in anesthesia and tumor prognosis from 2001 to 2021, export of results from the Online Analysis Platform of Literature Metrology (http://biblimetric.com). Bar chart reflects number of online articles online per year.

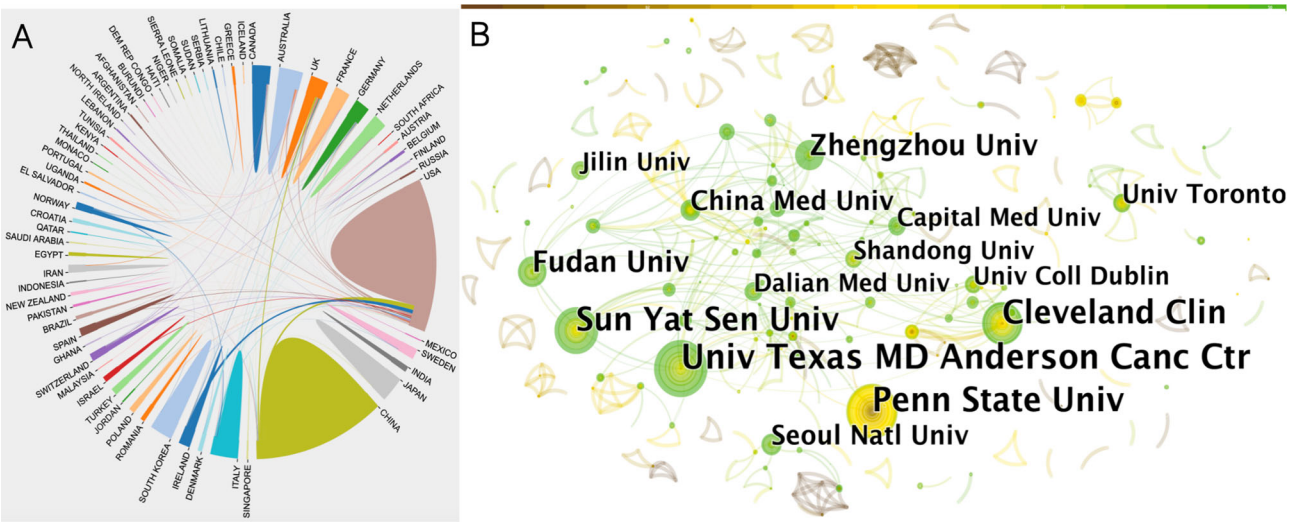

FIGURE 2 | **(A)** Collaboration efforts between 65 countries/regions on the topic of anesthesia and tumor prognosis from 2001 to 2020. Data output from the Online Analysis Platform of Literature Metrology (http://biblimetric.com). **(B)** Citespace network map of institutions involved in anesthesia and tumor prognosis research. Each circle represents an institution. Size of circle is positively correlated with the number of articles published by institutions, and links between two circles represents a collaboration between two institutions on the same article. Line thickness is positively correlated with frequency of collaborations. Top 10 institutions with the most publications are shown. (US Texas MD Anderson Cancer Center, Penn State Univ, Sun-Yat Sen Univ, Cleveland Clin, Fudan Univ, Zhengzhou Univ, China Med Univ, Seoul Natl Univ, Univ Toronto were top 9 and Dalian Medical University, Jilin University, University college Dublin, Shandong University and Capital Medical University tied for tenth). Timespan: 2001-2020; Slice length = 1.

strength of the cooperation. A network density of only 0.0036 indicated that cooperation between these organizations was not close enough. Interestingly, there was a closer connection between institutions with fewer publications (**Figure 2B**).

Co-Authorship Network and Core Author Distribution Analysis

In most cases, multiple researchers are required to collaborate on a study, and their contributions are represented as a ranking of authors. We can evaluate the core authors and their cooperation in a certain field by analyzing the characteristics of authors' cooperative networks. Results include 669 nodes and 722 links. The top 10 researchers and their teams in this research area are shown in **Figure 3**. Font size is positively associated with numbers of the authors' publication. IS Zagon (39 articles) and Patricia J Mclaughlin (39 articles), both from Pennsylvania State University, were first and second on the list, respectively (**Figure 3**). The other eight major research teams are also displayed in **Figure 3** (Juan P Cata from the University of Texas and MD Anderson Cancer Center, Donal J Buggy from Mater Misericordia University Hospital, Renee N Donahue from National Cancer Institute, Bethesda Maryland USA, Sebastiano Mercadante from La Maddalena Cancer Center, Palermo, Italy, Alessandra Casuccio from University of Palermo, Italy, Daqing Ma from Chelsea & Westminster Hospital, Eduardo Bruera from University of Edinburgh, Royal Infirmary and Changhong Miao from Zhongshan Hospital, Fudan University). Contrary to the results of the institution analysis, authors who published more articles tended to collaborate more closely with others.

Journal Analysis

The WoSCC showed that the 1,494 papers included in the current analysis were published in 604 different journals over the past 20 years (2001-2020). Bibliometrics online analysis platform was used to analyze journal influence. The top 10 most cited journals are listed in **Supplementary Table 1**. Four publishers of these 10 most cited journals are in the United States (USA), including the highest-ranking journal *Anesthesiology*; two are in the United Kingdom (UK); and the other four are in Greece, Ireland, France and Italy, respectively. *Anesthesiology*, which showed the highest number of total citations (628) with an IF of 7.892, ranked first in the research field of the influence of anesthesia on tumor prognosis.

Document Co-Citation and Clustered Network Analysis

Document co-citation is a method developed by bibliometric research to identify literature co-cited by a group of authors. In other words, this analysis is used to measure the relationship of two documents by visualizing their co-occurrence of citations (7). Citespace software was used to analyze 1494 original articles and their 36072 valid references to identify distinct homogenous clusters of highly cited documents in the anesthesia and tumor research field. The 36072 references included reviews and other *secondary literature*.

Figure 4A shows co-citations of the 36072 references, and the year and the first author of the top 10 most cited references. Each circle represents a reference. Circle size is positively correlated with the frequency of citations, and links between two circles

FIGURE 3 | Citespace network of co-authorship in the field of anesthesia and cancer research. Each circle represents one author. Size of circle is positively correlated with the number of articles published by authors, and links between two circles represents a collaboration between two authors on the same article. Line thickness is positively correlated with frequency of collaborations. Top 10 authors with the most publications are shown. Timespan: 2001-2020; Slice length = 1.

represent two references that were cited within the same article among the retrieved 1494 articles (citing articles) of the present study. Similarly, line thickness is positively correlated with the frequency of co-citations. Details of the top 10 most cited references are listed in **Supplementary Table 2**.

Results showed that the highest-ranking reference was a review published by *the British Journal of Anesthesia* in 2010 (8). It suggested that anesthetic technique and medication choice can interact with the cellular immune system and affect long-term outcomes. Results of limited human studies indicate that regional anesthesia may be beneficial, which is consistent with the results of the article with the second highest citation, a retrospective analysis published by *Anesthesiology* in 2008 (9). Researchers evaluated 225 patients with invasive prostatic carcinoma, and results showed that general anesthesia plus epidural analgesia, instead of general anesthesia plus opioid analgesia, has less risk of biochemical cancer recurrence. The third highest-ranking article was another retrospective study

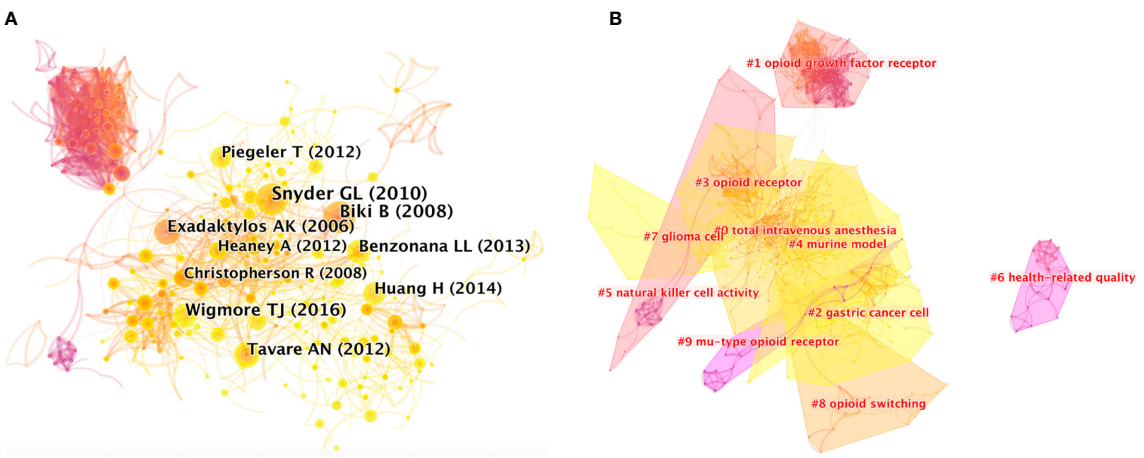

FIGURE 4 | **(A)** Citespce co-citation map of 36072 references on anesthesia and cancer research, filter option showing the largest connected component only. Each circle represents a reference. Size of circle is positively correlated with frequency of citations, and links between two circles represent two references that were cited in the same article. Year and first author of the top 10 most cited publications are shown. Timespan: 2001-2020; Slice length = 1. **(B)** Clustered networks of co-citation status of the investigated 36072 references and the 1494 citing articles *via* CiteSpace. The top 10 largest clusters of citing articles are shown.

published by *Anesthesiology* in 2016 (10), which analyzed 7030 patients undergoing elective surgery due to solid tumors. It concluded that total intravenous anesthesia (TIVA), compared with volatile inhalational (INHA), can improve long-term survival in patients presenting for elective surgery in a comprehensive cancer center over 3 years. Since literature is usually cited to support the opinions of authors, high citation would generally reflect that these references contain concept symbols which have received peer recognition, and it is an indication that they have made important past contributions in the field.

Clustered networks were then generated in a hierarchical order, based on the co-citation status of the 36072 references by the 1494 citing articles *via* CiteSpace, because if two publications have many references in common, they tend to be homogenous. The ten major clusters of the 1494 citing articles are shown in **Figure 4B**. Cluster labels were salient noun phrases extracted from titles of the citing articles using the LSR algorithm, including #0 total intravenous anesthesia, #1 opioid growth factor receptor, #2 gastric cancer cell, #3 opioid receptor, #4 murine model, #5 natural killer cell activity, #6 health-related quality, #7 glioma cell, #8 opioid switching, #9 mu-type opioid receptor (**Figure 4B**). The number of cluster tags are reversely correlated with the number of articles for each cluster included. In other words, the cluster of #0 contains the largest number of articles. A summary of clusters is listed in **Supplementary Table 3**.

Research Trend Analysis and Burst Detection With Keywords

In order to show the clusters of the citing articles more clearly, a timeline view of these clusters is shown in **Figure 5A**. A bolded timeline in **Figure 5A** indicates that the clustering topic was a hot spot during this period of time. Citation tree-rings of different sizes on the timeline represent key references with high citation rates. We found that in the field of *anesthesia and tumor*, mu-type opioid receptor has been a hot topic since 1993 until the end of 20th century. It has become a research focus again since around 2006, together with other types of opioid receptors, i.e., kappa-type, delta-type, etc. Total intravenous anesthesia has also attracted increasing attention in recent years. Furthermore, according to the clustering results, gastric cancer and glioma are two kinds of tumors that many people are interested in in recent studies. Natural killer cell activity may be the mechanism underlying the influence of anesthetics on tumor progression.

Keyword burst detection was another method to show research trends (**Figure 5B**). A red line indicates that the use of this keyword increased suddenly during this period. In contrast, a blue line represents relative unpopularity. Morphine, with a strength of 13.421, was ranked first, followed by cancer pain (9.8978), which were considered a research focus during the years from 2001 to 2010 and 2011. The impact that anesthesia methods (local anesthesia, anesthetic technique, epidural anesthesia, etc.) and drugs (such as volatile anesthetics, ropivacaine, etc.) exerted on cancer recurrence has drawn much more attention since 2008. In addition, we found that randomized controlled trials (RCTs) have become a research focus from 2017 onwards.

DISCUSSION

This current study visualized research articles in the field of *influence of anesthesia on tumor prognosis* from 2001 to 2020. The number of published articles on this topic rapidly increased after 2010 and reached almost 200 articles per year by 2020. However, the actual research topics were relatively diverse. Using an online bibliometric analysis platform and CiteSpace software, our study analyzed publications about *anesthesia and cancer* research from multiple dimensions, and showed a systematic view in understanding in this field over the past 20 years and provided guidance for future studies. Researchers new to this

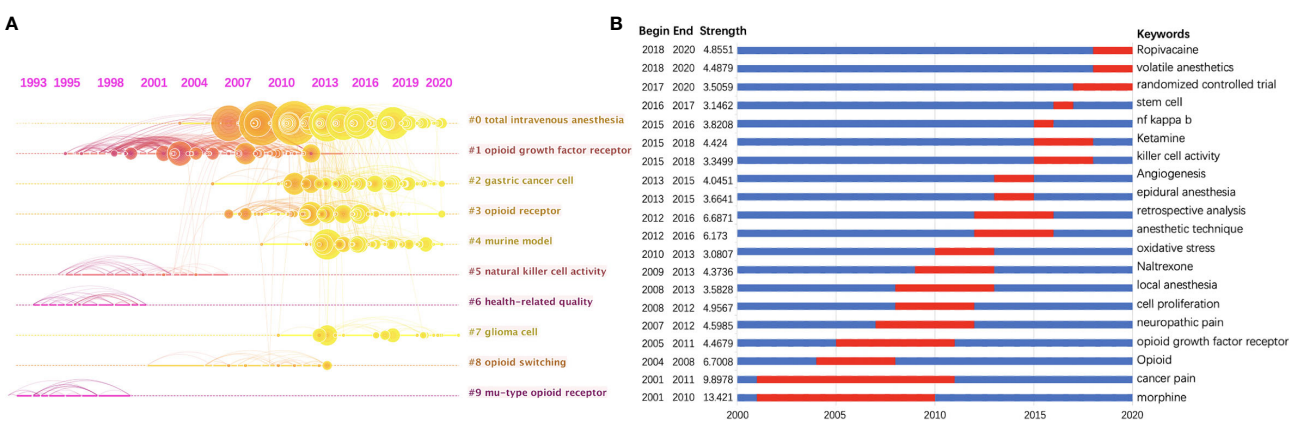

FIGURE 5 | **(A)** Timeline view of the top 10 largest clusters of citing articles in the field of anesthesia and cancer research. Right side = cluster labels. **(B)** Keywords with the strongest burst strength of the 1494 citing articles on anesthesia and cancer research between 2001 and 2020. Keywords marked in red indicates a sudden increase in usage frequency of this keyword during that period. Blue represents a relatively unpopular time period.

field of study now can easily get useful and relevant information with the help of our bibliometric analysis.

The top 3 countries that focus on the field of *influence of anesthesia on tumor prognosis* are the USA, China, and Japan. Notably, by 2015 the number of articles from China surpassed those from the USA, and China became the most prolific country of origin of publications in this field. However, China dropped out of the top 10 when quantifying the significance of their contributions compared to other countries. This suggests that Chinese researchers are quite interested in this topic, however, quality and influence of their research still needs improvement.

International cooperation, especially between the USA and China, was common. The top two most prolific research institutions were both located in the USA, followed by Sun Yat-sen University from China. Of the 604 different journals that published 1,521 papers in this field, 40% are from USA, making the USA the leading country in the field.

The timeline view of the 36072 related references and keyword burst detection both indicate trends in the field of *anesthesia and cancer* research. The effects of opioids, especially morphine, on tumor progression has attracted the researchers' attention since the beginning and lasted for decades (11–14). The effects of other anesthetics, including local anesthetics and volatile anesthetics, have also become a research interest in recent years.

At present, it is still a matter of debate how these different anesthesia methods and anesthetics influence the prognosis for patients undergoing tumor resection, but there is enough current evidence to generate hypotheses that they may affect long-term oncologic and survival outcomes. The current bibliometric analysis indicated that a plethora of retrospective clinical and experimental data has been published over the past 20 years, and most researchers showed that regional anesthesia is more favorable for a good prognosis for surgical oncology patients compared to general anesthesia (9, 15–23). Research also suggested that intravenous hypnotic drugs [like propofol (24–26)] and local anesthetics [like lidocaine (27) and ropivacaine (28)] provide an anti-cancer effect, whereas opioids (11) and volatile anesthetics (29, 30) may promote tumor development. However, the available evidence was not strong enough to change clinical practice. Consequently, as shown in the keyword burst detection results of this study, more RCTs are conducted by researchers in recent years in this research field to seek clinical evidence. Hopefully, they can address this important clinical question in an effective way.

Further work is required to fully understand the mechanisms driving the above mentioned phenomena. Consequently, this study found that immune response, such as the activity of natural killer cells, has been a research hot spot (31). It is now widely accepted that circulating immune cells in human blood can recognize tumor-associated antigens. This immunosurveillance protects the host against cancer development, and immunosuppressants are often associated with high incidences of cancer (32). Many preclinical and clinical studies found that anesthetic drugs can directly or indirectly modulate the immune system (33). Melamed et al. reported that ketamine, halothane, and thiopental can suppress natural killer cell activity and promote tumor metastasis. Conversely, propofol does not have these effects (34). In addition, opioids, widely used during anesthesia and perioperative period, were also proved to reduce activation of the pro-inflammatory transcription factor NF-κB, which was also detected by the Keywords burst detection in this study, and affect the adaptive immune system (33, 35). Recent studies showed that morphine promotes the migration of MCF-7 cells *via* the TLR4/NF-κB signaling pathway (36).

Anesthesia methods and drugs can also influence tumor progression by influencing the malignant biological behavior of tumor cells. The effect of opioids facilitating cancer cell proliferation was proven in both cell culture experiments and animal models (3, 37–39). Similar findings were reported when using volatile anesthetics (40, 41). In contrast, propofol and local anesthetics, i.e., lidocaine, have been shown to inhibit cell proliferation and migration in different kinds of cancer cells (24, 26, 27, 42, 43).

The current study has several limitations. First, the current analysis is based on citation data. Reviews and articles are two document types frequently cited, whereas very little citation data is available for books or conference papers. Therefore, some of these publications are not tracked by bibliometric searches. Second, only articles with English keywords or abstracts in the WOS database were considered in our analysis due to requirements by the CiteSpace software. High-quality articles in other languages, although few, were not cited. Third, bibliometrics is a quantitative analysis of scholarly publications, where high citation counts may not necessarily indicate quality. For example, a highly-cited article does not necessarily correspond to clinical demands, and sometimes it might even not be clinically relevant, especially for basic research articles focusing on a single molecule or a single pathway. In the future, we may use multimethod evaluations to gain a more in-depth understanding of this research field.

In conclusion, bibliometric analysis offers an objective and quantitative method for assessing publication performance between countries, researchers, research institutions, etc. Our results showed considerable interest in the field of *anesthesia and tumor prognosis* research in recent years, particularly the study of opioids and their receptors, local anesthetics and volatile anesthetics. Moreover, the keyword burst detection in this study also indicated that different anesthesia techniques and anesthetics have different effects on the prognosis of cancer patients, which would necessitate more RCTs. Their guidance for clinical practice may benefit countless patients with cancer.

AUTHOR CONTRIBUTIONS

JL and YS: These two authors contributed equally to this work helping raising the conception of the study, searching and screening articles, processing data of the study, drafting and reviewing the manuscript. XW, RZ, and SC: These authors contributed to the conception of the study, reviewed and

edited the manuscript. WY, DS, and JT: These authors helped to conceive the idea of the study, critically review and edit the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by grants from Shanghai Shenkang Hospital Development Center Three-year Funding for Major Clinical Research Projects (SHDC2020CR4062), Natural Science Foundation of Shanghai Science and Technology Committee (19ZR1430600) and Shanghai Municipal Key Clinical Specialty (shslczdzk03601).

ACKNOWLEDGMENTS

We thank Isabel Lambertz for English language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.683232/full#supplementary-material

Supplementary Figure 1 | Output of publications and growth prediction of anesthesia and cancer research. The number of publications from 2001 to 2020 are represented by large scatters; the dashed line represents the predicted curve, R^2 = 0.9877.

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frontiers in Oncology

ORIGINAL RESEARCH
published: 23 September 2021
doi: 10.3389/fonc.2021.686294

Effects of Serum From Radiofrequency Ablation Patients Receiving General Anesthesia or Local Anesthesia on Hepatocellular Carcinoma Cancer Cell Malignancy: A Prospective Randomized Controlled Trial

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OPEN ACCESS

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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

Received: *26 March 2021*
Accepted: *31 August 2021*
Published: *23 September 2021*

Citation:
Shi Y, Wu T, Wang T, Liu Y, Wang X, Luo J, Su D, Zhai B and Tian J (2021) Effects of Serum From Radiofrequency Ablation Patients Receiving General Anesthesia or Local Anesthesia on Hepatocellular Carcinoma Cancer Cell Malignancy: A Prospective Randomized Controlled Trial. Front. Oncol. 11:686294. doi: 10.3389/fonc.2021.686294

Background: Whether anesthesia methods affect malignant biological behavior of cancer remains unresolved. In this study, we aim to compare the effects of general anesthesia (GA) and local anesthesia (LA) on serum collected from primary hepatocellular carcinoma (HCC) patients presenting for radiofrequency ablation (RFA).

Methods: From August 2020 to December 2020, a prospective, randomized, and controlled study was conducted at Renji Hospital, which is affiliated with Shanghai Jiaotong University School of Medicine. 25 qualified patients from 18 to 65 years of age undergoing RFA were enrolled in the study and randomly assigned into two groups: the GA group (n = 14) and the LA group (n = 11). Venous blood was drawn from all patients preoperatively and 1 hour postoperatively. The serum collected was then used for the culturing of HepG2 cells. The malignant biological behaviors of HepG2 cells, including invasion, migration and proliferation, were observed after 24 hours of exposure to patients' serum. ELISA was used to compare expression levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and lymphokines (IFN-γ, IL-2) in patients' serum from both groups.

Results: HepG2 cells cultured with postoperative serum obtained from patients who received GA, but not LA, were associated with significantly increased cell invasion, migration and proliferation, compared to preoperative serum from the same patient group. Expression levels of pro-inflammatory cytokines were significantly higher, and lymphokines significantly lower in postoperative serum from GA patients compared to the corresponding preoperative serum.

Conclusion: GA affects the serum milieu of patients with HCC, promoting the malignant biological behavior of a human HCC cell line.

Keywords: general anesthesia, local anesthesia, hepatocellular carcinoma, serum milieu, cancer cell malignancy

INTRODUCTION

Hepatocellular carcinoma (HCC), one of the most aggressive cancers worldwide (1, 2), is the second leading cause of cancer-related death in China and has a poor prognosis (3). To date, surgical resection is the main treatment for HCC. However, recurrence and metastasis rate of HCC after surgery remains high, which greatly affects patient prognosis (4). Studies have shown that recurrence and metastasis rates of large HCC after resection is greater than 60% and that of small HCC is over 40% (5). Even liver transplantation cannot completely eliminate the recurrence of HCC (6, 7). Therefore, to effectively prevent HCC recurrence and metastasis is key to improve long-term survival of HCC patients.

The perioperative time period is a dangerous window for tumor metastasis (8), among which anesthesia contributes a significant part. On one hand, the use of anesthesia reduces patients' pain and relieves stress caused by the surgery; on the other hand, various anesthetics may affect cancer recurrence and metastasis. In recent years, a number of studies have shown that choice of anesthesia methods and drugs have a potential impact on the long-term prognosis of cancer patients (9–13). For example, Lin et al. found that patients who received general anesthesia (GA) combined with epidural anesthesia during ovarian serous adenocarcinoma surgery have a reduced mortality rate during the initial follow-up years compared to patients who received GA alone (10). However, most previous studies are comparisons between GA and GA combined with regional anesthesia, therefore it cannot be assessed whether the differences are due to a tumor-promoting effect of GA itself, or a protective effect of regional anesthesia.

Radiofrequency ablation (RFA) is a minimally invasive therapy for the treatment of HCC without damage to adjacent healthy tissue and with a shorter recovery period compared to surgical resection. It has become a safe and effective treatment for patients with HCC and is clinically widely used (14). Either GA or local anesthesia (LA) can be used to complete the operation, especially when the tumor size is still small. Our previous multi-center retrospective cohort study has shown that the anesthesia approach could influence the prognosis of HCC patients. GA patients undergoing RFA displayed a higher rate of tumor recurrence and shorter overall survival compared to HCC patients who received LA (15). However, how anesthesia methods influence the outcome of HCC patients receiving RFA surgery remains unclear.

To explore the potential mechanisms underlying the effects of anesthesia on clinical HCC patients, we performed a randomized clinical trial, allocating HCC patients to receiving either GA alone or LA alone. Serum was collected both pre- and postoperatively during the perioperative period. We then compared its effects on the malignant behaviors of HepG2 cells, a human HCC cell line, when culturing them using the collected patient serum. Our results show that for GA patients undergoing RFA, serum milieu was influenced such that GA increased the malignancy of HCC cells.

MATERIAL AND METHODS

Study Design

This was a prospective, randomized, and controlled study designed in accordance with the CONSORT recommendation.

Patients

The study was approved by the Institutional Human Ethics Committee of Renji Hospital (2015-064), located at 160 Pujian Road, Pudong New Area, Shanghai, China, and was registered at ClinicalTrials.gov (NCT04510935). It was conducted at the Renji Hospital, Shanghai Jiao Tong University School of Medicine between August 2020 and December 2020. Informed consents were obtained from all patients or legally authorized representatives. Only patients diagnosed with HCC and undergoing elective RFA surgery were enrolled in the study. Other inclusion criteria were: (a) between 18 and 65 years of age; (b) ASA Classes I-III; (c) the summary of the long diameter of all tumors was ≤3 cm; and (d) Child-Pugh degree A or B. Patients were excluded if they (a) had a previous elective RFA; (b) had severe systemic disease (heart, lung, kidney, or immune system); (c) INR>1.5 or platelet count <45,000 cells/mm^3; (d) were addicted to opioids; or (e) with known extension beyond the liver.

Randomization and Blinding

Eligible patients were randomly allocated 1:1 to receive either GA or LA according to computer-generated codes. The PROC program in SAS (version 9.0, SAS Institute Inc) was used to generate the sample randomization sequence with a 1:1 allocation. This was an open-label study, since blinding of either patients or investigators was not possible. The investigator who carried out the cell culture studies using the patients' serum, was blinded to the treatment assignment.

Procedures

Patients in the GA group were induced with 0.05-0.1mg/kg intravenous midazolam, 3-6μg/kg fentanyl, 1.0-2.5mg/kg propofol and 0.1-0.2mg/kg cisatracurium. A laryngeal mask was inserted for mechanical ventilation. Anesthesia was maintained with 4-8mg/kg/h propofol and 0.1-0.3μg/kg/min remifentanil, and additional non-depolarizing muscle relaxant when necessary. Patients recovered in a Post Anesthesia Care Unit (PACU), and were administered neostigmine combined with atropine routinely to reverse muscle relaxants.

Patients in the LA group were injected subcutaneously with ~10ml of 2% lidocaine at the surgical puncture points before insertion of laparoscopic needles. No propofol or other sedatives or narcotics were given. Patients were awake and breathing spontaneously during surgery.

Venous blood was obtained from patients from both groups immediately after entering the operating room and 1 hour postoperatively. Samples were centrifuged at 3000 rpm for 10 minutes at 4°C and serum was collected and stored at −80°C for future use.

Data Collection and Outcome Measures

Personal health records of the study participants were obtained from the hospital medical record system. Primary outcome was the mean percentage change from post- to preoperative values of the invasion ability of HepG2 cells cultured with the patients' serum for 24h. Secondary outcomes were the mean percentage change from post- to preoperative values of the migration and proliferation ability of HepG2 cells cultured with patients' serum for 24h, and expression levels of key cytokines, including interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), interferon gamma (IFN-γ) and interleukin-2 (IL-2), in pre- and postoperative serum.

Cell Culture

The human HCC cell line HepG2 was purchased from the FuHeng Cell Center (Shanghai, China). HepG2 cells were cultured at 37°C in a humidified incubator containing 5% CO_2, using high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 50 U/ml penicillin and 50μg/ml streptomycin. Cells were serum-starved in DMEM for 8-12h before treatment with DMEM plus 10% patient serum for 24h.

Transwell Assays

Cell invasion ability was determined using Transwell chambers with an 8μm pore size (Corning, USA) and Matrigel (BD Bioscience, China). Cells were incubated in the upper chamber at a density of 2×10^4 cells/chamber with 0.5mg/L Matrigel, and medium with patient serum in a final concentration of 10% was added to the lower chamber. After a 24h incubation, chambers were fixed with paraformaldehyde for 30 min and stained with 0.5% crystal violet for 20 min. Positive cells were visualized using a microscope. Three random fields per chamber were counted using the Image J1.54 software and averages were calculated to reflect invasion activity of the sample. Mean percentage change from post- to preoperative values for each individual patient was calculated and compared between the GA and LA groups. Mean percentage change = [(No. of invaded cells with postoperative serum) – (No. of invaded cells with preoperative serum)]/(No. of invaded cells with preoperative serum) ×100%. Representative fields were photographed with an Olympus fluorescence microscope at 100× magnification.

Wound Healing Assays

Migration activity of HepG2 cells was analyzed using scratch assays. 2×10^5 cells per well were seeded into 12-well plates and grown to 90% confluency. 200μl pipette tips were used to draw one straight scratch per well. Cells were then washed with PBS and cultured for 24h in medium containing 10% of the patients' serum. Three microscope images were taken of each set at 0h and 24h respectively, with the distance of cell migration measured for statistical analysis. Averages were calculated to reflect the migration activity of the sample: Recovery ratio = [(Blank area at 0h) - (Blank area at 24h)]/(Blank area at 0h) × 100%. Mean percentage change = [(Recovery ratio with postoperative serum) – (Recovery ratio with preoperative serum)]/(Recovery ratio with preoperative serum) ×100%.

Cell Counting Kit (CCK-8) Assays

Cell proliferation ability was measured with a CCK-8 Kit. Cells were seeded in 96-well plates at a density of 6×10^3 cells/well and using 10% of the patients' serum. After 24h or 48h of culture time, 10μl CCK-8 (Yeasen Biotech Co., Ltd. Shanghai, China) was added to the cultures and cells were incubated for 30 minutes. Optical density (OD) at 450 nm was detected by a microplate reader (Berthold Technologies-TriStar2LB942, German). Each treatment was performed in six replicates. Averages were calculated to reflect proliferation activity of the sample. The mean percentage change from post- to preoperative values for each individual patient was calculated and compared between the GA and LA groups. Mean percentage change = [(OD Value with postoperative serum) – (OD Value with preoperative serum)]/(OD Value with preoperative serum) ×100%.

EdU Assays

EdU assays were performed to investigate differences in HepG2 DNA synthesis and cell proliferation. HepG2 cells were seeded in 96-well plates at a density of 6×10^3 cells/well. EdU incorporation experiments were performed following the manufacturer's instructions (Yeasen Biotech Co., Ltd. China). Nuclei were stained with 4′,6- diamidino-2-phenylindole (DAPI). Cells were visualized using a confocal microscope (Olympus, Japan): EdU^+ cells (%) = number of positive EdU cells/the total number of nuclei×100%. The mean percentage change from post- to preoperative values for each individual patient was calculated and compared between the GA and LA groups. Mean percentage change = [(%EdU^+ cells with postoperative serum) – (%EdU^+ cells with preoperative serum)]/(%EdU^+ cells with preoperative serum) × 100%.

ELISAs

Serum levels of pro-inflammatory cytokines and lymphokines were measured using commercial ELISA kits (R&D Systems, Inc., USA) following the manufacturer's instructions. Catalog numbers are: interleukin-1β (IL-1β) (DLB50), tumor necrosis factorα (TNF-α) (DTA00D), interleukin-6 (IL-6) (D6050), interferon gamma (IFN-γ) (DIF50C) and interleukin-2 (IL-2) (D2050).

Statistical Analysis

PASS (version 11.0, NCSS, LLC) software was used for sample size calculations. Since evidence on the effects of GA on the invasion ability of cancer cells was lacking, we adopted a

conservative approach and assumed that the expected effect size (Cohen's d) between groups would be small (0.3). Thus, assuming that GA would result in an 18% increase in the mean percentage change from post- to preoperative invasion, with a SD of 10%, the study would require 8 patients per group to reach 90% power with an α equal to 0.05. When including an attrition rate of 10%, 9 patients per group should be included.

SPSS (version 24.0, SPSS Inc, Chicago, USA) software and GraphPad Prism 8 (GraphPad Software, San Diego, CA) were used for data analysis. Continuous variables were expressed as mean ± standard deviation (Mean ± SD) after they were proven to be normally distributed using the Kolmogorov-Smirnov (K-S) test. When comparing cell invasion, proliferation and migration ability in pre- or postoperative serum from the two groups, multiple comparisons were performed using two-way ANOVA. Mean percentage changes from post- to preoperative values between the two groups were calculated using Student's *t*-tests. Categorical variables were compared using the $\chi 2$ test with the Yates correction or Fisher's exact test (when total sample size was <40 or the expected frequency was <1). Two-sided tests were used and *P*-values <0.05 were considered significant.

RESULTS

From August 2020 to December 2020, a total of 28 patients were recruited and randomized into the LA or GA groups. Among these, two refused the postsurgical blood draw and one sample hemolyzed. Therefore, 25 patients were included in the final analysis (**Figure 1**). More than ten variables were analyzed and compared between the two groups, including general patient information, operation time, liver function variables and cancer characteristics, and none of the differences reached statistical significance (**Table 1**).

TABLE 1 | General characteristics of the thermal ablation surgery patients [Mean (SD) or number].

Variables	LA (*n* = 11)	GA (*n* = 14)	*P* value
Sex			
Male	9	11	1
Female	2	3	
Age (y)	54.8 (11.62)	52.0 (10.64)	0.54
Height (cm)	170.4 (7.10)	170.7 (7.20)	0.92
Weight (kg)	67.3 (11.95)	69.5 (16.53)	0.71
ASA (I/II/III)	0/11/0	0/14/0	1
Hypertension (yes/no)	1/10	2/12	1
Diabetes (yes/no)	1/10	1/13	1
Cirrhocsis (yes/no)	4/7	2/12	0.35
HBV/HCV infection (yes/no)	6/5	8/6	1
Child-pugh stage (A/B)	11/0	14/0	1
Adjuvant chemoradiotherapy* yes/no)	5/7	6/8	1
Tumor size (cm)	1.9 (0.54)	1.9 (0.62)	0.89
ALB (g/L)	44.3 (3.23)	42.4 (4.70)	0.26
ALT (U/L)	28.1 (14.88)	26.1 (16.03)	0.75
AST (U/L)	23.5 (5.25)	25.7 (13.10)	0.61
TBIL (mmol/L)	12.4 (6.44)	12.1 (6.50)	0.91
Duration of surgery(min)	12.4 (5.15)	12.6 (4.52)	0.99

*Variables are shown as "mean (SD)". *Adjuvant chemoradiotherapy is defined as patients received transcatheter arterial chemoembolization (TACE) or radioactive seed implantation simultaneously with or after TA surgery. ASA, American Society of Anesthesiologists; HBV, hepatitis B virus; HCV, hepatitis C virus; ALB, serum albumin; ALT, Alanine transaminase; AST, aspartate aminotransferase; TBIL, total bilirubin; SD, standard deviation; LA, local anesthesia; GA, general anesthesia.*

Serum From RFA Patients Receiving GA Facilitated Cell Invasion in HepG2 Cells

Transwell assays were used to investigate the invasion ability of HepG2 cells. As shown in **Figures 2A–E**, there was no significant difference in invasion ability of cells when they were treated with preoperative (pre-) serum from the GA or LA groups. Interestingly, it clearly showed that the number of cells invading to the lower surface was significantly greater when treated with

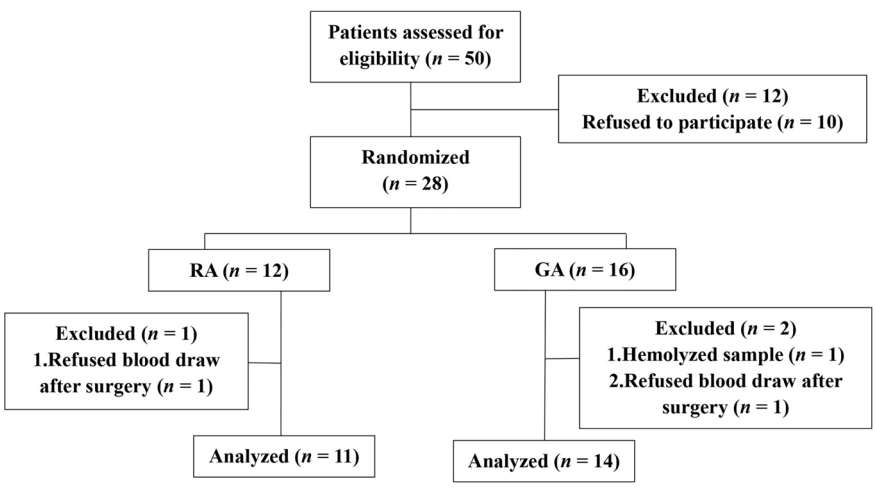

FIGURE 1 | Clinical trial flow diagram. 28 patients were recruited and randomized to the LA group or GA group. 25 patients were included in the final analysis, *n* = 11, LA group, and *n* = 14, GA group.

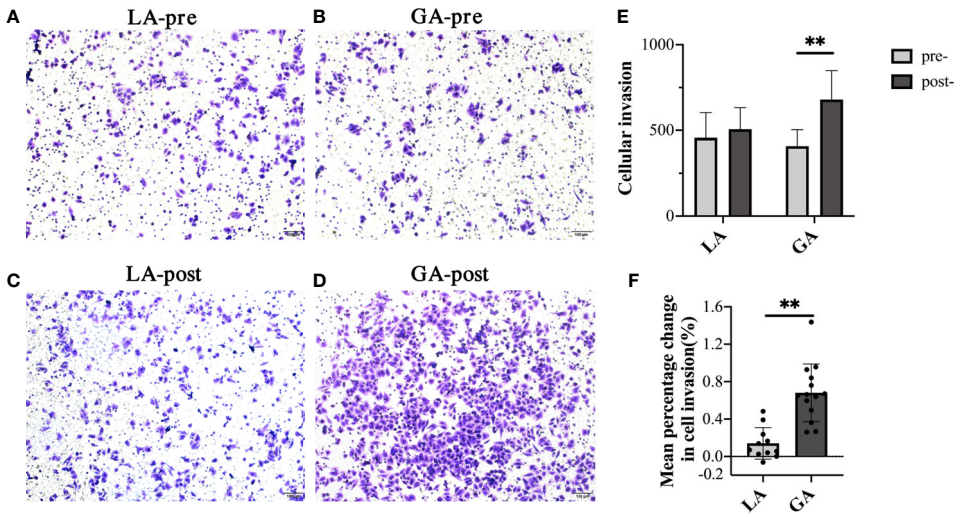

FIGURE 2 | Serum from RFA patients receiving GA facilitated cell invasion of HepG2 cells. **(A)** Representative image of pre-LA serum-treated HepG2 cells; **(B)** Representative image of pre-GA serum-treated HepG2 cells; **(C)** Representative image of post-LA serum-treated HepG2 cells; **(D)** Representative image of post-GA serum-treated HepG2 cells; Original magnification, 100X; **(E)** Graphical representation of cells that invaded the lower surface of the four groups; **(F)** Graphical representation of the mean percentage change from post- to preoperative values of invading cell numbers in the GA group *vs* the LA group. Values are expressed as mean ± SD. n = 11, LA group. n = 14, GA group. $^{**}P < 0.01$. GA, General Anesthesia; LA, Local Anesthesia.

serum from the postoperative (post-) GA group compared to the pre-GA group (404.74 ± 97.73 pre-GA *vs* 679.26 ± 169.32 post-GA, $P < 0.001$), whereas no differences were observed when comparing treatment with serum from the post-LA group to the pre-LA group (456.88 ± 146.78 pre-LA *vs* 506.67 ± 125.69 post-LA, $P = 0.40$). Furthermore, there was a statistically significant difference between the GA and LA groups when comparing the mean percentage change from post- to preoperative values in cell invasion (68.09% ± 30.83% in the GA group *vs* 14.07% ± 16.88% in the LA group, $P < 0.001$) (**Figure 2F**).

Serum From RFA Patients Receiving GA Facilitated Cell Migration of HepG2 Cells

Scratch assays were performed to examine cell migration ability of HepG2 cells treated with the different serum. Results were consistent with their respective invasion abilities, showing that while serum from pre- and postoperative LA patients had similar effects on the migration ability of HepG2 cells, postoperative serum from GA patients significantly promoted HepG2 invasion compared to preoperative serum (0.26 ± 0.05 pre-LA *vs* 0.27 ± 0.09 post-LA, $P = 0.86$; 0.25 ± 0.04 pre-GA *vs* 0.44 ± 0.07 post-GA, $P < 0.001$; 76.43% ± 18.96% change in the GA group *vs* 2.27% ± 33.17% change in the LA group, $P < 0.001$) (**Figure 3**).

Serum From RFA Patients Receiving GA Facilitated Cell Proliferation of HepG2 Cells After Long-Term, but Not Short-Term, Exposure

Next, CCK-8 assays were used to investigate the proliferative effects on HepG2 cells when using serum from both groups. Cellular proliferation did not differ between any of the groups when cultured for 24h in post- *versus* preoperative serum (**Figures 4A, C**). To explore whether this was an exposure time issue, we extended the incubation time to 48h. Interestingly, as shown in **Figures 4B, D**, cells cultured in postoperative GA serum for 48h displayed a modest, but still significantly higher OD value than those in preoperative GA serum (1.03 ± 0.07 pre-GA *vs* 1.15 ± 0.10 post-GA, $P = 0.001$). There still was no observable difference in OD values in cells exposed to postoperative LA serum for 48h *versus* preoperative LA serum (1.01 ± 0.11 pre-LA *vs* 1.02 ± 0.12 post-LA, $P = 0.81$). The mean percentage change from post- to preoperative values in cell proliferation after 48h of culture was also significantly increased in the GA group compared to the LA group (15.43% ± 10.40% in the GA group *vs* 1.55% ± 10.48% in the LA group, $P = 0.003$) (**Figure 4D**).

In order to further verify these results, EdU assays were carried out to detect the proportion of cells involved in the proliferation phase after culturing for 48h in pre- and postoperative patient serum. The results were in agreement with the CCK-8 assay results above, showing that long-term exposure to post- GA serum, but not LA serum, caused a significant increase in proliferation activity of HepG2 cells (**Figures 5A–C**).

Serum From RFA Patients Receiving GA Contained Increased Levels of Pro-Inflammatory Cytokines and Decreased Levels of Lymphokines

To understand whether GA leads to changes in the composition of certain molecules in patients' serum, we then examined expression levels of several cytokines in patient serum. There was no difference in the levels of IL-1β, IL-6, TNF-α, IFN-γ, and IL-2 in preoperative serum between the two groups. In patients

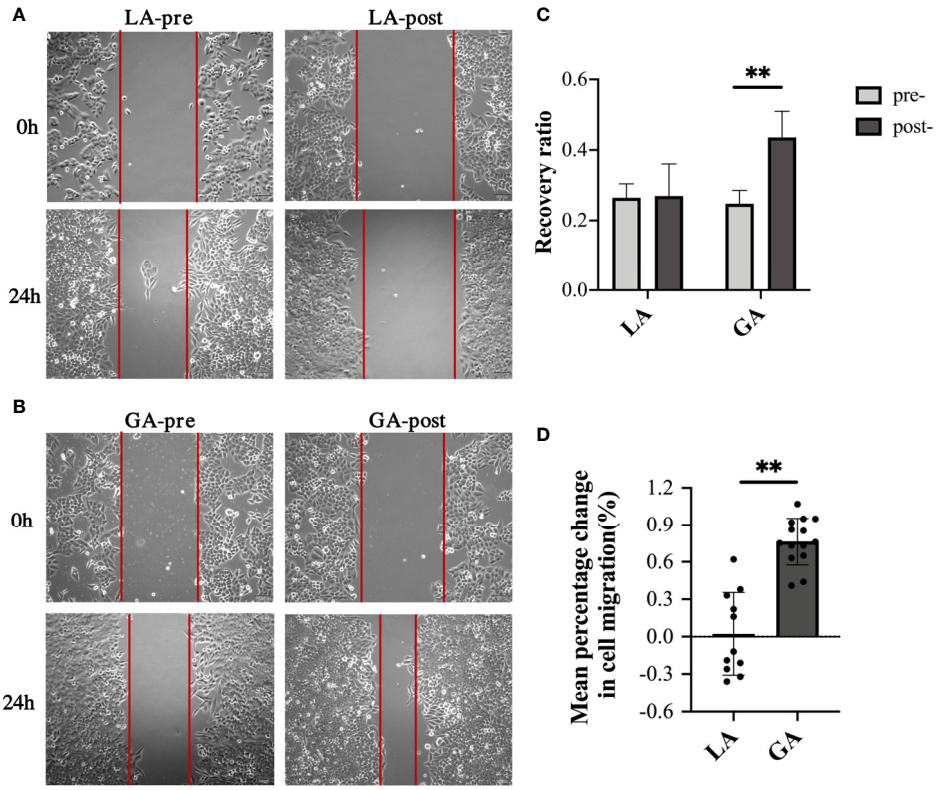

FIGURE 3 | Serum from RFA patients receiving GA facilitated cell migration of HepG2 cells. **(A)** Representative image of HepG2 cells cultured with preoperative or 1h postoperative serum from the LA group; **(B)** Representative image of HepG2 cells cultured with preoperative or 1h postoperative serum from the GA group; **(C)** Graphical representation of recovery ratios of the four groups in **(A, B, D)** Graphical representation of the mean percentage change from post- to preoperative values of recovery ratios in the GA group *vs* the LA group. Values are expressed as mean ± SD. *n* =11, LA group. *n* = 14, GA group. $^{**}P < 0.01$. GA, General Anesthesia; LA, Local Anesthesia.

undergoing GA, serum pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, increased significantly after surgery, while in the post- LA group, serum expression levels of these cytokines remained similar compared to the preoperative values from the same group. In addition, lymphokines including IFN-γ and IL-2 were significantly decreased after surgery in the GA group (**Table 2**).

DISCUSSION

In the present study, we conducted a randomized and controlled study using serum from patients with HCC undergoing RFA, who received either GA or LA. We found that postoperative serum from patients who received GA, but not those who received LA, were able to significantly promote the invasion, migration and proliferation ability of a human HCC cell line. HepG2 cells also displayed upregulated expression levels of pro-inflammatory cytokines and downregulated levels of lymphokines when treated with post- GA serum. Although a direct and definite causal relationship between anesthesia method and tumor-promoting features in serum remains to be verified, these findings suggest that GA is probably associated with a poorer prognosis for HCC patients receiving RFA surgery compared to patients who received only LA.

Whether anesthetic drugs and anesthesia methods influence the prognosis of cancer patients has been a topic of interest in recent years. Several retrospective clinical studies have shown that cancer patients receiving GA combined with regional anesthesia have a better prognosis than patients undergoing surgery under GA alone (10), whereas several large clinical studies published in recent years, both prospectively and retrospectively, show that anesthesia methods have no effect on patients' OS or RFS (16–18). Meanwhile, most fundamental studies focusing on anesthetics have proven that propofol, midazolam and local anesthetics exert potential anti-cancer properties, and in contrast, inhalants and opioids promote cancer development (19–24), which may be related to inhibition of the body's immune function and upregulation of tumor cell proliferation. While the exact effects of anesthesia remain to be elucidated, it is important to note that most prior clinical studies were comparisons between GA and GA combined with regional anesthesia [epidural anesthesia or peripheral nerve block]. Therefore, the differences found in their clinical patients could possibly be due to a negative impact of GA, or a protective effect of regional anesthesia, or a

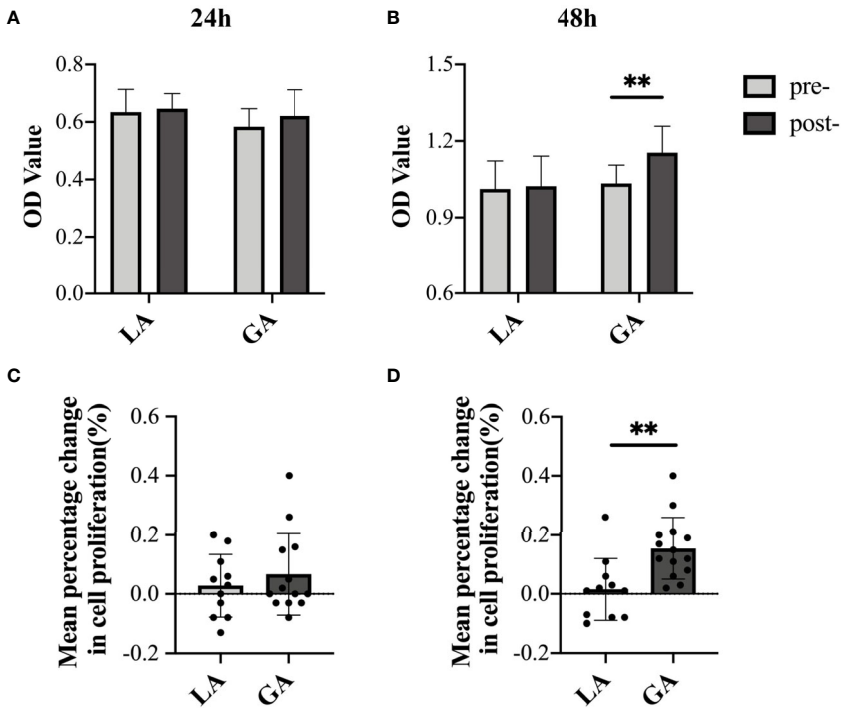

FIGURE 4 | Serum from RFA patients receiving GA facilitated cell proliferation of HepG2 cells after long-term, but not short-term, exposure. **(A)** CCK-8 assay OD values (culture time = 24h); **(B)** CCK-8 assay OD values (culture time = 48h); **(C)** mean percentage change from post- to preoperative OD value (culture time = 24h); **(D)** mean percentage change from post- to preoperative OD value (culture time = 48h). Values are expressed as mean ± SD. *n* = 11, LA group. *n* = 13~14, GA group. $^{**}P < 0.01$. GA, General Anesthesia; LA, Local Anesthesia.

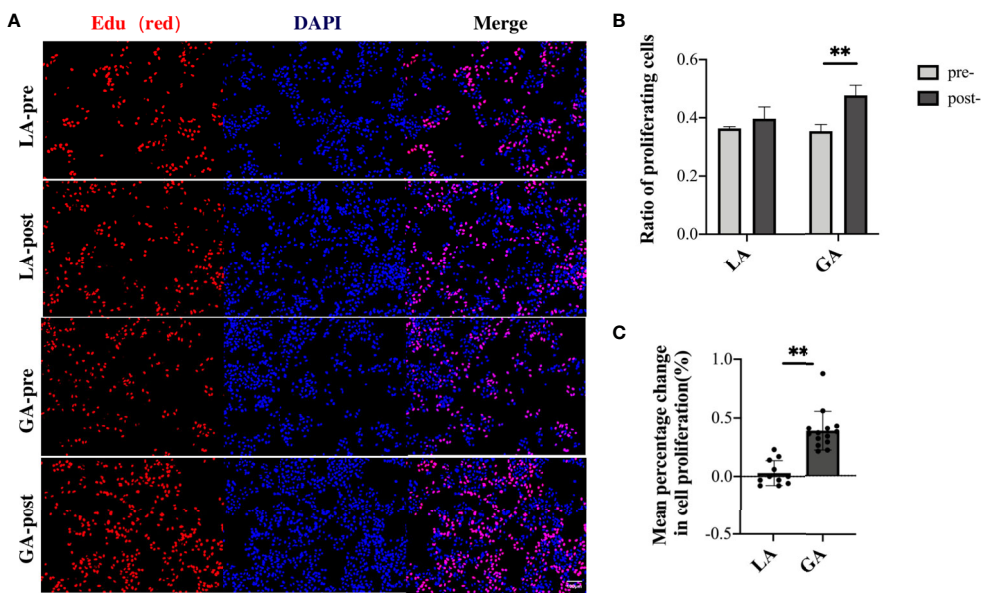

FIGURE 5 | Serum from RFA patients receiving GA facilitated cell proliferation of HepG2 cells after long-term exposure, demonstrated with EdU assays. **(A)** HepG2 cells cultured with preoperative and 1h postoperative serum from the LA and GA groups, respectively; proliferative cells were stained with EdU (red), and nuclei were counterstained with Hoechst 33342 (blue); Original magnification, 100X; **(B)** Graphical representation of the proportions of EdU positive cells of the four groups; **(C)** Graphical representation of the mean percentage change from post- to preoperative values in EdU positive cells in the GA *vs* LA groups. Values are expressed as mean ± SD. *n* = 11, LA group. *n* = 14, GA group. $^{**}P < 0.01$. GA, General Anesthesia; LA, Local Anesthesia.

TABLE 2 | Levels of pro-inflammatory cytokines and lymphokines of the thermal ablation surgery patients [Mean (SD)].

Cytokines	LA (*n* = 8)		*P* value	GA (*n* = 8)		*P* value
	Pre-	Post-		Pre-	Post-	
IL-1β (ng/ml)	0.45 (0.29)	0.50 (0.36)	0.73	0.62 (0.22)	2.69 (2.22)	0.02
IL-6 (ng/ml)	1.19 (0.86)	3.52 (4.10)	0.36	3.25 (1.90)	26.48 (17.82)	0.00
Ig (TNF-α) (ng/ml)	1.18 (0.70)	1.18 (0.61)	0.99	1.33 (0.50)	2.49 (0.64)	0.00
IFN-γ (ng/ml)	27.69 (3.52)	29.45 (3.19)	0.31	26.73 (6.43)	20.97 (3.48)	0.04
IL-2 (ng/ml)	24.77 (1.58)	25.34 (1.84)	0.52	25.26 (1.12)	23.52 (0.92)	0.01

Variables are shown as "mean (SD)". IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, Tumor necrosis factor-α; IFN-γ, interferon-γ; IL-2, interleukin-2; SD, standard deviation; LA, local anesthesia; GA, general anesthesia.

combination of both could possibly lead to no differences between groups. Therefore, in the present study, patients undergoing RFA surgery for HCC are included and studied, since RFA is a unique tumor surgery that can verify the association between GA *per se* and tumor malignancy.

According to our previous multi-center retrospective cohort study, HCC patients who received RFA surgery under GA have a higher rate of tumor recurrence and shorter OS than those who received LA, however, the mechanism behind this phenomenon still remains unclear. In this randomized clinical trial, we found that patients' serum from the post-GA group significantly promoted the invasion and migration ability of HepG2. The ability of cancer cells to migrate and invade directly relates to their degree of malignancy during cancer development (25). Increased invasion and migration abilities of cancer cells allow them to change position within tissues more easily, and once they arrive at suitable sites, such as bone and lung, metastasis occurs (26). Therefore, our findings that HepG2 cells display higher abilities of invasion and migration in serum from postoperative GA patients compared to preoperative patients, indicates that GA may promote remote metastasis of HCC, resulting in a poorer prognosis. In addition, long-term (48h), but not short-term (24h), exposure to postoperative serum from patients of the GA group caused a significant increase in proliferation activity of HepG2 cells. We speculated that this delayed increase is due to a much lower biological activity of human serum compared to fetal calf serum, which is normally used in cell culture experiments. An extended time frame is probably needed for HepG2 cells to reach the logarithmic phase when using adult patient serum.

The body's immune system plays an important role in resisting tumor recurrence and metastasis. Many studies have shown that the function of multiple immune cells, including natural killer cells, effector T cells, lymphocytes, dendritic cells and B cells, are suppressed after GA (27, 28). Lymphokines are a kind of cytokine derived from lymphocytes, which suppress tumor progression and metastasis (29–31). IFN-γ, one of the major lymphokines, acts as an important immune-activated factor in cancer (31). Another lymphokine, IL-2, also plays a vital role in promoting the secretion of T cell cytokines, enhancing the killing ability of Natural Killer (NK) cells, and promoting B cells to participate in humoral immunity. In recent years, many studies have confirmed that the enhanced function of IL-2 can inhibit tumor occurrence and development (30). Our study showed that post- GA, levels of IL-2 and IFN-γ in patients' serum decreased compared to pre- GA, suggesting that GA may lead to immunosuppression in patients through the inhibition of lymphocytes. This may arise from various anesthetics used during GA, especially opioids (32).

In contrast to lymphocytes, pro-inflammatory cytokines are associated with enhanced tumor development and spread (33). In this study, serum levels of pro-inflammatory cytokines IL-1β, IL-6, TNF-α were significantly increased after GA, whereas in the LA group, levels of these cytokines only increased slightly in postoperative serum compared to preoperative serum. We speculate that this slight increase in the LA group reflects an increased inflammatory response caused by surgical trauma (34), and the significant increase in the GA group was due to a combination of both surgery trauma and GA. In addition to pro-inflammatory cytokines, it has been reported that many anesthetics used during GA also increase synthesis of the vascular endothelial growth factor, hypoxia-inducible factor and matrix metalloproteinase, which ultimately stimulate the proliferation and migration capacities of tumor cells and increase stromal angiogenesis (20, 28, 35).

At present, whether the differences between the GA and LA groups were caused by the anesthesia technique, in other words, the state of GA *per se*, or by the anesthetics used, remains unknown. We speculate that the latter contributes more to the differences than the former. The most obvious change induced by the GA technique is a loss of state of consciousness. In an awake patient, the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system, whose activation is widely accepted to be associated with immune suppression (36, 37), should be more highly activated than in an anesthetized patient. Therefore, from this point of view, GA should have resulted in a better prognosis for cancer patients compared to patients receiving LA, which seems contradictory to the current findings. However, there are many other physiological changes during GA, which may contribute to tumor growth. Although unclear yet, their roles cannot be simply ruled out currently. On the other hand, various anesthetics have been reported to influence environmental signals that affect tumor outcome (38–40). For example, opioids, which are widely utilized in perioperative clinical practice for analgesia, could, after binding to their receptors (i.e., μ-opioid receptor), activate Akt and mTOR signaling, a well-defined pathway that contributes to tumor survival (38). Even though several studies have shown that propofol has potential anti-cancer properties (39), a recent study by Liu et al. (40) demonstrates that propofol augments

lung tumor metastasis by downregulating TRIM21 expression and consequently promoting adhesion and extension of tumor cells. Therefore, we assume that mixed influences from multiple anesthetics accounted for at least part of the differences that were observed in the current study between the two groups. Whether one or several medications among them played a major role remains to be elucidated by more studies.

This study has certain limitations. First, the sample size was relatively small. However, when taking into consideration the actual sample size of 25 and an observed difference of 54% in the primary outcome between the two groups, the actual calculated statistical power is much higher than the estimated power. Second, the objectives of this study were indirect indicators of tumor outcome. Follow-up studies of direct indicators, such as long-term RFS or OS, would provide convincing evidence whether anesthesia methods influence the prognosis of HCC patients.

In summary, these findings suggest that GA may affect the serum milieu of patients with HCC, thereby promoting the malignant biological behavior of HCC. These results provide important guidance for anesthesia method choice in HCC patients undergoing RFA surgery, and also indicate a necessity for large-scale, multicenter, and prospective clinical studies in such patients, to further verify the influence of anesthesia methods on their long-term prognosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Human Ethics Committee of Renji Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YS, TWu, and TWa have contributed equally to this work and share first authorship. BZ and JT were both corresponding authors. All authors contributed to the article and approved the submitted version.

FUNDING

This study received financial support from the Shanghai Science and Technology Committee Foundation (grant number 19ZR1430600), Shanghai Municipal Health Commission Foundation (grant number ZY (2018-2020)-ZYJS-48), Clinical Research Plan of SHDC (grant number SHDC2020CR4062), and Shanghai Municipal Commission of Health and Family Planning (grant number 201840241).

ACKNOWLEDGMENTS

We thank Isabel Lambertz for English language editing.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 24 September 2021
doi: 10.3389/fphar.2021.685265

Propofol-Based Total Intravenous Anesthesia is Associated with Better Survival than Desflurane Anesthesia in Epithelial Ovarian Cancer Surgery: A Retrospective Cohort Study

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OPEN ACCESS

Edited by:
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Taipei Medical University, Taiwan

Reviewed by:
Yuan-Wen Lee,
Taipei Medical University Hospital, Taiwan
Szu-Yuan Wu,
Asia University, Taiwan

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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Pharmacology

Received: *24 March 2021*
Accepted: *10 September 2021*
Published: *24 September 2021*

Citation:
Tseng W-C, Lee M-S, Lin Y-C, Lai H-C, Yu M-H, Wu K-L and Wu Z-F (2021) Propofol-Based Total Intravenous Anesthesia is Associated with Better Survival than Desflurane Anesthesia in Epithelial Ovarian Cancer Surgery: A Retrospective Cohort Study. Front. Pharmacol. 12:685265. doi: 10.3389/fphar.2021.685265

Background: Previous studies have shown that anesthetic techniques can affect outcomes of cancer surgery. We investigated the association between anesthetic techniques and patient outcomes after elective epithelial ovarian cancer surgery.

Methods: This was a retrospective cohort study of patients who received elective open surgery for epithelial ovarian cancer between January 2009 and December 2014. Patients were grouped according to the administration of propofol or desflurane anesthesia. Kaplan–Meier analysis was performed, and survival curves were constructed from the date of surgery to death. Univariate and multivariate Cox regression models were used to compare hazard ratios for death after propensity matching. Subgroup analyses were performed for age, body mass index, preoperative carbohydrate antigen-125 level, International Federation of Gynecology and Obstetrics staging, and operation and anesthesia time.

Results: In total, 165 patients (76 deaths, 46.1%) who received desflurane anesthesia and 119 (30 deaths, 25.2%) who received propofol anesthesia were eligible for analysis. After propensity matching, 104 patients were included in each group. In the matched analysis, patients who received propofol anesthesia had better survival with a hazard ratio of 0.52 (95% confidence interval, 0.33–0.81; $p = 0.005$). Subgroup analyses also showed significantly better survival with old age, high body mass index, elevated carbohydrate antigen-125 level, advanced International Federation of Gynecology and Obstetrics stage, and prolonged operation and anesthesia time in the matched propofol group. In addition, patients administered with propofol anesthesia had less postoperative recurrence and metastasis than those administered with desflurane anesthesia in the matched analysis.

Conclusion: Propofol anesthesia was associated with better survival in patients who underwent elective epithelial ovarian cancer open surgery. Prospective studies are

warranted to evaluate the effects of propofol anesthesia on oncological outcomes in patients with epithelial ovarian cancer.

Keywords: cancer surgery, desflurane, epithelial ovarian cancer, propofol, survival

INTRODUCTION

Ovarian cancer is the seventh most common cancer among women worldwide (Lheureux et al., 2019a; Lheureux et al., 2019b), and epithelial ovarian cancer (EOC) accounts for over 95% of ovarian malignancies (Lheureux et al., 2019a). Because EOC is often diagnosed at an advanced stage, the outcomes of the disease are complicated, making it the most lethal gynecological cancer, with a 5-year survival rate of 46% (Lheureux et al., 2019b). Surgery has been a mainstay of therapy for EOC and allows for accurate surgical staging and therapeutic effects by debulking the disease (Lheureux et al., 2019a). However, surgery-induced stress may lead to immunosuppression and upregulation of adhesion molecules through mechanisms involving inflammation, ischemia-reperfusion injury, sympathetic nervous system activation, and increased cytokine release (Chen et al., 2019). The combination of potential tumor cell dissemination and impaired immune response produces an environment favorable for the development of cancer recurrence and metastasis. Accordingly, there is increasing interest in the impact of the perioperative setting on cancer progression.

Accumulating evidence shows that different anesthetic agents or techniques can influence immune function and tumor development in various pathways (Snyder and Greenberg, 2010; Kim, 2018; Chen et al., 2019). Experimental studies showed that volatile anesthetics (VAs) may alter immunological processes that increase metastatic potential (Shapiro et al., 1981; Moudgil and Singal, 1997; Melamed et al., 2003), whereas propofol seemed to maintain the integrity of immunity and reduce the tendency toward cancer metastasis (Mammoto et al., 2002; Melamed et al., 2003; Kushida et al., 2007). Such effects of volatile and propofol anesthesia were also reported in clinically surgical settings, indicating the superiority of propofol over VAs in cancer surgery (Buckley et al., 2014; Zhang et al., 2014; Liu et al., 2016; Ai and Wang, 2020). In addition, results from retrospective studies reported that propofol-based anesthesia produced better long-term outcomes than VAs-based anesthesia after surgery in different types of cancers (Wigmore et al., 2016; Jun et al., 2017; Wu et al., 2018; Lai et al., 2019a; Lai et al., 2019b; Huang et al., 2020; Lai et al., 2020a; Lai et al., 2020b). However, some studies did not show definite effects of anesthetic agents on cancer immunity and outcomes (Lim et al., 2018; Oh et al., 2018; Huang et al., 2019; Yoo et al., 2019; Grau et al., 2020; Makito et al., 2020). Notably, a recent meta-analysis showed that propofol-based total intravenous anesthesia is generally associated with better overall survival than volatile anesthesia in cancer surgery, especially in patients who received desflurane anesthesia (Chang et al., 2021).

Previous studies have shown that intraoperative use of epidural anesthesia was associated with improved oncological outcomes in patients with ovarian cancer (de Oliveira et al., 2011; Tseng et al., 2018). To the best of our knowledge, there is a retrospective cohort study discussing the impacts of different VAs during anesthesia maintenance on survival outcomes after EOC surgery and concluding that patients with advanced EOC who were administered with desflurane anesthesia experienced a lower rate of disease recurrence and an improved disease-free survival after primary cytoreductive surgery compared with those who were administered with sevoflurane anesthesia (Elias et al., 2015). However, no known study has compared the effects between propofol and VAs on patient outcomes after EOC surgery. We hypothesized that propofol anesthesia was associated with greater overall survival than desflurane anesthesia as our previous studies (Wu et al., 2018; Lai et al., 2019a; Lai et al., 2019b; Huang et al., 2020; Lai et al., 2020a; Lai et al., 2020b). Therefore, we conducted a retrospective analysis to assess the relationship between the type of anesthesia and long-term outcomes after EOC surgery and to identify potential risk factors for mortality.

METHODS

Study Design and Setting

This retrospective cohort study was conducted at Tri-Service General Hospital (TSGH), Taipei, Taiwan.

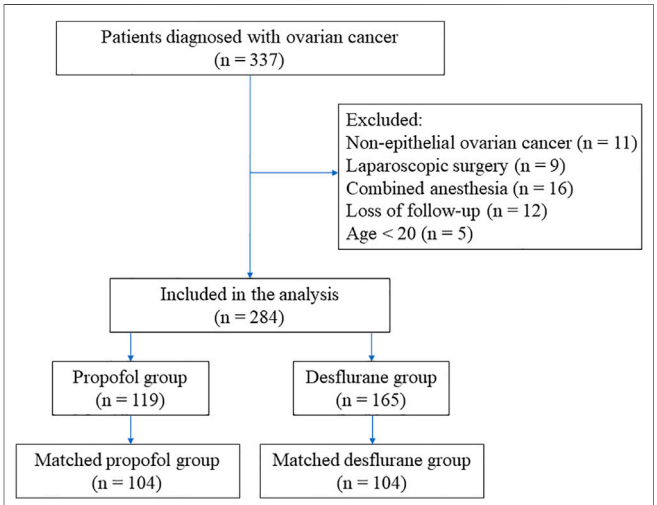

FIGURE 1 | Flow diagram detailing the selection of patients included in the retrospective analysis. Of the patients, 53 were excluded because of combined propofol anesthesia with inhalation anesthesia or regional analgesia, non-epithelial ovarian cancer, incomplete data, age <20 years old, or undergoing laparoscopic surgery.

Participants and Data Sources

The ethics committee of TSGH approved this retrospective study and waived the need for informed consent (TSGHIRB No: 2-106-05-132). Relevant information was retrieved from the medical records and electronic database at TSGH for 284 patients with an American Society of Anesthesiologists (ASA) score of II to III who had undergone elective EOC open surgery for International Federation of Gynecology and Obstetrics (FIGO) stage I to IV EOC between January 2009 and December 2014. Patients included for analysis were administered with either propofol anesthesia (n = 119) or desflurane anesthesia (n = 165), based on the anesthesiologist's preference.

Exclusion criteria were propofol anesthesia combined with VAs or regional analgesia, laparoscopic surgery, non-EOC, incomplete data, and age under 20 years. Ultimately, 53 cases were excluded from this analysis (**Figure 1**).

Anesthetic Technique

No premedication was given before the induction of anesthesia. Standard monitoring, including electrocardiography (lead II), noninvasive blood pressure, pulse oximetry, end-tidal carbon dioxide ($EtCO_2$), and direct radial arterial blood pressure measures, was instituted for each patient. Anesthesia was induced with fentanyl, propofol, and cisatracurium or rocuronium in all patients.

In the propofol group, anesthesia was maintained with a target-controlled infusion (TCI) pump (Orchestra® Base Primea, Fresenius Kabi AG, Bad Homburg, Germany) using propofol at an effect-site concentration of 3–4 mcg/ml in a fraction of inspired oxygen (FiO_2) of 100% oxygen at a flow rate of 300 ml/min. In the desflurane group, the desflurane vaporizer was set between 4 and 10% with 100% oxygen at a flow rate of 300 ml/min in a closed breathing system. Repetitive bolus injections of fentanyl and cisatracurium or rocuronium were administered as necessary during surgery. According to the hemodynamics, the maintenance of anesthesia with desflurane and the effect-site concentration of propofol using a TCI pump were adjusted upward and downward by 0.5–2% and 0.2–0.5 mcg/ml, respectively. The level of $EtCO_2$ was maintained at 35–45 mmHg by adjusting the ventilation rate in the volume control model with a tidal volume of 6–8 ml/kg and a maximum airway pressure <30 cmH_2O. After surgery, all patients were transferred to the post-anesthesia care unit for postoperative observation and care (Wu et al., 2018; Lai et al., 2019a; Lai et al., 2019b; Lai et al., 2020a; Lai et al., 2020b).

Variables

We retrospectively collected the following patient data: anesthetic technique; time since the earliest included patient serving as a surrogate of the calendar year; calendar period; age at the time of surgery; habitus; underlying disease; menstrual and reproductive factors; FIGO stage and histological grade of the primary tumor; presence of pleural effusion or ascites before surgery; and pretreatment serum level of carbohydrate antigen-125 (CA-125). For pretreatment serum CA-125 values, patients were grouped according to CA-125 values of ≥35 or <35 U/ml because a CA-125 level ≥35 U/ml was associated with poor survival in patients with EOC (Lin et al., 2020).

The Charlson comorbidity index (CCI) of 0 (least comorbidity) to 37 (highest comorbidity) was used to predict the 10-year survival in patients with multiple comorbidities. In addition, preoperative functional status was evaluated in metabolic equivalents (METs), and patients were grouped according to a functional status of ≥4 or <4 METs because perioperative cardiac and long-term risks increased in patients with a capacity of <4 METs during most normal daily activities (Fleisher et al., 2014).

Other data included the ASA physical status score from I (lowest morbidity) to V (highest morbidity); administration of neoadjuvant or adjuvant chemotherapy; need for intraoperative blood transfusion; use of postoperative nonsteroidal anti-inflammatory drugs (NSAIDs); operation and anesthesia time; total propofol dosage including induction dose; grade of surgical complications using the Clavien–Dindo classification scaled from 0 (no complication) to V (death); length of hospital stay; presence of postoperative recurrence or metastasis; and mortality. Postoperative recurrence could be identified by physical examination, radiological evidence, and serum CA-125 monitoring. Concerning levels of serum CA-125, postoperative recurrence was defined by the rise of more than twice the upper limit of normal (35 U/ml) 1) for patients with normal baseline CA-125 levels, or for those whose CA-125 levels had normalized during treatment; and 2) the rise of more than twice nadir value for patients whose CA-125 levels had not normalized. Postoperative metastasis was defined by the new development of 1) pleural effusion with positive cytology; 2) liver or splenic parenchymal metastasis; 3) metastasis to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside the abdominal cavity); and 4) transmural involvement of intestine, which was not detected before surgery (Lheureux et al., 2019b). Based on causes of death, patients who died at the follow-up period from the date of surgery to December 31, 2019 were recorded as all-cause or cancer-specific mortality. All-cause mortality was defined that patients died at the follow-up period under various causes including cancer-related or not; cancer-specific mortality was defined that patients died only from cancer-related causes at the follow-up period. Because these variables had been shown or posited to affect patient outcomes, they were chosen as potential confounders.

Statistical Methods

The primary outcome was overall survival compared between the propofol and desflurane groups. The survival time was defined as the interval between the date of surgery and the date of death or December 31, 2019, for patients who were censored. All data were presented as mean ± standard deviation (SD) or number (percentage).

Patient characteristics and mortality rates were compared between the groups treated with different anesthetics using Student's t-test or chi-squared test. The survival according to the anesthetic technique was depicted visually in a Kaplan–Meier survival curve. The association between the anesthetic technique (propofol or desflurane) and survival was analyzed using the Cox

TABLE 1 | Patient and treatment characteristics for overall group and matched group after propensity scoring.

Variables	Overall patients			Matched patients			SMD
	Propofol (*n* = 119)	Desflurane (*n* = 165)	*p* value	Propofol (*n* = 104)	Desflurane (*n* = 104)	*p* value	
Time since the earliest included patient (years), mean (SD)	3.28 (1.67)	3.36 (1.83)	0.723	3.33 (1.69)	2.37 (1.52)	<0.001	0.597
Calendar period, *n* (%)			0.054			0.008	0.443
2009–2010	30 (25.2)	45 (27.3)		26 (25.0)	45 (43.3)		
2011–2012	45 (37.8)	41 (24.8)		38 (36.5)	36 (34.6)		
2013–2014	44 (37.0)	79 (47.9)		40 (38.5)	23 (22.1)		
Age (years/o), mean (SD)	53.70 (11.28)	54.41 (12.17)	0.618	53.98 (11.34)	52.82 (12.19)	0.477	0.099
BMI (kg/m^2), mean (SD)	23.60 (3.88)	23.55 (3.84)	0.927	23.45 (3.62)	23.31 (3.74)	0.784	0.038
Charlson comorbidity index, mean (SD)	3.55 (1.67)	3.84 (1.96)	0.183	3.57 (1.59)	3.45 (1.70)	0.614	0.073
Underlying disease							
Diabetes mellitus	6 (5.0)	18 (10.9)	0.124	6 (5.8)	9 (8.7)	0.592	0.112
Coronary artery disease	8 (6.7)	13 (7.9)	0.891	7 (6.7)	6 (5.8)	1.000	0.037
Stroke	2 (1.7)	3 (1.8)	1.000	2 (1.9)	2 (1.9)	1.000	0.000
Chronic obstructive pulmonary disease	7 (5.9)	6 (3.6)	0.545	7 (6.7)	3 (2.9)	0.331	0.179
Liver disease	4 (3.4)	10 (6.1)	0.448	4 (3.8)	4 (3.8)	1.000	0.000
Peptic ulcer disease	4 (3.4)	11 (6.7)	0.337	4 (3.8)	5 (4.8)	1.000	0.049
ASA class, *n* (%)			0.195			1.000	0.025
II	96 (80.7)	121 (73.3)		83 (79.8)	84 (80.8)		
III	23 (19.3)	44 (26.7)		21 (20.2)	20 (19.2)		
Functional status, *n* (%)			0.195			1.000	0.025
≥4 METs	96 (80.7)	121 (73.3)		83 (79.8)	84 (80.8)		
<4 METs	23 (19.3)	44 (26.7)		21 (20.2)	20 (19.2)		
Menarche, *n* (%)			0.334			0.782	0.076
≥12 years/o	109 (91.6)	157 (95.2)		98 (94.2)	96 (92.3)		
<12 years/o	10 (8.4)	8 (4.8)		6 (5.8)	8 (7.7)		
Menopause, *n* (%)			0.483			0.940	0.048
≤50 years/o	35 (29.4)	41 (24.9)		29 (27.9)	27 (26.0)		
>50 years/o	41 (34.5)	68 (41.2)		38 (36.5)	38 (36.5)		
Not yet	43 (36.1)	56 (33.9)		37 (35.6)	39 (37.5)		
Parity, *n* (%)			0.713			1.000	0.020
0–1	32 (26.9)	40 (24.2)		30 (28.8)	29 (27.9)		
≥2	87 (73.1)	125 (75.8)		74 (71.2)	75 (72.1)		
FIGO stage of primary tumor, *n* (%)			0.103			0.095	0.253
I and II	63 (52.9)	70 (42.4)		54 (51.9)	41 (39.4)		
III and IV	56 (47.1)	95 (57.6)		50 (48.1)	63 (60.6)		
Histological grade of primary tumor, n (%)			0.511			0.988	0.015
I	14 (11.8)	13 (7.9)		10 (9.6)	10 (9.6)		
II	34 (28.6)	46 (27.9)		30 (28.9)	29 (27.9)		
III	71 (59.6)	106 (64.2)		64 (61.5)	65 (62.5)		
Pleural effusion, *n* (%)	21 (17.6)	37 (22.4)	0.403	17 (16.3)	19 (18.3)	0.855	0.053
Ascites, *n* (%)			0.721			0.652	0.085
None to mild	84 (70.6)	112 (67.9)		74 (71.2)	70 (67.3)		
Moderate to massive	35 (29.4)	53 (32.1)		30 (28.8)	34 (32.7)		
Preoperative CA-125 level, n (%)			0.106			1.000	0.030
≥35 U/ml	98 (82.4)	148 (89.7)		90 (86.5)	91 (87.5)		
<35 U/ml	21 (17.6)	17 (10.3)		14 (13.5)	13 (12.5)		
Neoadjuvant chemotherapy, *n* (%)	7 (5.9)	4 (2.4)	0.239	2 (1.9)	3 (2.9)	1.000	0.065
Adjuvant chemotherapy, *n* (%)	106 (89.1)	154 (93.3)	0.291	94 (90.4)	97 (93.3)	0.613	NA
Intraoperative transfusion, *n* (%)	57 (47.9)	88 (53.3)	0.433	50 (48.1)	57 (54.8)	0.405	NA
Postoperative NSAID, *n* (%)	28 (23.5)	42 (25.5)	0.817	26 (25.0)	27 (26.0)	1.000	NA
Operation time (min), mean (SD)	205.64 (69.24)	214.28 (81.85)	0.350	208.01 (70.38)	210.20 (84.88)	0.840	NA
Anesthesia time (min), mean (SD)	230.13 (69.34)	240.16 (81.39)	0.277	232.52 (70.40)	235.84 (84.50)	0.759	NA
Propofol dosage (mg), mean (SD)	1234.61 (347.68)	116.04 (20.09)	<0.001	1243.26 (349.61)	114.87 (18.99)	<0.001	NA
Grade of surgical complications, *n* (%)			0.849			0.762	NA
0	47 (39.5)	59 (35.8)		41 (39.4)	36 (34.6)		
I	14 (11.8)	17 (10.3)		12 (11.5)	10 (9.6)		
II	54 (45.4)	82 (49.7)		47 (45.2)	52 (50.0)		
III	4 (3.3)	7 (4.2)		4 (3.8)	6 (5.8)		
Length of hospital stay (days), mean (SD)	10.73 (7.09)	11.84 (6.52)	0.173	11.09 (7.30)	11.53 (6.54)	0.646	NA
Postoperative recurrence, *n* (%)	43 (36.1)	100 (60.6)	<0.001	42 (40.4)	64 (61.5)	0.004	NA
Postoperative metastasis, *n* (%)	27 (22.7)	68 (41.2)	0.002	26 (25.0)	43 (41.3)	0.018	NA
All-cause mortality, *n* (%)	30 (25.2)	76 (46.1)	0.001	29 (27.9)	50 (48.1)	0.004	NA
Cancer-specific mortality, *n* (%)	27 (22.7)	73 (44.2)	<0.001	26 (25.0)	49 (47.1)	0.001	NA

Data shown as mean ± SD or n *(%). Grade of surgical complications: Clavien–Dindo classification. ASA, American Society of Anesthesiologists; BMI, body mass index; FIGO, International Federation of Gynecology and Obstetrics; MET, metabolic equivalent; NA, not applicable; NSAID, nonsteroidal anti-inflammatory drug; SD, standard deviation; SMD, standardized mean difference.*

proportional-hazards model with and without adjustment for variables noted previously. Overall survival from the date of surgery grouped according to the anesthetic technique and other variables was compared separately in a univariate Cox model and subsequently in a multivariate Cox regression model. Variables that were significant in the univariate model proceeded to execute the multivariate analysis, but postoperative recurrence and metastasis were excluded to avoid multicollinearity. We also conducted subgroup analyses for age, body mass index (BMI), preoperative CA-125 level, FIGO stages, operation and anesthesia time, and disease progression between the 2 anesthetic techniques.

Propensity score (PS) matching using IBM SPSS Statistics 23.0 (IBM SPSS Inc., Chicago, IL) was applied to select the most similar PSs for preoperative variables (with caliper sets at 0.2 SD of the logit of the PS) across each anesthesia: propofol or desflurane in a 1:1 ratio, ensuring the comparability between propofol and desflurane anesthesia before surgery. Preoperative variables for performing PS matching included time since the earliest included patient; age; BMI; CCI; ASA class; menstrual and reproductive factors; FIGO stage and histological grade; presence of pleural effusion or ascites; preoperative serum level of CA-125; and administration of neoadjuvant chemotherapy. Because calendar period, underlying disease and functional status were highly correlated with time since the earliest included patient, CCI and ASA class, respectively, these variables were excluded to increase the rigorousness of PS matching. Two-tailed p values <0.05 were considered statistically significant.

RESULTS

Patient and Treatment Characteristics

Patient and treatment characteristics are shown in **Table 1**. The time since the earliest included patient; calendar period; age; BMI; CCI; underlying disease; ASA score; preoperative functional status; menstrual and reproductive factors; FIGO stage and histological grade of the primary tumor; presence of pleural effusion and ascites before surgery; baseline CA-125 level; administration of neoadjuvant and adjuvant chemotherapy; need for intraoperative blood transfusion; use of postoperative NSAIDs; operation and anesthesia time; grade of surgical complications; and length of hospital stay were not significantly different between the 2 anesthetic techniques (**Table 1**). Total propofol dosage in the propofol group was significantly more than that in the desflurane group (**Table 1**). In addition, no patient underwent postoperative radiotherapy.

The PS matching is an essential statistical method to minimize the effect of confounding in observational studies (Austin et al., 2018). Therefore, we used the PS from the logistic regression to adjust the baseline characteristics and the choice of treatment between the 2 anesthetic techniques. Altogether, 104 pairs were formed after matching. Patient characteristics and treatment factors of EOC were not significantly different between the matched groups except for time since the earliest included patient, calendar period and total propofol dosage (**Table 1**).

A greater percentage of patients in the desflurane group (60.6%) developed postoperative recurrence compared with the propofol group (36.1%; $p < 0.001$). The incidence of postoperative metastasis was also significantly higher in the desflurane group (41.2%) than in the propofol group (22.7%; $p = 0.002$) during follow-up. The all-cause mortality rate was significantly lower in the propofol group (25.2%) than in the desflurane group (46.1%; $p = 0.001$) during follow-up. Furthermore, the cancer-specific mortality rate was significantly lower in the propofol group (22.7%) than in the desflurane group (44.2%; $p < 0.001$) during follow-up. After PS matching, results were consistent between the 2 anesthetic techniques (**Table 1**). The median follow-up period was 5.86 years for the propofol group and 4.63 years for the desflurane group. Kaplan–Meier survival curves for the 2 anesthetic techniques are shown in **Figures 2A,B**. In addition, the cumulative incidence of cancer relapse is shown in **Figure 3**.

Risks of Overall Mortality

The risk of overall mortality associated with the administration of propofol and desflurane anesthesia during EOC open surgery is shown in **Table 2**. Patients who received propofol anesthesia had better overall survival than those who received desflurane anesthesia [overall survival, 74.8 versus 53.9%, respectively; hazard ratio (HR), 0.46; 95% confidence interval (CI), 0.30–0.70; $p < 0.001$]. In the multivariate model after adjustment for age at the time of surgery; CCI; ASA score; age at the time of menopause; FIGO stage; histological grade; presence of pleural effusion and ascites before surgery; preoperative CA-125 level; intraoperative blood transfusion; and grade of surgical complications, patients in the propofol group were also associated with improved overall survival than those in the desflurane group (HR, 0.53; 95% CI, 0.34–0.82; $p = 0.004$). Four other variables that significantly increased the mortality risk after the multivariate analysis were menopause at older age (>50 years old; $p = 0.010$), advanced FIGO stage ($p < 0.001$), moderate to massive ascites ($p = 0.044$), and higher baseline CA-125 level ($p = 0.046$) (**Table 2**).

Subgroup Analyses

The subgroup analyses for age, BMI, preoperative CA-125 level, FIGO stages, operation and anesthesia time, and disease progression are shown in **Table 3**. There was no interaction effect between the type of anesthesia and these factors on survival. All analyses were stratified based on age groups, BMI categories, serum CA-125 levels, different FIGO stages, and operation and anesthesia time.

Age

Elderly patients who received propofol anesthesia had better survival than those who received desflurane anesthesia. For patients with an age of <40 years old, the crude HR was 0.67 (95% CI, 0.16–2.71; $p = 0.570$), and the PS-matched HR was 0.68 (95% CI, 0.17–2.74; $p = 0.584$). For patients with an age of 40–59 years old, the crude HR was 0.48 (95% CI, 0.27–1.06; $p = 0.054$), and the PS-matched HR was 0.59 (95% CI, 0.30–1.13; $p = 0.111$). For patients with an age of ≥60 years old, the crude HR was 0.39 (95% CI, 0.19–0.77; $p = 0.007$), and the PS-matched HR was 0.37 (95% CI, 0.18–0.77; $p = 0.008$) (**Table 3**).

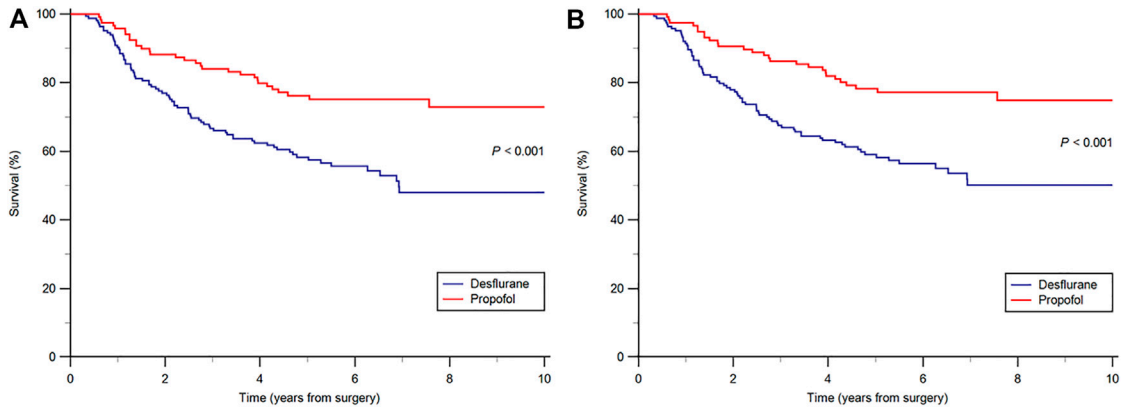

FIGURE 2 | (A) Overall **(B)** cancer-specific survival curves from the date of surgery by anesthesia type.

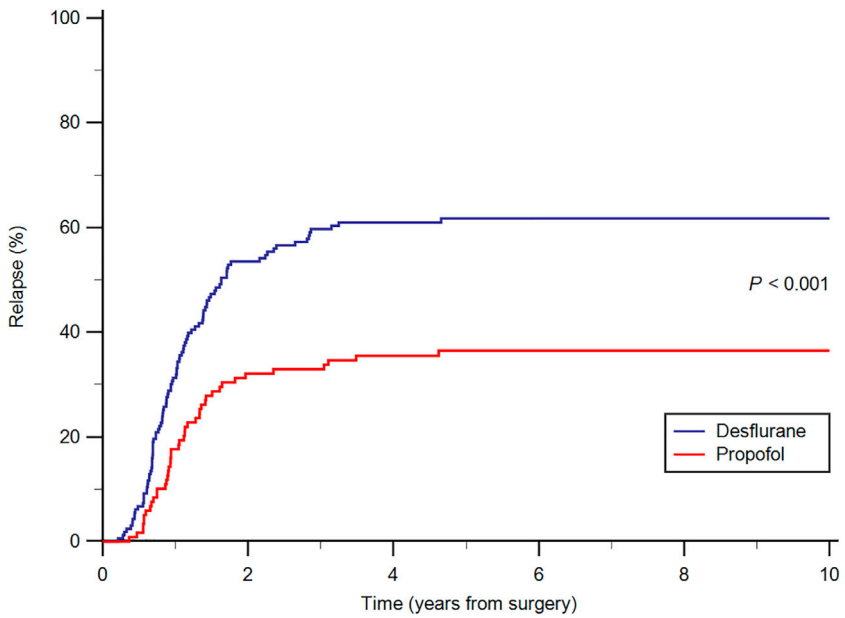

FIGURE 3 | Cumulative relapse curves from the date of surgery by anesthesia type.

Body Mass Index

Patients with overweight and obesity who received propofol anesthesia had better survival than those who received desflurane anesthesia. For patients with a BMI of <24 kg/m^2, the crude HR was 0.58 (95% CI, 0.34–1.01; $p = 0.051$), and the PS-matched HR was 0.66 (95% CI, 0.36–1.19; $p = 0.165$). For patients with a BMI of ≥24 kg/m^2, the crude HR was 0.33 (95% CI, 0.17–0.65; $p = 0.001$), and the PS-matched HR was 0.38 (95% CI, 0.18–0.78; $p = 0.008$) (**Table 3**).

Carbohydrate Antigen-125 Level

Patients with an elevated preoperative CA-125 level who received propofol anesthesia had better survival than those who received desflurane anesthesia. For patients with a CA-125 level of <35 U/ml, the crude HR was 1.70 (95% CI, 0.15–18.8; $p = 0.475$), and the PS-matched HR was 2.31 (95% CI, 0.34–21.1; $p = 0.664$). For patients with a CA-125 level of ≥35 U/ml, the crude HR was 0.46 (95% CI, 0.30–0.71; $p < 0.001$), and the PS-matched HR was 0.47 (95% CI, 0.29–0.74; $p = 0.001$) (**Table 3**).

International Federation of Gynecology and Obstetrics Stage

Patients with an advanced FIGO stage who received propofol anesthesia had better survival than those who received desflurane anesthesia. For patients with an early FIGO stage (I and II), the crude HR was 0.37 (95% CI, 0.12–1.16; $p = 0.089$), and the PS-matched HR was 0.53 (95% CI, 0.15–1.86; $p = 0.319$). For patients

TABLE 2 | Cox proportional hazards regression for mortality: univariate and multivariate models for overall patients.

Variables	Univariate		Multivariate	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Anesthesia, propofol (ref: desflurane)	0.46 (0.30–0.70)	<0.001	0.53 (0.34–0.82)	0.004
Time since the earliest included patient (years)	0.97 (0.87–1.08)	0.539		
Age (ref: <40)				
40–59 years/o	1.30 (0.64–2.63)	0.473	0.90 (0.39–2.05)	0.798
≧60 years/o	2.18 (1.06–4.46)	0.034	0.74 (0.27–2.01)	0.558
BMI (ref: <24)				
≧24 kg/m^2	1.09 (0.74–1.60)	0.671		
Charlson comorbidity index	1.27 (1.17–1.38)	<0.001	1.12 (0.99–1.26)	0.073
ASA, III (ref: II)	2.03 (1.36–3.02)	<0.001	0.76 (0.44–1.30)	0.310
Menarche, <12 years/o (ref: ≧12)	0.90 (0.40–2.06)	0.810		
Menopause (ref: ≦50)				
>50 years/o	1.81 (1.12–2.93)	0.015	1.96 (1.18–3.28)	0.010
Not yet	0.84 (0.48–1.45)	0.532	1.36 (0.69–2.65)	0.376
Parity, 0–1 (ref: ≧2)	0.99 (0.64–1.55)	0.972		
FIGO stage, III and IV (ref: I and II)	7.53 (4.35–13.0)	<0.001	4.94 (2.58–9.47)	<0.001
Histological grade (ref: I)				
II	4.51 (1.06–19.1)	0.041	1.83 (0.41–8.25)	0.429
III	7.94 (1.95–32.3)	0.004	2.30 (0.52–10.1)	0.273
Pleural effusion (ref: no)	2.74 (1.84–4.08)	<0.001	0.94 (0.55–1.61)	0.819
Ascites, moderate to massive (ref: none to mild)	3.43 (2.33–5.03)	<0.001	1.68 (1.01–2.79)	0.044
Preoperative CA-125, ≧35 U/ml (ref: <35)	6.77 (2.15–21.3)	0.001	3.57 (1.02–12.4)	0.046
Neoadjuvant chemotherapy (ref: no)	1.12 (0.46–2.76)	0.799		
Operation time (min)	1.00 (1.00–1.01)	0.063		
Anesthesia time (min)	1.00 (1.00–1.01)	0.072		
Intraoperative transfusion (ref: no)	1.70 (1.15–2.51)	0.008	0.09 (0.01–1.06)	0.056
Postoperative NSAID (ref: no)	0.98 (0.63–1.53)	0.943		
Grade of surgical complications (ref: 0)				
I	0.55 (0.23–1.32)	0.182	0.77 (0.31–1.93)	0.577
II	1.57 (1.03–2.38)	0.036	7.11 (0.61–83.3)	0.118
III	1.10 (0.39–3.11)	0.852	4.60 (0.52–40.3)	0.169
Postoperative recurrence (ref: no)	32.9 (13.4–81.1)	<0.001	NA	NA
Postoperative metastasis (ref: no)	8.52 (5.54–13.1)	<0.001	NA	NA

Hazard ratios in the multivariate analyses were adjusted by those variables having significance in the univariate analyses except for postoperative recurrence and metastasis. ASA, American Society of Anesthesiologists; BMI, body mass index; FIGO, International Federation of Gynecology and Obstetrics; HR, hazard ratio; NA, not applicable; NSAID, nonsteroid anti-inflammatory drug.

with a late FIGO stage (III and IV), the crude HR was 0.53 (95% CI, 0.33–0.83; $p = 0.006$), and the PS-matched HR was 0.60 (95% CI, 0.37–0.96; $p = 0.042$) (**Table 3**).

Operation Time

Patients with prolonged operation time who received propofol anesthesia had better survival than those who received desflurane anesthesia. For patients with operation time of <180 min, the crude HR was 0.68 (95% CI, 0.33–1.43; $p = 0.312$), and the PS-matched HR was 0.68 (95% CI, 0.32–1.44; $p = 0.312$). For patients with operation time of ≥180 min, the crude HR was 0.37 (95% CI, 0.22–0.63; $p < 0.001$), and the PS-matched HR was 0.43 (95% CI, 0.24–0.76; $p = 0.004$) (**Table 3**).

Anesthesia Time

Patients with prolonged anesthesia time who received propofol anesthesia had better survival than those who received desflurane anesthesia. For patients with anesthesia time of <180 min, the crude HR was 0.64 (95% CI, 0.27–1.50; $p = 0.302$), and the PS-matched HR was 0.70 (95% CI, 0.28–1.70; $p = 0.428$). For patients with anesthesia time of ≥180 min, the crude HR was 0.41 (95% CI, 0.25–0.67; $p < 0.001$), and the PS-matched HR was 0.46 (95% CI, 0.27–0.79; $p = 0.005$) (**Table 3**).

Disease Progression

Patients who received propofol anesthesia had less postoperative recurrence than those who received desflurane anesthesia. The crude HR was 0.47 (95% CI, 0.33–0.68; $p < 0.001$), and the PS-matched HR was 0.53 (95% CI, 0.36–0.78; $p = 0.001$). Patients who received propofol anesthesia had less postoperative metastasis than those who received desflurane anesthesia. The crude HR was 0.46 (95% CI, 0.30–0.72; $p = 0.001$), and the PS-matched HR was 0.53 (95% CI, 0.32–0.86; $p = 0.010$). Patients who received propofol anesthesia had less postoperative recurrence and metastasis than those who received desflurane anesthesia. The crude HR was 0.47 (95% CI, 0.30–0.74; $p = 0.001$), and the PS-matched HR was 0.52 (95% CI, 0.32–0.86; $p = 0.010$) (**Table 3**).

Due to the significant difference in the time since the earliest included patient between the two groups after PS matching, we adjusted the PS-matched HRs of above-mentioned subgroups by the variable, and the results were consistent with those without

TABLE 3 | Subgroup analyses for age, BMI, CA-125 level, FIGO stage, operation and anesthesia time, and disease progression.

Stratified variable	Anesthesia	Crude HR (95%CI)	*p* value	*p* value (interaction)	PS-matched HR (95%CI)	*p* value	PS-adjusted HR[a] (95% CI)	*p* value	PS-adjusted HR[b] (95% CI)	*p* value
Non-stratified										
	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.46 (0.30–0.70)	<0.001		0.52 (0.33–0.81)	0.005	0.54 (0.34–0.87)	0.011	0.48 (0.30–0.79)	0.004
Age				0.756						
<40 years/o	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.67 (0.16–2.71)	0.570		0.68 (0.17–2.74)	0.584	0.78 (0.19–3.23)	0.728	0.71 (0.16–3.12)	0.650
40–59 years/o	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.48 (0.27–1.06)	0.054		0.59 (0.30–1.13)	0.111	0.67 (0.34–1.35)	0.265	0.54 (0.24–1.22)	0.140
≥60 years/o	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.39 (0.19–0.77)	0.007		0.37 (0.18–0.77)	0.008	0.41 (0.19–0.87)	0.020	0.35 (0.16–0.74)	0.006
BMI				0.179						
<24 kg/m^2	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.58 (0.34–1.01)	0.051		0.66 (0.36–1.19)	0.165	0.68 (0.37–1.23)	0.201	0.62 (0.34–1.15)	0.127
≧24 kg/m^2	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.33 (0.17–0.65)	0.001		0.38 (0.18–0.78)	0.008	0.40 (0.19–0.87)	0.020	0.30 (0.12–0.70)	0.006
CA-125				0.323						
<35 U/ml	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	1.70 (0.15–18.8)	0.475		2.31 (0.34–21.1)	0.664	2.87 (0.61–28.3)	0.969	2.77 (0.56–27.4)	0.965
≧35 U/ml	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.46 (0.30–0.71)	<0.001		0.47 (0.29–0.74)	0.001	0.49 (0.30–0.80)	0.004	0.46 (0.28–0.76)	0.002
FIGO stage				0.582						
I and II	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.37 (0.12–1.16)	0.089		0.53 (0.15–1.86)	0.319	0.52 (0.14–1.91)	0.328	0.53 (0.13–2.28)	0.397
III and IV	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.53 (0.33–0.83)	0.006		0.60 (0.37–0.96)	0.042	0.61 (0.37–0.98)	0.048	0.59 (0.34–0.98)	0.047
Operation time				0.162						
<180 min	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.68 (0.33–1.43)	0.312		0.68 (0.32–1.44)	0.312	0.72 (0.33–1.56)	0.404	0.60 (0.26–1.37)	0.223
≧180 min	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.37 (0.22–0.63)	<0.001		0.43 (0.24–0.76)	0.004	0.44 (0.24–0.80)	0.007	0.41 (0.22–0.77)	0.005
Anesthesia time				0.355						
<180 min	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.64 (0.27–1.50)	0.302		0.70 (0.28–1.70)	0.428	0.72 (0.29–1.82)	0.488	0.47 (0.15–1.44)	0.186
≧180 min	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.41 (0.25–0.67)	<0.001		0.46 (0.27–0.79)	0.005	0.49 (0.29–0.85)	0.011	0.45 (0.25–0.80)	0.006
Disease progression										
Postoperative recurrence	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.47 (0.33–0.68)	<0.001		0.53 (0.36–0.78)	0.001	0.55 (0.37–0.81)	0.003	0.51 (0.34–0.77)	0.001
Postoperative metastasis	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.46 (0.30–0.72)	0.001		0.53 (0.32–0.86)	0.010	0.53 (0.32–0.87)	0.012	0.46 (0.28–0.78)	0.004
Postoperative recurrence + metastasis	Desflurane	1.00			1.00		1.00		1.00	
	Propofol	0.47 (0.30–0.74)	0.001		0.52 (0.32–0.86)	0.010	0.53 (0.32–0.87)	0.012	0.47 (0.28–0.78)	0.004

BMI, body mass index; CA-125, carbohydrate antigen-125; CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; HR, hazard ratio; PS, propensity score.

[a]*Adjusted by time since the earliest included patient.*

[b]*Adjusted by time since the earliest included patient, operation and anesthesia time.*

adjustment. Concerning the potential impacts of operation and anesthesia time, we also adjusted the PS-matched HRs of above-mentioned subgroups by the time since the earliest included patient, operation and anesthesia time, and found that the results were similar to those without adjustment (**Table 3**).

In summary, propofol anesthesia was associated with better survival outcomes in EOC patients with old age, high BMI, elevated CA-125 level, advanced FIGO stage, and prolonged operation and anesthesia time, which may imply its protective effects in patients with high risks or receiving complex surgery. In addition, patients who received desflurane anesthesia had poor disease progression than those who received propofol anesthesia.

DISCUSSION

The main finding in this study was that propofol anesthesia for EOC open surgery improved survival and reduced rates of postoperative recurrence and metastasis compared with desflurane anesthesia. These results were consistent with findings from previous studies that propofol anesthesia was associated with better outcomes compared with volatile anesthesia in some solid cancers (Wigmore et al., 2016; Jun et al., 2017; Wu et al., 2018; Lai et al., 2019a; Lai et al., 2019b; Huang et al., 2020; Lai et al., 2020a; Lai et al., 2020b). Nevertheless, there were retrospective studies reporting insignificant differences in survival between propofol and VAs in surgery for lung, breast, and digestive tract cancers as well as for glioblastoma (Oh et al., 2018; Huang et al., 2019; Yoo et al., 2019; Grau et al., 2020; Makito et al., 2020). As a result, the effects of anesthetic techniques on oncological outcomes from available data are still inconclusive.

Surgical resection is the mainstay of cancer treatment for potentially removable solid tumors. However, tumor cells may disseminate into the vascular and lymphatic systems during surgery and subsequently migrate to distant organs and initiate tumor regrowth and recurrence (Kim, 2018; Chen et al., 2019). Unlike for many cancers, survival rates for ovarian cancer have changed modestly for decades despite advances in screening, surgery, and treatment methods (Lheureux et al., 2019a). In addition, recurrence develops in approximately 75% of women who present with advanced disease (Lheureux et al., 2019b). Because postoperative recurrence and metastasis play important roles in survival and prognosis, discovering how to improve overall survival by reducing the incidence of relapse is requisite. The likelihood of tumor metastasis depends on the balance between the metastatic potential of the tumor and the anti-metastatic host defenses, of which cell-mediated immunity and natural killer cell function in particular are critical components (Snyder and Greenberg, 2010). Growing evidence from animal and human cancer cell line studies has shown that various anesthetics can affect the immune system in different ways and may therefore influence cancer outcomes (Shapiro et al., 1981; Moudgil and Singal, 1997; Mammoto et al., 2002; Melamed et al., 2003; Kushida et al., 2007).

In this study, we found a 48% lower mortality rate with propofol than with desflurane anesthesia in patients after open surgery for EOC. Moreover, propofol anesthesia was also shown to be associated with a lower incidence of postoperative recurrence and metastasis compared with desflurane anesthesia for patients with EOC, comparable with results in patients undergoing hepatocellular carcinoma; intrahepatic cholangiocarcinoma; and colon, prostate, pancreatic, and gastric cancer surgery (Wu et al., 2018; Lai et al., 2019a; Lai et al., 2019b; Huang et al., 2020; Lai et al., 2020a; Lai et al., 2020b). Elias and colleagues (Elias et al., 2015) compared cancer outcomes in patients with advanced EOC who received different VAs and reported the superiority of desflurane over sevoflurane anesthesia. However, no known study has compared the effects of propofol-based versus VAs-based anesthesia on patient outcomes after surgery for EOC. Although our results suggest a potential effect of anesthetics in humans, but it seems biologically implausible that something as complicated as cancer can be reduced by almost a-factor-of-two simply by anesthetic selection. Our results may overestimate the true treatment effect, which is common in retrospective studies. In addition, by contrast with propofol, VAs have very slow terminal elimination from the vessel-rich group and even slower elimination from the whole body, especially in lengthy anesthesia (Lockwood, 2010). Thus, the actual time interval that VAs act in cancer cell biology may be longer than the recorded anesthesia time. Of course, further investigations are warranted to determine the effects of anesthetic techniques on EOC recurrence and metastasis.

Regarding clinicopathological parameters associated with overall survival of patients with EOC, 4 other prognostic factors, including late menopause, advanced FIGO stage, moderate to massive ascites and elevated preoperative CA-125 level, were identified. This study showed that menopause at late age was associated with poor survival after EOC surgery. The finding may indicate at least a middle age (>50 years old) at the time of diagnosis for patients in this population. However, additional research is needed to determine the impact of late menopause on survival. We also found that a higher FIGO stage was associated with poor survival after open surgery for patients with EOC, as noted previously (Fu et al., 2014). Large volume of ascites at initial diagnosis was regarded as another significant factor related with worse oncological outcomes, which may be attributed to the reduced likelihood for complete resection of tumor (Szender et al., 2017). In addition, a higher preoperative CA-125 level was associated with poor survival for patients undergoing EOC surgery, which was consistent with findings from a previous study (Lin et al., 2020).

Laboratory data from human EOC cell lines support the influence of propofol on the behavior of EOC cells through different pathways (Wang et al., 2013; Su et al., 2014; Huang et al., 2016; Sun et al., 2020; Zeng et al., 2020). Using human EOC cell lines, Zeng et al. (2020) showed that propofol inhibited the proliferation and metastasis of EOC cells by enhancing miR-125a-5p, which targeted lin-28 homologue B. Sun et al. (2020) found that propofol could downregulate miR-374a and modulate the forkhead box O1 pathway to reduce the proliferation and cisplatin resistance in EOC cells. Similarly, Huang et al. (2016) reported that propofol hampered the invasion and proliferation

of EOC cells via upregulating miR-9 and suppressing NF-kB activation and its downstream matrix metalloproteinase 9 expression. Su et al. (2014) also reported that propofol facilitated the apoptosis of EOC cells through upregulating miR-let-7i. In addition, Wang et al. (2013) suggested that propofol impeded the invasion and metastasis and enhanced the paclitaxel-induced apoptosis in EOC cells through the suppression of the slug expression. Taken together, these findings suggest that propofol induces anti-tumor activity and may be an effective anesthetic agent for use in EOC surgery.

Research on the impacts of VAs on EOC cell biology is limited. Iwasaki et al. (2016) have reported that VAs including isoflurane, sevoflurane and desflurane enhanced the metastatic potential in EOC cells through the increased cellular signaling of chemokine receptor 2. Luo et al. (2015) suggested that isoflurane exposure significantly increased the expression of insulin-like growth factor 1 and its receptor, contributing to cell cycle progression and cell proliferation in EOC cells. A recent study also concluded that sevoflurane and desflurane enhanced cell proliferation and migration of EOC cells via the downregulation of miR-210 and miR-138 (Ishikawa et al., 2021). These studies suggest that VAs including desflurane may enhance the malignant potential of EOC cells. However, there was a previous report showing that sevoflurane could suppress the viability, cell cycle and progression and induce the apoptosis of EOC cells by downregulating stanniocalcin 1 (Zhang et al., 2019). Because of conflicting results, further studies are warranted to clarify the impacts of different VAs on EOC cell biology.

Hypoxia, one of the hallmarks of cancer, is caused by an insufficient oxygen supply, mostly due to a chaotic tumor microcirculation. Solid tumors generally exhibit hypoxia, which is a powerful stimulus for tumor angiogenesis and cancer metastasis; moreover, the hypoxia status of cancer cells may affect the cellular expression program and lead to the resistance to radiotherapy and chemotherapy (Han et al., 2019). Therefore, adaptation of tumor cells to a hypoxic environment may be associated with poor prognosis. Recently, hypoxia-inducible factors (HIFs) have been identified as key regulators of the response to hypoxic stress and are widely discussed. Previous studies have shown that HIF-1α overexpression in ovarian cancer was associated with poor overall survival (Shimogai et al., 2008; Braicu et al., 2014). As for the impacts of anesthetics on the expression of HIF-1α, volatile anesthetics generally upregulated HIF-1α, and propofol could inhibit HIF-1α activation (Kim, 2018). Although no study has been conducted to discuss the effects of anesthetics on the expression of HIFs in EOC cells, propofol anesthesia probably has beneficial effects on the expression of HIFs and subsequently provides better outcomes based on our results.

In addition to cellular signaling processes, the effect of anesthetic agents on components of the immune system is also an important pathway to determine tumor development. Generally, propofol provides its protective effects by increasing cytotoxic T-lymphocyte activity, decreasing pro-inflammatory cytokines, and inhibiting cyclooxygenase-2 and prostaglandin E_2 functions; in contrast, VAs have been shown to suppress nature killer cell cytotoxicity, induce T-lymphocyte apoptosis, and decrease the T-helper 1/2 ratio (Kim, 2018). The divergent effects on immune function between propofol and VAs may affect the level of surgery-induced immunosuppression and subsequent tumorigenesis. Therefore, in the present study, the mechanism of anesthetic agents contributing to the progression of EOC cells is mainly proposed by directly affecting signaling pathways of tumor cells and indirectly influencing neuroendocrine and immune function.

There were some limitations in this study. First, because this was a retrospective single-center observational study, our findings could not determine the causal relationship between anesthetics and oncological outcomes after EOC surgery; thus, it should be only deemed as hypothesis-generating. Second, the study was retrospective, and patients were not randomly allocated. We conducted PS matching to minimize confounding in this observational study (Austin et al., 2018). However, the small groups for PS matching may influence the reliability of the statistical significance in our study. Fortunately, regardless of the analytic approaches, the point estimation and significance of relative risk of propofol versus desflurane were consistent. Third, although we performed the multivariate analysis and PS matching analysis with many variables to obtain reliable results and valuable information, we could not exclude some unmeasured confounding factors that may be responsible for the result. Fourth, therapeutic methods for EOC patients have evolved over time, which may result in improved outcomes. Because detailed information about surgical techniques and cancer care were not available, we could not completely exclude the possibility that advances in cancer care and surgical techniques may influence survival outcomes. Fifth, there was a lack of data on the levels of immune cells and biomarkers in our study, so we could not confirm the definite relationship between anesthetics, immune and transcriptional factors, and the aggressiveness of the disease. Sixth, different VAs may have distinctive effects on EOC. We only included desflurane in our analysis because it is the most frequently used VA in our hospital. Seventh, we analyzed only the diagnosis of EOC accounting for the majority of ovarian malignancies (Lheureux et al., 2019a), and did not refine the histologic subtypes due to incomplete data. Eighth, we excluded EOC patients undergoing laparoscopic surgery ($n = 9$) to increase the consistency of patient characteristics, although there was no significant difference in oncological outcomes between minimally invasive and open procedures (Jochum et al., 2020). Finally, epidural use has been linked to better survival in patients with ovarian cancer (de Oliveira et al., 2011; Tseng et al., 2018). In our hospital, we do not routinely use epidural anesthesia and analgesia during EOC open surgery because of the risk of life-threatening complications such as neurological deficits and epidural hematoma (Bos et al., 2017). Despite these limitations, our results may have an important clinical implication for EOC management if the relationship between anesthetics and oncological outcomes after cancer surgery is indeed causal.

CONCLUSION

Propofol anesthesia was associated with better survival than desflurane anesthesia in open surgery for EOC. Propofol anesthesia also showed better outcomes in EOC patients with old age, high BMI, elevated CA-125 level, advanced FIGO stage, and prolonged operation and anesthesia time compared with desflurane anesthesia. In addition, patients given propofol anesthesia had significantly less postoperative recurrence and metastasis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board, Tri-Service General Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Study design: W-CT, Z-FW; conduct of the study: W-CT, H-CL, Z-FW; data analysis: M-SL, Y-CL, M-HY, K-LW; data collection: W-CT, H-CL, M-HY, K-LW; preparation of the article: W-CT, M-SL; writing of the article: W-CT, Z-FW; all authors read and approved the final version of the article.

FUNDING

The study was supported by grants from Tri-Service General Hospital (TSGH-C108-221), Taiwan.

ACKNOWLEDGMENTS

The authors thank the Cancer Registry Group of Tri-Service General Hospital for the clinical data support.

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frontiers in Pharmacology

REVIEW
published: 07 December 2021
doi: 10.3389/fphar.2021.770694

Mechanisms of Cancer Inhibition by Local Anesthetics

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The use of local anesthetics during surgical treatment of cancer patients is an important part of perioperative analgesia. In recent years, it has been showed that local anesthetics can directly or indirectly affect the progression of tumors. *In vitro* and *in vivo* studies have demonstrated that local anesthetics reduced cancer recurrence. The etiology of this effect is likely multifactorial. Numerous mechanisms were proposed based on the local anesthetic used and the type of cancer. Mechanisms center on NaV1.5 channels, Ras homolog gene family member A, cell cycle, endothelial growth factor receptor, calcium Influx, microRNA and mitochondrial, in combination with hyperthermia and transient receptor potential melastatin 7 channels. Local anesthetics significantly decrease the proliferation of cancers, including ovarian, breast, prostate, thyroid, colon, glioma, and histiocytic lymphoma cell cancers, by activating cell death signaling and decreasing survival pathways. We also summarized clinical evidence and randomized trial data to confirm that local anesthetics inhibited tumor progression.

Keywords: local anesthetics, cancer cells, cellular mechanisms, lidocaine, bupivacaine, ropivacaine

OPEN ACCESS

Edited by:
Daqing Ma,
Imperial College London,
United Kingdom

Reviewed by:
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Cathay General Hospital, Taiwan
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Pharmacology

Received: *04 September 2021*
Accepted: *15 November 2021*
Published: *07 December 2021*

Citation:
Zhang Y, Jing Y, Pan R, Ding K, Chen R and Meng Q (2021) Mechanisms of Cancer Inhibition by Local Anesthetics. Front. Pharmacol. 12:770694. doi: 10.3389/fphar.2021.770694

INTRODUCTION

The high morbidity and mortality of malignant tumors is a difficult problem for human life and health. Although radical surgery, radiotherapy, chemotherapy, immunotherapy, and hormone therapy are used, the recurrence and metastasis of cancer remain key problems. Surgery is the primary method to treat malignant tumors. However, there is growing evidence that surgical treatment may actually promote cancer recurrence and metastasis. Whether these events occur largely depends on the ability of the tumor to spread and the host's immune and inflammatory responses.

Surgery provides an opportunity to eradicate tumors, but it also allows residual cancer cells to proliferate and invade. Surgery increases the shedding of malignant cells into the blood and lymph circulation, inhibits their apoptosis, and enhances their invasive ability (Neeman and Ben-Eliyahu, 2013). Surgery also increases the lever of tumor vascular-related and growth factors, and supports local and distant metastasis and tumor recurrence. Significant changes in the immune, endocrine, and inflammatory systems in response to surgery promote cancer progression (Gottschalk et al., 2010). Psychological distress (anxiety, stress, and depression) associated with surgery releases stress hormones, lowers cellular immunity, reduces the host's immune response, and increases the risk of metastasis (Thornton et al., 2010) (**Figure 1**).

There is increasing evidence that anesthesia techniques and other perioperative factors potentially influence long-term outcomes after malignant tumor surgery. Local anesthetics (LAs) can inhibit the development of tumors and limit tumor metastasis *via* a variety of mechanisms. Local anesthetics block voltage-gated sodium channels (VGSCs) on nerve cell membranes, and these channels are

present on tumor cell membranes and are associated with the invasion and metastasis of tumor cells (Lirk et al., 2012). Local anesthetics have indirect effects on cancer biology. The following anti-tumor mechanisms were proposed(Neeman and Ben-Eliyahu, 2013): anti-tumor cell proliferation and metastasis(Gottschalk et al., 2010), induction of cell apoptosis(Thornton et al., 2010), improvement of chemotherapeutic efficacy(Lirk et al., 2012), reduction of the demand for opioids (Cata et al., 2020). Opioids are immunosuppressive, and their use may reduce a patient's resistance to tumor metastasis (Dan et al., 2018).

OVERVIEW OF LOCAL ANESTHETICS AND CLINICAL IMPLICATIONS IN CANCER TREATMENT

Since the appearance of cocaine in 1884, LAs have been widely used in all types of surgeries to relieve pain. LAs primarily block voltage-dependent Na^+ and K^+ channels, which blocks nerve transmission and produces local anesthesia. The chemical formula of LAs consists of aromatic rings, amino groups and intermediate chains. According to different intermediate chains, LAs divided into esters such as procaine, tetracaine, etc., and amides such as lidocaine, ropacaine, and bupivacaine. LAs may be used alone or in combination with general anesthetics. Combination therapy reduces the dose of general anesthetics, improves the anesthetic effect and reduces the neuroendocrine stress response and perioperative immunosuppression, and may directly inhibit the proliferation and metastasis of tumor cells (Dubowitz et al., 2018).

Thirty female cervical cancer patients who received radical hysterectomy were treated with lidocaine (1.5 mg/kg iv followed by 1.5 mg/kg ivpump and discharge). The interferon-gamma (IFN-γ)/Interleukin-4 (IL-4) ratio of the lidocaine group was better than the control group, and the apoptosis of lymphocytes was weaker than the control group. These results suggest that lidocaine has a protective effect on anti-cell-mediated immunity (CMI) in patients with radical hysterectomy of cervical cancer. This treatment may help reduce the incidence of postoperative septic complications and the formation of tumor metastasis (Wang et al., 2015). Lidocaine also decreased the viability of all breast cancer cell lines, inhibited the migration of tumor breast epithelium, and inhibited the immobile growth of triple-negative cells. Intraperitoneal injection of lidocaine improved the survival rate of MDA-MB-231 mice with peritoneal carcinomatosis. The dose of lidocaine is consistent with the current clinical analgesia setting (10 mg/ml) (Chamaraux-Tran et al., 2018).

EFFECTS OF LOCAL ANESTHETICS ON CANCER CELLS

Inhibition of NaV1.5 Channels

Cancer cells and tissues express VGSCs, and VGSCs activity increases the lateral motility and invasion of tumor cells *in vitro* (Fraser et al., 2005). VGSCs play an important role in the occurrence and development of tumors (Pedersen and Stock, 2013; Hofschröer et al., 2020), and functionally expressed in many types of tumor cells (epithelial carcinoma), including breast, cervical, ovarian, prostate, colon, skin, and lung cancers (Fraser et al., 2014). The overexpression of these channels enhances the metastasis cascade and tumor cell metastasis. LAs inhibit VGSCs function, and prevent VGSCs activity during and after surgery, which reduces the ability of cancer cells to escape and metastasize from the perioperative range of surgery. These effects reduce cell proliferation and indirectly increase patient survival.

Ropivacaine inhibited the invasion of SW620 colon cancer cells in a concentration range of 10–100 uM, which was similar to the current effect on the Nav1.5 mutant channel of the neonatal isoform, and this range is related to LAs blockade of sodium channels (Baptista-Hon et al., 2014). Shilpa Dutta et al. found that Nav1.5 was overexpressed in the highly invasive human breast cancer cell line MDA-MB-231. 1uM Nav1.5 blocker inhibited the invasion of MDA-MB-231 cells, and the rate of invasion inhibition was 30.3 ± 4.5%, and fortunately, cell viability was not affected (Dutta et al., 2018). LAs can block the VGSCs which blocks channels in resting, open, and inactivated states (inactivated states have the highest binding affinity) (Grandhi and Perona, 2020). Amide LAS, especially lidocaine, have more systemic anti-inflammatory benefits and effects on immune cells than other LAS agents (Van Der Wal et al., 2015).

The expression of the Nav1.5 can be blocked by Lidocaine in highly metastatic human breast cancer MDA-MB-231 cells (Fraser et al., 2014). Tetrodotoxin (TTX), which is a blocker of VGSCs, was used as a local anesthetic for the treatment of pain in cancer patients in clinical trials, and it showed significant anticancer effects *in vivo* and *in vitro* (Makarova et al., 2019).

Inhibition of Ras Homolog Gene Family Member A Migration

Rho and RAC GTP enzymes regulate all types of cell migration (Zheng et al., 2020), and one of the activators of the Ras homolog gene family member A (RhoA) pathway is neuroepithelial cell gene 1 (NET1) (Pang et al., 2021). Rho-associated protein kinase (Rock) is a downstream effector of the RhoA pathway. Rock activates myosin phosphatase targeting subunit 1 (MYPT1), and it phosphorylates myosin on myosin light chain 1 (MLC1) (Dan et al., 2018). Low concentrations of bupivacaine (10–50 mM) reduced the migration of gastric cancer cells *via* the RhoA and MLC1 pathways but had no significant effect on tumor growth or survival (Dan et al., 2018). Bupivacaine decreased the phosphorylation of MYPT1 and MLC1, which reduced the migration of gastric cancer cells. Research also suggests that bupivacaine inhibits the migration of cancer cells by stimulating NET1 (Pang et al., 2021). NET1 is significantly up-regulated in gastric and breast cancers, which suggests that bupivacaine is involved in tumor migration *via* this pathway to inhibit tumor migration. Levobupivacaine and ropivacaine reduced tumor cell invasion and migration by reducing RhoA protein levels (Castelli et al., 2020).

Some studies showed that sodium channel blockade contributed to the anticancer activity of LAs (Fraser et al., 2014), but these studies also demonstrated that bupivacaine inhibited the migration of gastric cancer cells *via* the sodium-independent channel blocker RhoA and NET1 inhibition (Dan et al., 2018; Zheng et al., 2020; Pang et al., 2021). These results suggest that LAs are associated with the RhoA and NET1 pathways in addition to sodium channel blockade.

Cell Cycle

Cell cycle progression is a hallmark of cancer, primarily because Cyclins D1, E, and B2 are key regulators of the cell cycle, and are dysregulated in different cancers, including breast, esophageal, bladder, skin, and lung cancers (Icard et al., 2019). Treatment of breast cancer and melanoma cells with bupivacaine and lidocaine significantly decreased cell cycle proteins, which promoted cell cycle arrest (Castelli et al., 2020). Ropivacaine arrested liver cancer cells in the G2 phase (Le Gac et al., 2017). P53 is a cancer marker that induces cell cycle arrest at G2/M or G0/G1 and activates the expression of the cyclin-dependent kinase inhibitor p21. P27 is a tumor suppressor that regulates the G0 to S phase, and increased P53 phosphorylation (active form) and P27 were observed after treatment with LAs, which indicated impaired cell proliferation.

Golgi apparatus transporter 1A (GOLT1A) is significantly elevated in patients with lung adenocarcinoma, and it was associated with prognosis and pathological staging (Yang et al., 2018a). The down-regulation of GOLT1A inhibits cell proliferation and induces cell cycle arrest. After 2 mmol/L lidocaine for 24 h, the expression of GOLT1A, Cyclin D1, and Cyclin E1 was decreased, which prevented the cells from transitioning from the G1 phase to the S phase (Zhang et al., 2017). Notably, GOLT1A also modulates the sensitivity of breast cancer cells to tamoxifen and improved prognosis (Zhang et al., 2017).

Epidermal Growth Factor-Associated Effects

Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor that regulates cell proliferation and differentiation of epithelial cells and tumors, including head and neck, breast, colorectal, lung, and pancreatic cancers (Mitchell et al., 2018). EGFR may be incorrectly activated by a variety of mechanisms: Ligand-dependent dimerization, point mutation, partial deletion, or overexpression. EGFR is expressed in tumor and non-tumor cells in the tumor microenvironment (TME). EGFR plays a role in the stimulation of vascular endothelial growth factor (VEGF), fibroblast growth factor and interleukin-8 (IL-8), which suggests that it supports tumor cell proliferation, angiogenesis, and metastasis (Grapa et al., 2019).

EGFR is activated by specific ligands, such as pro-epidermal growth factor and transforming growth factor-beta (TGF-β). Once activated, EGFR dimers stimulate intrinsic protein tyrosine kinase activity in cells, which results in the automatic phosphorylation of several tyrosine residues within EGFR-expressing cells. Therefore, several signal transduction pathways, including mitogen-activated protein kinase (MAPK), protein kinase B (Akt) and c-jun N-terminal kinase (JNK), are initiated, which leads to DNA synthesis and cell proliferation (Sakaguchi et al., 2006). Lidocaine inhibited the proliferation of human tongue cancer cells (CAL27 strain). 400 microM Lidocaine inhibited serum- and EGF-induced CAL27 proliferation *via* inhibition of the auto-phosphorylation of EGFR tyrosine residues without cytotoxicity. With the increase of clinical concentration, 4,000 microM lidocaine inhibited the proliferation of CAL27 cells by inhibiting the activity of EGFR (Sakaguchi et al., 2006).

LAS preferentially induced the EGFR pathway in breast cancer cells (MCF-7) *via* exogenous and endogenous caspase-dependent apoptosis. The activation of EGFR leads to an increase in the downstream activity of caspase 8 and 9, which leads to the apoptosis of breast cancer cells (Li et al., 2018). Treatment of human hepatoma cells (HEP G2 cells) with 1 mM or 5 mM lidocaine showed a continuous increase in caspase 3 concentrations, which reached a maximum level after 24 h (Xing et al., 2017).

Reduction of Calcium Influx

The increased activity of TME ion channels leads to an increase in intracellular calcium concentration. An increased level of Ca^{2+} in the cytosol promoted the formation of podosomes/invadopodia, which facilitated the invasion of cancer cells (Hantute-Ghesquier et al., 2018). Calcium is also a key regulator of cell invasion. Lidocaine inhibits chemokine-induced tumor cell migration *via* the direct inhibition of CXCR4 activity. Calcium signaling controls the progression and apoptosis of cancer cells *via* the transient receptor potential subfamily member 6 (TRVP6) channel of the transient receptor potential channel V subfamily (Xuan et al., 2016; Liu et al., 2020a). TRVP6 mRNA and protein were detected in ovarian, breast, prostate, thyroid cancer and colon cancers. These results suggest that TRVP6 plays an important role in tumorigenesis, progression and prognosis. After treatment with 100 μm lidocaine for 12 h, the expression of TRPV6 was reduced 50–80%, and the survival rate, migration and cell division of MDA-MB-231 cells were decreased (Xuan et al., 2016). TRPM7 also affects the activity of cancer cells, and it is involved in Ca^{2+} and Mg^{2+} steady-state ion channels (Liu et al., 2020a). Lidocaine inhibited glioma cell proliferation and metastasis *via* the blockade of TRPM7 channels, which prevented the cell cycle and induced protective autophagy (Leng et al., 2017).

Regulation of microRNA and Mitochondrial Inhibition

MicroRNAs (miRNAs) play important roles in gene silencing and post-transcriptional regulation (Bartel, 2004). Several cancer-related miRNAs were sensitive to LAs *in vitro*, including miR-21, miR-145, miR-520a-3p, and miR-539 (Xuan et al., 2016; Yang et al., 2018b; Yang et al., 2018c; Yang et al., 2019). Lidocaine enhanced the toxicity of cisplatin in lung cancer *via* miR-21 regulation (Xuan et al., 2016), and inhibited the growth, migration and invasion of

gastric cancer cells *via* the up-regulation of miR-145 (Sui et al., 2019). Adenosine triphosphate (ATP) levels are strongly associated with tumor cell growth and survival. Mitochondrial metabolism limits tumor proliferation and metastasis by altering ATP levels. Ropivacaine inhibited breast tumor proliferation and metastasis by destroying mitochondrial complexes I and II but not III or IV (Gong et al., 2018). Bupivacaine inhibited mitochondrial complexes I and III to inhibit thyroid tumor proliferation (Chang et al., 2014). Bupivacaine (1 mm and 5 mm) inhibited mitochondrial complexes I and II to induce a decrease in ATP. There was no similar decline in ATP levels or activity after bupivacaine administration in mutant gastric cancer cells without mitochondria (Gong et al., 2018). Bupivacaine inhibits the growth of tumor cells by reducing the level of ATP in mitochondria.

In Combination With Hyperthermia

Hyperthermia is a non-invasive, localized cancer treatment option that induces targeted cancer cell death. Local hyperthermia induces cell damage in the tumor area with minimal damage to the surrounding tissue (usually 40–44°C) (Markowitz and Bressler, 2021). Under high temperature alone, human histiocytic lymphoma (U937) cells showed a certain degree of DNA fragmentation and nuclear fragmentation, but the degree of nuclear fragmentation was enhanced in a dose-dependent manner when amides were used in combination. LAS with higher liposolubility had a greater promotion effect of heat-induced apoptosis. Intracellular Ca^{2+} concentrations are elevated during high-temperature-induced apoptosis, and the Ca^{2+}-chelating agents (BAPTAAM) inhibit DNA fragmentation, which suggests that calcium-dependent pathways are involved in hyperthermia-induced apoptosis (Arai et al., 2002). Moderate temperature (42°C) combined with a low concentration of lidocaine (0.2%) significantly increased skin cancer cell death (Raff et al., 2019). Previous studies also showed that cells in the intermediate and advanced phase of S are more sensitive to high temperatures, with an increased proportion of cells in S phase compared to five types of cancer cells (fibroblasts, keratocytes, melanoma, cervical cancer, basal cell carcinoma) were more sensitive to combination therapy (Raff et al., 2019). The causes of LAS combined with high temperature-induced cancer cell death include the formation of superoxides, a decrease in mitochondrial membrane potential, the activation of caspase 3 and an increase in intracellular Ca^{2+} (Raff et al., 2019).

Inhibition of Transient Receptor Potential Melastatin 7 Channels

Transient receptor potential melastatin 7 channels (TRPM7) participate in Ca^{2+} and Mg^{2+} steady-state ion balance, which affects cell viability. TRPM 7 is not regulated in many cancers, including head and neck, breast, thyroid, lung, and pancreatic cancers (Grandhi and Perona, 2020). TRPM7 overexpression in bladder cancer cells promoted the proliferation of cancer cells. Regulation of Ca^{2+} homeostasis is associated with cancer development (Gao et al., 2017). Some studies showed the migration of pancreatic cancer cells by TRPM7 *via* the regulation of Mg^{2+}-dependent mechanisms (Rybarczyk et al., 2012). Another *in vitro* study showed that TRPM7 was associated with the growth of the human breast cancer cell line MCF-7 (Guilbert et al., 2009). Lidocaine inhibited the proliferation and metastasis of breast cancer cells by inhibiting the function of TRPM7 channels in breast cancer cell lines (Liu et al., 2021). Lidocaine inhibited TRPM7 channels in a concentration-dependent manner. The inhibition rate was 20% at 1 mM and 50% at 3 mM (Leng et al., 2017), but the dose of lidocaine in this study was higher than the clinical dose. Lidocaine prevented the cell cycle and induced protective autophagy in glioma cells by blocking TRPM7 channels (Leng et al., 2017). Another calcium channel, TRPV6, is also expressed in MDA-MB-231 human breast cancer cells, PC-3 prostate cancer cells and ES-2 ovarian cancer cells (Jiang et al., 2016). Lidocaine inhibited the migration and invasion of MDA-MB-231 cells by inhibiting this channel (Jiang et al., 2016).

CLINICAL TRIALS OF LOCAL ANESTHETICS IN TUMOR SUPPRESSION

LAs inhibit the occurrence and development of tumors. Some reliable clinical evidence and randomized trial data also support relevant conclusions. The effect of intravenous lidocaine on tumor-related outcomes after cancer resection was studied using *in vivo* tumor models. Levels of MMP-2, which is a key protein in the metastatic potential of breast cancer cells (Wall et al., 2019) and lung metastasis colonies (Freeman et al., 2019) were reduced after intravenous administration of lidocaine compared to sevoflurane. Lidocaine inhibited tumor growth and increased sensitivity to cisplatin in a xenograft model of hepatocellular carcinoma (Xing et al., 2017). Clinical studies have not investigated the effect of perioperative intravenous lidocaine on the long-term prognosis of cancer. However, Toner et al. showed that intravenous lidocaine was safe, effective and feasible in patients undergoing breast cancer surgery (Toner et al., 2021), and the VAME-C trial (NCT04316013, ~5,376 patients) is an international randomized controlled trial to compare propofol-TIVA with inhaled sevoflurane and intravenous lidocaine/placebo using a 2 × 2 trial design for colorectal and lung cancer surgery (Dubowitz et al., 2021). These studies should provide high-level evidence for the significant role of lidocaine in tumor anesthesia. A few clinical studies investigated the effects of local anesthesia and intravenous lidocaine on the post-operative inflammatory response. It is suggested that intravenous lidocaine during the procedure until 1 h postoperatively can decrease the inflammatory cytokine (IL-1, IL-6, IL-10, TNF-α, and IFN-γ) and increase the anti-inflammatory cytokine (IL-10) (Ortiz et al., 2016). The Association for the Promotion of Postoperative Recovery guidelines for perioperative care during elective colorectal surgery strongly recommends lidocaine infusion during colorectal surgery (Gustafsson et al., 2019). However, intravenous lidocaine has a risk of toxicity, and guidelines and rational perioperative administration are essential.

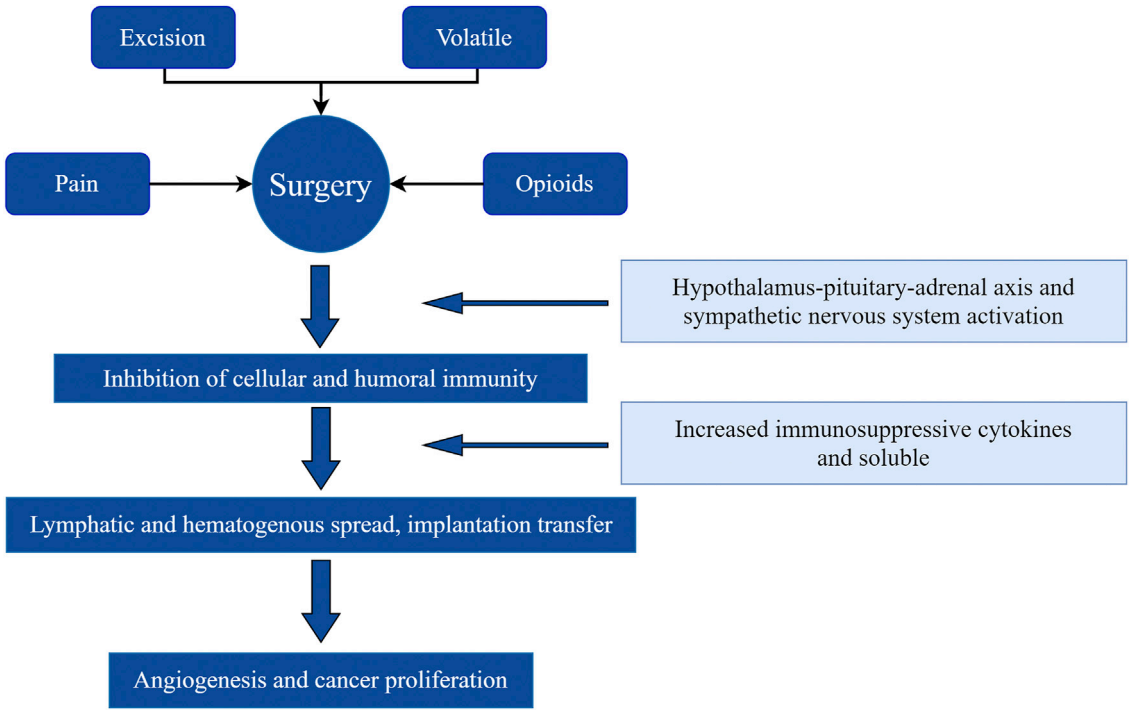

FIGURE 1 | Operative critical factors leading to angiogenesis and cancer cell proliferation.

The preliminary evidence is compelling, but there is insufficient high-quality evidence to fully explain the role of LAs in tumor regulation and support changes in current clinical practice. With some large prospective trials underway, our understanding of the impact of anesthesia on cancer-related outcomes should improve rapidly in the future. There will also be stronger evidence that LAs inhibit tumor progression, and relevant clinical guidelines will be developed to provide best practice guidelines for tumor anesthesia and treatment.

DISCUSSION

Evidence is mounting to address the effects of anesthesia, anesthetics, anesthesia techniques, and surgical stress on long-term cancer outcomes. Surgery remains one of the main treatments of tumors, especially early benign tumors. It is obvious that the choice of anesthesia methods and anesthetics are key to the treatment of cancer. Current research on LAs in oncology suggests that the role of LAs is not independent, and these agents are more likely to be used as chemosensitizers or synergistic therapies. For example, lidocaine increases the sensitivity of breast cancer cells to tamoxifen by down-regulating the expression of GOLT1A, which enhanced prognosis (Zhang et al., 2017), Lidocaine also enhances the toxicity of the cancer drugs mitomycin C, pirarubicin, softening lotion and cisplatin (Sui et al., 2019; Gong et al., 2018). This article reviewed the basic pharmacology of LAs, their mechanisms of action on tumor cells, and their current clinical application. LAs primarily inhibit the proliferation and metastasis of tumor cells, induce apoptosis, improve the efficacy of chemotherapy, and reduce the need for opioids to fight against cancer. These mechanisms interact to form a local anesthetic anti-tumor mechanism network (**Figure 2**). Liu et al. showed that all LAs were toxic to cancer cells at high concentrations, but different anesthetics have different effects, and the same tumor cell line had a different local anesthetic, such as bupivacaine > lidocaine > ropivacaine (Liu et al., 2020b). Laboratory and human studies showed that lidocaine reduced the levels of the tumor markers IL-1, TNF-α and IL-8 and had a direct effect on cancer cells *via* blockade of voltage-gated sodium channels or other mechanisms. Lidocaine reduced the viability and migration of cancer cells in laboratory studies and increased the survival rate of breast cancer mice (Li et al., 2018).

Despite extensive experimental evidence of the potentially beneficial effects of the perioperative use of regional and LAs, the exact role and impact of the use of these substances in cancer surgery are not clear. The drug concentrations in many animal and cell experiments were significantly higher than clinical use (Leng et al., 2017), but there were also consistent results with clinical concentrations (Chamaraux-Tran et al., 2018). There is still a lack of support from clinical data from randomized controlled trials. Studies *in vitro* and animal models do not always fit human clinical conditions perfectly. There is growing evidence that different types of anesthetics promote or inhibit metastasis, depending on the type of tumor cell and the type, dose and regimen of anesthetics used. Continuous intravenous infusion of lidocaine during the perioperative

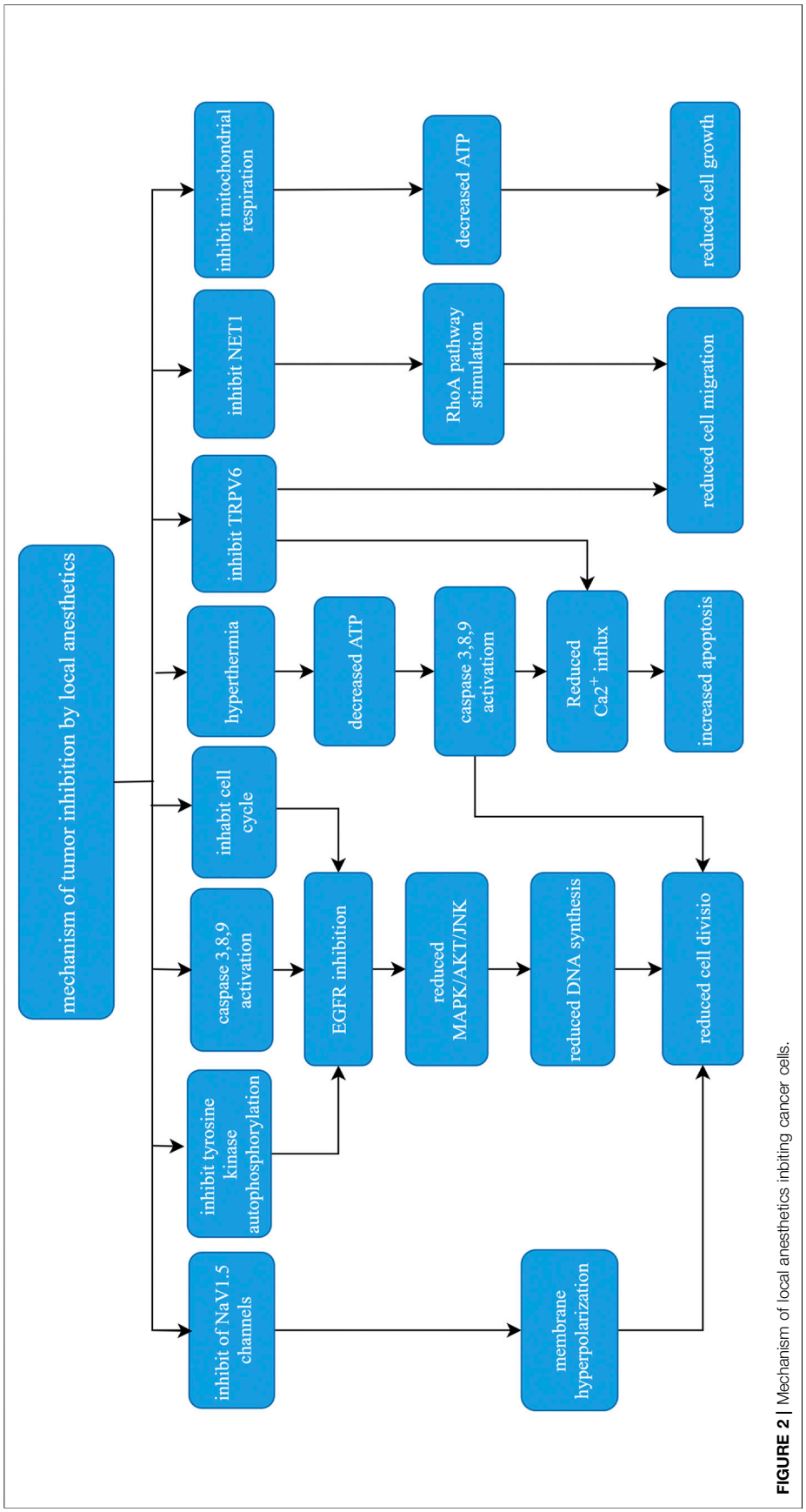

FIGURE 2 | Mechanism of local anesthetics inbiting cancer cells.

period has been safely used to reduce systemic inflammation and intestinal dysfunction (Gustafsson et al., 2019), after abdominal surgery, and the results of this study also provide a basis and guidance for the role of LAs in tumor therapy. Although lidocaine is currently a local anesthetic that may be administered intravenously and is most widely used in anesthesiology clinics, it has not always been the most effective anticancer agent *in vitro* studies. Intravenous lidocaine is also not without risk. Perhaps future studies focusing on the *in vivo* and *in vitro* effects and mechanisms of certain types of tumors may yield better results in the pharmaceutical field for the development of new intravenous LAs with high anti-tumor effects and low toxicity as a more attractive solution.

Future research into the effects and mechanisms of LAs on cancer cells is promising and necessary. This review boldly conceived and summarized some of the solutions and research priorities:

1) More vivo studies. We encourage the addition of more animal experiments in this area. Animal experiments are closer to the reality of the human body than cell experiments. These studies simulate human cancer surgery conditions in animal models and evaluate tumor suppression, metastasis, and related endpoints for animal survival, which will help elucidate the systemic effects of LAs.
2) Standardization of experimental methods. There is no consistency between research groups on the effects of LAs on cancer cells. The reason for the inconsistent results may be that the experimental methods have not been well standardized or due to some experimental difficulties caused by the drugs themselves, such as the low solubility of some LAs and local anesthetic toxicity during intravenous administration. Appropriate controls and standardization are required to eliminate the potential effects of different experimental protocols to produce consistent and repeatable results.
3) Investigations of the effects of LAs on tumor stem cells. The presence of tumor stem cells raises the question of whether these cells or differentiated cancer cells drive tumorigenesis (Dawood et al., 2014). Previous studies showed that lidocaine, ropivacaine and bupivacaine were effective inhibitors of leukemic stem cell colony formation, and non-cancer stem cells are unaffected by these LAs (Ni et al., 2018). Although these studies are sporadic, they provide a reference for the treatment of tumors and a feasible idea for the mechanism of LAs in inhibiting the development of tumors. If LAs are found to have a more consistent ability to interfere with cancer stem cells, the widely studied differences between differentiated cancer cell types may be reconciled.
4) Investigation of the specificity of tumors. It is generally difficult to kill most tumors *via* the intravenous administration of drugs at the site of surgery or at the time of surgery. Specific tumors or types of cancer cells that may be particularly sensitive to certain LAs should be identified. It is even possible to compare the effects of LAs on cancer and non-cancer cells to help determine the specificity of LAs.
5) Use bioinformatics. Advanced gene sequencing techniques and bioinformatics tools may be applied to problems related to LAs and tumors. This use may help identify multiple mechanisms of tumorigenesis and reveal new targets of LAs. Identification of the correlation between the two may lead to a better solution for the treatment of tumors.

AUTHOR CONTRIBUTIONS

All authors read and approved the final manuscript. YZ: Conducted the study and critically revised the manuscript. YJ: Conducted the study and critically revised the manuscript. RP: Provided input to protocol development, and critically revised the manuscript. KD: Provided protocol development input, and critically revised the manuscript. RC: Made the figures, and critically revised the manuscript. QM: Conceived the study, led the proposal and protocol development, and critically revised the manuscript.

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frontiers in Oncology

ORIGINAL RESEARCH
published: 06 January 2022
doi: 10.3389/fonc.2021.689832

Effects of Intravenous Infusion of Lidocaine on Short-Term Outcomes and Survival in Patients Undergoing Surgery for Ovarian Cancer: A Retrospective Propensity Score Matching Study

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Background: Intravenous lidocaine has been shown to reduce opioid consumption and is associated with favourable outcomes after surgery. In this study, we explored whether intraoperative lidocaine reduces intraoperative opioid use and length of stay (LOS) and improves long-term survival after primary debulking surgery for ovarian cancer and explored the correlation between SCN9A expression and ovarian cancer prognosis.

Methods: This retrospective study included patients who underwent primary debulking surgery(PDS) for ovarian cancer from January 2015 to December 2018. The patients were divided into non-lidocaine and lidocaine [bolus injection of 1.5 mg/kg lidocaine at the induction of anaesthesia followed by a continuous infusion of 2 mg/(kg·h) intraoperatively] groups. Intraoperative opioid consumption, the verbal numeric rating scale (VNRS) at rest and LOS were recorded. Propensity score matching was used to minimize bias, and disease-free survival (DFS) and overall survival (OS) were compared between the two groups.

Results: After propensity score matching(PSM), the demographics were not significantly different between the groups. The intraoperative sufentanil consumption in the lidocaine group was significantly lower than that in the non-lidocaine group (Mean: 35.6 μg vs. 43.2 μg, P=0.035). LOS was similar between the groups (12.0 days vs. 12.4 days, P=0.386). There was a significant difference in DFS between the groups (32.3% vs. 21.6%, P=0.015), and OS rates were significantly higher in the lidocaine group than in the non-lidocaine group (35.2% vs. 25.6%, P=0.042). Multivariate analysis indicated that intraoperative lidocaine infusion was associated with prolonged OS and DFS.

OPEN ACCESS

Edited by:
Daqing Ma,
Imperial College London,
United Kingdom

Reviewed by:
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

Received: *01 April 2021*
Accepted: *13 December 2021*
Published: *06 January 2022*

Citation:
Zhang H, Gu J, Qu M, Sun Z, Huang Q, Cata JP, Chen W and Miao C (2022) Effects of Intravenous Infusion of Lidocaine on Short-Term Outcomes and Survival in Patients Undergoing Surgery for Ovarian Cancer: A Retrospective Propensity Score Matching Study. Front. Oncol. 11:689832. doi: 10.3389/fonc.2021.689832

Conclusion: Intraoperative intravenous lidocaine infusion appears to be associated with improved OS and DFS in patients undergoing primary debulking surgery for ovarian cancer. Our study has the limitations of a retrospective review. Hence, our results should be confirmed by a prospective randomized controlled trial.

Keywords: lidocaine infusion, SCN9A, ovarian cancer, overall survival, disease-free survival

INTRODUCTION

Ovarian cancer ranks third among the most common gynaecologic tumours and is the eighth leading cause of cancer-related death in women in developing countries (1). Despite significant progress in the treatment of ovarian cancer, the 5-year survival rate remains lower than 50% (2). This worrisome statistic highlights the urgent need to find therapies that can contribute to reducing ovarian cancer progression and thus improve patient survival. Preclinical and clinical studies suggest that anaesthetic agents may influence cancer biology and outcomes (3). Anaesthetics and inadequate pain control might also be associated with the risk of metastatic recurrence after ovarian cancer surgery (4–6). Therefore, current research is focused on improving the survival of women with ovarian cancer by optimizing perioperative management.

Lidocaine is a widely used amide local anaesthetic that can reduce general anaesthetic use, minimize opioid consumption and provide adequate analgesia when given systemically during oncological surgery (7). In addition, lidocaine has shown promising anticancer properties. Different mechanisms have been described as being responsible for the antimetastatic effects of lidocaine, including TRPV6 receptor inhibition (8), reduced epidermal growth factor activity (9), and time- and dose-dependent deoxyribonucleic acid demethylation in different cancer cell lines (10). Moreover, voltage-gated sodium channels (VGSCs) are expressed in cancer cells, controlling important mechanisms of the metastatic process (11). In support of these preclinical findings, the results of our previous study indicate that perioperative intravenous infusion of lidocaine is associated with longer overall survival (OS) in patients with pancreatic cancer (12).

It is unclear whether intravenous lidocaine infusion during ovarian cancer surgery is associated with any improvement in surgical recovery and long-term oncologic outcomes. Thus, the aim of this retrospective study was to evaluate the association between intravenous infusion of lidocaine during ovarian cancer surgery and intraoperative sufentanil consumption, postoperative analgesia scores, and long-term patient survival outcomes. Since lidocaine acts on sodium voltage channels, we also explored the association between *SCN9A* gene expression and long-term prognosis in ovarian cancer patients.

METHODS

Patients Enrolment

This study was approved by the Ethics Committee of Fudan University (no. 20200206). From January 2015 to December 2018, patients undergoing primary debulking surgery (Ro resection) for ovarian cancer were enrolled as a retrospective cohort. The inclusion criteria were as follows: (a) 18 years or older; (b) received combined general-epidural anaesthesia and (c) complete clinicopathological and follow-up data. Patients were excluded if they (a) underwent emergency surgery, (b) had any history of another malignant tumour, (c) died within 30 days of surgery from postoperative complications or (d) were lost to follow-up.

We collected demographic information [age, body mass index and American Society of Anaesthesiologists (ASA) physical status], Charlson Comorbidity Index (CCI), pathological details (tumour size, histologic diagnosis, FIGO stage and tumour differentiation), surgical details (surgical complexity, estimated blood loss volumes and operative time), preoperative CA125 levels and postoperative chemotherapy. We obtained one to five years of follow-up (every three months for the 1st and 2nd years and every six months for the 3rd year) from medical records and telephone contacts.

Endpoints

The primary endpoints of interest were disease-free survival (DFS) and overall survival (OS). OS was defined as the period from the patient's date of surgery to the time of death or last follow-up. DFS was defined as the interval between the date of surgery and the date of tumour recurrence or December 31, 2019. Follow-up was continued until December 31, 2019, or until the patient died. The secondary endpoints included intraoperative sufentanil consumption, the verbal numeric rating scale (VNRS) at rest and the length of postoperative hospital stay.

Exposure Variable

We sought to determine the effect of lidocaine on short- and long-term outcomes after primary debulking surgery. Patients in the lidocaine group received an initial bolus of lidocaine (1.5 mg/kg) at the induction of general anaesthesia, followed by a continuous infusion of 2 mg/(kg·h) intraoperatively that was stopped at the end of surgery. In the non-lidocaine group, the patients in non-lidocaine group receive continuous bolus injection of 1.5 mg/kg saline at the induction of anaesthesia followed by a continuous infusion of 2 mg/(kg·h) intraoperatively. Epinephrine was not used combined with lidocaine infusion. The decision to initiate lidocaine therapy was made by the attending anaesthesiologist assigned to the case and based on clinical judgement.

Anaesthesia Care

Upon entering the operating room, all patients were monitored according to ASA monitoring standards. In all patients, general

anaesthesia was induced with propofol (target-controlled infusion, effect-site concentration: 3.0-4.0 μg/ml), sufentanil (0.3-0.5 μg/kg), and rocuronium (0.6 mg/kg). The patients were then endotracheally intubated, and general anaesthesia was maintained with 2.0-3.0% sevoflurane in an oxygen/air mixture. Repeated bolus injections of sufentanil and rocuronium were given as necessary throughout the operation. All patients received 5ml of 0.375% ropivacaine after the induction of general anaesthesia, plus 4ml of ropivacaine every 50 minutes until the end of surgery. All patients in the study received epidural analgesia with an infusion of 0.375% ropivacaine *via* an epidural catheter placed at the mid-thoracic level (T12-S1). At the end of the operation, all patients in both groups received a patient-controlled epidural analgesia (PCEA) pump (0.1% ropivacaine and 0.5 μg/ml sufentanil, background: 2-3 ml/h, bolus: 3-4 ml, lockout time: 15 min) for 48 h.

SCN9A mRNA Expression Analysis

The Kaplan-Meier (KM) Plotter database contains reliable *SCN9A* mRNA expression for 675 patients (13). Patients with *SCN9A* mRNA tumour information in the database were identified from Cancer Biomedical Informatics Grid (http://cabig.cancer.gov/), Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and The Cancer Genome Atlas (http://cancergenome.nih.gov) cancer datasets. We analysed progression-free survival and OS as provided by the KM plotter database.

Statistical Analysis

Patient demographics, disease status, intraoperative variables and outcomes were summarized through descriptive statistics. Categorical data were analysed with the chi-square test, and the results are expressed as N (%); continuous data are expressed as the mean ± standard deviation (SD), and two independent samples were analysed with the t-test. Fisher's exact test or the chi-square test was used to evaluate associations between categorical variables. T-tests or Mann-Whitney U tests were employed to compare continuous variables between patient groups. The Kaplan-Meier method was applied to calculate OS and DFS. To assess the prognostic value of *SCN9A*, each percentile (of expression) between the lower and upper quartiles and the median threshold was used as the final cut-off in univariable Cox regression analysis. To evaluate the impact of lidocaine on survival, Cox proportional hazards regression models were used to compare risk factors between the different groups by using univariate models. Variables that were significant in the univariate analysis were entered into a multivariate model using the forward conditional method, which was used to fit the multivariate model. We performed propensity score matching analysis to adjust for selection bias in the observational study. The following variables were entered in our propensity model: age, ASA grade, Charlson comorbidity, FIGO stage, histologic diagnosis, tumour differentiation, residual disease, and adjuvant chemotherapy. Statistical analyses were performed with SPSS 17.0 (SPSS Inc., Chicago, IL, U.S.A.), and a P-value <0.05 was considered statistically significant.

RESULTS

A total of 702 patients who underwent primary debulking surgery for ovarian cancer were enrolled in this study, with 376 in the non-lidocaine group and the remaining 326 in the lidocaine infusion group. After propensity score matching, 302 patients remained in the non-lidocaine infusion group and 302 in the lidocaine infusion group. The patients' demographics, including age, ASA physical status, operative variables, and FIGO stage, were similar between the groups (**Table 1**).

Primary Endpoint

In this study, the median follow-up time for all patients was 46.8 months. The Kaplan–Meier survival curves for the lidocaine infusion and non-lidocaine infusion groups are displayed in **Figure 1**. The Kaplan-Meier curves for OS suggest that patients who were treated with lidocaine had a significant improvement in survival. Indeed, OS rates at 3 years and 5 years after surgery were significantly higher among the patients in the lidocaine infusion group than in the non-lidocaine infusion group [3-year OS: 45.2%, vs. 37.5%, P<0.001, and 5-year OS: 35.2% vs. 25.6%, P=0.011, respectively, **Figure 1A**]. In addition, univariate Cox regression analysis showed that age, ASA score, CCI, tumour differentiation, FIGO stage, residual disease, surgical complexity, ascites, intraoperative blood loss, adjuvant chemotherapy and lidocaine infusion were associated with OS (**Table 2**). According to multivariate analysis before propensity score matching, the following covariates were significantly associated with unfavourable OS: poor tumour differentiation (HR: 1.36, 95% CI: 1.02, 1.78, P=0.011), residual disease (HR: 1.28, 95%CI: 1.23, 1.58, P<0.001), and no adjuvant chemotherapy (HR: 1.56, 95% CI: 1.41, 1.62, P<0.001) (**Table 3**). Intravenous infusions of lidocaine were associated with prolonged OS (HR: 0.93, 95% CI: 0.82, 0.98, P=0.026). After propensity score matching, the association between lidocaine infusion and OS remained statistically significant (HR: 0.86, 95% CI: 0.62, 0.98, P=0.038). The following covariates were also statistically significant: tumour differentiation (HR: 1.26, 95% CI: 1.22, 1.73, P=0.011), residual disease (HR: 1.21, 95%CI: 1.02, 1.48, P=0.029), and no adjuvant chemotherapy (HR: 1.16, 95% CI: 1.12, 1.43, P=0.016) (**Table 3**).

Moreover, DFS rates at 3 and 5 years after surgery differed between patients in the lidocaine group and those in the non-lidocaine infusion group [3-year DFS: 42.5%, vs. 34.6%, P<0.001, and 5-year DFS: 32.6% vs. 21.3%, P=0.011, respectively, **Figure 1B**]. Univariate Cox regression analysis showed that age, ASA score, CCI, tumour differentiation, FIGO stage, residual disease, surgical complexity, ascites, intraoperative blood loss, postoperative chemotherapy, and lidocaine infusion were associated with DFS (**Table 2**). In the multivariate Cox proportional hazards model before propensity score matching, poor tumour differentiation (HR: 1.36, 95% CI: 1.02, 1.78, P=0.011), residual disease (HR: 1.28, 95%CI: 1.23, 1.58, P<0.001), and no adjuvant chemotherapy (HR: 1.56, 95% CI: 1.41, 1.62, P<0.001) were independent factors of unfavourable DFS (**Table 3**). Intravenous infusions of lidocaine were associated with prolonged DFS (HR: 0.93, 95% CI: 0.82, 0.98,

TABLE 1 | Patient and treatment characteristics for both groups.

Variable	Original cohort		*P*	Matched cohort		*P*	Standard difference (%)
	Non-lidocaine group (n=376)	Lidocaine group (n=326)		Non-lidocaine group (n=302)	Lidocaine group (n=302)		
Age (years)	53.6±10.6	54.2±11.2	0.460	53.2±10.2	53.4±10.6	0.813	3.26
BMI (kg/m2)	27.6±6.5	28.6±6.2	0.382	27.3±6.2	27.4±6.3	0.844	–
ASA (n, %)			0.893			0.927	4.12
I-II	283 (75.2%)	243 (74.6%)		242 (80.1%)	241 (79.8%)		
III-IV	93 (24.8%)	83 (25.4%)		80 (19.9%)	81 (20.2%)		
Patients enrolled			<0.001			0.998	–
2015	93 (24.7%)	76 (23.2%)		73 (24.1%)	71 (23.5%)		
2016	92 (24.5%)	78 (23.8%)		72 (23.8%)	73 (24.2%)		
2017	95 (25.3%)	80 (24.5%)		81 (26.8%)	81 (26.9%)		
2018	96 (25.5%)	92 (28.5%)		76 (25.3%)	77 (25.4%)		
CCI (n, %)			0.942			0.964	5.26
0	62 (16.5%)	55 (16.9%)		59 (19.5%)	58 (19.3%)		
1	172 (45.7%)	152 (46.5%)		159 (52.6%)	157 (51.9%)		
≧2	142 (37.8%)	119 (36.6%)		84 (27.9%)	87 (28.8%)		
Histologic diagnosis			0.903			0.930	4.25
Serous histology	236 (62.8%)	207 (63.5%)		210 (69.5%)	208 (68.9%)		
Non-serous histology	140 (37.2%)	119 (36.5%)		92 (30.5%)	94 (31.1%)		–
Tumor size			0.801			0.932	
>5	218 (57.9%)	185 (56.8%)		195 (64.6%)	194 (64.2%)		
<5	158 (42.1%)	141 (43.2%)		107 (35.4%)	108 (35.8%)		
FIGO stage (n, %)			0.950			0.995	3.28
I	32 (8.5%)	30 (9.3%)		26 (8.6%)	25 (8.4%)		
II	43 (11.4%)	35 (10.7%)		35 (11.6%)	36 (11.8%)		
III	168 (44.7%)	141 (43.5%)		159 (52.6%)	157 (51.9%)		
IV	133 (35.4%)	120 (36.5%)		82 (27.2%)	84 (28.2%)		
Tumor differentiation			0.991			0.965	6.25
Well	36 (9.6%)	32 (9.8%)		29 (9.6%)	28 (9.3%)		
Moderate	215 (57.2%)	185 (56.9%)		165 (54.6%)	163 (53.9%)		
Poor	125 (33.2%)	109 (33.3%)		108 (35.8%)	111 (36.8%)		
Residual disease			0.919			0.878	4.65
No visible disease	186 (49.5%)	159 (48.7%)		156 (51.6%)	154 (51.0%)		
<1cm residual disease	132 (35.1%)	119 (36.4%)		113 (37.4%)	111 (36.9%)		
>1cm residual disease	58 (15.4%)	48 (14.9%)		33 (11.0%)	37 (12.1%)		
Surgical complexity			0.829			0.986	–
Low	62 (16.5%)	56 (17.2%)		43 (14.2%)	44 (14.5%)		
Intermediate	200 (53.2%)	178 (54.6%)		162 (53.6%)	160 (52.9%)		
High	114 (30.3%)	92 (28.2%)		97 (32.2%)	98 (32.6%)		
Operation time (min)	213±63	209±59	0.388	202±61	206±62	0.424	–
Ascites (ml)			0.929			0.961	–
<200	62 (16.5%)	56 (17.3%)		45 (14.9%)	44 (14.6%)		
>200	53 (14.1%)	45 (13.9%)		43 (14.2%)	44 (14.5%)		
Estimated blood loss (n, %)			0.730			0.953	–
≤ 400 ml	219 (58.2%)	195 (59.7%)		195 (64.6%)	194 (64.2%)		
> 400 ml	157 (41.8%)	131 (40.3%)		107 (35.4%)	108 (35.8%)		
Blood transfusion			0.912			0.934	–
No	235 (62.5%)	206 (63.2%)		185 (61.3%)	183 (60.5%)		
Yes	141 (37.5%)	120 (36.8%)		117 (38.7%)	119 (39.5%)		
Pre CA125 (U/ml)	635 (182-1126)	663 (172-1047)	0.462	626 (194-985)	610 (202-798)	0.541	–
Postop-Chemotherapy (n, %)			0.902			0.931	6.25
No	123 (32.7%)	109 (33.5%)		100 (33.1%)	98 (32.5%)		
Yes	253 (67.3%)	217 (66.5%)		202 (66.9%)	204 (67.5%)		
Postop-Chemotherapy across year (n, %)			0.981			0.937	–
2015	62 (16.5%)	51 (15.8%)		53 (17.5%)	52 (17.3%)		
2016	64 (17.0%)	54 (16.7%)		50 (16.6%)	48 (15.8%)		
2017	68 (18.1%)	58 (17.8%)		53 (17.5%)	52 (17.2%)		
2018	59 (15.7%)	54 (16.2%)		46 (15.3%)	52 (17.2%)		

BMI, Body Mass Index; ASA, American Society of Anesthesiologists score; CCI, Charlson Comorbidity Index; FIGO, Federation International of Gynecology and Obstetrics.

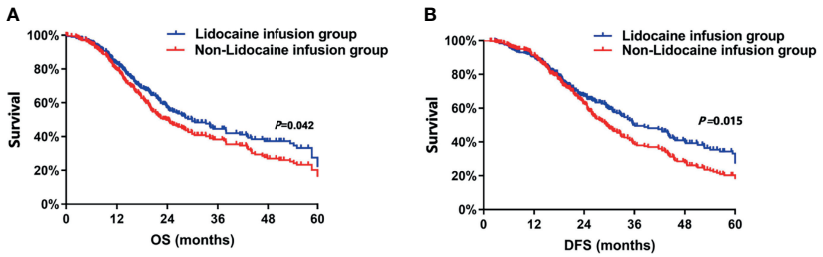

FIGURE 1 | **(A)** Overall survival curves from the date of surgery according to the use of intraoperative intravenous lidocaine infusion. **(B)** Disease-free survival curves from the date of surgery according to the use of intraoperative intravenous lidocaine infusion. DFS, disease-free survival; OS, overall survival.

TABLE 2 | Univariate analysis of OS and DFS.

Variables	OS		DFS	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years)	1.06 (1.02-1.12)	0.025	1.12 (1.08-1.26)	0.013
BMI (kg/m2)	1.00 (0.95-1.05)	0.265	1.02 (1.00-1.16)	0.352
ASA score (III-IV)	1.48 (1.22-1.73)	0.016	1.23 (1.14-1.28)	0.036
CCI (>2)	1.26 (1.20-1.56)	0.035	1.32 (1.11-1.42)	0.019
Histologic diagnosis (Non-serous histology)	1.35 (1.16-1.65)	<0.001	1.42 (1.32-1.63)	<0.001
Tumor differentiation (poor)	1.46 (1.12-1.68)	0.035	1.46 (1.26-1.83)	0.042
FIGO stage (III-IV)	1.68 (1.15-2.15)	<0.001	1.59 (1.12-1.78)	<0.001
Residual disease (>1cm)	1.82 (1.45-2.16)	0.028	1.95 (1.26-2.06)	0.014
Surgical complexity	1.15 (1.08-1.26)	0.015	1.26 (1.22-1.56)	0.036
Ascites (ml)	1.56 (1.42-1.98)	0.005	1.15 (1.10-1.48)	0.002
Estimated blood loss (ml)	1.22 (1.15-1.42)	0.042	1.20 (1.16-1.62)	0.034
Postop-Chemotherapy (no)	1.95 (1.24-2.16)	<0.001	2.16 (1.62-2.42)	<0.001
Lidocaine infusion (yes)	0.85 (0.78-0.94)	0.026	0.76 (0.62-0.88)	0.032

BMI, Body Mass Index; ASA, American Society of Anesthesiologists score; CCI, Charlson Comorbidity Index; FIGO, Federation International of Gynecology and Obstetrics OS, Overall Survival; DFS, Disease free Survival.

TABLE 3 | Multivariable Cox proportional of OS and DFS.

Variables	OS (Before matching)		OS (After matching)		DFS (Before matching)		DFS (After matching)	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Lidocaine infusion (yes)	0.93 (0.82-0.98)	0.026	0.86 (0.62-0.98)	0.038	0.80 (0.62-0.92)	0.019	0.73 (0.62-0.88)	0.046
Tumor differentiation (poor)	1.36 (1.02-1.78)	0.011	1.26 (1.22-1.73)	<0.001	1.76 (1.62-1.88)	0.021	1.63 (1.142-1.78)	0.035
Residual disease (>1cm)	1.28 (1.23-1.58)	<0.001	1.21 (1.02-1.48)	0.029	1.83 (1.62-1.98)	0.026	1.66 (1.22-1.53)	<0.001
Postop-Chemotherapy (no)	1.56 (1.41-1.62)	<0.001	1.16 (1.12-1.43)	0.016	1.93 (1.32-2.28)	<0.001	1.76 (1.12-1.83)	<0.001

OS, Overall Survival; DFS, Disease free Survival.

P=0.026), and the association between lidocaine infusion and OS remained statistically significant after propensity score matching (HR: 0.86, 95% CI: 0.62, 0.98, P=0.038). The following covariates were associated with worse DFS: tumour differentiation (HR: 1.26, 95% CI: 1.22, 1.73, P=0.011), residual disease (HR: 1.21, 95%CI: 1.02, 1.48, P=0.029), and adjuvant chemotherapy (HR: 1.16, 95% CI: 1.12, 1.43, P=0.016) (**Table 3**).

Secondary Outcomes

Intraoperative sufentanil consumption was significantly lower in the lidocaine group (Mean (standard deviation, SD),35.6 μg ± 4.8 μg *vs.*43.2 μg ± 4.6 μg, P<0.001, **Figure 2A**) than in the non-lidocaine group. Also, the average VNRS score after surgery on postoperative day 1 in lidocaine infusion group was lower compared with non-lidocaine infusion group. (4.0 ± 1.3 *vs.* 4.7 ± 1.1, P<0.001, **Figure 2C**). In terms of length of hospital stay, the median duration (interquartile) in the non-lidocaine group was 12.4 days [10.0, 13.7], whereas the mean LOS was 12.0 days in the lidocaine group [10.0, 13.1] (P=0.386, **Figure 2B**).

SCN9A as a Predictor of Ovarian Cancer Survival

For mRNA SCN9A expression analysis, we included women with all types of ovarian cancer (stages 1-4) who might

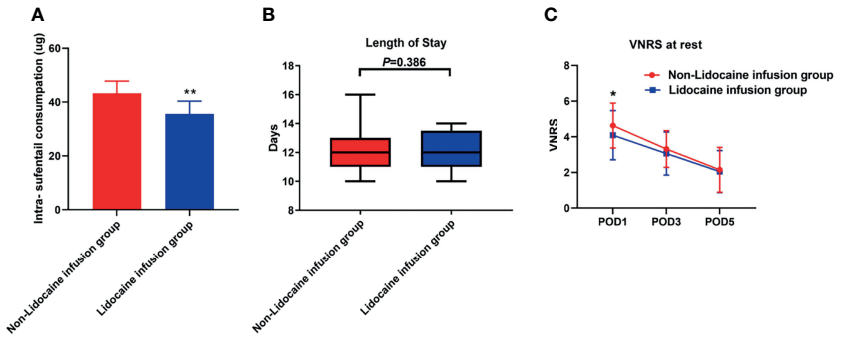

FIGURE 2 | **(A)** Intraoperative sufentanil consumption between groups. **(B)** Length of postoperative hospital stay between groups. **(C)** The verbal numeric rating scale at rest between groups. *P < 0.05, **P < 0.001.

have received primary debulking surgery and adjuvant chemotherapies. The mRNA expression analysis demonstrated that *SCN9A* is expressed in ovarian cancer tissues, though at slightly lower levels than in controls (**Figure 3A** and **Table 4**). Kaplan-Meier curves also indicated significantly shorter progression-free survival and OS among women with tumours expressing higher than the median *SCN9A* mRNA level (**Figure 3B**).

DISCUSSION

The theory that intraoperative administration of lidocaine can improve perioperative outcomes and survival in cancer patients has been the focus of extensive debate and controversy (14). In the present work, we demonstrate that the systemic infusion of lidocaine during ovarian cancer debulking surgery is not only associated with lower opioid use and adequate analgesia but also with improved survival when compared to no intraoperative administration of the local anaesthetic. Our findings deserve several considerations. First, this is the first report suggesting that intraoperative infusion of lidocaine is associated with survival benefits in women with ovarian cancer. Other investigations have been conducted to assess the impact of regional anaesthesia on ovarian cancer progression; however, the results of those studies are mixed (15, 16). In our study, systemic lidocaine was added to epidural analgesia. De Oliveira et al. suggested that adequate and robust intraoperative and postoperative analgesia is associated with better survival in a cohort of women with ovarian cancer (15). Therefore, we speculate that strong modulation of the stress response associated with the use of lidocaine in combination with epidural analgesia may have conferred survival benefits in our cohort of patients. Second, we cannot rule out a direct effect of lidocaine on minimal residual disease. It has been theorized that the long-term effects of anaesthetics in the tumour niche of minimal residual disease are more important in terms of oncological outcomes than is the

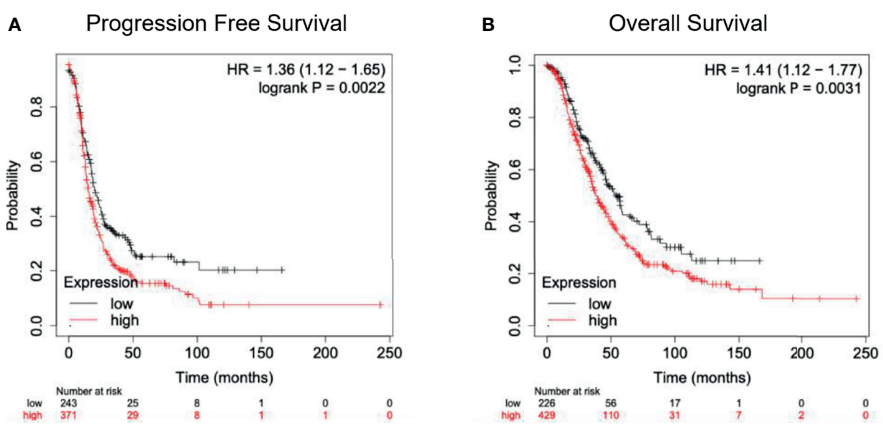

FIGURE 3 | **(A)** Progression-free survival curves from the date of treatment according to SCN9A expression. **(B)** Overall survival curves from the date of treatment according to SCN9A expression.

TABLE 4 | mRNA expression of SCN9A in normal versus ovarian cancer tissue.

Gene	Tissue	Min	Q1	Median	Q3	Max	P value
SCN9A	Normal (n=5)	10	43	52	54	57	0.137
	Cancer (n=1,648)	1	15	32	58	706	

Min, minimum; Max, Maximum; Q, Quartile.

potential impact in the primary disease. In this regard, lidocaine can modulate several mechanisms involved in cancer cell survival and proliferation as well as enhance the effect of cells involved in immune surveillance. For instance, lidocaine inhibits the invasion and migration of ovarian cancer ES-2 cells, exhibits antiangiogenic effects and stimulates the cytotoxic function of natural killer cells at lower than clinical concentrations (8, 17, 18).

Last, lidocaine is an inhibitor of voltage-gated sodium channels. These channels are expressed at higher levels in ovarian cancer cells with high metastatic potential than in those with low metastatic properties. Our study also showed that the Nav1.7 channel encoded by the gene *SCN9A* may play a role in ovarian cancer cell biology: Patients with high expression of this gene showed worse survival. Therefore, lidocaine might induce apoptosis in minimal residual disease by acting on voltage-gated sodium channels, as indicated in previous studies (19).

Our work has several limitations that are mostly with regard to its retrospective design. Therefore, significant bias and confounding factors related to unknown and unmeasured variables may have influenced our findings, such as postoperative complications and time to adjuvant chemotherapy initiation. We cannot exclude the possibility that the women in the lidocaine group started adjuvant chemotherapy earlier than those in the non-lidocaine group. Additionally, we did not include women who did not receive epidural analgesia. Therefore, it remains unknown whether the associated beneficial effects of lidocaine can be extended to women unable to receive epidural analgesia.

In conclusion, intraoperative intravenous lidocaine infusion during ovarian cancer surgery is associated with a reduction in intraoperative sufentanil consumption, adequate postoperative analgesia and longer PFS and OS. We consider that it is necessary to test our hypothesis under the scientific rigour of a randomized controlled trial.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Fudan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conception and design: HZ, WC, JC, and CM. Administrative support: HZ, JC, and ZS. Provision of study materials or patients: HZ, JG, and WC. Collection and assembly of data: HZ, JG, JC, and ZS. Data analysis and interpretation: HZ, JG, JC and ZS. Manuscript writing: All authors. Final approval of manuscript: All authors.

FUNDING

This research was supported by the National Natural Science Foundation of China (NO. 82102253, 81871591), Clinical Research Plan of SHDC (NO. SHDC2020CR4064), the Shanghai Municipal 2021 "Science and Technology Innovation Action Plan" (NO. 21S31902600), Natural Science Foundation of Shanghai (NO. 21ZR1413400), Shanghai Sailing Program (NO. 21YF1406800), 2019 Fudan University Zhuo-Xue Project (NO. JIF159607).

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 09 February 2022
doi: 10.3389/fphar.2022.794109

No Difference Among Inhaled Anesthetics on the Growth and Metastasis of Murine 4T1 Breast Cancers in a Mouse Model of Spontaneous Metastasis

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Objective: This study evaluates the effect of the commonly used inhaled anesthetics isoflurane, sevoflurane, and desflurane on the viability and migration of murine 4T1 breast cancer cells, the growth, and lung metastasis in a syngenetic model of spontaneous metastasis.

Methods: The murine 4T1 breast cancer cells were exposed to isoflurane (2%), sevoflurane (3.6%), or desflurane (10.3%) for 3 h. Cell viability was measured using the MTT assay. The migratory capacity of 4T1 cells was assessed using a scratch assay after 24 h incubation. Female balb/c mice were subjected to orthotopic implantation of 4T1 cells under anesthesia with one of the inhaled anesthetics: 2% isoflurane, 3.6% sevoflurane, or 10.3% desflurane. Subsequently, resection of primary tumors was performed under the identical anesthetic used during implantation for 3 h. Three weeks later, the mice were euthanized to harvest lungs for *ex vivo* bioluminescent imaging and histological analysis. Blood was collected for serum cytokine assays by ELISA.

Results: There was no difference in cell viability among isoflurane, sevoflurane, desflurane, and control groups (n = 180 for each group, P = 0.648). Sevoflurane but not isoflurane or desflurane significantly increased the migration of 4T1 cells compared to the control group (n = 18, P = 0.024). There was no difference in the growth of the orthotopically implanted primary tumors (n = 12 for the isoflurane group, n = 11 for the sevoflurane group, and for the desflurane group, P = 0.879). Surgical dissection of primary tumors in mice under anesthesia with isoflurane, sevoflurane, or desflurane led to no difference in lung metastasis following surgery (P = 0.789). No significant difference was observed among isoflurane, sevoflurane, and desflurane groups in the serum levels of IL-6 (P = 0.284), CCL-1 (P = 0.591), MCP-1 (P = 0.135), and VEGF (P = 0.354).

Conclusion: Our study demonstrated that sevoflurane increased the migration of 4T1 breast cancer cells *in vitro*. Inhaled anesthetics isoflurane, sevoflurane, and desflurane had no difference on the growth of primary tumor and the lung metastasis of 4T1 cells in the mouse model of spontaneous metastasis with surgical removal of primary tumors.

Keywords: isoflurane, sevoflurane, desflurane, cell viability, migration, breast cancer, tumor growth, metastasis

Abbreviations: CCL-1, chemokine ligand-1; CXCR-2, CXC chemokine receptor type-2; ELISA, enzyme-linked immunosorbent assay; HIF, hypoxia-inducible factor; IL-6, interleukin-6; OPN, osteopontin; MCP-1, mono-cyte chemotactic protein-1; MMP-11, matrix metallopeptidase-11; TGF-β, transforming growth factor-β; TGF-βRII, transforming growth factor-β receptor type II; VEGF-A, vascular endothelial growth factor-A.

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Edited by:
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Pharmacology

Received: *13 October 2021*
Accepted: *14 January 2022*
Published: *09 February 2022*

Citation:
Liu Q, Li R and Lin J (2022) No Difference Among Inhaled Anesthetics on the Growth and Metastasis of Murine 4T1 Breast Cancers in a Mouse Model of Spontaneous Metastasis. Front. Pharmacol. 13:794109. doi: 10.3389/fphar.2022.794109

INTRODUCTION

Breast cancer is one of the most common malignancies in women and the second most frequently occurring newly diagnosed cancers worldwide (Wörmann, 2017). Surgical resection greatly improves the patient outcome (Ferlay et al., 2019), but tumor recurrence or metastasis after surgery is still the main cause of cancer patient death. The perioperative period carries many risks for cancer patients such that surgical procedures may disseminate cancer cells into the circulation and surrounding tissues (Camara et al., 2006). The number of circulating tumor cells has been shown correlating to the outcome of patients (Barbazan et al., 2014; Bortolini Silveira et al., 2021). The viability and motility of those cancer cells released from primary tumors may determine the spread and the development of clinical metastasis.

Inhaled anesthetics are routinely used for the maintenance of general anesthesia, and the choice of a particular anesthetic is at the discretion of the anesthesia provider. Isoflurane, sevoflurane, and desflurane are the most widely used inhaled anesthetics and have been suggested to influence the patient outcome following oncologic surgery (Buggy et al., 2015). Some retrospective studies have suggested that inhaled anesthetics may increase cancer recurrence, but not confirmed by other retrospective studies and a prospective clinical study (Enlund et al., 2014; Wigmore et al., 2016; Kim et al., 2017; Yoo et al., 2019; Sessler et al., 2019). Laboratory research has shown that inhaled anesthetics may change the microenvironment in healthy organs (Sakamoto et al., 2005) and alter mRNA expression in cancer cells (Jiao et al., 2018). It has also been shown that inhaled anesthetics promoted ovarian cancer cell migration and expression of metastasis-related genes and protein, which included VEGF-A, MMP-11, CXCR2, and TGF-β with a magnitude order of desflurane, sevoflurane, and isoflurane (Iwasaki et al., 2016). Our previous study found that sevoflurane was associated with more postoperative lung metastasis than intravenous anesthetic propofol in mouse models of spontaneous metastasis, of which the mechanism was linked to inflammatory cytokine IL-6 (Li et al., 2020). Thus, the difference of inhaled anesthetics on the cancer biology may lead to clinical significance.

No study has analyzed the difference among the commonly used inhaled anesthetics on the tumor growth and metastasis. The potential difference in inhaled anesthetics is important in evaluating the results of animal and human studies and selecting anesthetics in clinical studies or practice. Therefore, we hypothesized that inhaled anesthetics isoflurane, sevoflurane, and desflurane differentially affect the metastatic function of breast cancers at clinically relevant concentration. We tested our hypothesis in a preclinical mouse model of spontaneous metastasis using 4T1 cells as the primary endpoint and cellular functions of 4T-1 cells *in vitro* as secondary endpoints. Since IL-6 was associated with the promoting effect of sevoflurane on lung metastasis (Li et al., 2020), we measured the levels of IL-6 and other inflammatory cytokines including CCL-1, MCP-1, and VEGF as well.

METHODS

Ethics Statement

All of the mice used in these experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Stony Brook University (917821). Balb/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME United States) and maintained in accordance with federal guidelines. Mice were housed in sterilized plastic cages under pathogen-free conditions (21–25°C, 12/12 light/dark cycle). Food and water were offered *ad libitum*. Mice were euthanized using CO_2 overdose followed by cervical dislocation to ameliorate the suffering of mice.

Test Gas Exposure

The treatment with different gases was conducted in a purpose-built 1.5 L airtight gas chamber equipped with inlet and outlet valves (Iwasaki et al., 2016). All gases were delivered to the gas chamber at a rate of 1 L/min and monitored using an anesthetic analyzer (POET IQ Anesthesia Gas Monitor, CRITICARE Systems ING) until the desired anesthetic concentrations were achieved. Then the chamber of gases was sealed and placed in an incubator at 37°C for the duration of 3 h. The experimental gases were air (medical grade) or one of the inhaled anesthetics in air: 2% isoflurane (Baxter, Deerfield, IL, United States), 3.6% sevoflurane (Baxter, Deerfield, United States), or 10.3% desflurane (Baxter, Deerfield, United States). The concentrations of the anesthetic gases are the equivalence of 1.7 minimum alveolar concentrations (MAC) in humans. After exposure, cells were returned to the normal cell culture incubator for further study.

Cell Culture and Survival Assay

The murine breast cancer cell line 4T1-LUC was purchased from the American Type Culture Collection (Rockville, MD, United States) and cultured in RPMI 1640 supplemented with 10% FBS (Weene, l, United States), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Weene, l, United States) in 5% CO_2 humidified atmosphere at 37°C. For the survival assay, cells were divided into a 96-well plate and incubated at 37°C for 24 h, and then treated with air (control) or one of the tested anesthetic gases in air for 3 h. Cell viability was measured using the MTT assay after 24 h incubation as previously described (Li et al., 2020). In brief, the culture medium was removed, and 100 μL MTT/medium solution(2.5 mg/ml) were added to each well and incubated for 3 h; then the medium was removed, and 100 μl aliquot of DMSO were added to each well to solubilize the formazan crystals. Absorbance was measured at 571 nm using a microplate reader (BioTek, Winooski, VT, United States). The percentage of cell viability was expressed relative to the control.

Migration Assay

A wound healing assay was employed to evaluate the effects of isoflurane, sevoflurane, or desflurane on the cell migratory ability. The 4T1 cells were seeded at a density of 2×10^6 cells/well in 6-well plates and incubated for 12 h at 37°C to allow adherence to take place. The scratches were then made using a 100-μl yellow tip (time 0), transferred to the low-serum culture medium, and treated with 2% isoflurane, 3.6% sevoflurane, or 10.3% desflurane for 3 h. The distances of migrating cells were measured from pictures (five fields) taken at 24 h after the initial wound, and the distance of each measurement was calculated by using ImageJ (NIH, Bethesda, MD, United States). Each experiment was independently repeated at least three times.

Animal Models and Surgery

Female balb/c mice in each group were subjected to orthotopic implantation of 4T1 cells (2×10^5 cells per mice) in the mammary fat pad. Implantations were conducted with one of the inhaled anesthetics (2% isoflurane, 3.6% sevoflurane, or 10.3% desflurane) within 10 min. The growth of 4T1 tumors was monitored by non-invasive bioluminescent imaging (IVIS Lumina III, PerkinElmer, Waltham, MA). The volume of tumors was measured using a caliper every week and calculated using formula $V = (\text{Width}^2 \times \text{Length}) \times 2^{-1}$. When the volume of the primary tumor reached around 500 mm^3, the primary tumors were dissected under the identical anesthetic used for cancer cell implantation, and the anesthesia was maintained for 3 h. During surgery, the delivery of inhaled anesthetics was maintained using a SomnoSuite Rodent Anesthesia System (Kent Scientific Corporation, Torrington, CT, United States), and the oxygen saturation and heart rate were monitored by using the PhysioSuite (Kent Scientific Corporation, Torrington, CT, United States) with a pulse oximeter. The mice were placed on the warming pad for temperature control with the SomnoSuite. After surgery, lung metastasis was monitored by using non-invasive bioluminescent imaging after 3 weeks. Three weeks later, the mice were euthanized to harvest lungs for *ex vivo* bioluminescent imaging and histological analysis. Blood was collected for the serum cytokine assay.

Hematoxylin and Eosin Staining and Nodule Counting

Harvested mouse lungs were rinsed in PBS buffer to remove the blood and then fixed in 4% paraformaldehyde overnight at 4°C. Tissues were embedded in paraffin, and a sampling of sections was taken across the lung as follows: two consecutive 5 μm sections were taken, and then a number of consecutive 5 μm sections were discarded (typically 20–40 depending on the size of the tumor nodules) before collecting another two consecutive 5 μm sections. This process was repeated along with the entire lung. The consecutive sections were then stained using H&E, and metastatic nodules were counted on each H&E paraffin section using a phase contrast microscope. The sum of microscopic counting was taken as the final number of lung metastatic nodules.

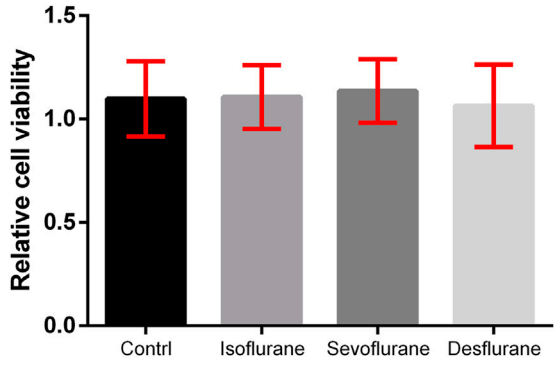

FIGURE 1 | Effect of inhaled anesthetics on the viability of 4T1 cells *in vitro*. The 4T1 cells were treated with air (control), 2% isoflurane, 3.6% sevoflurane, or 10.3% desflurane for 3 h. Cell viability was determined using the MTT assay. There was no significant difference between the four groups ($n = 180$, $P = 0.648$). Isoflurane vs. control, $P = 0.684$; sevoflurane vs. control, $P = 0.541$; desflurane vs. control, $P = 0.363$; one-way ANOVA + Tukey's multiple comparisons test.

ELISA Assay

Mouse serum was subjected to IL-6, CCL-1, MCP-1, and VEGF ELISA assays according to the manufacturer instructions (R&D Systems, Minneapolis, MN, United States). The concentrations of IL-6, CCL-1, MCP-1, and VEGF in serum were calculated according to the volume of serum.

Statistical Analysis

For the animal experiment, 11 mice per group would provide 80% power to detect 30% difference in the total burden of metastasis among three groups treated with inhaled anesthetics at the α level of 0.05, based on a sample size calculation using JMP by SAS (version 10). Statistical analysis was performed using GraphPad Prism 7.0. All the values were expressed as means ± SD. The data were analyzed using ANOVA. Differences were considered significant at $p < 0.05$.

RESULTS

Inhaled Anesthetics Have no Significant Effect on the Viability of 4T1-Luc Cells

The 4T1 LUC cells were treated with air, 2% isoflurane, 3.6% sevoflurane, or 10.3% desflurane ($n = 180$ for each group). The viability (%) of 4T1 cells treated with inhaled anesthetics for 3 h and the statistical differences between groups are illustrated in **Figure 1**. There is no significant difference in the viability of the 4T1 cell among the control, isoflurane, sevoflurane, and desflurane groups ($n = 180$ for each group, $P = 0.648$).

Sevoflurane Promotes the Migration of 4T1-Luc Cells

Wound healing assays were used to evaluate the effects of inhaled anesthetics on cell migration. There is a tendency that the gap

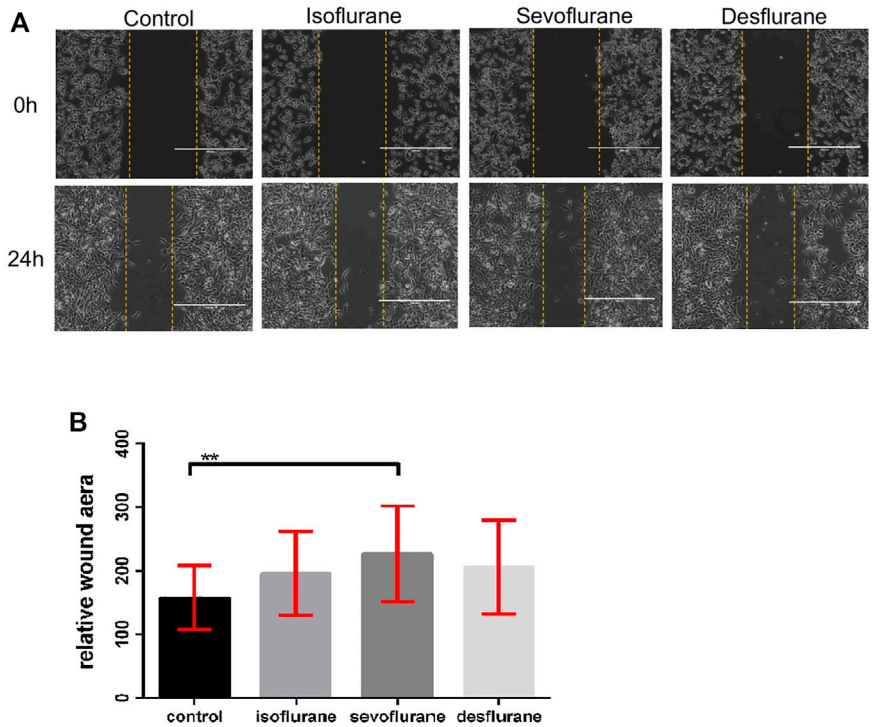

FIGURE 2 | Effect of inhaled anesthetics on the migratory capacity of 4T1 cells *in vitro*. **(A)** The 4T1 cells were wounded by a sterile pipette tip to create a cell-free path, and then they were treated with air (control), 2% isoflflurane, 3.6% sevoflflurane, or 10.3% desflflurane for 3 h (n = 18 for each group). **(B)** Relative wound distance was measured for statistical analysis. The differences between the control group and the sevoflflurane group was signifificant (P = 0.024). There was no signifificant difference between isoflflurane or desflflurane and control group. Isoflflurane vs. control, P = 0.153; desflflurane vs. control, P = 0.465; one-way ANOVA + Tukey's multiple comparisons test.

closures were accelerated by treatment with 2% isoflurane, 3.6% sevoflurane, or 10.3% desflurane compared to the control at 24 h post-exposure (n = 18 for each group, **Figure 2**). Only sevoflurane significantly affected the migration of 4T1 cells in comparison with the control group (P = 0.024).

Effect of Inhaled Anesthetics on Lung Metastases in 4T1 Murine Cancer Mouse Model

The implantation of murine 4T1-Luc cells stably expressing luciferase in the unilateral mammary fat pad of balb/c mice was carried out under one of the inhaled anesthetics: 2% isoflurane, 3.6% sevoflurane, or 10.3% desflurane (n = 12 for isoflurane group, n = 11 for sevoflurane group, and n = 11 for desflurane group). Surgical dissection was conducted under the same anesthetic for 3 h when the volume of the primary tumor reached around 500 mm^3. There is no significant difference in primary tumor volumes in 3 groups (P = 0.789, **Figure 3A**). Three weeks after surgical removal of the primary tumor, no significant difference in the burden of lung metastasis was observed in the mice receiving different anesthetics (**Figure 3B**), which was confirmed by histology analysis of nodule counts (**Figures 3C,D**).

We analyzed the effect of inhaled anesthetics on the serum levels of inflammatory cytokines. No significant difference was observed among isoflurane, sevoflurane, and desflurane groups in serum levels of IL-6, CCL-1, MCP-1, and VEGF (**Figure 4**). The desflurane group has a trend of lower MCP-1 than the other two anesthetics, but it was not statistically significant.

DISCUSSION

The role of anesthesia in patient outcome remains to be defined. One question is whether there is a difference among inhaled anesthetics on cancer cell biology that may affect the patient outcome. Our results suggest there is no significant difference in metastatic functions of murine breast cancers and support the practice that groups all inhaled anesthetics together in retrospective clinical studies. Our data are also informative to the animal studies involving the use of general anesthetics. The limitation of our study should be noted; however, as our results were obtained from murine breast cancer, it may not be applicable to all other cancer types or human cancers.

A significant finding of this study is that sevoflurane at the clinically relevant concentration increased migration of 4T1-luc

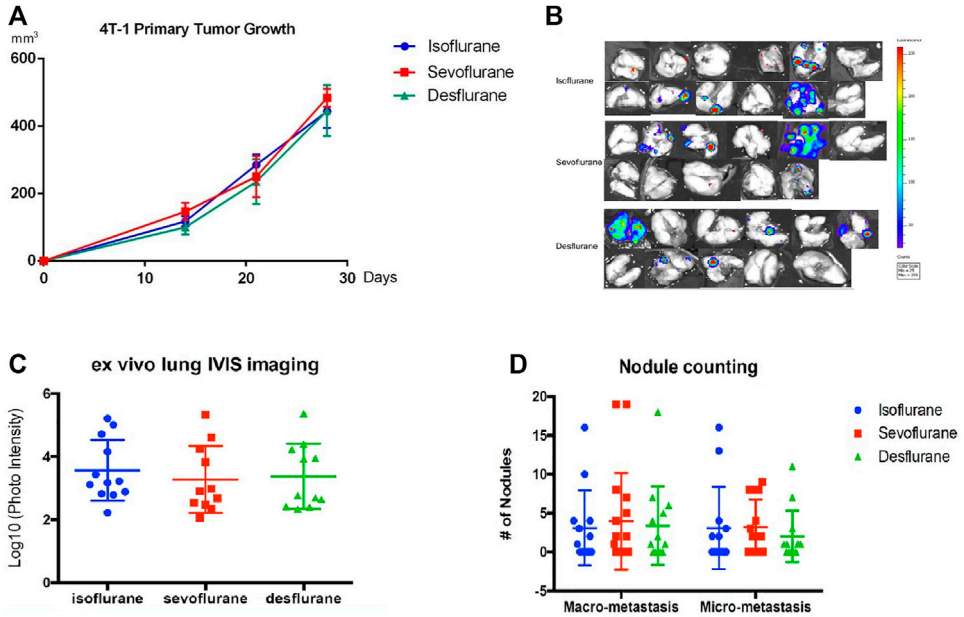

FIGURE 3 | No significant difference in the lung metastasis following mastectomy with different inhaled anesthetics. Mice bearing primary tumors were generated by orthotopical implantation with the luciferase-tagged murine 4T1 breast cancer cells in the mammary fat pads of balb/c mice (n = 12 for isoflurane group, n = 11 for sevoflurane group, and n = 11 for desflurane group). Surgical dissection of primary tumor with 2% isoflurane, 3.6% sevoflurane, or 10.3% desflurane. Mastectomy was performed in mice models, and lung metastases were evaluated 3 weeks after surgery. **(A)** There was no difference in the primary tumor volumes among isoflurane, sevoflurane, or desflurane groups (isoflurane vs. sevoflurane, P = 0.901; isoflurane vs. desflurane, P = 0.847; sevoflurane vs. desflurane P = 0.645; one-way ANOVA + Tukey's multiple comparisons test), **(B)** *ex vivo* lung bioluminescent imaging, and **(C)** photon intensity of them showed no significant difference in lung metastasis among isoflurane, sevoflurane, or desflurane groups (isoflurane vs. sevoflurane, P = 0.778; isoflurane vs. desflurane, P = 0.899; sevoflurane vs. desflurane, P = 0.971). **(D)** The examination of number and size of metastatic nodules showed no significant difference among isoflurane, sevoflurane, or desflurane groups (isoflurane vs. sevoflurane, P = 0.996; isoflurane vs. desflurane P = 0.993; sevoflurane vs. desflurane, P = 0.986).

breast cancer cells *in vitro*. Migration is the basic biological process that is essential for tumor cells to metastasize. There are reports showing inhaled anesthetics enhance the malignancy of cancer cells by different mechanisms. Sevoflurane increased cell viability, migration, and chemoresistance of renal carcinoma by upregulating TGF-βRII and OPN (Ciechanowicz et al., 2018). Sevoflurane increased the migration and colony formation of human glioblastoma cells *via* the expression of CD44 (Lai et al., 2019). Sevoflurane promoted the proliferation and migration of immortalized cervical cancer cells through the activation of phosphatidylinositide 3-kinase/AKT- and ERK1/2-signaling pathway activation (Zhang et al., 2020). Sevoflurane increased migrations of breast cancer estrogen receptor (ER)-positive MCF7 cells and ER-negative MDA-MB- 231 cells (Ecimovic et al., 2013). Isoflurane activated the expression of HIF-1α and its downstream effectors in prostate PC3 cancer cells, leading to increased migration (Huang et al., 2014). In addition, isoflurane increased the levels of HIF-1α, HIF-2α, and VEGF in primary renal cell carcinoma (Benzonana et al., 2013). Indeed, we found that sevoflurane enhanced 4T1 cell migration significantly, but we did not observe any significant effect of sevoflurane on the viability of 4T1 cells. We found a tendency of increase in migration with isoflurane and desflurane. All three inhaled anesthetics did not have a significant effect on viability. Thus, the effects of inhaled anesthetics on the biology of cancer cells appear to vary among types of cancers.

A variety of factors regulate cancer cell migration including matrix-degrading enzymes and cell–cell adhesion molecules. As the change of cell viability and migration *in vitro* do not always translate to the effect of tumor growth and metastasis *in vivo* (Li et al., 2020), which is more clinically relevant to our hypothesis, we elected to analyze the effect of the inhaled anesthetics in a mouse model of spontaneous metastasis. This orthotopically implanted model is a preclinical model with a high clinical predictive value (Shan et al., 2005; Bailey-Downs et al., 2014). Surgery to remove primary tumor was incorporated to closely mimic the clinical scenario. Inflammatory cytokines such as IL-6, CCL-1, MCP-1, and VEGF play a vital role in cancer progression and metastasis (Kaplan et al., 2005; Gril et al., 2018; Li et al., 2020). We have shown, in our previous report, that sevoflurane increased the activity of the IL-6 pathway, leading to more lung metastatic burden than propofol (Li et al., 2020). In this study, we did not observe any significant difference of primary tumor growth and the lung metastasis in the mice receiving different inhaled anesthetics after surgery, nor in the levels of pro-inflammatory cytokines (IL-6, MCP-1, CCL-1, or VEGF). The desflurane group had a trend of lower MCP-1 than the other two

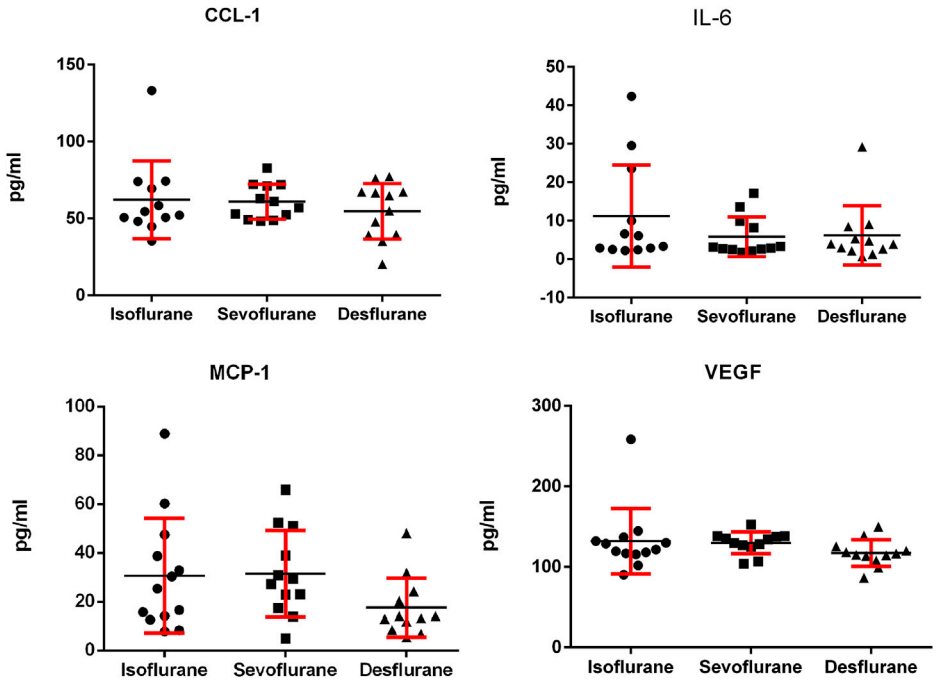

FIGURE 4 | Serum levels of IL-6, MCP-1, CCL-1, and VEGF in the murine 4T1 breast tumor bearing mice 3 weeks following mastectomy with isoflurane, sevoflurane, or desflurane. There was no significant difference in serum levels of IL-6, MCP-1, CCL-1, and VEGF among 3 groups (n = 12 for isoflurane group, n = 11 for sevoflurane group, and n = 11 for desflurane group; one-way ANOVA + Tukey's multiple comparisons test). IL-6: isoflurane vs. sevoflurane, P = 0.508; isoflurane vs. desflurane, P = 0.317; sevoflurane vs. desflurane, P = 0.445. MCP-1: isoflurane vs. sevoflurane P = 0.212; isoflurane vs. desflurane, P = 0.134; sevoflurane vs. desflurane, P = 0.106. CCL-1: isoflurane vs. sevoflurane P = 0.504; isoflurane vs. desflurane, P = 0.334; sevoflurane vs. desflurane, P = 0.590. VEGF: isoflurane vs. sevoflurane, P = 0.354; isoflurane vs. desflurane, P = 0.457; sevoflurane vs. desflurane, P = 0.389.

anesthetics, and it was however not statistically significant. Therefore, we conclude that there is no significant difference among inhaled anesthetics on the primary tumor growth and postoperative metastasis in our models. Taken together, our data and literature show that inhaled anesthetics affect cancer cells *in vitro* differently but suggest no significant difference in the primary tumor growth and the metastasis *in vivo*.

LIMITATION

One limitation of our study is to evaluate the possibility of difference in inhaled anesthetics on tumor metastasis in one cell line and one animal model. Second, different doses or time courses may produce more anesthetic effects on cancer cells, even the non-specific effects of volatile drugs. Another limitation of this study is the relatively small sample size to detect a small change, and that populations are more at risk of such obese mice have not been studied.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Stony Brook University (917821).

AUTHOR CONTRIBUTIONS

QL helped in conceptualization, methodology, performing experiment, data analysis, and original draft preparation. RL helped in methodology, data analysis, and review and editing the draft. JL helped in conceptualization, project administration, funding acquisition, supervision, and review and editing the draft. All authors have read and agreed to the published version of the manuscript.

FUNDING

This work was supported by a grant from the Department of Anesthesiology (Stony Brook University Award 56562) and New York State Department of Health Peter T. Rowley Breast Cancer Scientific Research Projects (DOH01-C33915GG).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 10 February 2022
doi: 10.3389/fonc.2022.837959

Potential Influence of Anesthetic Interventions on Breast Cancer Early Recurrence According to Estrogen Receptor Expression: A Sub-Study of a Randomized Trial

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OPEN ACCESS

Edited by:
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Imperial College London,
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Reviewed by:
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Imperial College London,
United Kingdom
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

Received: *17 December 2021*
Accepted: *14 January 2022*
Published: *10 February 2022*

Citation:
Li M, Zhang Y, Pei L, Zhang Z, Tan G and Huang Y (2022) Potential Influence of Anesthetic Interventions on Breast Cancer Early Recurrence According to Estrogen Receptor Expression: A Sub-Study of a Randomized Trial. Front. Oncol. 12:837959. doi: 10.3389/fonc.2022.837959

Background: Effects of anesthetic interventions on cancer prognosis remain controversial. There is evidence that estrogen receptor (ER)-negative breast cancer patients have an early recurrence peak. We aimed to assess the potential benefit of regional anesthesia-analgesia versus general anesthesia regarding early recurrence in breast cancer according to ER expression.

Methods: Based on a multicenter randomized controlled trial (clinicaltrials.gov, NCT00418457), we included all the patients from Peking Union Medical College Hospital research center in this study. The primary outcome was breast cancer recurrence after surgery. The Cox proportional hazard model was used to compare recurrence between groups.

Results: In total, 1,253 breast cancer patients were included in this sub-study, among whom the median follow-up time was 53 months. In this sub-study, 320 patients were ER-negative, and 933 were ER-positive. As for ER-negative patients, the recurrence risk in the PPA (paravertebral blocks and propofol general anesthesia) group showed no statistical difference compared with the GA (sevoflurane and opioids general anesthesia) group (19.1% versus 23.4%; adjusted HR: 0.80, 95% CI: 0.50–1.30; $P = 0.377$). In the first 18 months after breast cancer surgery, which is considered as the classical early peak of recurrence, after adjustment for menstruation and the pathological stage of tumor, the decrease of early recurrence observed in the PPA group was not significant compared with the GA group (adjusted HR: 0.63, 95% CI: 0.34–1.14; $P = 0.127$).

Conclusions: In our study, the effects of early recurrence after breast cancer surgery in both ER-negative and ER-positive patients were similar between regional anesthesia-analgesia and general anesthesia. Large samples of ER-negative patients will be needed to clarify the effects of anesthetic interventions.

Keywords: anesthesia, breast cancer, estrogen receptor, recurrence, propofol

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer type and is also the leading cause of cancer death in women (1). Despite the evolving process of treatment methods, breast cancer recurrence remains a major problem that affects patient prognosis. Cell phenotype affects recurrence. The annual hazard rate of recurrence in estrogen receptor (ER)-positive patients is higher beyond 5 years than that in ER-negative patients. Conversely, during the first 5 years, the annual hazard rates of recurrence are higher in ER-negative patients (2). An early peak of recurrence could be seen classically in the first 18 months after breast cancer surgery (3, 4), which is in accordance with the annual hazard rate peak observed in ER-negative breast cancer around years two and three after initial diagnosis (5).

Various research demonstrated that the surgical stress response may increase the risk of breast cancer dissemination and metastasis during and after surgery (6). Regional anesthesia-analgesia is thought to prevent cancer recurrence by influencing angiogenesis, moderating the neuroendocrine system, and affecting immunity (7). Moreover, some studies indicate that compared to sevoflurane, propofol attenuates the inflammatory response, which may finally reduce the risk of cancer recurrence (8). However, these findings were only observed in animal studies and retrospective clinical research (9–11), but not in prospective clinical trials (12, 13), which makes the relationship between anesthetic interventions and breast cancer recurrence controversial. It is worth noting that ER-negative breast cancer cells were usually used to explore the relationship between anesthesia and cancer recurrence in both *in vivo* and *in vitro* studies, but in clinical studies, the subtypes of breast cancer were rarely considered. Only few clinical trials were focused on different breast cancer subtypes (14, 15).

Previous studies indicate that the early recurrence peak of breast cancer may be resulted from dormant cell division and angiogenesis induced by operation (16), while the late peak is considered to be the result of metastasis dormancy and which is most common in ER-positive subtype (4). Compared to ER-positive breast cancer cells, ER-negative cells tend to be associated with more invasion and more related to early recurrence (17). Biological studies showed that anesthetic interventions may influence breast cancer early recurrence. It can increase cell apoptosis and reduce proliferation (18, 19) and alter the angiogenesis factors and cancer immunomodulatory cytokines in serum, thereby affecting the functions of ER-negative breast cancer cells (20, 21). Furthermore, the decrease of methylation can reactivate suppressor genes and lead to the inhibition of cancer (22). Ropivacaine could decrease methylation in ER-negative cells rather than ER-positive cells (23). Therefore, it is logically reasonable to hypothesize that ER-negative patients could benefit more from regional anesthesia-analgesia especially in early recurrence.

Current clinical studies have seldom reported the effects of anesthetic interventions in specific cancer cell phenotypes. Considering the gap of current research, we tried to test whether patients according to ER expression status would have an increased benefit on early recurrence from regional anesthesia-analgesia compared with general anesthesia.

METHODS

Patients

This study was based on a previous multicenter randomized controlled trial (clinicaltrials.gov, NCT00418457) (12). The Peking Union Medical College Hospital (PUMCH) ethics committee approved the protocol of the original randomized controlled trial (S-638) on January 23rd, 2014, and all patients understood and signed informed consent for participation in the previous study. Patients receiving primary breast cancer surgery at PUMCH who met the following inclusion criteria were enrolled: aged 18–85 years, and American Society of Anesthesiologists physical status I–III. Patients with contraindications for either anesthetic approach were excluded.

Anesthetic Interventions

Patients were randomly assigned at a 1:1 ratio *via* a computer-generated random sequence to regional anesthesia-analgesia (PPA) group or general anesthesia (GA) group, and received either paravertebral blocks and propofol or sevoflurane and opioids respectively (24). Thirty minutes prior to the induction of anesthesia, patients of the PPA group underwent a single thoracic paravertebral nerve block under ultrasound guidance, using a multipoint method (T1~T5) to inject 5 ml 0.75% ropivacaine at each puncture point. Patients of the GA group were positioned in a similar manner as those of the PPA group, while received 0.2 ml 1% lidocaine injections at each puncture point for local infiltration anesthesia only. Analgesia in the PPA group was primarily based on paravertebral blocks, and maintained using propofol target-controlled infusion (effect site concentration: 2.5–4.0 μg/ml, Marsh model). In the GA group, general anesthesia was induced with 2 mg/kg propofol, and maintained with 2% sevoflurane. Both groups received 1–2 μg/kg fentanyl and 0.4–0.6 mg/kg rocuronium at the induction of anesthesia to facilitate laryngeal mask insertion. During each patient's operation, additional intravenous fentanyl and rocuronium were provided intermittently, and blood pressure and heart rate within a 20% range of basic values were maintained (25).

Outcome

The primary outcome was breast cancer recurrence, which was assessed by contacting patients or the specialist every 6 months. Time to recurrence was measured from the date of surgery to the earliest date that recurrence was detected at any site. Clinical evidence such as radiographic examinations or pathologic findings was provided to confirm recurrence. Medical records were provided including demographic characteristics, clinical factors, and pathological factors related to breast cancer recurrence.

Statistical Analysis

The analysis was performed by the intention-to-treatment principle. Patients lost to follow-up were censored at the time of last contact. To assess the validity of our hypothesis, that there is an interaction between anesthetic interventions and cell phenotype, the data analysis was performed based on ER

status. Breast cancer recurrence rates were analyzed using Poisson regression and Cox proportional hazard models, and were adjusted for confounders. Prognostic factors for recurrence that were unequally distributed among intervention groups in the PUMCH population were regarded as confounders. A standardized mean difference was used to assess distributions of prognostic factors among groups, and a threshold of < 0.1 was considered a negligible difference (26). In addition to confounders identified using the standardized mean difference, other factors were considered clinically important for breast cancer recurrence. Hence, the following two models were devised: Model 1, which was created by adjusting for confounders that were unequally distributed between the two groups; and Model 2, which considered predetermined factors including age, tumor-node-metastasis (TNM) stage, nuclear grade, postoperative radiotherapy, and chemotherapy.

The proportional hazard assumption was tested by evaluating the statistical significance of the anesthesia group-by-time interaction. Because the classical early peak of recurrence is usually observed 18 months post-surgery, we used a split function to explore time-varying coefficients using 18 months as a prespecified cut-off time point (27). Different time splitting points were tested *via* sensitivity analyses. One patient died before recurrence. Therefore, a competing risk analysis was conducted.

This was a sub-study of a randomized controlled trial, which included patients from a single study site. Therefore, the sample size was predetermined. We estimated the statistical power of the study using the available sample size. In the sub-study, recurrence was observed in 68 patients of the ER-negative population. Our sub-study was able to detect a 20% reduction in ER-negative breast cancer recurrence using an event-driven design with a statistical power of 15%.

All statistical tests were two-sided. The significance level was set at 0.05. Statistical analyses were performed using R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria; URL: https://www.R-project.org/.) with "cmprsk", "gsDesign", "prodlim", "stats", "survival", "survminer", "tableone" and "tidyverse" packages. Plots were created using GraphPad PRISM 8.2.0 (GraphPad Software company, San Diego, California, USA; URL: https://www.graphpad.com/scientific-software/prism/).

RESULTS

From February 8th, 2014, to December 8th, 2016, 1,253 patients from PUMCH research center were included in this sub-study. Patients were followed-up till December 8th, 2019, except for 11 patients who were lost to follow-up, patients either reported recurrence or completed at least 3 years of follow-up. The median follow-up time was 53 (IQR 44-62) months. In total, 624 patients were assigned to the PPA group, and 629 patients were assigned to the GA group. Some exposures were unequally distributed between the two groups, and were therefore considered as potential confounders when the association between anesthesia method and cancer recurrence was assessed. These included menstruation status and pathological stage of tumor (**Supplementary Table 1**). Since recurrence was infrequently observed in T0 and TNM stage 0 patients, pathological stage of tumor and tumor TNM stage were regrouped as binary variables.

When the full dataset of the PUMCH population was considered, anesthetic interventions did not affect recurrence after breast cancer surgery (unadjusted hazard ratio [HR]: 0.96, 95% confidence interval [CI]: 0.71–1.30; $P = 0.778$). In Model 1, menstruation and pathological stage of tumor were considered confounders of the multivariable Cox regression, and the HR of regional anesthesia-analgesia compared with general anesthesia was 0.92 (95% CI: 0.68–1.26; $P = 0.612$). After adjusting for predetermined factors (Model 2) including age, tumor TNM stage, nuclear grade, postoperative chemotherapy and radiotherapy, the HR of regional anesthesia-analgesia was 0.92 (95% CI: 0.67–1.26; $P = 0.598$).

In this sub-study, 320 and 933 patients were ER-negative and -positive, respectively, based on pathological results (**Table 1**). We observed a peak in early recurrence in ER-negative patients (**Figure 1**). Recurrence risk was higher in ER-negative patients than ER-positive patients (21.3% versus 10.4%, respectively; adjusted relative risk [RR]: 1.91; 95% CI: 1.39-2.61; $P < 0.001$). Further analyses were conducted in ER-negative and ER-positive subgroups separately.

158 ER-negative patients were placed in the GA group, and 162 were placed in the PPA group. Recurrence risk among those in the PPA group was not reduced versus the GA group (19.1% versus 23.4%, respectively; adjusted RR: 0.86, 95% CI: 0.53–1.39; $P = 0.542$), and the adjusted HR was 0.80 (95% CI: 0.50–1.30; $P = 0.377$) (**Figures 2A, D**). No violation of the proportional hazard assumption was observed ($P = 0.122$). To assess the potential benefit of regional anesthesia-analgesia on early recurrence in ER-negative patients, a step function with a predefined time splitting point of 18 months was used to perform an extended Cox regression analysis. Throughout a period of < 18 months after surgery, the unadjusted HR for regional anesthesia-analgesia was 0.62 (95% CI: 0.34–1.13; $P = 0.122$). After adjusting for confounders using Model 1, the HR for early recurrence was 0.63 (95% CI: 0.34–1.14; $P = 0.127$). For periods exceeding the classical recurrence peak of 18 months, the effect of regional anesthesia seemed limited (adjusted HR: 1.33, 95% CI: 0.57–3.12; $P = 0.513$).

The same result was obtained using Model 2. Although it was not statistically significant (**Table 2**), the incidence curve revealed that anesthetic interventions may influence rates of early recurrence in ER-negative patients (**Figures 2B, E**). We also tested other splitting points *via* sensitivity analyses, since the early peak in recurrence was reported to occur between the first and third year (5); however, results were unaffected. The multivariable Cox regression model satisfied the proportional hazard assumption in the ER-positive group ($P = 0.859$). Both models showed that anesthetic interventions did not significantly affect recurrence (Model 1: adjusted HR, 1.03; 95% CI 0.69–1.53; $P = 0.888$; Model 2: adjusted HR, 1.06; 95% CI, 0.71–1.60; $P = 0.764$). The recurrence curve of ER-positive group was not affected by anesthetic interventions (**Figures 2C, F**).

TABLE 1 | Demographic and clinical characteristics according to ER status.

	ER status = negative (n = 320)		ER status = positive (n = 933)	
	PPA (n = 162)	**GA (n = 158)**	**PPA (n = 462)**	**GA (n = 471)**
Demographics				
Age, yr	50 ± 10	48 ± 10	48 ± 10	49 ± 9
Menstruation, n (%)				
Premenopausal	58 (35.8)	61 (38.6)	221 (47.8)	215 (45.6)
Perimenopausal	11 (6.8)	21 (13.3)	52 (11.3)	63 (13.4)
Postmenopausal	93 (57.4)	76 (48.1)	189 (40.9)	193 (41.0)
Body mass index, kg/m^2	23.9 ± 3.3	23.9 ± 3.6	23.6 ± 3.1	23.7 ± 3.3
ASA physical status, n (%)				
I	113 (69.8)	111 (70.3)	334 (72.3)	318 (67.5)
II	49 (30.2)	47 (29.7)	127 (27.5)	151 (32.1)
III	0 (0.0)	0 (0.0)	1 (0.2)	2 (0.4)
Neoadjuvant, n (%)	9 (5.6)	8 (5.1)	16 (3.5)	14 (3.0)
Primary tumor				
Tumor side, n (%)				
Left	89 (54.9)	79 (50.0)	225 (48.7)	236 (50.1)
Right	71 (43.8)	77 (48.7)	233 (50.4)	224 (47.6)
Bilateral	2 (1.2)	2 (1.3)	4 (0.9)	11 (2.3)
Nuclear grade, n (%)				
1/2	57 (38.3)	47 (30.9)	328 (76.3)	334 (76.8)
3	92 (61.7)	105 (69.1)	102 (23.7)	101 (23.2)
Unknown	13 (8.0)	6 (3.8)	32 (6.9)	36 (7.6)
PR status, n (%)				
Negative	146 (90.1)	148 (93.7)	49 (10.6)	63 (13.4)
Positive	16 (9.9)	10 (6.3)	413 (89.4)	407 (86.6)
Unknown	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.2)
HER2 status, n (%)				
Negative	72 (44.4)	69 (43.7)	299 (64.7)	321 (68.2)
Positive	84 (51.9)	84 (53.2)	115 (24.9)	104 (22.1)
Equivocal	6 (3.7)	5 (3.2)	48 (10.4)	46 (9.8)
Pathology stage, tumor (T), n (%)				
T0 or Tis	10 (6.2)	6 (3.9)	12 (2.6)	14 (3.0)
T1	70 (43.5)	73 (47.1)	262 (57.1)	293 (62.3)
T2	74 (46.0)	61 (39.4)	172 (37.5)	151 (32.1)
T3	7 (4.3)	12 (7.7)	12 (2.6)	11 (2.3)
T4	0 (0.0)	3 (1.9)	1 (0.2)	1 (0.2)
Pathology stage, nodes (N), n (%)				
N0	92 (56.8)	84 (53.2)	249 (53.9)	261 (55.5)
N1	37 (22.8)	24 (15.2)	111 (24.0)	129 (27.4)
N2	16 (9.9)	19 (12.0)	46 (10.0)	42 (8.9)
N3	17 (10.5)	31 (19.6)	56 (12.1)	38 (8.1)
Tumor TNM stage, n (%)				
0	9 (5.6)	5 (3.2)	9 (2.0)	14 (3.0)
1	43 (26.7)	47 (29.7)	169 (36.8)	190 (40.5)
2	74 (46.0)	54 (34.2)	175 (38.1)	184 (39.2)
3	35 (21.7)	52 (32.9)	106 (23.1)	81 (17.3)
Intraoperative				
Surgery type, n (%)				
Simple mastectomy	27 (16.7)	21 (13.3)	49 (10.6)	66 (14.0)
Modified radical	120 (74.1)	109 (69.0)	324 (70.1)	324 (68.8)
Wide local excision with node dissection	9 (5.6)	23 (14.6)	59 (12.8)	50 (10.6)
Others	6 (3.7)	5 (3.2)	30 (6.5)	31 (6.6)
Drugs				
Propofol, mg	531 [434, 677]	120 [100, 130]	502 [430, 650]	120 [100, 130]
Fentanyl, μg	100 [50, 100]	200 [185, 250]	100 [50, 100]	200 [190, 250]
Lidocaine, mg	20 [0, 40]	40 [0, 40]	20 [0, 40]	30 [0, 40]
NSAIDS, n (%)	6 (3.7)	6 (3.8)	10 (2.2)	6 (1.3)
Postoperative treatment				
Radiotherapy, n (%)	55 (34.0)	73 (46.2)	200 (43.3)	164 (34.8)
Chemotherapy, n (%)	148 (91.4)	144 (91.1)	336 (72.7)	322 (68.4)
Endocrine therapy, n (%)	16 (9.9)	13 (8.2)	393 (85.1)	399 (84.7)
Herceptin, n (%)	46 (28.4)	47 (29.7)	73 (15.8)	59 (12.5)
Recurrence, n (%)	31 (19.1)	37 (23.4)	50 (10.8)	47 (10.0)

Results presented as $\bar{x}$ ± s or median (P_{25}, P_{75}) or n (%).

ER, estrogen receptor; PPA, paravertebral block with propofol general anesthesia; GA, fentanyl with sevoflurane general anesthesia; ASA, American Society of Anesthesiologist; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

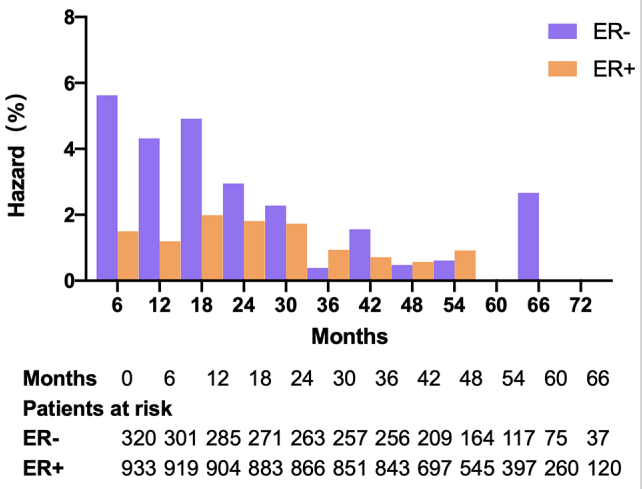

FIGURE 1 | Hazard rate per 6 months in ER-negative patients and ER-positive patients.

TABLE 2 | Extended Cox regression model with estimated recurrence hazard ratios of PPA *vs.* GA in ER-negative group.

Variables	HR (95% CI)	*P*
Unadjusted Model[a]		
PPA *vs.* GA	0.77 (0.48-1.25)	0.290
PPA *vs.* GA (T ≤ 18m)[b]	0.62 (0.34-1.13)	0.122
PPA *vs.* GA(T>18m)[b]	1.16 (0.51-2.66)	0.717
Model 1		
PPA *vs.* GA	0.80 (0.50-1.30)	0.377
PPA *vs.* GA(T ≤ 18m)[b]	0.63 (0.34-1.14)	0.127
PPA *vs.* GA(T>18m)[b]	1.33 (0.57-3.12)	0.513
Model 2		
PPA *vs.* GA	0.82 (0.49-1.36)	0.438
PPA *vs.* GA(T ≤ 18m)[b]	0.72 (0.39-1.34)	0.309
PPA *vs.* GA(T>18m)[b]	1.05 (0.44-2.54)	0.908

Model 1 was adjusted for menstruation and pathology stage of tumor (binary) using multivariable extended Cox regression.
Model 2 was adjusted for age, Tumor TNM stage (binary), nuclear grade, postoperative radiotherapy and postoperative chemotherapy using multivariable extended Cox regression.
PPA, paravertebral block with propofol general anesthesia; GA, fentanyl with sevoflurane general anesthesia; HR, hazard ratio; CI, confidence interval.
[a]*Analyzed with univariable Cox regression model.*
[b]*Analyzed with step function model.*

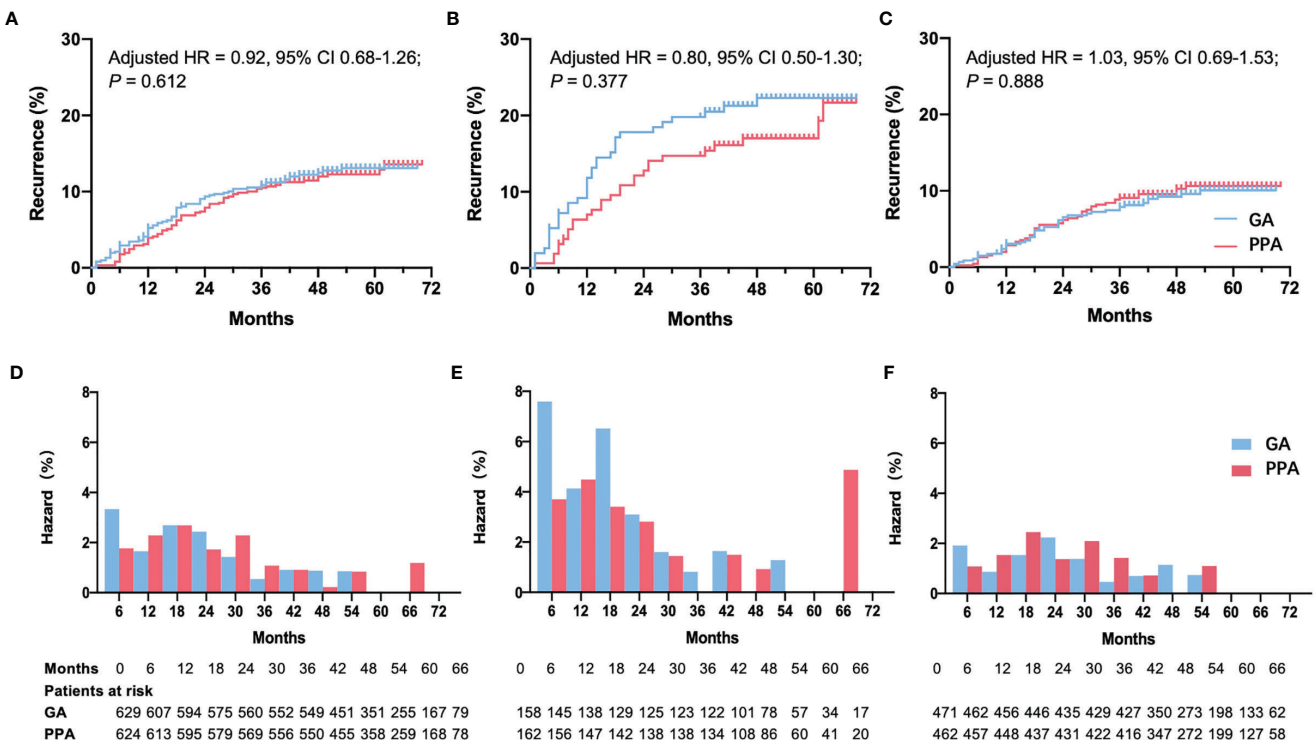

FIGURE 2 | Recurrence curve and hazard rate per 6 months among patients who were given PPA or GA. Recurrence curve in all patients **(A)**, ER-negative patients **(B)**, and ER-positive patients **(C)**. Hazard rate per 6 months in all patients **(D)**, ER-negative patients **(E)**, and ER-positive patients **(F)**. ER, estrogen receptor; PPA, paravertebral block with propofol general anesthesia; GA, fentanyl with sevoflurane general anesthesia; HR, hazard ratio; CI, confidence interval. HR, 95% CI and *P* values were reported using adjusted multivariable Cox regression Model 1 adjusted for menstruation and pathology stage of tumor (binary).

One death in an ER-negative patient occurred due to heart disease prior to cancer recurrence. The competing risk model showed that the difference of recurrence was not significant in ER-negative patients between PPA and GA group (Model 1: adjusted HR, 0.81; 95% CI, 0.50–1.31; P = 0.390; Model 2: adjusted HR, 0.83; 95% CI, 0.50–1.39; P = 0.480).

DISCUSSION

Several studies have investigated the effects of anesthetic interventions on cancer prognosis in recent years (10, 28–34). Although some *in vitro* studies and observational analyses have reported beneficial effects of regional anesthesia on cancer recurrence, most randomized studies have shown that regional anesthesia does not improve breast cancer recurrence-free survival. In our study, similar to findings of the original multicenter randomized trial (12), regional anesthesia-analgesia showed no statistical difference in risk of early recurrence after breast cancer surgery in the ER-negative population. However, an early recurrence peak was clearly observed among the ER-negative group, and the trend of reduced risk could also be seen in the ER-negative patients rather than ER-positive patients under regional anesthesia-analgesia.

Although the use of regional anesthesia-analgesia for breast cancer surgery minimizes the alteration of cytokines and inflammation, and improves the immune response, there is no convincing clinical evidence that supports or refutes the clinical use of regional anesthesia-analgesia to reduce the risk of cancer recurrence (35–37). One possible reason why results of *in vitro* studies cannot be reproduced in clinical trials is that cells are subjected to prolonged exposure to local anesthetics for 72 hours *in vitro* (23), while short-term exposure of anesthetics in the clinical context seems negligible. Another reason for the discrepancy may be related to the fact that trauma due to breast cancer surgery is relatively less significant than that of other types of surgeries (38, 39). However, a range of randomized trials focusing on major surgeries have also revealed a similar effect in reducing recurrence due to the administration of regional anesthesia-analgesia (28, 29). More importantly, intrinsic biological characteristics of tumors and treatments can also affect recurrence besides anesthetic interventions (40).

However, although there is no statistically significant difference in the risks of recurrence between PPA and GA groups when assessed according to ER expression status, a trend of reduced recurrence hazard could be observed under regional anesthesia-analgesia during the first 18 months in the ER-negative patients in our study, but not in ER-positive patients. Breast cancer cell phenotypes have distinct biological behaviors, with differing recurrence curves (5), of which ER-negative patients rather than ER-positive patients have a higher early recurrence peak (2), consistent with our results. The early recurrence peak is often interpreted as a break in dormancy, which is induced by growth stimulating factors after surgery (41), and may be reduced by regional anesthesia due to its immune-preserving and anti-inflammatory qualities (3, 42). The effects of anesthetic interventions on cancer recurrence may vary among different cytotypes (43). Lirk P et al. found that the demethylation effect of local anesthetics is more significant in ER-negative cells compared with ER-positive cells, leading to the inhibition of cancer. As a result, impacts of regional anesthesia-analgesia on cancer recurrence may be greater in ER-negative patients than ER-positive patients.

A strength of this randomized study is the large proportion of patients who were successfully followed up. Secondly, the patients were from the same study center, so that to circumvent bias with a similar genetic background. However, it does have some limitations. Most importantly, the sample size of ER-negative patients may not have been large enough to assess the influence of regional anesthesia-analgesia on recurrence. In the original multicenter randomized controlled trial, the sample size was selected to assess a 30% reduction in cancer recurrence. However, a recent meta-analysis focusing on late-stage patients revealed only a slight benefit from regional anesthesia use, decreasing cancer recurrence by 4%–12% (32). Similarly, a nationwide retrospective cohort study also revealed a slight decrease of total intravenous anesthesia compared with volatile anesthesia in recurrence-free survival (2%–13%) (34). Although regional anesthesia-analgesia seemed to benefit ER-negative patients in this study with a decrease of 20% in cancer recurrence, far more ER-negative patients should be considered. Also, this study included two factors of regional anesthesia vs. general anesthesia, and propofol intravenous anesthesia vs. sevoflurane inhalation anesthesia simultaneously. As a result, the effect is combined and difficult to identify if it is regional anesthesia or propofol has any potential influence on the early recurrence of ER-negative breast cancer patients.

In summary, rates of early recurrence in both ER-negative and ER-positive breast cancer were similar between regional anesthesia-analgesia using paravertebral blocks and propofol and general anesthesia by sevoflurane and opioids. However, the recurrence curve revealed a potential benefit of regional anesthesia-analgesia in ER-negative patients. Large samples of high-risk patients (such as ER-negative patients) will be needed to clarify the influence of anesthetic interventions.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the datasets were obtained from the hospital information system, which is not openly accessible to other individuals or institutions. Requests to access the datasets should be directed to Lijian Pei, hazelbeijing@vip.163.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Peking Union Medical College Hospital ethics committee (S-638). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LP and YH contributed to the study design. ML, ZZ, GT, and LP contributed to the data acquisition. ML and YZ contributed to the data analysis. ML and LP wrote the report. All authors reviewed the report and approved it for publication.

FUNDING

This study was supported by Peking Union Medical College Hospital Precipitation and Integration Foundation (ZC201906511).

ACKNOWLEDGMENTS

We thank the Breast Cancer Recurrence Collaboration, the Department of Anesthesiology, and the Department of Breast Surgery, PUMCH, for making this study possible.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.837959/full#supplementary-material

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 22 February 2022
doi: 10.3389/fphar.2022.821779

Metabolomic Impact of Lidocaine on a Triple Negative Breast Cancer Cell Line

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OPEN ACCESS

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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Pharmacology

Received: *24 November 2021*
Accepted: *24 January 2022*
Published: *22 February 2022*

Citation:
Chamaraux-Tran T-N Muller M, Pottecher J, Diemunsch PA, Tomasetto C, Namer I-J and Dali-Youcef N (2022) Metabolomic Impact of Lidocaine on a Triple Negative Breast Cancer Cell Line. Front. Pharmacol. 13:821779. doi: 10.3389/fphar.2022.821779

Background: Metabolomics and onco-anesthesia are two emerging research fields in oncology. Metabolomics (metabolites analysis) is a new diagnostic and prognostic tool that can also be used for predicting the therapeutic or toxic responses to anticancer treatments. Onco-anesthesia studies assess the impact of anesthesia on disease-free and overall survival after cancer surgery. It has been shown that local anesthetics (LA), particularly lidocaine (LIDO), exert antitumor properties both *in vitro* and *in vivo* and may alter the biologic fingerprints of cancer cells. As LA are known to impair mitochondrial bioenergetics and byproducts, the aim of the present study was to assess the impact of LIDO on metabolomic profile of a breast cancer cell line.

Methods: Breast cancer MDA-MB-231 cells were exposed for 4 h to 0.5 mM LIDO or vehicle (n = 4). The metabolomic fingerprint was characterized by high resolution magic angle spinning NMR spectroscopy (HRMAS). The multivariate technique using the Algorithm to Determine Expected Metabolite Level Alteration (ADEMA) (Cicek et al., PLoS Comput. Biol., 2013, 9, e1002859), based on mutual information to identify expected metabolite level changes with respect to a specific condition, was used to determine the metabolites variations caused by LIDO.

Results: LIDO modulates cell metabolites levels. Several pathways, including glutaminolysis, choline, phosphocholine and total choline syntheses were significantly downregulated in the LIDO group.

Discussion: This is the first study assessing the impact of LIDO on metabolomic fingerprint of breast cancer cells. Among pathways downregulated by LIDO, many metabolites are reported to be associated with adverse prognosis when present at a high titer in breast cancer patients. These results fit with the antitumor properties of LIDO and suggest its impact on metabolomics profile of cancer cells. These effects of LIDO are

of clinical significance because it is widely used for local anesthesia with cutaneous infiltration during percutaneous tumor biopsy. Future *in vitro* and preclinical studies are necessary to assess whether metabolomics analysis requires modification of local anesthetic techniques during tumor biopsy.

Keywords: lidocaine, onco-anesthesia, perioperative period, anesthesia, cancer surgery, metabolomics, cancer progression

INTRODUCTION

In 2020, female breast cancer was the most diagnosed cancer in the world (2,261,419 cases). As almost 685,000 women die of this cancer each year (Sung et al., 2021), breast cancer care is still challenging (Burguin et al., 2021), particularly the triple negative breast cancer (TNBC) subtype which is very aggressive (Bianchini et al., 2021). Customizing care to patient's phenotypic and/or genotypic background could be an approach to TNBC issues (Burstein et al., 2021; Li et al., 2021).

An emerging strategy to improve survival by personalized medicine and treatment is using metabolomics, an "-omic" approach based on Nuclear Magnetic Resonance (NMR). This technology is an interesting tool for personalized care (Vignoli et al., 2021). Indeed, NMR may provide clues to determine the best therapeutic strategy to follow in patient care and monitoring. High resolution magic angle spinning (HR-MAS) NMR spectroscopy can simultaneously analyze approximately 40 metabolites in biological samples without altering them and can determine tumor metabolomic fingerprints. Many studies have reported a significant association between those fingerprints and clinicopathological status (Choi et al., 2012; Cao et al., 2014; Chae et al., 2016; Tayyari et al., 2018; Vignoli et al., 2021), response to chemotherapy (Cao et al., 2012; Choi et al., 2013) and survival (Giskeødegård et al., 2010, 2012; Cao et al., 2012). Some metabolites are of particular interest: Cao et al. (2012) have demonstrated a significant decrease of glycerophophocholine, phosphocholine, choline and total choline level in survivors in response to treatment compared to non-survivors in breast cancer. Higher levels of glycine and lactate were found to be associated with lower survival rates in breast cancer (Giskeødegård et al., 2012).

Another emerging field of research in cancer care is called onco-anesthesia (Wigmore et al., 2016; Hiller et al., 2017; Cata et al., 2020). Onco-anesthesia investigates the potential impact of anesthesia practices on cancer progression after surgery. Many anesthetic and analgesic drugs used during perioperative period may have a significant impact on immune responses but can also interfere with signaling pathways.

Lidocaine is a commonly used local anesthetics which is often required for local anesthesia before performing fine needle aspiration biopsy or core needle biopsy. It is also employed for regional anesthesia in breast cancer surgery, remote from the surgical site when performing paravertebral block or closer to the wound through plane blocks (pectoral nerves block, serratus blocks, erector spinae plane block ...) (Elshanbary et al., 2021; Gabriel et al., 2021).

In addition to its anesthetic effects, lidocaine can also be administrated intravenously (i.v.) for postoperative analgesia (Beaussier et al., 2018). In breast cancer surgery LIDO contributes to prevent both acute and chronic pain after breast cancer surgery (Grigoras et al., 2012). And it was shown to have anticancer properties (Chamaraux-Tran and Piegeler, 2017; Chamaraux-Tran et al., 2018; Wall and Buggy, 2021; Zhang et al., 2021).

It is also well-know that anesthetic and analgesic drugs do have an impact on cell metabolism (Nouette-Gaulain et al., 2011; Jose et al., 2012). Energy metabolism modulation properties of local anesthetics may stand for a potential therapy to decrease cancer cell proliferation (Jose et al., 2012).

Given the emerging role of metabolomics in breast cancer care, the antitumor properties of local anesthetics and their impact on cell metabolism, we sought to evaluate the impact of lidocaine in metabolomics fingerprints. In an *in vitro* study, we assessed the impact of lidocaine on a triple negative breast cancer human cell line.

MATERIALS AND METHODS

Cells and Cell Culture

MDA-MB-231 (ER and PGR double negative, no amplification of erbB-2 oncogene) human breast cancer cell line representative of the triple negative subtype used throughout this study was obtained from the American Type Culture Collection (ATCC) biological resource center (http://www.atcc.org). The detailed characteristics of the tumor cell line are described elsewhere (Lacroix and Leclercq, 2004). MDA-MB-231 cells were grown in RPMI 1640 medium without HEPES and enriched with 10% fetal calf serum (FCS) and gentamicin (40 µg/ml). Subculturing was routinely carried out every week using diluted trypsin solution (0.25%) in Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium (pH 7.2) and cell cultures were kept in a 5% CO_2 incubator at 37°C.

Drug Treatment

To perform *in vitro* experiments, lidocaine hydrochloride monohydrate was obtained in a pure lyophilized form (MW 288.81, Sigma-Aldrich, St. Louis, MO). A stock solution (50 mg/ml in H_2O) was freshly prepared and increasing drug concentrations (0.1, 0.5, 1, 5, 10 mM) were obtained by diluting the stock solution in cell culture medium. Final pH of lidocaine-containing or -free (control) mediums were controlled and were found to be equivalent.

MTT Assay for Cell Viability

This rapid colorimetric assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue (MTT) was elaborated by Mosmann *et al.* to assess cellular growth and survival (Mosmann, 1983). Exponentially growing cells were enzymatically detached and a single tumor cell suspension in culture medium at a density of 30×10^3 cells/ml was prepared. Cells were seeded in 24-well microtiter plates (1 ml/well) and allowed to attach for 48 h under the previous specified conditions. Culture medium in each well was aspirated and replaced with fresh culture medium containing the different lidocaine concentrations and allowed to grow for a further 4 h. Triplicate wells were used for controls (H_2O as vehicle alone) and each concentration of lidocaine. Cell viability was then determined using the MTT assay (Marks et al., 1992) with minor modifications. In brief, 100 µl of MTT (5 mg/ml in DPBS) (3-(4,5 dimethylthiazol-2-yl) 2,5 diphenyl-tetrazolium bromide) were added and the plates were incubated at 37°C for 1 h in the dark. This assay is based on the cleavage of the tetrazolium salt by viable cells and the accumulation of a water insoluble formazan salt proportional to the number of living cells in the well. After careful aspiration of the culture medium, 100 µl of DMSO were added to each well and the plates were incubated for a further 1 h. Absorbances were then measured for each treatment condition at a wavelength of 550 nm with reference to the appropriate blank (DMSO only) in a 96-wells microplate spectrophotometer (ELx808 Absorbance Microplate Reader, Biotek Instruments and Gen5 Data Analysis Software 1.06) and compared to control untreated cells.

^{1}H-High Resonance Magic Angle Spectroscopy (^{1}H-HRMAS) Metabolomic Data Acquisition and Processing

For this experiment, 10^7 MDA-MB-231 cells were seeded in 750 ml cell culture flask with a polystyrene growth area of 175 cm^2 for 24 h. Culture medium was then aspirated and replaced with fresh culture medium containing lidocaine (at concentration of 0.5 mM, $n = 5$) or the same volume of H_2O ($n = 4$). After 4-h incubation at 37°C, medium was removed, and cells were washed by phosphate-buffered saline (PBS 1M). Cells were trypsin-detached and centrifugated at 1,200 rpm to throw supernatant. Cell pellet was then homogenized and 20 µl of the cap was put into a cryotube. Manual centrifugation was performed to remove any air bubbles and the cryotube was immediately placed in liquid nitrogen for rapid freezing. Five microliters of deuterium oxide were added before −20°C storage.

NMR HRMAS data acquisition and processing have been previously detailed (Battini et al., 2016). Briefly, NMR HRMAS assay was performed by 500 MHz Bruker Avance III spectrometer. A 1-dimensional (1D) proton spectrum using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was acquired for each sample with a 285 µs interpulse delay and a 76 min acquisition time for each tissue sample. The number of loops was set at 328, giving the CPMG pulse train a total length of 93 ms. The chemical shift was calibrated to the peak of the methyl proton of L-lactate at 1.33 parts per million (ppm).

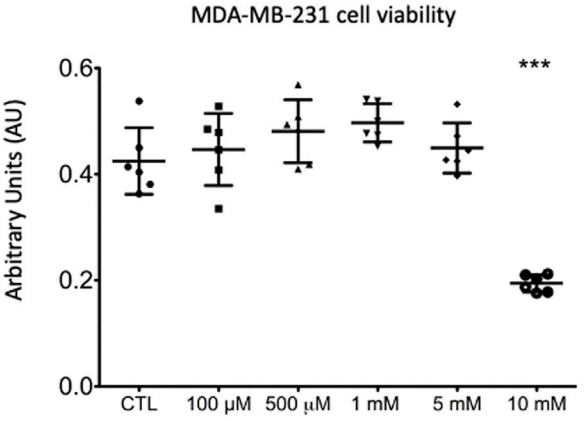

FIGURE 1 | MDA-MB-231 cell viability exposed for 4 h to increased concentrations of lidocaine (from 0.01 to 10 mM) compared to cells exposed to vehicle alone (purified water, CTL). ANOVA: F (5,30) = 28.16; $p < .0001$ (***: $p < .001$).

Unidimensional (1D) acquisition was immediately followed by a 2-dimensional (2D) heteronuclear experiment (in order to confirm resonance assignments). Heteronuclear Single Quantum Coherence (HSQC) spectrum was acquired during 15 hs and 22 mns (time acquisition: 0.073s (1H)/0.006s (13C), 136 scans, spectral window: 7,002 Hz (1H)/20,833 Hz (13C), relaxation time: 1.5 s). Metabolites were assigned using a standard metabolite chemical shift table available in the literature (Martínez-Bisbal et al., 2004; Wishart et al., 2007). Metabolite quantification was performed using an external reference standard of lactate (19,3 nM), scanned under the same analytical conditions. Spectra were normalized according to sample weight. Peaks of interest were automatically defined by an in-house program using MATLAB (MATLAB R2010; MathWorks, Natik, MA).

Statistical Analysis

Data Are Expressed as Mean ± Standard Deviation

MTT *in vitro* assay was performed in triplicate and at least three times. Results were compared with one-way repeated measures ANOVA followed by a Dunnett test. GraphPad InStat statistics software (GraphPad Software, Inc., La Jolla, CA) was used for these analyses. p values < .05 were considered statistically significant.

Network analysis was obtained using the Algorithm to Determine Expected Metabolite Level Alterations Using Mutual Information (ADEMA) which has been applied on metabolite quantification values. ADEMA processing has been previously detailed (Cicek et al., 2013; Battini et al., 2016; Bender et al., 2020). Briefly, this method allows for the comprehensive analysis of variations in a pathway of metabolites within cells exposed or not to lidocaine. Instead of analyzing the metabolites one by one, ADEMA integrates them into the topology of the metabolic network that was built according to the Kyoto Encyclopedia of Genes and Genomes (Kanehisa and Goto, 2000) and Salway's work (Salway, 2016).

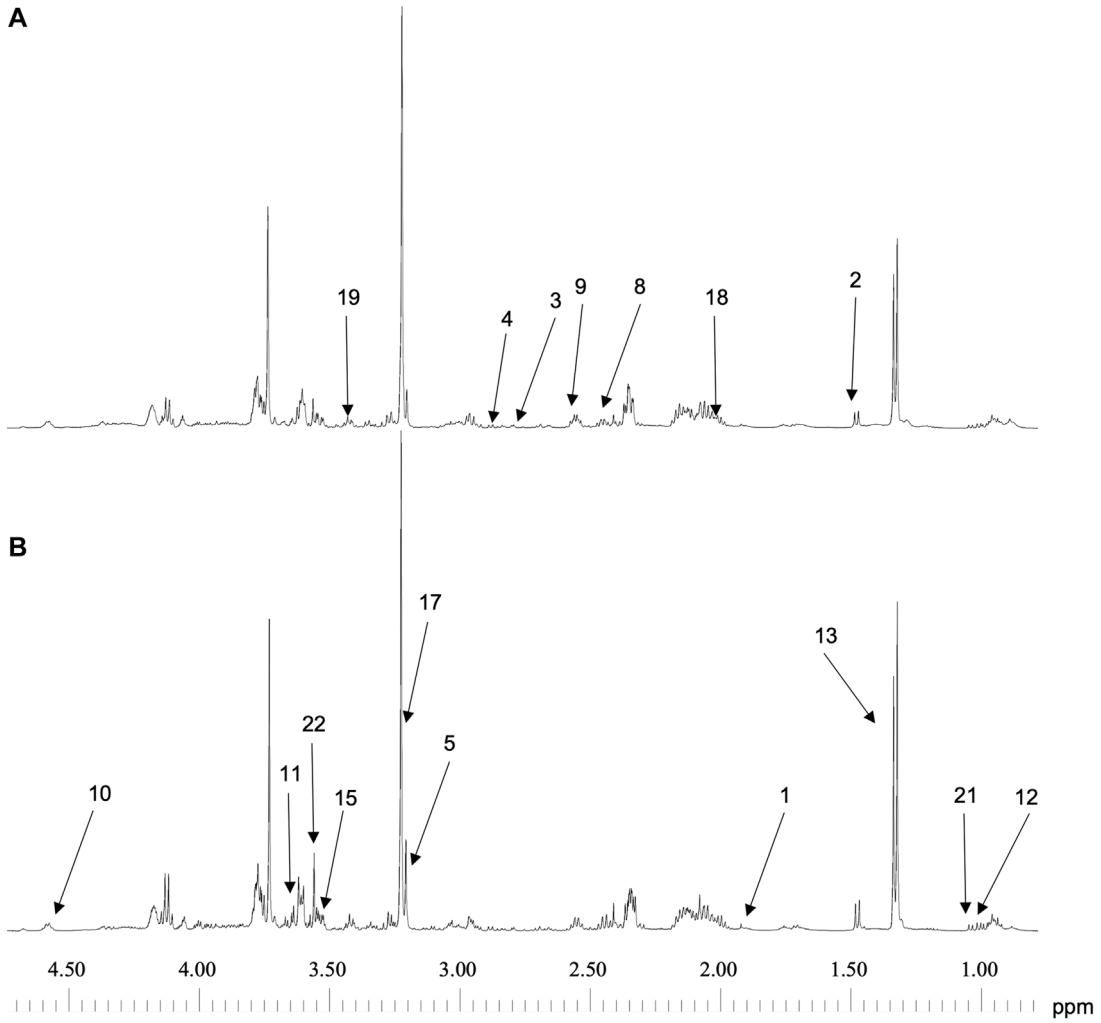

FIGURE 2 | 1D ^{1}H CPMG HRMAS spectra of MDA-MB-231 exposed to vehicle **(A)** or to lidocaine (0.5 mM) **(B)**. Spectra can be compared because they were normalized to the sample weight. Peaks are identified as below: 1-Acetate 2-Alanine 3-Asparagine 4-Aspartate 5-Choline 8-Glutamate 9-Glutamine 10-Glutathione 11-Glycerol 12-Isoleucine 13-Lactate 15-myo-Inositol 17-Phosphocholine 18-Proline 19-Taurine 21-Valine 22-Glycine.

RESULTS

High Concentration of Lidocaine Impairs Cell Viability

As compared to untreated cells, MDA-MB-231 cell viability was significantly impaired when treated with lidocaine at the concentration of 10 mM (45% reduction, **Figure 1**) (0.194±0.016 AU versus 0.425± 0.06 AU in control group, $p < .0001$ in Dunnet test). Because of its negative effect on cell viability, the lidocaine concentration of 0.5 mM was selected for the ^{1}H-HRMAS assay.

Quality of Spectra Acquisitions

Spectra of the 9 samples collected (5 for cells exposed to lidocaine and 4 for control group) were of high quality. **Figure 2** represents 1D ^{1}H CPMG HRMAS spectra of MDA-MB-231 cells exposed or not to lidocaine. **Figure 3** represents a 2D ^{1}H-^{13}C HSQC spectrum of MDA-MB-231 cells exposed to lidocaine.

Twenty-two metabolites were quantified for the experiment: Alanine, Asparagine, Aspartate, Choline, Creatine, Fumarate, Glutamate, Glutamine, Glutathione, Glycerol, Isoleucine, Lactate, Malate, myo-Inositol, Phenylalanine, Phosphocholine, Proline, Taurine, Total Choline, Valine and *Glycine*. Mean values are presented in **Table 1**. Glucose and glycerophosphocholine were not measurable in both groups. There were no peaks of lidocaine in the samples, thus confirming efficient cell washing.

Lidocaine Modulates Metabolic Pathways and Decreases Cell Proliferation Potential in Triple Negative Breast Cancer Cells

Network analysis using the ADEMA algorithm shows an impairment in several metabolic pathways in MDA-MB-231

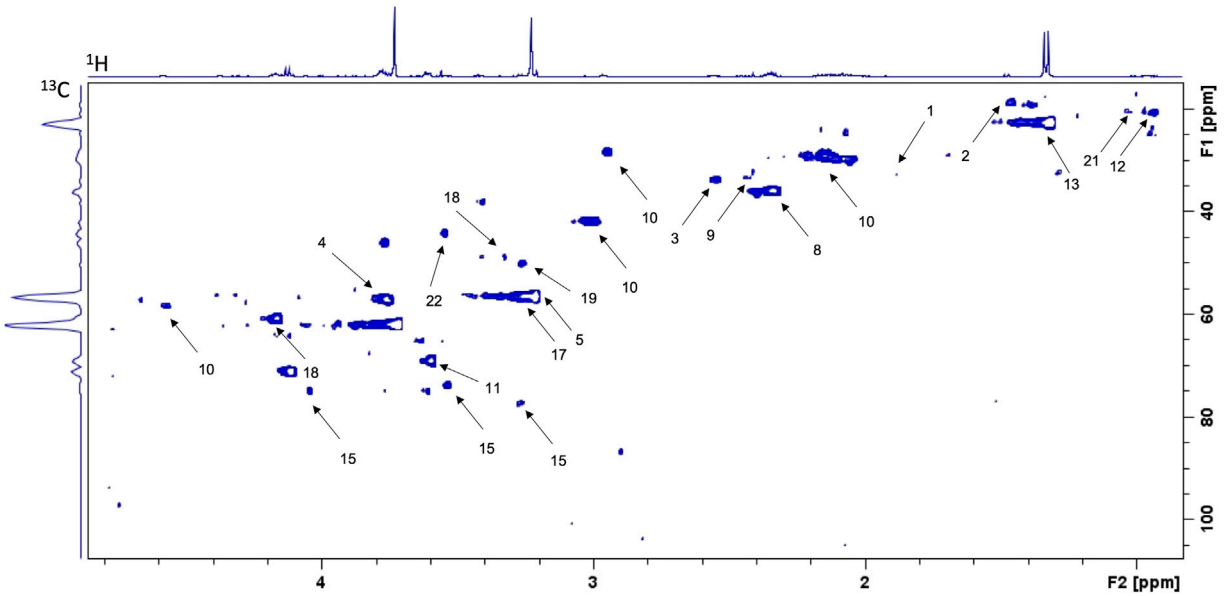

FIGURE 3 | Example of 2D ^{1}H-^{13}C HSQC spectrum of MDA-MB-231 cells exposed to lidocaine (0.5 mM for 4 h). Spots are identified as below: 1-Acetate 2-Alanine 3-Asparagine 4-Aspartate 5-Choline 8-Glutamate 9-Glutamine 10-Glutathione 11-Glycerol 12-Isoleucine 13-Lactate 15-myo-Inositol 17-Phosphocholine 18-Proline 19-Taurine 21-Valine 22-Glycine.

TABLE 1 | Metabolite quantification in MDA-MB-231 cells exposed or not to lidocaine, expressed in mM [mean ± standard deviation (SD)].

	Lido *n* = 5 mean (mM)	±SD	Control *n* = 4 mean (mM)	± SD
Acetate	0,139	0,030	0,137	0,019
Alanine	0,758	0,276	0,847	0,081
Asparagine	0,727	0,264	0,828	0,196
Aspartate	0,620	0,287	0,788	0,287
Choline	0,363	0,293	0,354	0,083
Creatine	0,311	0,118	0,386	0,093
Fumarate	0,043	0,020	0,061	0,017
Glutamate	5,882	1,751	6,812	1,396
Glutamine	1,519	0,520	1,692	0,116
Reduced Glutathion	2,367	0,404	2,805	0,316
Glycerol	1,213	0,736	1,214	0,258
Isoleucine	0,260	0,081	0,242	0,027
Lactate	9,599	1,468	9,466	1,646
Malate	1,349	0,190	1,985	0,431
myo-Inositol	1,453	0,591	1,645	0,241
Phenylalanine	0,131	0,041	0,133	0,022
Phosphocholine	3,777	0,949	4,121	0,670
Proline	2,102	0,744	2,314	0,333
Taurine	1,492	0,363	1,607	0,134
TotalCholine	1,943	0,521	2,109	0,356
Valine	0,166	0,063	0,144	0,018
Glycine	1,086	0,605	0,985	0,089

cells (**Figure 4**). Cell exposure to 0.5 mM of lidocaine for 4 h yielded predicted decrease in levels of metabolites involved in phospholipids metabolism and cell membrane proliferation: total choline, choline and phosphocholine. A predicted decrease in the levels of taurine, asparagine, aspartate, malate, fumarate, alanine, myoinositol, glutathione, glutamine, glutamate, proline, and creatine was also observed. On the other hand, valine, isoleucine levels were predicted to increase. Lactate, glycine and acetate levels were similar in the 2 groups (**Figure 4**). The metabolomic profiles indicate that lidocaine treatment of MDA-MB-231 cells at a 0.5 mM concentration impairs choline and glutaminolysis pathways and the tricarboxylic acid (TCA) cycle.

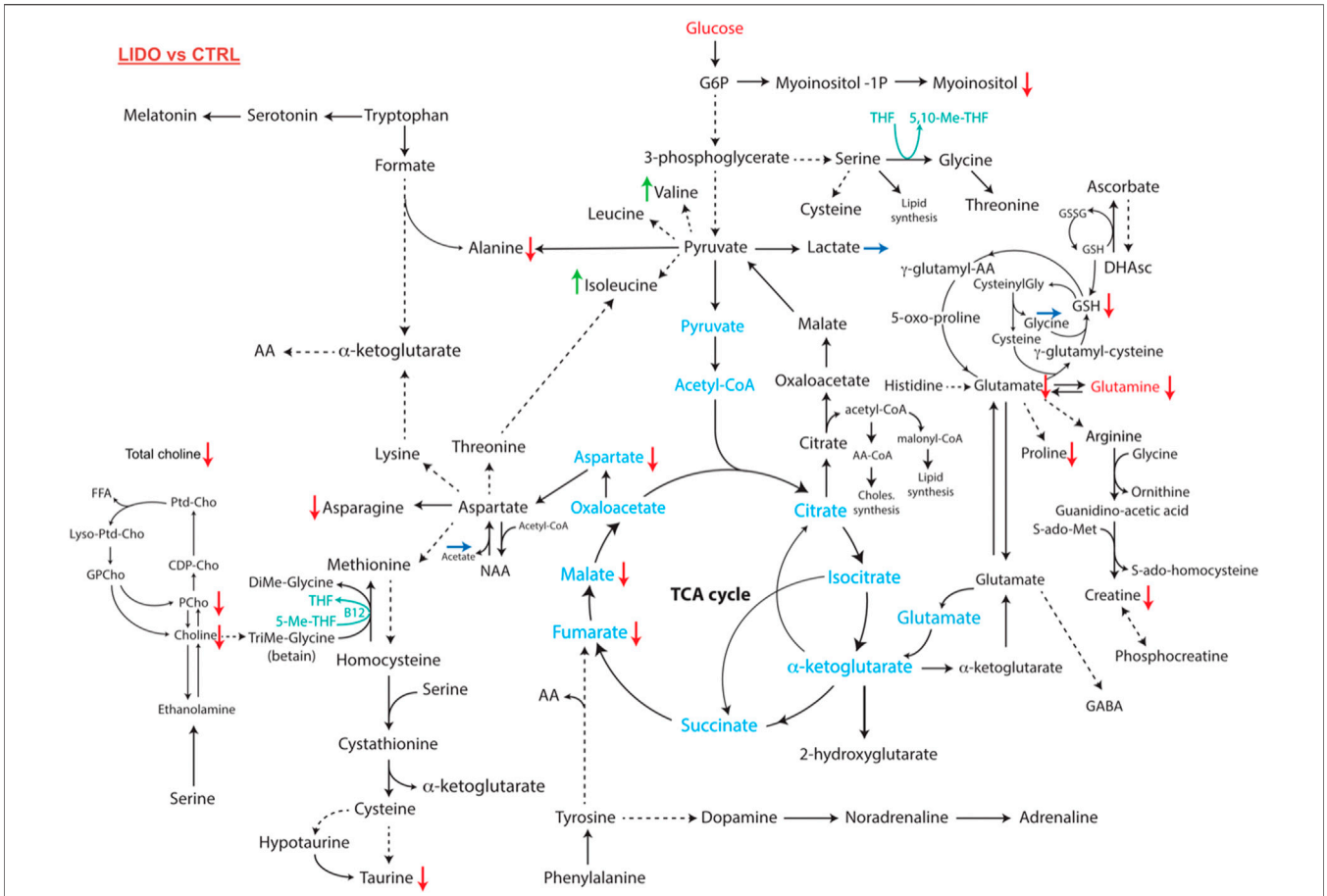

FIGURE 4 | Metabolomic network of MDA-MB-231 cells exposed to 0.5 mM of lidocaine or vehicle as control for 4 h. Several pathways which promote proliferation, invasion and metastasis (glutaminolysis, choline, phosphocholine and total choline syntheses) were significantly downregulated in lidocaine group. Red, green and blue arrows indicate the decreased, the increased or the unchanged levels of metabolite after exposure to lidocaine compared to control, respectively.

DISCUSSION

To our knowledge, this is the first study reporting the metabolic impact of lidocaine on the metabolomic fingerprint in cancer cells. We have demonstrated that lidocaine, at concentration of 0.5 mM for 4 h, can significantly alter metabolites levels and some metabolic pathways which are active in highly proliferative tumors.

First, our viability assay supports previous works showing a decrease in the proliferation of MDA-MB-231 cells exposed to lidocaine (Chamaraux-Tran and Piegeler, 2017; Chamaraux-Tran et al., 2018; D'Agostino et al., 2018). It was mandatory for us to determine a lidocaine concentration with no significant effect on cell viability to have the same quantity of cells for the HR-MAS NMR assay. In this experiment we used lidocaine hydrochloride monohydrate to avoid absolute ethanol as solvent which might compromise NMR assay. As higher concentrations of lidocaine hydrochloride monohydrate were needed to decrease cell viability [10 versus 0.5 mM of lidocaine prepared in absolute ethanol (Chamaraux-Tran et al., 2018)], it confirms that excipient may have a direct antitumor effect (Chamaraux-Tran and Beloeil, 2018). To note, higher concentrations of lidocaine (10 mM) were needed in our experiment compared to previous studies on MDA-MB-231 cells, independently to solvent. Jiang et al. (2016) and Li et al. (2018) have demonstrated that lidocaine from 1 mM was able to significantly decreased cell viability in a concentration-dependent manner. These results could be explained by shorter exposure in our study (4 h versus 24 or 48 h, respectively).

The HR-MAS NMR assay finds similar metabolomic fingerprints for MDA-MB-231 cells to a previous work (Maria et al., 2015). To note, glucose was not measurable in both groups due to the highly intense glucose uptake and glycolysis in most solid tumors compared to normal tissues. High levels of choline-rich metabolites are mainly due to increased phospholipid turnover and cell membrane synthesis in proliferative cells. Intense glutaminolysis promotes tumor proliferation and chemoresistance, in part through activation of the PI3K/AKT/mTORC1 pathway (Vignoli et al., 2021). Lidocaine causes a decrease in the metabolites of these two pathways, which reflects its impact on the proliferative potential of cancer cells. These results confirm previous experiments on lung cancer (Sun

and Sun, 2019) and hepatocellular carcinoma cells (Zhang et al., 2020) showing an inhibitor effect of lidocaine on PI3K/AKT/mTORC1 pathway, evidenced by assessing the phosphorylation levels of PI3K and AKT by western blot. The metabolomic impact of lidocaine choline pathway is comparable to the effects of some chemotherapies on this triple negative cell line: Maria et al. (2017) have demonstrated that cisplatin and tamoxifen could significantly reduce phosphocholine content suggesting a direct antiproliferative effect.

The tricarboxylic cycle (Krebs cycle) of MDA-MB-231 cells is also affected by lidocaine. There is a decrease in fumarate, malate and alanine. For instance, fumarate inhibits prolyl-hydroxylases, which leads to an increase in HIF-1α levels and allows, among other things, the survival of cancer cells exposed to hypoxia (Kuo et al., 2016). Thus, lidocaine could modulate the HIF-1-induced proliferation pathway as it was suggested in other studies. Indeed, western blot and/or gene expression experiments showed that lidocaine impairs HIF-1 pathway in renal and neuronal cells (Okamoto et al., 2017) or in human hepatoma and neuroblastoma cell lines (Nishi et al., 2005).

Our results showed a decrease in glutathione in its reduced form (GSH), which could be linked to the decrease in myoinositol. The level of glutamate, which is a precursor of GSH, is lowered; its synthesis may thus also be compromised by lidocaine. Another likely hypothesis would be glutathione consumption in response to increased oxidative stress. Indeed, a previous work investigating the impact of lidocaine on yeast cells observed an initial decline in GSH at H+1 but a gradual increase in this antioxidant from H+2, which may be a counter-regulation mechanism against oxidative stress induced by lidocaine (Boone et al., 2017). Similarly, an *in vitro* study showed that lidocaine caused a decrease in mitochondrial membrane potential and an increase in free radical production in non-small cell lung cancer cells (Wang et al., 2016). Furthermore, the absence of glucose and glycerophosphocholine in our samples and the similar levels of lactates in the control and lidocaine groups indicate that the glycolysis pathway and the choline pathway remain highly active in MDA-MB-231 cells despite lidocaine treatment. Indeed the increase of glycolysis in the tumor cell is a well-known phenomenon described as the Warburg effect (Wishart et al., 2016). It is linked to tumor overexpression of glucose membrane transporters, to the increase in hexokinase activity involved in glucose phosphorylation, and to the increase in anaerobic cellular glycolysis by inhibition of the oxidative pathway. Similarly, while glycerophosphocholine (GPC) levels still cannot be measured under lidocaine treatment, the fact that phosphorylcholine (PC) levels are lowered supports an increase in the GPC/PC ratio, which is considered a good prognosis factor in a cohort study in patients with *in situ* root canal carcinoma biopsies (Chae et al., 2016) and in a cohort of patients with gliomas (Dali-Youcef et al., 2015) or oligodendroglioma (Bund et al., 2019).

The effects of decreased myoinositol by lidocaine should be further investigated. In fact, inositols have important antiproliferative properties (Bizzarri et al., 2016). For instance, they can interfere with cell proliferation by decreasing the PI3K level or inhibiting pRB phosphorylation or Akt activation and therefore NF-kB. They can also interfere with cell invasion and the epithelial-mesenchymal transition (Bizzarri et al., 2016).

We have shown here that lidocaine at a concentration of 0.5 mM for 4 h can modulate the metabolism of triple negative cells. This concentration is compatible with clinical use of lidocaine infiltration for local anesthesia as lidocaine is frequently used at the concentration of 10 mg/ml (=42 mM, MW = 234,34 g·mol−1) but not with systemic intravenous administration. Indeed, over a plasma concentration of 21 μg/ml (90 μM) (Beaussier et al., 2018), patients may experience the systemic toxicity of lidocaine with neurologic symptoms ranging from cognitive disorders to seizures and cardiovascular compromise ranging from rhythm disorders to cardiac arrest. Moreover, lidocaine plasma levels following its intravenous administration are in a range of 1,4–6 μg/ml (25 μM) (Beaussier et al., 2018).

Therefore, the deepening of our study, investigating dose-effects and time-effects curves, would allow us to determine the molecular mechanisms at play and potential clinical use given the antitumoral properties of lidocaine. Similarly, *in vitro* studies in breast cancer (Li et al., 2014) and *in vivo* findings in hepatocellular carcinoma (Xing et al., 2017) showed that lidocaine can have a synergistic effect with cisplatin. It would thus be worth studying these combined effects in metabolomics to reach lidocaine doses below its toxic thresholds allowing its systemic use and repositioning lidocaine in chemotherapy.

Finally, as metabolomic profiles in oncology are established to develop prognostic strategies capable of classifying different breast cancers or therapeutic strategies in personalized medicine, it appears important to continue this work. Thus, studying the impact of lidocaine on an *in vivo* model (such as PDX xenograft) would get closer to physiological conditions, that could be transposed into the clinical arena. Indeed, if the cell culture allows for a simple experimental approach, both the nutritional conditions (excess glucose in the culture medium) and the oxygen concentrations (ranging from hyperoxia to hypoxia in some parts of the flask if it is not agitated) often do not allow extrapolation of experimental results to clinical use. Moreover, our *in vitro* study doesn't assess the impact of lidocaine on the microenvironment while it has been shown that local anesthetics could affect viability and differentiation capacity of adult stem/progenitor cells (Kim et al., 2020; Kubrova et al., 2021). Those effects on mesenchymal stem cells could influence wound healing or tumor spreading after surgery (Lucchinetti et al., 2012). Similarly, the study should be extended to other anesthesia drugs (hypnotics and analgesics in particular) that may also affect tumor progression (Sekandarzad et al., 2017). It would allow for a standardization of tumor sampling protocols in breast cancer surgery. Indeed, if percutaneous biopsies and clips are systematically performed under local anesthesia by lidocaine, the dose administered is not standardized. Similarly, wire localization by ultrasound before surgical excision can be done the day before or the morning of the surgery and the local anesthesia is not systematic. It depends for example on the expected difficulties, the anatomic structures that will be crossed, the patient's wish or the type of localization device. Finally, in the context of

multimodal analgesia during surgery, patients can benefit from regional anesthesia (plane or paravertebral blocks) or intravenous lidocaine administration. Similarly, *in vivo* studies have shown that other analgesic drugs such as morphine or hypnotics needed for general anesthesia can affect cellular metabolism (Sonnay et al., 2017). Hu *et al.* have recently shown that propofol, the most commonly used anesthetic drug, could alter metabolism of lung cancer cells (Hu et al., 2021). In this study, propofol increased intracellular glutamate and glycine but decreased acetate and formate in A549 cell line. Considering the results of this study and our own, it would be interesting to find a protocol which could have a direct protective effect against circulating cells or micro-metastasis, which development may be favored during the perioperative time (Benish and Ben-Eliyahu, 2010). All those parameters should also be considered to establish metabolomic fingerprints.

CONCLUSION

Our *in vitro* study showed that, under our experimental conditions, lidocaine at clinical concentrations useful for surgical site infiltration inhibits the proliferation of a high dose triple negative breast cancer cell line. At lidocaine concentrations that do not affect cell viability *a priori*, there is an inhibition of several overactive metabolic pathways in oncogenesis. This effect could have interesting clinical applications in several respects: 1) for local tumor recurrence, lidocaine may prevent the proliferation of a possible remnant of malignant cells at the surgical site; 2) for metastases, this local anesthetic may limit the spread of tumor cells.

On the other hand, the concentrations studied in our work were higher than systemic toxic thresholds. Further works are needed to refine the dose-response relationship of the observed effects and possibly to find a synergistic effect with conventional antiproliferative drugs. Our experimental results will need to be supplemented and tested in prospective multi-year clinical studies using either infiltration or intravenous analgesia. Our *in vitro* data are also interesting because they are part of the current trend of over-specialization in «onco-anesthesia». In this context, anesthesiologists should be made aware of the impact of their management as specialists in perioperative medicine on the long-term oncological outcomes of patients anesthetized for cancer surgery. Additionnally the impact of local anesthetics should be considered to establish metabolomic fingerprints in cancer.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

T-NC-T, I-JN, and ND-Y conceived the project. CT, I-JN, and ND-Y supervised the project. T-NC-T performed and analyzed all cell biology experiments, with the help of CT. I-JN, and ND-Y performed and analyzed NMR experiments. JP and PD assisted in designing and interpreting experiments. T-NC-T, MM, and ND-Y wrote the manuscript. All authors commented on the manuscript.

FUNDING

This study was supported by the grant ANR-10-LABX-0030-INRT, a French State fund managed by the Agence Nationale de la Recherche under the frame program Investissements d'Avenir ANR-10-IDEX-0002-02. T-NC-T was an IGBMC International PhD Programme fellow supported by LabEx INRT funds and has also received grants from the SFAR (French Society of Anesthesia & Intensive Care Medicine).

ACKNOWLEDGMENTS

The Authors would like to thank Betty Heller, Corinne Wendling and Amélie Jaulin (IGBMC institute) for their help with the cell culture and Elisa Ruhland for her help in 1H-HRMAS NMR experiments.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 23 February 2022
doi: 10.3389/fphar.2021.806954

Ropivacaine Inhibits Lung Cancer Cell Malignancy Through Downregulation of Cellular Signaling Including HIF-1α *In Vitro*

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Background: Ropivacaine is widely used to induce regional anesthesia during lung cancer surgery. Previous studies reported that amide-linked local anesthetics, e.g., ropivacaine, affected the biological behavior of lung adenocarcinoma cells, but the conclusion is controversial and warrants further study. This study set out to investigate the biological effects of ropivacaine on cultured lung cancer cells and underlying mechanisms.

Methods: Lung cancer cell lines (A549 and H1299) were cultured and then treated with or without ropivacaine (0.5, 1, and 2 mM) for 48 or 72 h. Their proliferation, migration, and invasion together with cell death and molecules including hypoxia inducible factor (HIF)-1α, VEGF, matrix metalloproteinase (MMP)-1, MMP-2, and MMP-9 expression associated with these changes were determined.

Results: Ropivacaine significantly inhibited proliferation and migration, invasion, and cell death in a concentration-dependent manner in both cell lines. Ropivacaine also promoted cell death and induced a concentration- and time-dependent cell arrest towards the G0/G1 phase. Expression of VEGF, MMP-1, MMP-2, MMP-9, and HIF-1α in both cell lines was also inhibited by ropivacaine in a concentration-related manner.

Conclusion: Our data indicated that ropivacaine inhibited lung cancer cell malignancy, which may be associated with downregulation of cell-survival-associated cellular molecules. The translational value of the current work is subjected to further study.

Keywords: ropivacaine, HIF-1α, non-small-cell lung cancer (NSCLC), local anesthetics, proliferation, migration, invasion

OPEN ACCESS

Edited by:
Daqing Ma,
Imperial College London,
United Kingdom

Reviewed by:
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Pharmacology

Received: *01 November 2021*
Accepted: *28 December 2021*
Published: *23 February 2022*

Citation:
Shen J, Han L, Xue Y, Li C, Jia H and Zhu K (2022) Ropivacaine Inhibits Lung Cancer Cell Malignancy Through Downregulation of Cellular Signaling Including HIF-1α In Vitro. *Front. Pharmacol. 12:806954. doi: 10.3389/fphar.2021.806954*

INTRODUCTION

Lung cancer is one of the most common malignant cancers and causes the highest death among all cancers worldwide. Recent studies estimated that in 2020, the 5-year survival rate of lung cancer was only 19%, just behind pancreatic and liver cancer (Siegel et al., 2020). With the lung cancer screening strategy implemented recently, more and more early-stage lung cancer can be diagnosed, and patients can receive earlier surgical resection. However, lung cancer recurrence after surgery is still a clinical challenge. Perioperative risk factors including anesthetic use during surgery may contribute

to cancer recurrence after surgery (Tavare et al., 2012; Wall et al., 2019), which may be due to anesthetics; in particular, inhalational anesthetics significantly modulated cell signaling changes, including hypoxia inducible factor (HIF)-1α (Huang et al., 2014; Unwith et al., 2015; Zhang W. et al., 2020). Conversely, it has been reported that local anesthetics (bupivacaine and levobupivacaine) have antitumor (colon, ovarian, and prostate cancer) properties (Xuan et al., 2015; Xuan et al., 2016; Li et al., 2019). Furthermore, clinical retrospective data also suggested that paravertebral anesthesia and analgesia for breast cancer surgery reduces the risk of recurrence or metastasis during the initial years of follow-up (Exadaktylos et al., 2006). Open prostatectomy surgery with general anesthesia, substituting epidural analgesia, was associated with substantially less risk of cancer recurrence (Biki et al., 2008). Patients who received paravertebral or high-pleural epidural anesthesia combined with sedation or light general anesthesia had a lower incidence of local or metastatic recurrence of breast cancer after surgery (Sessler et al., 2008; Snyder and Greenberg, 2010).

Ropivacaine is the most used amide-linked local anesthetic for regional anesthesia and acute pain, chronic pain, and cancer pain relief use (Yanagidate and Strichartz, 2007). Ropivacaine was reported to significantly inhibit the proliferation of gastric cancer cells, which was associated with reduction of the phosphorylation of EKR1/2 (Yang et al., 2018) and promoted liver cancer cell death *via* impaired mitochondrial function and caspase-3 activation (Wang et al., 2019). To understand the effect of ropivacaine on lung cancer malignancy and underlying mechanisms, the current study was set to investigate the effects of different concentrations of ropivacaine on proliferation, invasion, and metastasis of non-small-cell lung cancer cell lines and associated molecular changes.

MATERIALS AND METHODS

Cell Culture

Human lung cancer cell lines (A549 and H1299), which are two of the common lung cancer phenotypes clinically, were purchased from the Cellular Biology Institute of the Shanghai Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (GIBCO, United States) supplemented with 10% bovine serum (Biological Industries, Beit HaEmek, Israel). The cells were grown in monolayer at 37°C in a humidified atmosphere supplemented with 5% CO_2. Ropivacaine (AstraZeneca AB, Sweden) was dissolved in normal saline, with the pH adjusted to 7.4, and kept at −20°C. The cultured lung cancer H1299 and A549 cells at 90% confluence were treated with ropivacaine at 0.5, 1, and 2 mM. Cells treated with saline served as controls. Cobalt chloride ($CoCl_2$) (Sigma-Aldrich, St Louis, MO, United States) at 100 μM was used to induce cellular hypoxia and increase HIF-1α expression in both A549 and H1299 cells treated with ropivacaine.

Cell Proliferation Assessment

Approximately 5×10^3 cells/well were placed in a 96-well plate and then treated with 0, 0.5, 1, and 2 mM ropivacaine for 48 or 72 h. Subsequently, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] solution (Promega, Madison, WI, United States) (20 μl/well) was added to the cultures, which were incubated in the dark at 37°C for 2 h. The absorbance was measured at 492 nm with a microplate reader (Thermo Fisher Scientific Inc., MA, United States).

Transwell Assay

The Boyden chambers (pore size 8 μm) (Collaborative Biomedical, Becton Dickinson Labware, Bedford, MA, United States) covered with or without 200 μg/ml Matrigel (Beyotime Biotechnology) were used to evaluate cell invasion or migration ability. A549 or H1299 cells (1×10^5) were seeded in the upper chamber with 0.2 ml of RPMI 1640 medium without serum, while 0.6 ml medium with 10% FBS was added to the lower chamber. After incubation for 18 h, nonmigratory cells remaining above the membrane were removed with a cotton swab, and cells penetrating below the membrane are stained with crystal violet. Cells that penetrated the membrane were counted through a microscope in five randomly selected fields.

Cell-Cycle Analysis and Apoptosis Analysis

The effect of ropivacaine in A549 and H1299 on cell-cycle progression was evaluated by flow cytometric analysis followed by propidium iodide (PI) staining. Cells were seeded in six-well plates and cultured for 24 h, and then the medium was replaced with no-serum medium for a further 24 h to synchronize the cell cycle at the G0/G1 phase; then the medium was replaced by ropivacaine (0, 0.5, 1, and 2 mM) for 48 or 72 h. After which, cells were stained with PI (MULTI SCIENCES, Hangzhou, China) according to the manufacturer's instructions. The stained cells were incubated for 20 min at 37°C and then analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lake, NJ, United States).

Approximately 5×10^5 cells were gleaned and washed twice with phosphate-buffered saline (PBS) and then resuspended with 500 μl of 1× binding buffer. FITC annexin V and PI were added to the solution, which was then incubated in the dark for 5 min at room temperature. The cells were gently vortexed for flow cytometric analysis.

Wound Healing Assay

Wound healing assay was performed to determine cell migration. The cells (5×10^5) were seeded in six-well plates and cultured in the medium without bovine serum. When the cell confluence reached ≥80%, a 200 μl pipette was used to scratch a line on the monolayer gently. Then the medium was replaced by ropivacaine (0, 0.5, 1, and 2 mM) dissolved in culture medium. The micrographs of scratches were recorded randomly under an inverted microscope at 0, 24, and 48 h after being scratched.

Western Blot Analysis

The proteins from the variously treated cells were extracted using RIPA buffer containing protease inhibitors. Lysates were centrifuged, and proteins were denatured through heating. The concentration of proteins was measured using the BCA assay (Beyotime, Nanjing, China). Total proteins (40 μg) were separated by 10% SDS-PAGE and then transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, MA, United States). The membranes were blocked with 5% BSA for 2 h at room temperature and then were incubated overnight at 4°C with antibodies against matrix metalloproteinase (MMP)-2, MMP-

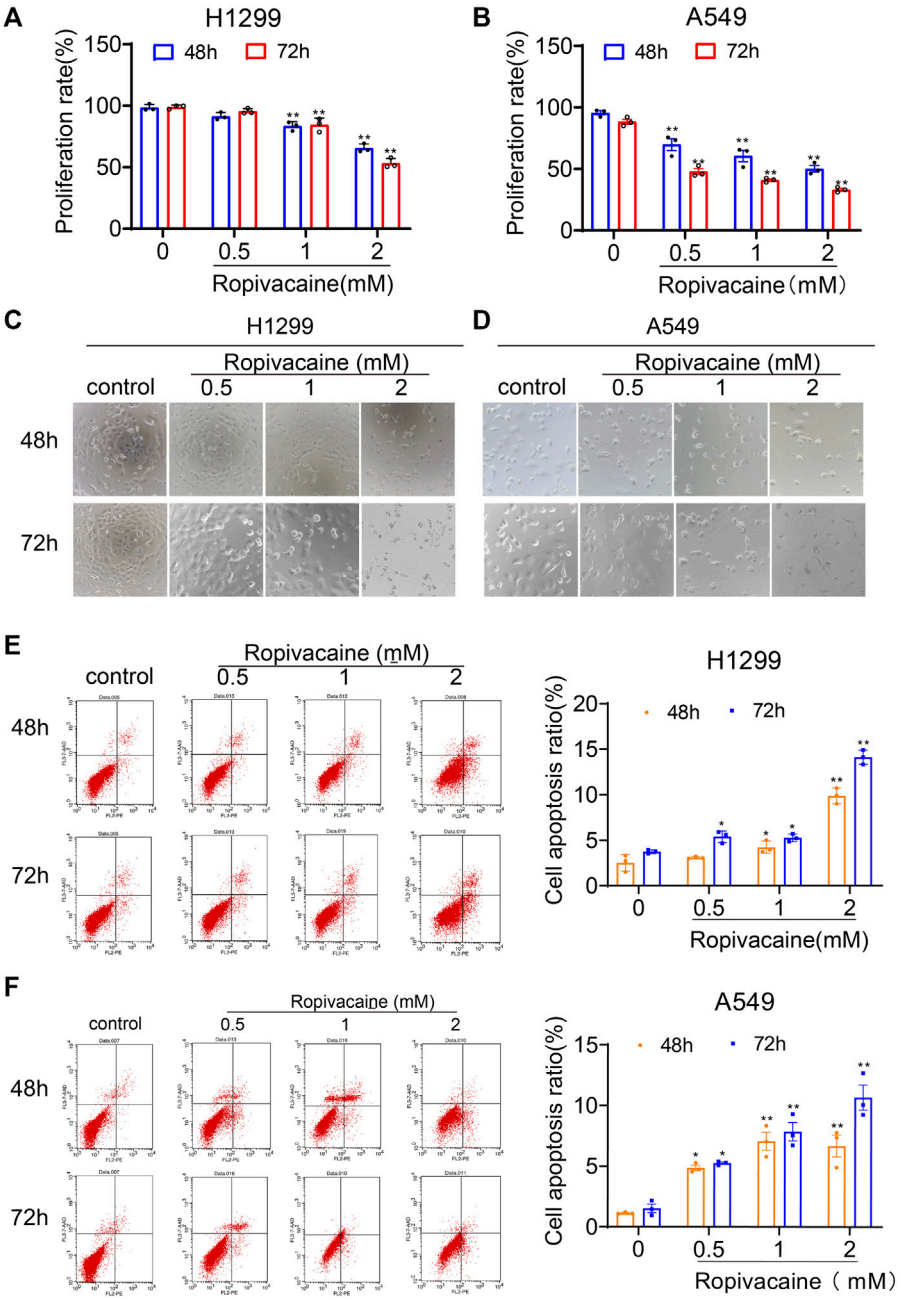

FIGURE 1 | Ropivacaine inhibited proliferation and promoted apoptosis of lung cancer cells. H1299 **(A)** and A549 **(B)** cells treated with ropivacaine (0.5, 1, and 2 mM) for 48 or 72 h. Morphological changes of H1299 **(C)** and A549 **(D)** cells treated with ropivacaine for 48 or 72 h. Apoptotic death of lung cancer H1299 cells **(E)** and A549 cells **(F)** measured by flow cytometry analysis following a 48 or 72 h treatment. Independent experiments were repeated three times. Data are presented as the mean ± SD. *$p < 0.05$ and **$p < 0.01$ vs. the control group.

9, MMP-1, VEGF, HIF-α, or GAPDH. Anti-rabbit IgG (Cell Signaling Technology, Boston, MA, United States) was visualized by Odyssey imaging (LI-COR, Lincoln, NE, United States). Antibodies against MMP-2, MMP-9, and MMP-1 were purchased from Cell Signaling Technology (CST, CA, United States). Antibodies against VEGF (polyclonal) and HIF-α were purchased from Bioss Biotechnology Co., Ltd. (Beijing, China), and GAPDH antibodies were obtained from Abcam (Cambridge, United Kingdom).

Statistical Analysis

All data were expressed as mean ± standard deviation, and then comparisons to the mock treatments (controls) were made with nonparametric ANOVA first and followed with

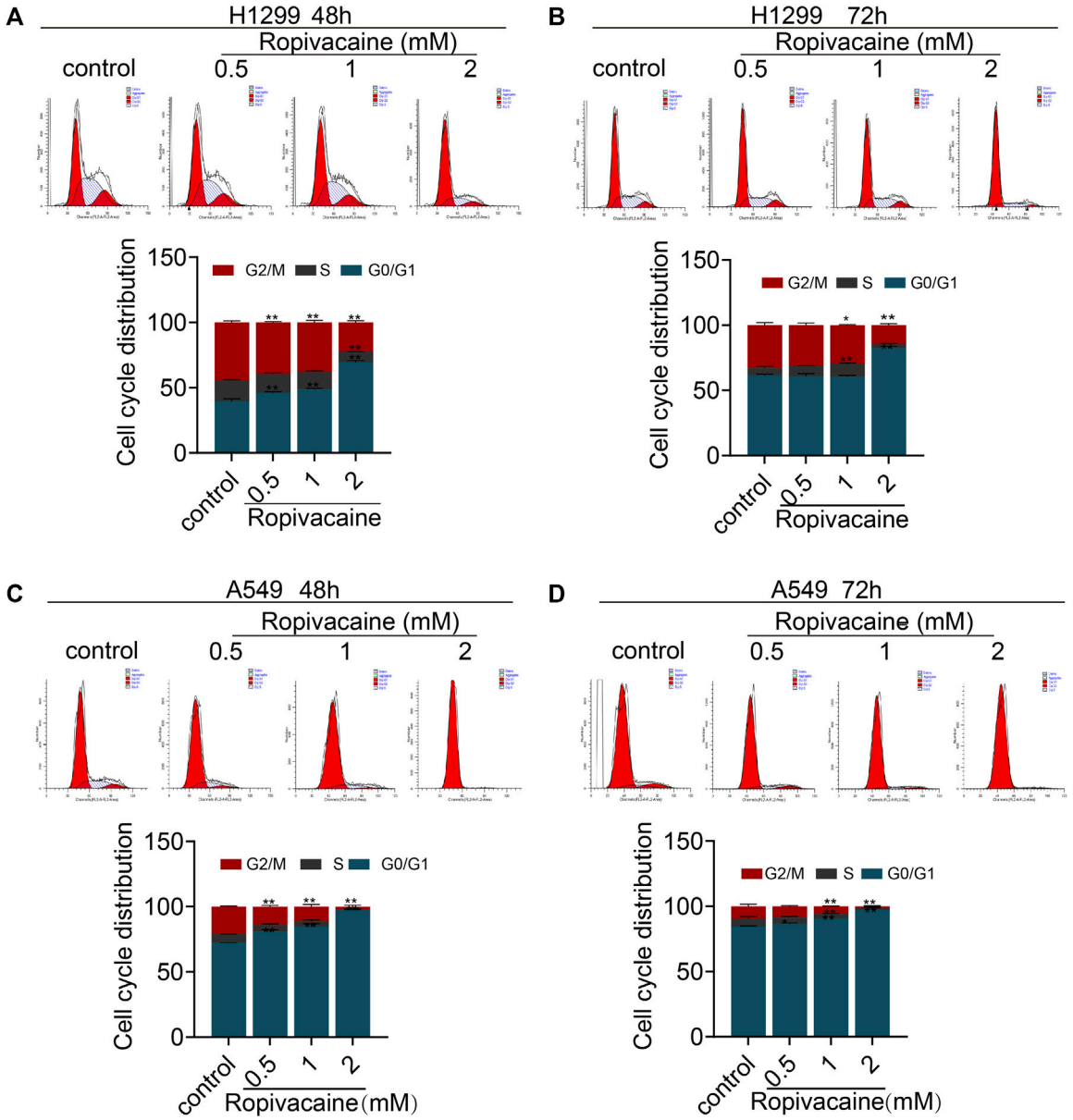

FIGURE 2 | Cell cycle changes induced by ropivacaine. H1299 cells **(A,B)** and A549 cells **(C,D)** were treated with different concentrations of ropivacaine for 48 or 72 h. The cell cycle distribution was measured with PI staining and assessed with flow cytometry. Data are presented as the mean ± SD from three independent experiments. *$p < 0.05$ and **$p < 0.01$ vs. controls.

post-hoc Tukey's test (SPSS version 19.0). A two-tailed *p*-value of less than 0.05 was considered statistically significant.

RESULT

Ropivacaine Suppresses Proliferation and Causes Apoptosis of Lung Cancer Cells

Ropivacaine inhibited the proliferation of lung cancer cells (**Figure 1**). For comparison, H1299 cells (**Figure 1A**) were less sensitive to ropivacaine exposure for either 48 h or 72 h than A549 cells (**Figure 1B**). The number of H1299 and A549 lung cancer cells was significantly decreased with an increase of ropivacaine concentration (**Figures 1C,D**). These changes, at least in part, were because ropivacaine caused the death of both types of cells via apoptosis (**Figures 1E,F**).

Ropivacaine Induces Cell Cycle Arrest of Lung Cancer Cells

Considering that the ropivacaine inhibited the proliferation of H1299 and A549 cells, we further investigated its effect on cell cycle changes of lung cancer cells. Ropivacaine, in particular at 2 mM,

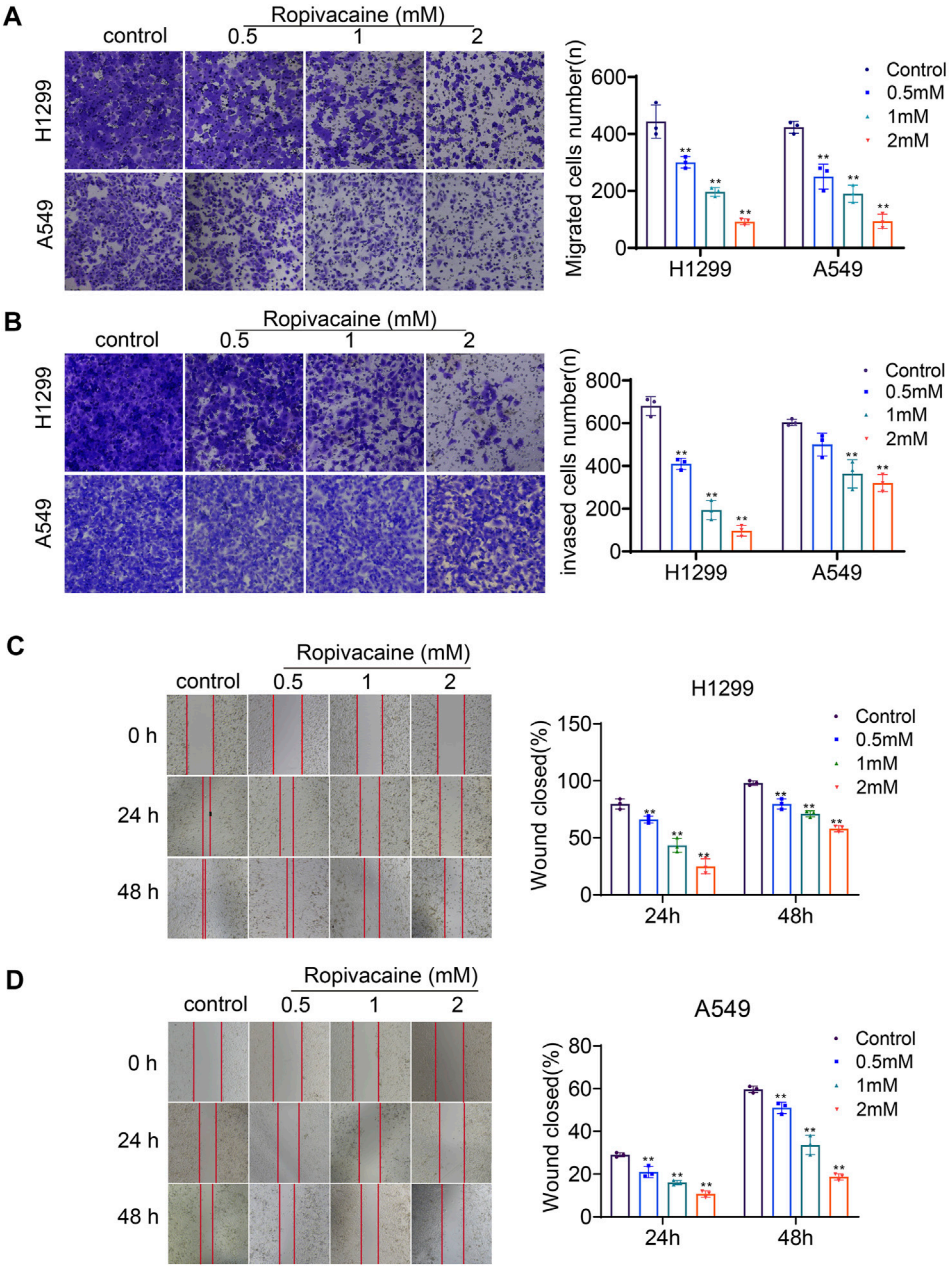

FIGURE 3 | Ropivacaine treatment induced migration and invasion changes. **(A,B)** Effects of ropivacaine treatment on migration and invasiveness of H1299 and A549 cells were investigated using transwell and Matrigel assays. The number of cells that migrated or invaded was counted in five different fields. **(C,D)** Wound healing assays were performed to detect the migratory ability of H1299 and A549 cells, and the migratory ratio was determined by dividing the wound area by the total area. The data are expressed as mean ± SD from three independent experiments. *$p < 0.05$ and **$p < 0.01$ vs. controls.

induced cell cycle arrest in the G0/G1 phase, and this effect was more readily detectable in A549 than H1299 cells (**Figures 2A–D**).

Ropivacaine Inhibited Lung Cancer Cell Migration and Invasion

The transwell assay and wound healing assay were applied to determine the effects of ropivacaine on the migration and invasion of H1299 and A549 cells. Cell migration assay data showed that the numbers of H1299 and A549 cells that migrated into transwell filters after treatment with ropivacaine at concentrations of 0.5, 1, and 2 mM were significantly reduced compared to those of the control group ($p < 0.05$) (**Figure 3A**). The invasion experiments showed that H1299 cells treated with 0.5, 1, or 2 mM ropivacaine had a significant decrease in the number of cells passing through Matrigel-coated membranes ($p < 0.05$) (**Figure 3B**). Similarly, the invasion ability of A549 cells treated with 1 or 2 mM ropivacaine was significantly decreased ($p < 0.05$) (**Figure 3B**). In the wound healing experiment,

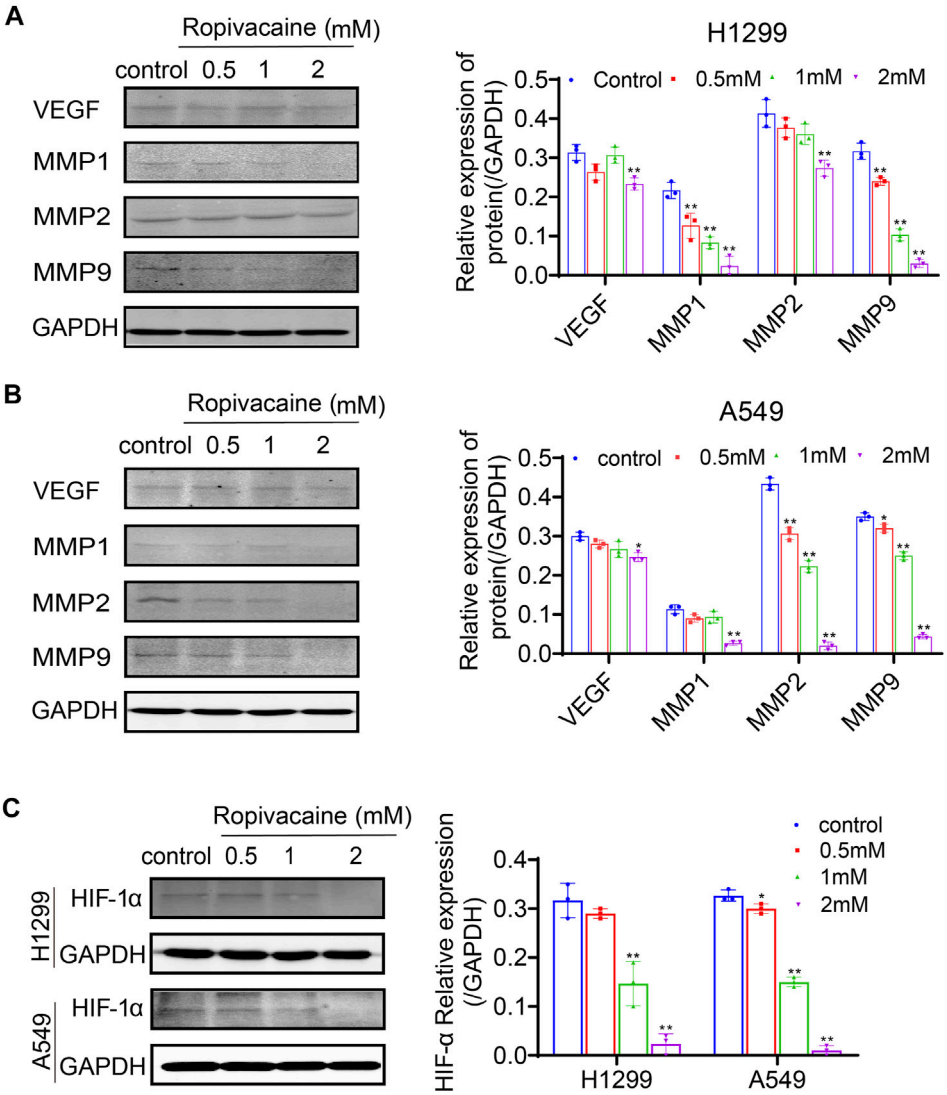

FIGURE 4 | Ropivacaine treatment reduced expression of VEGF, MMPs, and HIF-1α in lung cancer cells. **(A)** H1299 cells and **(B)** A549 cells were treated with ropivacaine (0.5, 1, or 2 mM) for 48 h. The control group was treated with a no-FBS medium for 48 h. VEGF, MMP-1, MMP-2 and MMP-9 proteins in the cell lysates were assayed by western blot. GAPDH proteins were used as internal controls. **(C)** H1299 and A549 cells were treated with ropivacaine (0.5, 1, or 2 mM) and $CoCl_2$ (100 μM) for 48 h. The control group was treated with a no-FBS medium and $CoCl_2$ (100 μM) for 48 h. The protein expression of HIF-1α in H1299 cells and A549 cells was measured by western blot. The independent experiments were repeated three times. The data are expressed as the mean ± SD from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. controls.

the wound healing process of H1299 and A549 cells was delayed significantly after treatment with 0.5, 1, or 2 mM ropivacaine at 24 and 48 h ($p < 0.05$) (**Figures 3C,D**).

Ropivacaine Decreased VEGF, MMPs, and HIF-1α Expression of Lung Cancer Cells

VEGF and the MMP family were reported to be closely related to lung cancer malignancy and even metastasis (Merchant et al., 2017; Zhang et al., 2020a). To determine the mechanisms for why ropivacaine inhibited the invasion and metastasis of lung cancer cells, we detected by western blot the VEGF and MMP protein expression levels in H1299 and A549 cells after ropivacaine treatment. It was found that the protein expression level of VEGF in both H1299 and A549 cells treated with 2 mM ropivacaine was significantly lower than that in the controls ($p < 0.05$). The expression of MMP-1, MMP-2, and MMP-9 was also significantly decreased in a contraction-related manner after ropivacaine treatment in both H1299 and A549 cells (**Figures 4A,B**). VEGF and MMPs were downstream effectors of HIF-1α (Wang et al., 1995; Jiang et al., 1996). Therefore, we further detected the changes of HIF-1α in H1299 and A549 cells after ropivacaine treatment. To enhance HIF-α expression, cobalt chloride ($CoCl_2$) was used to treat H1299 and A549 cells. Compared with that in the control group, the expression of HIF-1α in all treatment groups was significantly decreased, and the HIF-1α expression was decreased with the increase of ropivacaine concentration, especially at 1 and 2 mM ($p < 0.05$) (**Figure 4C**).

DISCUSSION

In the current *in vitro* study, we found that ropivacaine suppressed proliferation and invasion of lung cancer cells. Ropivacaine also promoted lung cancer cell death via apoptosis. Our data also demonstrated that ropivacaine significantly decreased migration ability of both lung cancer cell types. The cell cycle data indicated that ropivacaine arrested lung cancer H1299 and A549 cells staying in the G0/G1 phase. All these changes induced by ropivacaine may be associated with the decrease of MMPs, VEGF, and HIF-1α expression.

Tumor microenvironmental changes by any factors, e.g., inflammation induced by surgery, may promote cancer development and reoccurrence after surgery (Wall et al., 2019). Studies have shown that abnormal tumor microenvironments, such as hypoxia, pH value changes, and low glucose concentration, all affect the occurrence and development of tumors (Arneth, 2019). A hypoxic microenvironment is conducive to tumor growth and metastasis and plays a role in tumor initiation and progression (Liao et al., 2007). HIF-1α is a major subtype identified in the tumor microenvironment and has been found to be a key regulator of tumor growth (Pezzuto and Carico, 2018). In a normal-oxygen microenvironment, HIF-1α is hydroxylated by proline hydroxylase. Hydroxylated HIF-1α is suitable for binding to the tumor suppressor Von Hippel Lindau protein (VHL) in the cytoplasm that is being degraded by the protein body. Conversely, under hypoxia (1% O_2 tension), proline hydroxylase is inactivated, and HIF-1α undergoes stabilization, nuclear translocation, and aggregation mechanisms and also evades decomposition via co-activator signals such as the C-terminal trans-activation domain (C-TAD) binding protein (CBP) (Kuschel et al., 2012). HIF-1α regulates a significant number of genes involved in many biological processes, including angiogenesis, glycolytic metabolism, and cell survival and invasion (Semenza, 2003). Overexpression of HIF-1α has been shown in many cancers. A previous study found that HIF-1α expression affected tumor proliferation and apoptosis in surgically resected lung cancer (Takasaki et al., 2016). HIF-1α is stable under low oxygen tension, so in our experiment, lung cancer cells were treated with cobalt chloride to obtain a hypoxic environment (Huang et al., 2003), and as a result, HIF-1α was upregulated in A549 and H1299 cells but decreased by ropivacaine in our study.

A significant association between MMP-9 and HIF-1α expression was reported in studies of lung cancers (Swinson et al., 2004; Chang et al., 2017). MMPs are zinc-dependent endopeptidases that participate in extracellular matrix degradation and play an important role in tumorigenesis, cell adhesion, and epithelial–mesenchymal transition. Among them, MMP-1, MMP-2, and MMP-9 are closely related to tumor invasion and metastasis (Nabeshima et al., 2002; Toth et al., 2012). It has been reported that ropivacaine can block tumor cell invasion and MMP-9 secretion (Piegeler et al., 2015). Our results showed that ropivacaine not only reduced the expression of MMP-9 but also inhibited the expression of MMP-2 and MMP-1 simultaneously. VEGF is one of the downstream effectors of HIF-1α (Jones et al., 2001; Pezzuto and Carico, 2018) and plays an important role in tumor development and even invasion and metastasis. These results suggested that ropivacaine effectively inhibited HIF-1α, VEGF, and MMP cellular signaling in human lung cancer cells and hence caused a decrease in malignant lung cancer cells. Similar to our results, previous studies also showed that ropivacaine inhibited the proliferation of breast cancer, cervical cancer, and thyroid papillary cancer cells; suppressed the invasion and metastasis of gastric cancer and thyroid papillary cells; and decreased the generation of tumor blood vessels (Zhang N. et al., 2020; Chen et al., 2020; Qin et al., 2020).

Our data may indicate that ropivacaine can change the lung cancer microenvironment; in particular, ropivacaine can potentially destroy new vascular formation and hence decrease the energy substrates supporting cancer cell development. Perhaps the most importance finding in our study is that ropivacaine directly suppressed lung cancer cell proliferation, migration, and invasion and promoted lung cancer cell death. All these effects may decrease the risk of lung cancer recurrence after surgery although this requires the direct application of ropivacaine to the cancer resection area, which is not a very common clinical practice during cancer surgery (Tavare et al., 2012; Wall et al., 2019). On the other hand, local anesthetics are often used for regional anesthesia, which can significantly block pain signal traveling through the pain pathway into the central nervous system, which causes surgical stress (Iwasaki et al., 2015; Xuan et al., 2015; Xuan et al., 2016). To this end, local anesthetics may have multi-beneficial effects for cancer patients.

Our work is not without limitations. Firstly, this study is a pure *in vitro* work, which is far from clinical settings. More clinical studies including animal studies are needed. Secondly, we found that ropivacaine was ineffective in both cancer cells in the μM concentration range found in our preliminary study. However, as stated above, local anesthetics are often given for local infiltration injection, and its concentration can reach to more than the mM range concentration. Hence, our data are clinically relevant although their translational value is subject to further study. Lastly, the causal relationship between the inhibitive effects of ropivacaine and molecular changes found in this study cannot be established and warrants further study.

In conclusion, our study suggested that ropivacaine inhibited the expression of HIF-1α in H1299 and A549 lung cancer cells, hence reducing the expression of its downstream effectors VEGF and MMPs and decreasing the ability of lung cancer invasion and metastasis potential *per se*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JS and LH were in charge of the experimental design, YX and KZ were in charge of data collection, and HJ was in charge of funding. CL and HJ revised the manuscript. All authors listed wrote the manuscript and approved it for publication.

FUNDING

This work was supported by the Hebei Province introducing foreign intelligence project (No. 2019YX010A), China.

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frontiers in Oncology

ORIGINAL RESEARCH
published: 03 March 2022
doi: 10.3389/fonc.2022.708632

Anesthesia With Propofol Sedation Reduces Locoregional Recurrence in Patients With Breast Cancer Receiving Total Mastectomy Compared With Non-Propofol Anesthesia

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Purpose: We examined locoregional recurrence (LRR) in patients with breast invasive ductal carcinoma (IDC) receiving total mastectomy (TM) under propofol-based paravertebral block-regional anesthesia (PB-RA) versus sevoflurane-based inhalational general anesthesia (INHA-GA) without propofol. All-cause death and distant metastasis were secondary endpoints.

Patients and Methods: Patients with breast IDC receiving TM were recruited through propensity score matching and categorized into INHA-GA with sevoflurane and PB-RA with propofol groups. Cox regression analysis was performed to calculate hazard ratios (HRs) and 95% confidence intervals (CIs).

Results: In the multivariate Cox regression analysis, the adjusted HR (aHR; 95% CI) of LRR for the PB-RA with propofol group was 0.52 (0.28–0.96) compared with the INHA-GA with sevoflurane group. The aHRs of LRR for differentiation grade II, grade III, the American Joint Committee on Cancer clinical stage II, stage III, pathological tumor (pT) stage 2, pT stage 3–4, pathological nodal (pN) stage 1, and pN stage 2–3 were 1.16 (1.04–2.08), 1.28 (1.07–2.12), 3.71 (1.82–7.59), 4.67 (1.65–13.18), 1.09 (1.02–1.21), 1.17 (1.03–2.16), 1.10 (1.03–1.33), and 1.22 (1.06–2.41), respectively, compared with

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Edited by:
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

Received: *12 May 2021*
Accepted: *07 February 2022*
Published: *03 March 2022*

Citation:
Zhang J, Chang C-L, Lu C-Y, Chen H-M and Wu S-Y (2022) Anesthesia With Propofol Sedation Reduces Locoregional Recurrence in Patients With Breast Cancer Receiving Total Mastectomy Compared With Non-Propofol Anesthesia. Front. Oncol. 12:708632. doi: 10.3389/fonc.2022.708632

differentiation grade I, clinical stage I, pT1, and pN0. The aHR of LRR for adjuvant RT was 0.88 (0.64–0.94) compared with that for no adjuvant RT.

Conclusion: PB-RA with propofol might be beneficial for reducing LRR in women with breast IDC receiving TM compared with INHA-GA without propofol.

Keywords: propofol, general anesthesia, survival, invasive ductal carcinoma, total mastectomy

INTRODUCTION

Many preclinical studies including *in vivo* or *in vitro* have suggested an association between anesthetic drugs and techniques and the activity and survival of cancer cells; this association can result from changes in the immune response, modulation of the neuroendocrine stress response to surgery, or effects on cancer cell signaling (1–7). However, few studies have reported high-quality clinical outcomes. Most existing clinical studies are retrospective in nature (8–11), and most prospective trials were initially designed to study outcomes other than cancer recurrence (12–14).

Sevoflurane is one of the most widely used volatile anesthetic agents. Sevoflurane exhibited chemoresistance to cisplatin (15) and led to an increased expression of metastasis-related genes (16). By contrast, propofol is the most commonly used intravenous induction agent and is often used for maintaining anesthesia (7). In a laboratory study, propofol exhibited antitumor effects (7). However, investigating the effects of anesthetics, such as sevoflurane and propofol, on patients with cancer in a clinical trial is difficult (17, 18) because patients generally require a combination of anesthetic agents (19, 20). Patients are often managed with either inhalation agents and opioids or propofol as the anesthetic agent and regional anesthesia as the analgesic agent (19, 20). Moreover, performing surgery without providing perioperative pain relief or solely under regional anesthesia to examine the effects of specific anesthetic modalities would be unethical (19, 20). In addition, interpretation of these findings from controversial conclusions in previous studies is limited by heterogeneity resulting from the different extents of surgery, cancer types, and patient characteristics as well as other limitations associated with the retrospective nature of most studies (21). Therefore, conflicting conclusions have been reported in preclinical and clinical studies (1–7, 19, 20).

To address this crucial problem, we chose a consistent extent of surgery (total mastectomy [TM]) for patients with breast invasive ductal carcinoma (IDC), consistent anesthesia (propofol-based paravertebral block-regional anesthesia [PB-RA] vs. sevoflurane-based inhalational general anesthesia [INHA-GA]), and the primary endpoint of locoregional recurrence (LRR) to investigate LRR between INHA-GA without propofol and PB-RA with propofol in patients with breast cancer who underwent TM through propensity score matching (PSM).

Abbreviations: OS, overall survival; LRR, locoregional recurrence; DM, distant metastasis; IDC, invasive ductal carcinoma; TM, total mastectomy; PB-RA, paravertebral block-regional anesthesia; GA, general anesthesia; INHA, inhalational; HR, hazard ratio; aHR, adjusted hazard ratio; CI, confidence interval; RCT, randomized controlled trial; PSM, propensity score matching; TCRD, Taiwan Cancer Registry database; SD, standard deviation; AJCC, American Joint Committee on Cancer; HR, Hormone Receptor; HER2, Human Epidermal Growth Factor Receptor-2; RT, radiotherapy; ASA, American Society of Anesthesiology; CCI, Charlson comorbidity index; ICD-9-CM, International Classification of Diseases, Ninth Revision, Clinical Modification; TNM, Tumor, Node, and Metastasis; T, tumor; N, nodal; pT, pathological tumor stage; pN, pathological nodal stage; NCCN, National Comprehensive Cancer Network; ALND, axillary lymph node dissection; SLNB, sentinel lymph node biopsy.

PATIENTS AND METHODS

Study Cohorts

This retrospective study was conducted using data from the Health and Welfare Data Center (HWDC) established by Taiwan's Ministry of Health and Welfare. The HWDC consolidates data gathered by the Taiwanese government from various sources. These data are then deidentified and made available for research purposes based on case-by-case approval. In particular, we used the Taiwan Cancer Registry, which includes the detailed staging and treatment information of patients with cancer, the Cause of Death database, which lists all death certificates issued in Taiwan (22), and the National Health Insurance Research Database, which contains billing information on all National Health Insurance (NHI)-reimbursed examinations, medications, and treatments. We have confident are that no evidence of death is evidence of life, because all death certificates issued is the Government system-specific judgment. A death certificate is required for property inheritance, abandonment of inheritance to the court, burial or cremation. The NHI program has been implemented since 1995 and covers more than 99% of Taiwan's population.

We established a cohort consisting of female patients with breast IDC by using data from the Taiwan Cancer Registry Database (TCRD), which is maintained by the Collaboration Center of Health Information Application. We enrolled patients who received a diagnosis of IDC between January 1, 2009, and December 31, 2018, and underwent TM. The follow-up duration was from the index date to December 31, 2019. The index date was the date of TM. The mean follow-up duration was 43.3 months (standard deviation [SD], 29.8 months) and 55.9 months (22.6 months) for patients receiving INHA-GA without propofol and those receiving PB-RA with propofol, respectively. The TCRD contains detailed cancer-related information including the clinical or pathological stage (according to the American Joint Committee on Cancer [AJCC], seventh edition), anesthesia modalities, hormone receptor (HR) status, human epidermal growth factor receptor-2 (HER2) status, and radiotherapy (RT)

and chemotherapy regimens used (23–27). The study protocols were reviewed and approved by the Institutional Review Board of Tzu-Chi Medical Foundation (IRB109-015-B). Patient diagnoses were confirmed on the basis of pathological data, and patients who received a new diagnosis of breast IDC were confirmed to have no other cancers and no distant metastasis. In the PB-RA with propofol group, propofol was initially used as target-controlled infusion for conscious sedation during paravertebral block and TM (28). The optimal propofol target concentration was ≥0.8 mg/ml at least for the PB-RA with propofol group (29). In the INHA without propofol group, anesthesia was continued with sevoflurane in 100% oxygen at a flow rate of ≥5 L/min in a circle system, and the end-tidal concentration of sevoflurane was maintained at a minimum alveolar concentration of approximately ≥2 (30). Our propofol doses in our study were similar with the previous studies (20, 31). There is no association of the cost of propofol, cost of treatment, and not affected by insurance or decision to in the chose either type of anesthesia. All surgical procedures and propofol cost of treatment for breast cancer were all covered by NHI. Propofol was not used in the INHA-GA group. Other inclusion criteria were age ≥20 years and AJCC clinical stage I–III. Patients with metastasis, missing sex data, age <20 years, nonstandard adjuvant breast RT (contrast with standard adjuvant RT, consisting of irradiation to both the chest wall/whole breast and regional nodes with a minimum of 50 Gy), neoadjuvant chemotherapy, unclear differentiation of tumor grade, missing HR status, missing HER2 status, or unclear pathological tumor, node, and metastasis (TNM) staging were excluded. Adjuvant treatments such as adjuvant RT, adjuvant chemotherapy, hormone therapy, and target therapy were allowed on the basis of National Comprehensive Cancer Network (NCCN) guidelines for breast cancer in Taiwan (32). Furthermore, we excluded patients with unclear surgical procedures, ill-defined nodal surgery, unclear HR status, unclear HER2 status, unknown pathologic TNM stages, unknown American Society of Anesthesiology (ASA) physical status, unclear Charlson comorbidity index (CCI), unclear grade of differentiation, or nonrecorded hospital type (33) (academic center or community hospital) from our cohort. HR positivity was defined as ≥1% of tumor cells demonstrating positive nuclear staining through immunohistochemistry (34) and HER2 positivity was defined as an immunohistochemistry score of 3+ or a fluorescence *in situ* hybridization ratio of ≥2 (33, 35). Finally, we enrolled patients with breast IDC receiving TM under PB-RA with propofol or INHA-GA without propofol during perioperative anesthesia. Comorbidities were assessed using the CCI (36, 37). The CCI has prognostic significance for all-cause death in patients with breast cancer (38, 39). Only comorbidities observed 6 months before the index date were included, and new-onset comorbidities diagnosed within 6 months before the index date were excluded. On the basis of the inclusion criteria, we examined the effects of long-term comorbidities on the survival of patients. Comorbidities were identified according to primary *International Classification of Diseases, Ninth Revision, Clinical Modification* (ICD-9-CM) codes; diseases present at the first admission and those identified more than twice during outpatient visits were included as comorbidities.

PSM and Covariates

After adjustment for confounders, we used a Cox proportional-hazards model to model time from the index date to LRR (primary endpoint) for patients with IDC receiving TM. To reduce the effects of potential confounders when LRR was compared between different anesthesia groups, PSM was performed. Matching variables used were age, menopausal status, diagnosis year, CCI score, differentiation, AJCC clinical stage, pathological tumor (pT) stage, pathological nodal (pN) stage, ASA physical status, adjuvant chemotherapy, adjuvant RT, HR status, HER2 status, nodal surgery, and hospital level. We matched the cohorts at a ratio of 1:1 by using the greedy method, with age, diagnosis year, menopausal status, CCI score, differentiation, AJCC clinical stage, pT, pN, adjuvant RT, HR status, HER2 status, and nodal surgery completely matched with a propensity score within a caliper of 0.2 (40). Matching is a common technique used for selecting controls with identical background covariates as study participants to minimize differences between individuals that the investigator believes must be controlled. A Cox model was used to regress all-cause death and distant metastasis (DM; secondary endpoints) on different anesthesia statuses, and a robust sandwich estimator was used to account for clustering within matched sets (41). Multivariate Cox regression analysis was performed to calculate hazard ratios (HRs) to determine whether factors such as different anesthesia modalities, age, menopausal status, diagnosis year, CCI score, differentiation, AJCC clinical stage, pT, pN, ASA physical status, adjuvant chemotherapy, adjuvant RT, HR status, HER2 status, nodal surgery, and hospital level are potential independent predictors of all-cause death, LRR, or DM. Potential predictors were controlled for in the analysis (**Table 1**), and LRR was the primary endpoint in both anesthesia groups. All-cause death and DM were the secondary endpoints in our study.

Statistics

All analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC, USA). In a two-tailed Wald test, $p < 0.05$ was considered significant. Overall survival (OS), LRR-free survival, and DM-free survival were estimated using the Kaplan–Meier method, and differences between the INHA-GA without propofol and PB-RA with propofol groups were determined using the stratified log-rank test to compare survival curves (stratified according to matched sets) (42).

RESULTS

PSM and Study Cohort

The matching process yielded a final cohort of 1,414 patients (707 and 707 in the INHA-GA without propofol and PB-RA with propofol groups, respectively) eligible for further analysis; their characteristics are summarized in **Table 1**. Age distribution was

TABLE 1 | Demographics of propensity score-matched patients with breast cancer receiving total mastectomy under PB-RA with propofol or INHA-GA without propofol.

		INHA-GA without propofol *N* = 707		PB-RA with propofol *N* = 707		*p*-value
		n	(%)	*n*	(%)	
Age (years)	Mean (SD)	56.4	(12.4)	56.1	(12.4)	0.9999
	Median (Q1–Q3)	56	(47-64)	55	(47-64)	
	20–49	236	(33.4)	236	(33.4)	1.0000
	50+	471	(66.6)	471	(66.6)	
Diagnosis year	2009–2013	210	(29.7)	210	(29.7)	1.0000
	2014–2018	497	(70.3)	497	(70.3)	
Menopausal status	Premenopausal	282	(39.9)	282	(39.9)	1.0000
	Postmenopausal	425	(60.1)	425	(60.1)	
CCI scores	0	478	(67.6)	476	(67.3)	0.6530
	1	148	(20.9)	149	(21.1)	
	2+	81	(11.5)	82	(11.6)	
Differentiation	I	68	(9.6)	68	(9.6)	1.0000
	II	486	(68.7)	486	(68.7)	
	III	153	(21.6)	153	(21.6)	
AJCC clinical stage	I	206	(29.1)	206	(29.1)	1.0000
	II	382	(54.0)	382	(54.0)	
	III	119	(16.8)	119	(16.8)	
pT	pT1	269	(38.0)	269	(38.0)	1.0000
	pT2	345	(48.8)	345	(48.8)	
	pT3–4	93	(13.2)	93	(13.2)	
pN	pN0	369	(52.2)	369	(52.2)	1.0000
	pN1	184	(26.0)	184	(26.0)	
	pN2–3	154	(21.8)	154	(21.8)	
ASA physical status	ASA I	400	(56.6)	384	(54.3)	0.5510
	ASA II	167	(23.6)	172	(24.3)	
	ASA III–IV	140	(19.8)	151	(21.4)	
Adjuvant chemotherapy	No	254	(35.9)	243	(34.4)	0.7214
	Yes	453	(64.1)	464	(65.6)	
Adjuvant RT	No	410	(58.0)	419	(59.3)	0.3952
	Yes	297	(42.0)	288	(40.7)	
HR status	No	373	(52.8)	375	(53.0)	0.7520
	Yes	334	(47.2)	332	(47.0)	
HER2 status	No	577	(81.6)	586	(82.9)	0.5149
	Yes	130	(18.4)	121	(17.1)	
Nodal surgery	ALND	510	(72.1)	508	(71.9)	0.8629
	SLNB	197	(27.9)	199	(28.1)	
Hospital level	Academic centers	553	(78.2)	553	(78.2)	1.0000
	Nonacademic	154	(21.8)	154	(21.8)	
Follow-up time, months	Mean (SD)	55.9	(26.6)	43.3	(29.8)	0.7298
All-cause death		79	(11.2)	66	(9.3)	0.0901
Locoregional recurrence		44	(6.2)	27	(3.8)	0.0110
Distant metastasis		82	(11.6)	61	(8.6)	0.0521

IQR, interquartile range; PB-RA, paravertebral block-regional anesthesia; GA, general anesthesia; INHA, inhalational; SD, standard deviation; AJCC, American Joint Committee on Cancer; HER2, Human Epidermal Growth Factor Receptor-2; RT, radiotherapy; ASA, American Society of Anesthesiology; CCI, Charlson comorbidity index; T, tumor; N, nodal; pT, pathological tumor stage; pN, pathological nodal stage; ALND, axillary lymph node dissection; SNLB, sentinel lymph node biopsy.

balanced between the two groups (**Table 1**). Menopausal status, diagnosis year, CCI score, differentiation, AJCC clinical stages, pT, pN, hospital level, adjuvant RT, adjuvant chemotherapy, ASA physical status, HR status, HER2 status, and nodal surgery were similar after head-to-head PSM in the two cohorts, and no significant differences were observed in the variables between the two cohorts. The follow-up duration, LRR, DM, or all-cause death was not matched because oncological outcomes were inconsistent between the two groups (**Table 1**). The crude primary endpoint of LRR in women with breast IDC receiving TM under INHA-GA without propofol and PB-RA with propofol varied significantly ($p = 0.0110$; **Table 1**).

Prognostic Factors for All-Cause Death After Multivariate Cox Regression Analysis

No significant differences in OS were observed in explanatory variables except for age ≥ 50 years, differentiation grade II (moderate differentiation), grade III (poor differentiation), AJCC clinical stage II–III, pT2, pT3–4, pN1, and pN2–3 (**Table 2**). In the multivariate Cox regression analysis, the adjusted HR (aHR; 95% CI) of all-cause death for PB-RA with propofol compared with INHA-GA without propofol was 1.01 (0.68–1.51). The aHRs (95% CIs) of all-cause death for age ≥ 50 years, differentiation grade II, grade III, AJCC clinical stage II, clinical stage III, pT2, pT3–4, pN1, and pN2–3 were 1.64 (1.03–

2.62), 2.85 (1.13–7.15), 3.83 (1.48–9.93), 1.42 (1.12–2.45), 1.56 (1.28–3.13), 1.70 (1.07–2.72), 3.06 (1.72–5.43), 1.74 (1.07–2.83), and 3.55 (2.10–6.01), respectively, compared with age < 50 years, differentiation grade 1, AJCC clinical stage I, pT1, and pN0. The aHR of all-cause death for adjuvant chemotherapy was 0.40 (0.27–0.60) compared with no adjuvant chemotherapy.

Prognostic Factors for LRR After Multivariate Cox Regression Analysis

The aHR (95% CI) of LRR for the PB-RA with propofol group was 0.52 (0.28–0.96) compared with the INHA-GA without propofol group (**Table 3**). The aHRs of LRR for differentiation grade II, grade III, clinical stage II, stage III, pT2, pT3–4, and pN2–3 were 1.16 (1.04–2.08), 1.28 (1.07–2.12), 3.71 (1.82–7.59), 4.67 (1.65–13.18), 1.09 (1.02–1.21), 1.17 (1.03–2.16), 1.10 (1.03–1.33), and 1.22 (1.06–2.41), respectively, compared with differentiation grade I, clinical stage I, pT1, and pN0. The aHR of LRR for adjuvant RT was 0.88 (0.64–0.94) compared with that for no adjuvant RT.

Prognostic Factors for DM After Multivariate Cox Regression Analysis

The aHR (95% CI) of DM for the PB-RA with propofol group was 0.74 (0.49–1.10) compared with the INHA-GA without propofol group (**Table 4**). The aHRs of DM for clinical stage II, stage III, pT2, pT3–4, pN1, pN2–3, and HER2 positivity were 1.15 (1.06–2.46), 1.35 (1.12–2.92), 1.12 (1.02–2.21), 2.01 (1.12–3.59), 1.24 (1.11–2.29), 2.11 (1.22–3.64), and 2.06 (1.07–3.52), respectively, compared with clinical stage I, pT1, pN0, and HER2 negativity. The aHR of DM for adjuvant chemotherapy was 0.70 (0.46–0.96) compared with that for no adjuvant chemotherapy.

DISCUSSION

Most existing clinical studies were retrospective in nature or included a small sample, and meta-analyses included heterogeneous cancers, surgical techniques, patient

TABLE 2 | Multivariate analysis of all-cause death for propensity score-matched patients with breast cancer receiving total mastectomy under PB-RA with propofol or INHA-GA without propofol.

		All-cause death		
		aHR*	**(95% CI)**	**p-value**
Anesthesia	INHA-GA	ref		0.9497
	Propofol	1.01	(0.68–1.51)	
Age (years)	20–49	ref		0.0386
	50+	1.64	(1.03–2.62)	
Diagnosis year	2009–2013	ref		0.1900
	2014–2018	0.75	(0.49–1.15)	
Menopausal status	Premenopausal	ref		0.7093
	Postmenopausal	1.09	(0.75–1.54)	
CCI scores	0	ref		0.0807
	1	0.89	(0.54–1.46)	
	2+	1.56	(0.91–2.69)	
Differentiation	I	ref		0.0172
	II	2.85	(1.13–7.15)	
	III	3.83	(1.48–9.93)	
AJCC clinical stage	I	ref		0.0051
	II	1.42	(1.12–2.45)	
	III	1.56	(1.28–3.13)	
pT	pT1	ref		0.0007
	pT2	1.70	(1.07–2.72)	
	pT3–4	3.06	(1.72–5.43)	
pN	pN0	ref		<0.0001
	pN1	1.74	(1.07–2.83)	
	pN2–3	3.55	(2.10–6.01)	
Nodal surgery	ALND	ref		0.3374
	SLNB	1.06	(0.73–1.31)	
ASA	I	ref		0.1308
	II	1.03	(0.62–1.69)	
	III–IV	1.58	(0.93–2.68)	
Adjuvant chemotherapy	Yes	0.40	(0.27–0.60)	<0.0001
Adjuvant RT	Yes	0.96	(0.62–1.49)	0.8469
HR	Positive	0.98	(0.67–1.43)	0.9121
HER2	Positive	1.10	(0.72–1.68)	0.6563
Hospital level	Academic centers	ref		0.2536
	Nonacademic	1.29	(0.83–2.00)	

PB-RA, paravertebral block-regional anesthesia; GA, general anesthesia; INHA, inhalational; aHR, adjusted hazard ratios; CIs, confidence intervals; AJCC, American Joint Committee on Cancer; HR, Hormone Receptor; HER2, Human Epidermal Growth Factor Receptor-2; RT, radiotherapy; ASA, American Society of Anesthesiology; CCI, Charlson comorbidity index; T, tumor; N, nodal; pT, pathological tumor stage; pN, pathological nodal stage; ALND, axillary lymph node dissection; SNLB, sentinel lymph node biopsy; ref, reference group.
All covariates mentioned in **Table 2 were adjusted.*

TABLE 3 | Multivariate analysis of locoregional recurrence for propensity score-matched patients with breast cancer receiving total mastectomy under PB-RA with propofol or INHA-GA without propofol.

		LRR		
		aHR*	(95% CI)	p-value
Anesthesia	INHA-GA	ref		0.0365
	Propofol	0.52	(0.28–0.96)	
Age (years)	20–49	ref		0.9111
	50+	0.97	(0.55–1.72)	
Diagnosis year	2009–2013	ref		0.2513
	2014–2018	1.13	(0.90–3.75)	
Menopausal status	Premenopausal	Ref		0.7081
	Postmenopausal	0.81	(0.71–1.30)	
CCI scores	0	ref		0.1309
	1	1.04	(0.80–1.06)	
	2+	1.07	(0.76–2.49)	
Differentiation	I	ref		0.0099
	II	1.16	(1.04–2.08)	
	III	1.28	(1.07–2.12)	
AJCC clinical stage	I	ref		0.0012
	II	3.71	(1.82–7.59)	
	III	4.67	(1.65–13.18)	
pT	pT1	ref		0.0260
	pT2	1.09	(1.02–1.21)	
	pT3–4	1.17	(1.03–2.16)	
pN	pN0	ref		0.0022
	pN1	1.10	(1.03–1.33)	
	pN2–3	1.22	(1.06–2.41)	
Nodal surgery	ALND	ref		0.3066
	SLNB	1.55	(0.72–3.36)	
ASA	I	ref		0.2221
	II	1.16	(0.57–2.38)	
	III-IV	1.89	(0.90–3.96)	
Adjuvant chemotherapy	Yes	1.26	(0.71–2.25)	0.4343
Adjuvant RT	Yes	0.88	(0.64–0.94)	0.0413
HR	Positive	0.88	(0.68–3.28)	0.2252
HER2	Positive	1.64	(0.89–3.02)	0.1103
Hospital level	Academic centers	ref		0.1078
	Nonacademic	0.56	(0.28–1.13)	

PB-RA, paravertebral block-regional anesthesia; GA, general anesthesia; INHA, inhalational; aHR, adjusted hazard ratios; CIs, confidence intervals; AJCC, American Joint Committee on Cancer; HR, Hormone Receptor; HER2, Human Epidermal Growth Factor Receptor-2; RT, radiotherapy; ASA, American Society of Anesthesiology; CCI, Charlson comorbidity index; T, tumor; N, nodal; pT, pathological tumor stage; pN, pathological nodal stage; ALND, axillary lymph node dissection; SNLB, sentinel lymph node biopsy; ref, reference group.
All covariates mentioned in **Table 2 were adjusted.*

populations, and follow-up (8, 9, 11, 16). Multiple factors can be responsible for differences in study findings; for instance, the characteristics and treatments varied among patients with breast IDC in clinical studies, whereas fixed conditions were examined in preclinical studies (22–26, 43–45). Factors affecting breast cancer prognosis are diverse and complex (43–45). For example, adjuvant chemotherapy is indicated for women with advanced pathological stages of breast IDC receiving breast surgery (46, 47); however, no adjuvant chemotherapy was administered in preclinical studies (1–7). Clinical covariates including molecular status (HR or HER2 status) and adjuvant treatment (adjuvant RT or chemotherapy) might result in inconsistent findings in preclinical and clinical studies (1–7, 22–26, 43–45). The only published randomized controlled trial (RCT) including breast-conserving surgery (BCS) or TM for breast cancer showed that the administration of INHA-GA without propofol or PB-RA with propofol exerted no effect on the primary endpoint of cancer recurrence including LRR and DM in patients with breast cancer (20). Moreover, the findings of this RCT are different from those of preclinical studies (1–7). Thus, to address these problems, we included LRR as the primary endpoint and performed PSM to control for all potential covariates in this study with the consistent surgical procedure.

The novelty of our study is the inclusion of LRR as the primary endpoint. No study has included LRR as a study endpoint. We controlled for all the potential covariates of LRR (**Table 1**) and observed no bias between the INHA-GA without propofol and PB-RA with propofol groups through PSM. Additionally, the various extent of surgery might be associated with different hypoxia time related with local recurrence (48, 49). Thus, in our study we maintain a consistent surgical procedure (all patients receiving TM) for breast IDC patients. Our results revealed that patients with breast IDC receiving TM under PB-RA with propofol had a significantly decreased risk of LRR compared with those receiving TM under INHA-GA (sevoflurane) without propofol (**Table 3**). A similar benefit was not observed for OS,

TABLE 4 | Multivariate analysis of distant metastasis for propensity score-matched patients with breast cancer receiving total mastectomy under PB-RA with propofol or INHA-GA without propofol.

		DM		
		aHR*	(95% CI)	p-value
Anesthesia	INHA-GA	ref		0.1369
	Propofol	0.74	(0.49–1.10)	
Age (years)	20–49	ref		0.7548
	50+	0.94	(0.62–1.41)	
Diagnosis year	2009–2013	ref		0.2296
	2014–2018	0.77	(0.50–1.18)	
Menopausal status	Premenopausal	ref		0.4711
	Postmenopausal	0.79	(0.68–1.51)	
CCI scores	0	ref		0.8673
	1	0.88	(0.54–1.43)	
	2+	0.90	(0.47–1.72)	
Differentiation	I	ref		0.7573
	II	1.26	(0.62–2.54)	
	III	1.33	(0.63–2.82)	
AJCC clinical stage	I	ref		0.0089
	II	1.15	(1.06–2.46)	
	III	1.35	(1.12–2.92)	
pT	pT1	ref		0.0015
	pT2	1.12	(1.02–2.21)	
	pT3–4	2.01	(1.12–3.59)	
pN	pN0	ref		0.0073
	pN1	1.24	(1.11–2.29)	
	pN2–3	2.11	(1.22–3.64)	
Nodal surgery	ALND	ref		0.2283
	SLNB	1.09	(0.88–3.25)	
ASA	I	ref		0.9537
	II	1.07	(0.59–1.58)	
	III–IV	1.12	(0.62–1.80)	
Adjuvant chemotherapy	Yes	0.70	(0.46–0.96)	0.0157
Adjuvant RT	Yes	0.81	(0.52–1.26)	0.3475
HR	Positive	0.96	(0.73–1.55)	0.7624
HER2	Positive	2.06	(1.07–3.52)	<0.0001
Hospital level	Academic centers	ref		0.4898
	Nonacademic	0.85	(0.53–1.36)	

PB-RA, paravertebral block-regional anesthesia; GA, general anesthesia; INHA, inhalational; aHR, adjusted hazard ratios; CIs, confidence intervals; AJCC, American Joint Committee on Cancer; HR, Hormone Receptor; HER2, Human Epidermal Growth Factor Receptor-2; RT, radiotherapy; ASA, American Society of Anesthesiology; CCI, Charlson comorbidity index; T, tumor; N, nodal; pT, pathological tumor stage; pN, pathological nodal stage; ALND, axillary lymph node dissection; SNLB, sentinel lymph node biopsy; ref, reference group.
All covariates mentioned in **Table 2 were adjusted.*

possibly because adjuvant treatments might have masked the benefits of PB-RA with propofol; studies with longer follow-up duration should be conducted to examine the effect on OS. In addition, the proportion of patients who developed LRR in our study was small (3.8% and 6.2% for non-propofol and propofol groups, respectively); a larger sample size would be necessary to examine OS. However, our study is the first to investigate the effect of the administration of INHA-GA without propofol or PB-RA with propofol on LRR in patients with breast IDC receiving TM. Our findings for LRR are different from those reported by Sessler et al. who included DM and LRR together to examine cancer recurrence (20). Moreover, to maintain a consistent extent of surgery, we enrolled patients who received TM only and matched them at a ratio of 1:1 by using the greedy method (**Table 1**). In theory, the consistent time and the same extent of surgery related with similar levels of hypoxia (49) could be more consistent between the two anesthesia techniques in our study than Sessler et al.'s study (20). Tissue hypoxia causes an upregulated expression of the transcription factor hypoxia-inducible factor 1-alpha, which is crucial for the promotion of cellular pathways for angiogenesis, cell proliferation, and metastasis (48). Moreover, preclinical studies have reported that propofol exhibits the anticancer property by exerting an immune effect (4, 50, 51). Patients receiving PB-RA with propofol demonstrated an increased level of immune cell infiltration into the breast cancer tissue, an increased level of cancer cell apoptosis, and preserved cytotoxicity of natural killer cells (4, 50, 51). The advantages of PB-RA with propofol observed in preclinical studies were reproduced in our clinical study through head-to-head PSM. Our clinical study indicated differentiation grade II–III, clinical stage II–III, pT2, pT3–4, and pN2–3 as independent poor prognostic factors of LRR; this finding is compatible with those of previous clinical studies (22–26) Adjuvant RT reduced the risk of LRR in patients with breast IDC receiving TM (**Table 3**); this result is also in agreement with that of a previous clinical study (52).

In our study, we examined OS (the secondary endpoint) in patients with breast IDC receiving TM under INHA-GA without propofol and PB-RA with propofol (**Table 2**). We observed that the administration of INHA-GA without propofol or PB-RA with propofol did not exert any effect on the OS of these patients; this finding is compatible with those of previous clinical studies (**Table 2**) (8, 11, 53). All existing studies examining the endpoint of OS were retrospective in nature and included a small sample size, heterogeneous cancers, various surgical techniques, different patient populations, and short follow-up durations (8, 9, 11, 16). A meta-analysis conducted in 2014 found no difference in OS among patients with breast, prostate, colon, and gastroesophageal cancers who received general epidural anesthesia versus GA alone (8). Similarly, in 2017, a meta-analysis of 28 studies (retrospective, observational, and randomized) reported that OS was similar in patients with various cancers who underwent surgery under RA with or without GA and those who underwent surgery under GA alone (11). A meta-analysis of 10 retrospective studies including approximately 13,760 patients who underwent radical prostatectomy for cancer found that RA with or without GA was associated with improved OS but similar cancer recurrence compared with GA alone (9). Furthermore, a meta-analysis suggested that RA was associated with improved OS, particularly in patients with colorectal cancer, as well as a reduced risk of cancer recurrence (10). Therefore, conflicting results have been reported in clinical studies including different cancer types, extents of surgery, and adjuvant treatments (8–11, 16). The inconsistency in the results of clinical and preclinical studies might be attributed to the use of different therapeutic modalities, such as adjuvant chemotherapy, hormone therapy, and tyrosine kinase inhibitors, and different surgical procedures, which might have masked the effects of different anesthesia techniques (RA with propofol or sevoflurane-based INHA-GA) on patients' OS (22–26, 43–45). By contrast, the findings of multivariate analysis performed in our study indicated that old age, moderate-poor differentiation (grade II–III) (54), clinical stage II–III, pT2, pT3–4, pN1, and pN2–3 were independent poor prognostic factors for all-cause death; this finding is compatible with those of previous clinical studies (20, 22–26). Adjuvant chemotherapy was associated with better OS in patients with breast IDC receiving TM (**Table 2**); this finding is also in accordance with those of previous clinical studies (46, 47, 52). Because the trend of oncological outcomes and prognostic factors for OS in our study was similar to that reported in other studies (20, 22–26, 46, 47, 52, 54–56), the effect of the administration of INHA-GA without propofol or PB-RA with propofol on oncological outcomes (OS, LRR, and DM) in patients with IDC receiving TM might truly exist in real-world clinical practice, although clinical outcomes might vary for different molecular breast types, adjuvant treatments, or extents of surgery. In the current study, most confounding factors like molecular breast types, adjuvant treatments, or extents of surgery (BCS or TM) were consistent or adjusted in our analysis.

As shown in **Table 4**, we observed that the risk of DM was not associated with the administration of INHA-GA without propofol or PB-RA with propofol in patients with IDC receiving TM; this finding differs from those of previous preclinical studies (1–7). Although many preclinical studies have reported that volatile anesthetics can enhance metastasis, such as by exerting direct survival-enhancing effects on cancer cells, suppressing immune cell functions, and killing tumor cells (2–4, 51), no association of DM with the administration of INHA-GA without propofol or PB-RA with propofol in patients with breast IDC receiving TM was observed in our clinical study. In laboratory studies, propofol exhibited antitumor effects by directly regulating key ribonucleic acid pathways and signaling in cancer cells (7). In addition, propofol exerts anti-inflammatory and antioxidative effects (1, 6, 50), which may protect against perioperative immune suppression. Although many preclinical studies have shown that propofol might inhibit cancer metastasis and INHA-GA can enhance cancer metastasis (1–4, 6, 50, 51), these phenomena were not observed in our study (**Table 4**). This difference might be attributed to the use of different adjuvant treatments and the inclusion of various breast cancer molecular types that might have obscured the effects of propofol and sevoflurane (43–45). However, other independent poor or better prognostic factors such as clinical stage II–III, pT2, pT3–4, pN2–3, HER2 positivity, and adjuvant chemotherapy determined in this study are compatible with those observed in previous clinical studies (22–26). **Supplementary Figures 1A–C** present survival curves for OS and LRR-free and DM-free survival obtained using the Kaplan–Meier method for the propensity score-matched cohort of patients with breast IDC receiving TM under PB-RA with propofol or INHA-GA without propofol. The crude LRR-free survival without adjustment for PB-RA with propofol was not significantly longer than that for INHA-GA without propofol for all patients with breast IDC receiving TM ($p = 0.1430$).

The strength of our study is that this is the first and largest cohort study to estimate the primary endpoint of LRR for patients with breast IDC receiving TM under INHA-GA without propofol and PB-RA with propofol. The covariates between the two anesthesia techniques were homogenous for women with breast IDC receiving TM; no selection bias was observed for the two anesthesia techniques through PSM (**Table 1**). No study has examined the effect of PB-RA with propofol on LRR in patients with breast cancer receiving TM, and all prognostic factors including clinical and pathologic stages and molecular types were evaluated. Poor prognostic factors for OS, LRR, or DM determined in patients with breast cancer receiving TM in the present study, namely, moderate-poor differentiation, advanced clinical stages, advanced pathologic TN stages, HER2 positivity, adjuvant RT, and adjuvant chemotherapy (**Tables 2–4**), are similar to those reported in previous studies (57–61). In patients with breast IDC receiving TM, adjuvant RT reduced the risk of LRR and adjuvant chemotherapy reduced the risk of DM. However, PB-RA with propofol in patients with breast IDC receiving TM was beneficial only for LRR instead of all-cause death and DM. This is the first study to show that PB-RA with propofol reduced the risk of LRR. Previous studies did not focus on recurrence; thus, LRR and DM could not be distinguished (20, 57–65). Our study is the first to

examine the effects of INHA-GA without propofol or PB-RA with propofol on LRR and DM individually instead of breast cancer recurrence including LRR and DM. Our findings should be considered in future clinical practice and prospective clinical trials.

This study has some limitations. First, because all patients with breast IDC were enrolled from an Asian population, the corresponding ethnic susceptibility compared with the non-Asian population remains unclear; hence, our results should be cautiously extrapolated to non-Asian populations. However, no evidence has demonstrated differences in oncological outcomes between Asian and non-Asian patients with breast IDC receiving TM. Second, recently, the propensity score could be currently recommended as a standard tool for investigators trying to estimate the effects of intervention in studies where any potential bias may exist. Although the main advantage of the propensity score methodology is in its contribution to the more precise estimation of intervention response, PSM cannot control for factors not accounted for in the model and is predicated on an [explicit selection bias] of those whom could be a match (i.e., those who could not be matched are not part of the scope of inference). Third, the diagnoses of all comorbid conditions were based on ICD-9-CM codes. Nevertheless, the Taiwan Cancer Registry Administration randomly reviews charts and interviews patients to verify the accuracy of diagnoses, and hospitals with outlier chargers or practices may be audited and subsequently be heavily penalized if malpractice or discrepancies are identified. Accordingly, to obtain crucial information regarding population specificity and disease occurrence, a large-scale randomized trial comparing carefully selected patients undergoing suitable extent of surgery, consistent molecular types, and treatments is essential. Finally, the Taiwan Cancer Registry database does not contain information regarding dietary habits, lifestyle factors, socioeconomic status, or body mass index, all of which may be risk factors for LRR or mortality. However, considering the magnitude and statistical significance of the observed effects in this study, these limitations are unlikely to affect the conclusions.

CONCLUSIONS

PB-RA with propofol might be beneficial in reducing LRR in women with breast IDC receiving TM compared with INHA-GA without non-propofol. INHA-GA without propofol or PB-RA with propofol was not associated with the risk of OS or DM in patients with breast IDC receiving TM.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because we used data from the National Health Insurance Research Database (NHIRD) and Taiwan Cancer Registry database. The authors confirm that, for approved reasons, some access restrictions apply to the data underlying the findings. The data utilized in this study cannot be made available in the manuscript, the **Supplementary Material**, or in a public repository due to the "Personal Information Protection Act" executed by Taiwan's government, starting from 2012. Requests for data can be sent as a formal proposal to obtain approval from the ethics review committee of the appropriate governmental department in Taiwan. Specifically, links regarding contact info for which data requests may be sent to are as follows: http://nhird.nhri.org.tw/en/Data_Subsets.html#S3 and http://nhis.nhri.org.tw/point.html. Requests to access the datasets should be directed to http://nhird.nhri.org.tw/en/Data_Subsets.html#S3 and http://nhis.nhri.org.tw/point.html.

ETHICS STATEMENT

Our study protocols were reviewed and approved by the Institutional Review Board of Tzu-Chi Medical Foundation (IRB109-015-B). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conception and Design: JZ, C-LC, C-YL, H-MC, and S-YW. Collection and Assembly of Data: H-MC and S-YW. Data Analysis and Interpretation: JZ, C-LC, and S-YW. Administrative Support: S-YW. Manuscript Writing: JZ, C-LC, and S-YW. Final Approval of Manuscript: All authors. All authors contributed to the article and approved the submitted version.

FUNDING

Lo-Hsu Medical Foundation, LotungPoh-Ai Hospital, supports Szu-Yuan Wu's work (Funding Number: 10908, 10909, 11001, 11002, 11003, 11006, and 11013).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.708632/full#supplementary-material

Supplementary Figure 1 | (A) Kaplan–Meier overall survival curves of propensity score–matched patients with breast cancer receiving total mastectomy under PB-RA with propofol or INHA-GA without propofol. **(B)** Kaplan–Meier locoregional recurrence-free survival curves of propensity score–matched patients with breast cancer receiving total mastectomy under PB-RA with propofol or INHA-GA without propofol. **(C)** Kaplan–Meier distant metastasis-free survival curves of propensity score–matched patients with breast cancer receiving total mastectomy under PB-RA with propofol or INHA-GA without propofol.

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frontiers in Oncology

ORIGINAL RESEARCH
published: 28 March 2022
doi: 10.3389/fonc.2022.837974

Propofol Inhibits Proliferation and Augments the Anti-Tumor Effect of Doxorubicin and Paclitaxel Partly Through Promoting Ferroptosis in Triple-Negative Breast Cancer Cells

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OPEN ACCESS

Edited by:
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Imperial College London, United Kingdom

Reviewed by:
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

Received: *17 December 2021*
Accepted: *04 March 2022*
Published: *28 March 2022*

Citation:
Sun C, Liu P, Pei L, Zhao M and Huang Y (2022) Propofol Inhibits Proliferation and Augments the Anti-Tumor Effect of Doxorubicin and Paclitaxel Partly Through Promoting Ferroptosis in Triple-Negative Breast Cancer Cells. Front. Oncol. 12:837974. doi: 10.3389/fonc.2022.837974

Background: Triple-negative breast cancer (TNBC) is relatively common in women and is associated with a poor prognosis after surgery and adjuvant chemotherapy. Currently, the mechanism underlying the relationship between propofol and breast cancer is controversial and limited to cell apoptosis. Moreover, there are only a few studies on the effect of propofol on the chemotherapeutic sensitivity of TNBC cells. Therefore, this study explored whether propofol and its commonly used clinical formulations affect the proliferation and chemotherapeutic effects on TNBC cells by regulating cell ferroptosis.

Methods: We selected MDA-MB-231 cells, and the effects of propofol, propofol injectable emulsion (PIE), or fospropofol disodium, alone or combined with doxorubicin or paclitaxel on cell viability, apoptosis, intracellular reactive oxygen species (ROS) accumulation, ferroptosis-related morphological changes, intracellular Fe^{2+} levels, and the expression and localization of ferroptosis-related proteins were investigated.

Results: We found that propofol significantly inhibited MDA-MB-231 cell proliferation, and all three propofol formulations augmented the anti-tumor effects of doxorubicin and paclitaxel. The results from the ROS assay, transmission electron microscopy, intracellular Fe^{2+} assay, western blotting, and multiplex immunohistochemistry revealed that propofol not only induced apoptosis but also triggered ferroptosis-related changes, including morphological changes of mitochondria, increased intracellular ROS levels, and intracellular iron accumulation in MDA-MB-231 cells. The ferroptosis-related p53-SLC7A11-GPX4 pathway was also altered under different treatment propofol, doxorubicin, or paclitaxel regimens.

Conclusion: Propofol showed anti-proliferation effects on TNBC cells and could be a potential adjuvant to enhance the chemotherapeutic sensitivity of TNBC cells partly by promoting cell ferroptosis.

Keywords: propofol, triple-negative breast cancer, ferroptosis, propofol injectable emulsion, fospropofol disodium

INTRODUCTION

Breast cancer is the most common cancer in women and the second leading cause of cancer death (1, 2). Among breast cancers, triple-negative breast cancer (TNBC), which lacks the expression of hormone receptors and human epidermal growth factor receptor 2 (HER2), is associated with a higher rate of recurrence and poorer prognosis after surgery and adjuvant chemotherapy (3). Therefore, it is necessary to optimize treatment strategies for TNBC.

Surgery is currently the primary treatment for breast cancer. As one of the most commonly used intravenous anaesthetics (4), propofol (2,6-diisopropylphenol) has also shown many non-anesthetic effects, such as inhibitory effect on tumor in recent investigations (5). Most studies have found that propofol inhibits proliferation, migration, and invasion of cancer cells and promotes cell apoptosis *in vitro* by regulating the expression of various signaling pathways and non-coding microRNAs (6, 7), suppressing tumor growth and metastasis *in vivo* (8, 9), and increasing sensitivity to chemotherapeutics (10, 11).

The current findings remain controversial in terms of breast cancer. In clinical research, some studies demonstrated that compared with inhalation anesthetics, using propofol during surgery could improve the prognosis of patients with breast cancer (12); however, this effect has not been observed in other studies (13). As for *in vitro* studies, most studies showed that propofol inhibited the proliferation of breast cancer cells through different pathways. For example, propofol induces MDA-MB-435 cell apoptosis by downregulating the miR-24 signaling pathway (14). In addition, by suppressing miR-21 expression, propofol could inhibit the proliferation and epithelial-mesenchymal transition of MCF-7 cells (15). However, researchers also found that propofol may promote the proliferation and metastasis of breast cancer (16, 17). Besides the controversy, the proposed underlying mechanisms in the aforementioned *in vitro* studies are mainly based on cell apoptosis and related pathways, and they only choose propofol itself in their experiments, without including the commonly used clinical propofol formulations, such as propofol injectable emulsion (PIE), limiting the clinical value. Moreover, there are few studies on the effect of propofol on the chemotherapeutic sensitivity of TNBC cells.

Ferroptosis is an iron-dependent form of regulated cell death driven by excessive lipid peroxidation, which is morphologically, biochemically, and genetically distinct from apoptosis, necrosis, and autophagy (18). Furthermore, it is reported to be involved in various diseases such as cancer, stroke, and diabetes (19–22). Ferroptosis is characterized by the accumulation of intracellular iron and lipid reactive oxygen species (ROS) and is regulated by several pathways related to intracellular redox reactions such as the SLC7A11-GPX4 (23) and the ubiquinone-FSP1-ubiquinol axes (24, 25). In the past decade, ferroptosis has been implicated in the development and therapeutic response of various tumor types. Previous studies have suggested that inducing ferroptosis may be an effective strategy for tumor treatment and preventing acquired resistance to multiple chemotherapeutic agents (19).

In this study, we aimed to explore whether propofol, PIE, and fospropofol disodium affect the proliferation of TNBC cells and chemotherapeutic effects by regulating cell ferroptosis, hoping to provide novel treatment strategies for patients with TNBC.

MATERIALS AND METHODS

Cell Lines and Reagents

The human breast cancer cell line MDA-MB-231, a TNBC cell line, was obtained from China Infrastructure of Cell Line Resource (Beijing, China). Propofol was obtained from Sigma-Aldrich (1572503; St. Louis, MO, USA), with PIE from AstraZeneca (Cambridge, UK) and fospropofol disodium from Yichang Humanwell Pharmaceutical Co., Ltd. (LB52200301; Hubei, China). Doxorubicin (A1832) and paclitaxel (A4393) were purchased from Applied Biosystems (Houston, TX, USA).

Cell Culture and Drug Application

MDA-MB-231 cells were cultured in Roswell Park Memorial Institute 1640 medium (C11875500BT; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (AQmv09900; Analysis Quiz, Uruguay, South America) and 1% penicillin-streptomycin (15140-122; Gibco) in an incubator with 5% CO_2 at 37°C. Propofol and fospropofol disodium were first dissolved in dimethyl sulfoxide (DMSO) (D2650; Sigma Aldrich) and normal saline respectively to prepare 50mg/ml store solution, and then diluted into 2, 5, 10 and 20 μg/ml using complete cell culture medium. PIE, 10mg/ml originally, was directly diluted in complete medium at 2, 5, 10, and 20 μg/ml. Doxorubicin and paclitaxel were also dissolved in DMSO and then in complete medium at 0.01, 0.1, 0.5, 1, 5, and 10 μg/ml. The final concentration of DMSO was less than 0.1%. All drugs were administered at different concentrations when cell growth reached 60-70% confluence, and treatment continued for 24 h.

Cell Viability Assay

The viability of MDA-MB-231 cells was determined using a Cell Counting Kit-8 (CCK-8, CK04; Dojindo Laboratories, Japan). Briefly, the cells were digested with 0.05% trypsin-EDTA (25300-062; Gibco) and then inoculated into Costar® 96-well plates (Corning Inc., Corning, NY, USA) at a cell density of 2×10^4 cells

per well. When the fusion rate of cells increased to 60-70% after overnight culture, the cells were replenished with fresh medium containing drugs at the indicated concentrations. The cells were then incubated for 24 h and subsequently replenished with fresh medium containing 10 μl of CCK-8 reagent for each well. After fully reacting in a 37°C incubator in the dark for another 2 h, an Epoch Microplate Reader (BioTek, Winooski, VT, USA) was used to measure the optical density (OD) of the cells in each well at 450 nm. The percentage of viable cells was determined using the following formula: cell viability (%) = (treatment group OD – blank group OD)/(control group OD – blank group OD) × 100%. Each experiment was conducted in triplicate.

Flow Cytometry

An Annexin V-FITC Apoptosis Detection Kit (AD10; Dojindo Laboratories, Kumamoto, Japan) was used to analyze MDA-MB-231 cell apoptosis. Briefly, the cells were seeded into Costar® 6-well plates (Corning Inc.) and digested with 0.05% trypsin after treatment with the drugs for 24 h, then collected and washed twice with ice-cold phosphate-buffered saline (PBS, 1×; Hyclone Laboratories Inc., Logan, UT, USA). Thereafter, a 1× Annexin V binding solution was added to make a cell suspension at a final concentration of 1×10^6 cells/ml. The cells were then stained with Annexin V-FITC and propidium iodide (PI) for 15 min in the dark at room temperature (RT). After adding 400 μl of 1× Annexin V binding solution into each tube of the cell suspension, the cells were loaded onto a flow cytometer (Accuri C6 Plus; BD BioSciences, Franklin Lakes, NJ, USA) within 1 h. The results of three independent experiments were analyzed using BD Accuri C6 Plus software, according to the manufacturer's instructions.

Intracellular Reactive Oxygen Species (ROS) Assay

The DCFDA/H2DCFDA-Cellular ROS Assay Kit (ab113851; Abcam, Cambridge, UK) was used to analyze intracellular ROS levels in MDA-MB-231 cells. Briefly, the cells were grown in eight-chambered slides (155411; Nalge Nunc International Corporation, Rochester, NY, USA) to an appropriate density before treatment with the drugs. After 24 h of cultivation in 5% CO_2 at 37°C, cells were washed twice with 1× buffer and stained with diluted DCFDA solution for 45 min at 37°C in the dark. Subsequently, the cells were washed twice with 1× buffer and viewed using a real-time live-cell laser scanning confocal microscope (UltraVIEW VOX; PerkinElmer, Waltham, MA, USA) with a filter set appropriate for fluorescein (FITC) under low light conditions. Intracellular ROS-positive cells were counted, and their ratio was calculated by comparing them with total cells in each microscope field.

Transmission Electron Microscopy (TEM)

MDA-MB-231 cells were trypsinized and centrifuged at 1 000 rpm for 5 min, followed by fixation in 2.5% glutaraldehyde (EM Grade, P1126; Solarbio Life Sciences, Beijing, China) at 4°C overnight. Next, the cell samples were fixed in 1% osmium acid, dehydrated, and embedded in molds in a standard fashion. Afterwards, appropriate areas were selected, and ultrathin sections (0.08 μm) were stained with lead citrate and uranyl acetate for 5-10 min at approximately 95°C. Finally, the sections were analyzed by TEM (JEM-1400Plus; JEOL Ltd., Tokyo, Japan).

FerroOrange Iron Assay

MDA-MB-231 cells were inoculated into eight-chambered slides and cultivated until the fusion rate reached 60-70%. Next, the original medium was discarded and replaced with fresh medium containing the drugs at the indicated concentrations. After incubation at 37°C for 24 h, the cells were washed with serum-free medium thrice. Subsequently, 1 μmol/L FerroOrange working solution (F374; Dojindo Laboratories Inc.) was added into each chamber and cultivated in 5% CO_2 at 37°C for 30 min. Thereafter, cell samples were imaged using a real-time live-cell laser scanning confocal microscope (UltraVIEW VOX; PerkinElmer, Inc.). The relative mean fluorescence intensity (MFI) of intracellular ferrous ions in different microscopic fields was calculated and analyzed using ImageJ software.

Western Blotting

MDA-MB-231 cells were lysed in RIPA buffer (R0020; Solarbio Life Sciences) for 30 min at 4°C after treatment with the drugs for 24 h. The lysates were centrifuged at 12 000 rpm for 15 min at 4°C, and the supernatant containing total protein was harvested and then denatured. Next, protein concentrations were determined using a bicinchoninic acid (BCA) assay system (Beyotime, Shanghai, China). Protein samples were then separated by 10 or 15% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis, according to molecular weight, and then electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (with 0.45-μm diameter pores). After 2 h of blocking with 5% skim milk at RT, the PVDF membranes were incubated in primary antibodies diluted in universal antibody diluent (WB500D; NCM Biotech, Suzhou, China) at 4°C overnight; the primary antibodies included anti-caspase-3 (1:5 000; Abcam, ab32351), anti-Bcl-2 (1:1 000; Abcam, ab182858), anti-GPX4 (1:2 000; Abcam, ab125066), anti-SLC7A11 (1:2 000; Abcam, ab175186), anti-p53 (1:1 000; CST, #2524), anti-ubiquinol-cytochrome C reductase core (1:4 000; Abcam, ab110252), anti-ubiquinone (1:500; Proteintech, 17812-1-AP), and anti-FSP1 (1:500; Proteintech, 20886-1-AP). Thereafter, the membranes were incubated with goat anti-mouse/anti-rabbit secondary antibodies (1:5 000; Proteintech, SA00001-1, SA00001-2) conjugated with horseradish peroxidase (HRP) at RT for 2 h, followed by detection using an enhanced chemiluminescence detection kit (P10100, NCM Biotech), and images were captured using a chemiluminescence imaging system (Tanon 5800, Tanon Science & Technology Co., Ltd. Shanghai, China). β-actin (1:20 000; Proteintech, 66009-1-Ig) was used as an internal control.

Multiplex Immunohistochemistry (mIHC)

The expression and localization of proteins involved in apoptosis and ferroptosis were identified using mIHC modified for adherent cells (patent pending), using a PANO Multiplex IHC Kit (0001100100; Panovue, Beijing, China).

Statistical Analysis

Quantitative variables were expressed as the mean ± standard deviation, and Student's *t*-test was used to analyze the differences between the two groups. $P < 0.05$ was considered statistically significant. All data were analyzed using GraphPad Prism 8.2.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Propofol Inhibited Proliferation, and Propofol/PIE/Fospropofol Disodium Enhanced Sensitivity to Doxorubicin and Paclitaxel of MDA-MB-231 Cells

The viability of MDA-MB-231 cells was determined using a CCK-8 assay after treatment with 0-20 μg/ml propofol, PIE, or fospropofol disodium, or 0.01-10 μg/ml doxorubicin or paclitaxel for 24 h. As shown in **Figure 1**, exposure to propofol resulted in a significant dose-dependent decrease in cell proliferation compared with control (**Figure 1A**), while proliferation was inhibited following treatment with PIE or fospropofol disodium, although not significantly (**Figures 1B, C**). Cell proliferation was inhibited in a dose-dependent manner by either doxorubicin or paclitaxel (**Figures 1D, E**).

To investigate whether propofol/PIE/fospropofol disodium has synergistic effects with doxorubicin or paclitaxel, we administered 10 μg/ml propofol/PIE/fospropofol disodium together with 0.5, 1, and 5 μg/ml doxorubicin or paclitaxel to MDA-MB-231 cells. As shown in **Figures 1F–K**, combination with propofol induced significant inhibition of proliferation compared with that of doxorubicin alone (cell viability, 72.83 ± 3.22% vs. 56.90 ± 1.42%, $P < 0.001$; 69.09 ± 3.24% vs. 58.64 ± 3.38%, $P < 0.01$; 43.75 ± 2.92% vs. 38.93 ± 2.22%, $P < 0.05$, respectively). Similarly, propofol combined with paclitaxel significantly inhibited cell proliferation compared with that of paclitaxel alone (cell viability, 75.73 ± 2.15% vs. 64.68 ± 3.76%, $P < 0.001$; 72.21 ± 3.35% vs. 59.94 ± 4.43%, $P < 0.001$; 62.29 ± 3.67% vs. 54.61 ± 5.60%, $P < 0.05$, respectively). Consistently, similar results were obtained from PIE and fospropofol disodium when combined with doxorubicin or paclitaxel. Based on the results above, we selected 10 μg/ml propofol/PIE/fospropofol disodium together with 1 μg/ml doxorubicin or paclitaxel for further experiments.

Propofol/PIE/Fospropofol Disodium Induced MDA-MB-231 Cell Apoptosis

We analyzed MDA-MB-231 cell apoptosis following different drug treatments. Apoptotic morphological changes, such as chromatin condensation, apoptotic bodies, and nuclear fragmentation, were observed using TEM in all drug-treated groups (**Figure 2A**).

Flow cytometry was used to determine the percentage of apoptotic cells (**Figures 2B, C**). Considering the spontaneous fluorescence of doxorubicin, this analysis was not performed in the doxorubicin-treated groups. As can be seen from **Figure 2C**, compared with the control group, cells exposed to 10 μg/ml propofol/PIE/fospropofol disodium alone were associated with a significantly higher percentage of early apoptotic cells (3.88 ± 0.39% vs. 1.10 ± 0.20%, $P < 0.001$; 2.61 ± 0.29% vs. 1.10 ± 0.20%, $P < 0.01$; 2.57 ± 0.26% vs. 1.10 ± 0.20%, $P < 0.01$, respectively), late apoptotic cells (2.46 ± 0.27% vs. 0.31 ± 0.03%, $P < 0.001$; 3.17 ± 0.09% vs. 0.31 ± 0.03%, $P < 0.001$; 4.92 ± 0.43% vs. 0.31 ± 0.03%, $P < 0.001$, respectively), and necrotic cells (0.72 ± 0.13% vs. 0.17 ± 0.04%, $P < 0.01$; 1.04 ± 0.13% vs. 0.17 ± 0.04%, $P < 0.001$; 2.74 ± 0.27% vs. 0.17 ± 0.04%, $P < 0.001$, respectively).

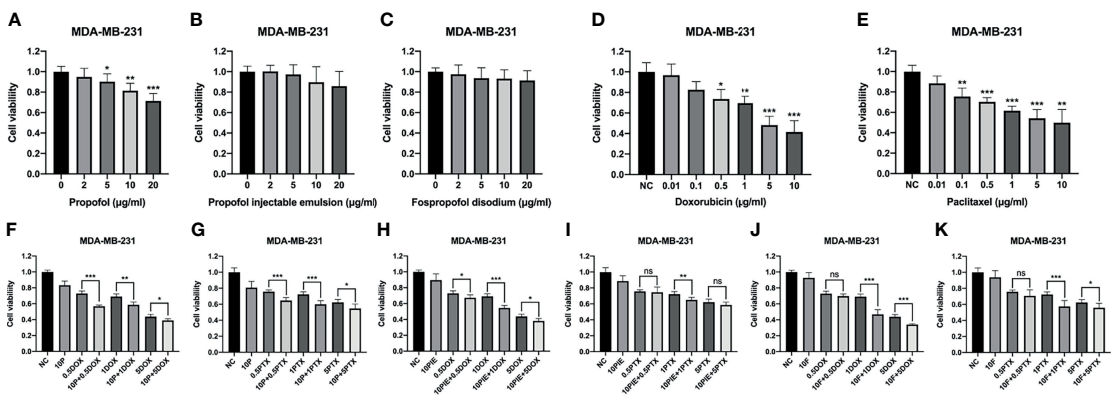

FIGURE 1 | Cell viability of MDA-MB-231 cells treated with propofol/PIE/fospropofol disodium, doxorubicin/paclitaxel, or drug combinations at different concentrations. **(A–C)** Effects of propofol, PIE or fospropofol disodium on MDA-MB-231 cell viability. **(D, E)** Effects of doxorubicin or paclitaxel on MDA-MB-231 cell viability. **(F, G)** Effects of propofol with doxorubicin or paclitaxel on MDA-MB-231 cell viability. **(H, I)** Effects of PIE with doxorubicin or paclitaxel on MDA-MB-231 cell viability. **(J, K)** Effects of fospropofol disodium with doxorubicin or paclitaxel on MDA-MB-231 cell viability. Cell viability was determined using CCK-8 assays, and each experiment was repeated at least three times. NC, normal control; 10P, 10 μg/ml propofol; 10PIE, 10 μg/ml propofol injectable emulsion; 10F, 10 μg/ml fospropofol disodium; 0.5DOX, 0.5 μg/ml doxorubicin; 1DOX, 1 μg/ml doxorubicin; 5DOX, 5 μg/ml doxorubicin; 0.5PTX, 0.5 μg/ml paclitaxel; 1PTX, 1 μg/ml paclitaxel; 5PTX, 5 μg/ml paclitaxel. ns, no significant difference. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

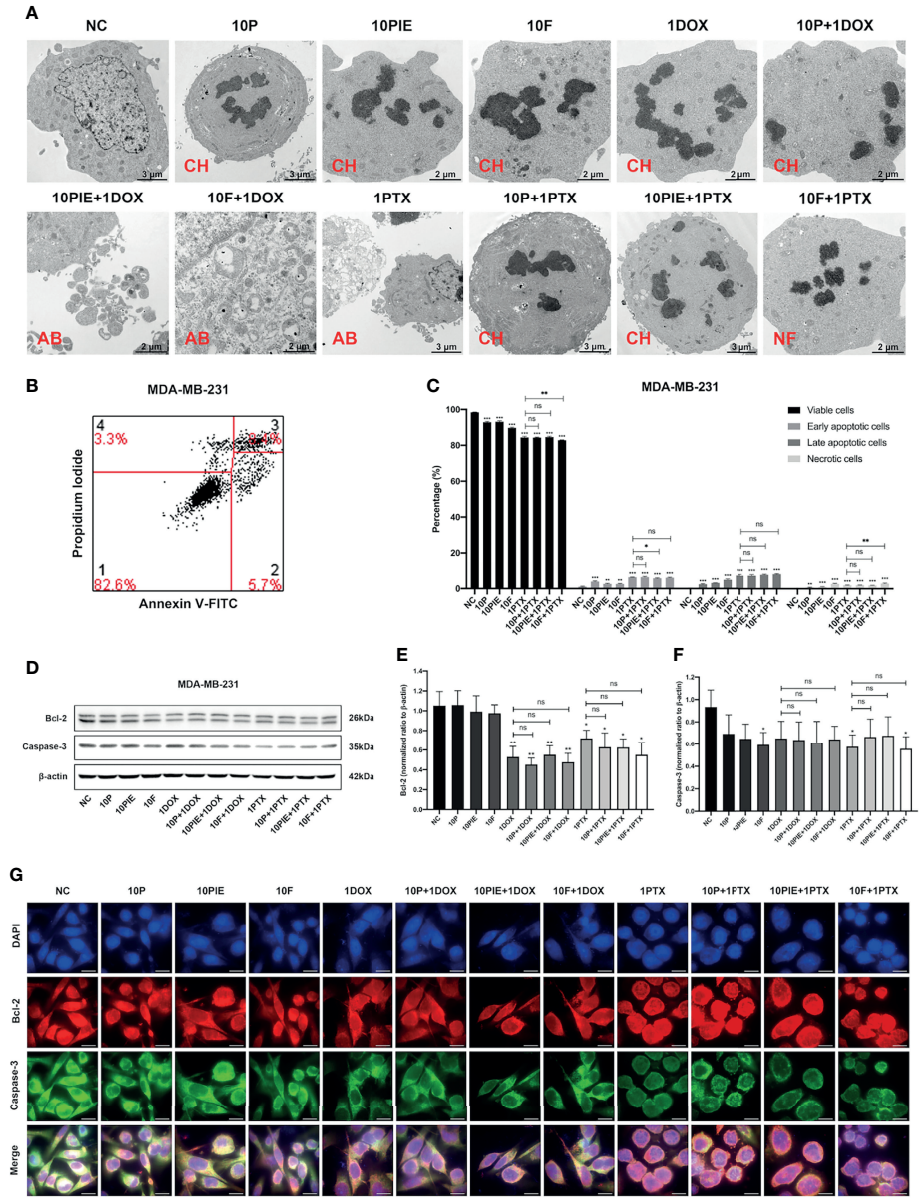

FIGURE 2 | Apoptosis analyses of MDA-MB-231 cells exposed to propofol/PIE/fospropofol disodium with or without doxorubicin/paclitaxel. **(A)** Apoptotic morphological changes of MDA-MB-231 cells treated with different drugs, observed *via* TEM. CH, chromatin condensation; AB, apoptotic bodies; NF, nucleus fragmentation. **(B)** Gating strategy for cell apoptosis assessment. Representative plots were obtained from MDA-MB-231 cells administered fospropofol disodium (10 μg/ml) plus paclitaxel (1 μg/ml). **(C)** Percentage of viable, early and late apoptotic, and necrotic MDA-MB-231 cells exposed to propofol/PIE/fospropofol disodium with or without paclitaxel. **(D)** The expression level of Bcl-2 and caspase-3 in MDA-MB-231 cells under treatment with different drugs was determined by western blotting. β-actin was used as the internal reference. **(E, F)** The statistical analyses of western blotting bands of Bcl-2 and caspase-3. Each experiment was repeated at least three times. **(G)** The expression and subcellular localization of Bcl-2 and caspase-3 in MDA-MB-231 cells after different drug treatments was determined by mIHC. DAPI was used to stain cell nuclei. Bar = 14 μm. NC, normal control; 10P, 10 μg/ml propofol; 10PIE, 10 μg/ml propofol injectable emulsion; 10F, 10 μg/ml fospropofol disodium; 1DOX, 1 μg/ml doxorubicin; 10P+1DOX, 10 μg/ml propofol combined with 1 μg/ml doxorubicin; 10PIE+1DOX, 10 μg/ml PIE combined with 1 μg/ml doxorubicin; 10F+1DOX, 10 μg/ml fospropofol disodium combined with 1 μg/ml doxorubicin; 1PTX, 1 μg/ml paclitaxel; 10P+1PTX, 10 μg/ml propofol combined with 1 μg/ml paclitaxel; 10PIE+1PTX, 10 μg/ml PIE combined with 1 μg/ml paclitaxel; 10F +1PTX, 10 μg/ml fospropofol disodium combined with 1 μg/ml paclitaxel. ns, no significant difference. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

The percentage of early apoptotic cells significantly decreased after treatment with 10 μg/ml PIE plus 1 μg/ml paclitaxel, compared with that of paclitaxel treatment alone (5.75 ± 0.20% vs. 6.30 ± 0.13%, $P < 0.05$), while the other two combination treatment groups did not significantly differ from paclitaxel treatment alone. No significant difference was observed in late apoptotic cells between the combination treatment groups and the paclitaxel alone group. In addition, although more necrotic

cells were found in the 10 μg/ml fospropofol disodium plus 1 μg/ml paclitaxel treatment than with paclitaxel only (2.95 ± 0.28% vs. 1.97 ± 0.17%, $P < 0.01$), the other two combination treatment groups did not significantly differ with the paclitaxel alone treatment.

The expression and localization of apoptosis-related proteins were determined using western blotting and mIHC. As shown in **Figures 2D–F**, the expression levels of Bcl-2 and caspase-3 decreased in the groups treated with different drugs compared with control group. Both Bcl-2 and caspase-3 were mainly expressed in the cytoplasm (**Figure 2G**).

Propofol or PIE Increased Intracellular ROS Level of MDA-MB-231 Cells With or Without Doxorubicin or Paclitaxel

Intracellular ROS plays a critical role in cell death, it can not only activate the apoptotic signaling pathways, but also induce cell ferroptosis (26). ROS levels in MDA-MB-231 cells were analyzed using fluorescent assay and presented as the proportion of cells marked by green fluorescence (ROS-positive cells) in each merged field. As shown in **Figure 3A**, ROS-positive cells increased after treatment with propofol/PIE (10 μg/ml), 1 μg/ml paclitaxel, or a combination of propofol/PIE (10 μg/ml) and doxorubicin/paclitaxel (1 μg/ml) for 24 h. Intracellular ROS level was significantly higher in MDA-MB-231 cells administered propofol/PIE/fospropofol disodium (10 μg/ml) alone than those in the control (ROS proportion, 16.82 ± 3.31% vs. 0.00 ± 0.00%, $P < 0.001$; 8.36 ± 3.59% vs. 0.00 ± 0.00%, $P < 0.001$; 4.40 ± 2.56% vs. 0.00 ± 0.00%, $P < 0.01$, respectively; **Figure 3B**). As for the combination drug treatments, there was a significant increase in intracellular ROS level in cells treated with propofol/PIE (10 μg/ml) plus doxorubicin (1 μg/ml) compared with doxorubicin alone (ROS proportion, 14.74 ± 4.05% vs. 0.88 ± 2.15%, $P < 0.001$; 9.26 ± 5.71% vs. 0.88 ± 2.15%, $P < 0.01$, respectively; **Figure 3B**), and so did propofol/PIE (10 μg/ml) plus paclitaxel (1 μg/ml) versus paclitaxel alone (ROS proportion, 23.36 ± 2.84% vs. 13.29 ± 5.02%, $P < 0.001$; 19.45 ± 4.61% vs. 13.29 ± 5.02%, $P < 0.05$, respectively; **Figure 3B**).

Interestingly, the effects of fospropofol disodium on intracellular ROS levels relatively differed from those of propofol and PIE. Although fospropofol disodium alone was associated with higher ROS levels in MDA-MB-231 cells relative to the control (4.40 ± 2.56% vs. 0.00 ± 0.00%, $P < 0.01$), it showed no significant difference between fospropofol disodium plus doxorubicin and doxorubicin alone (1.85 ± 3.10% vs. 0.88 ± 2.15%, $P = 0.541$, **Figure 3B**). Moreover, ROS-positive cells were fewer after treatment with fospropofol disodium plus paclitaxel than paclitaxel alone (4.05 ± 2.33% vs. 13.29 ± 5.02%, $P < 0.001$, **Figure 3B**).

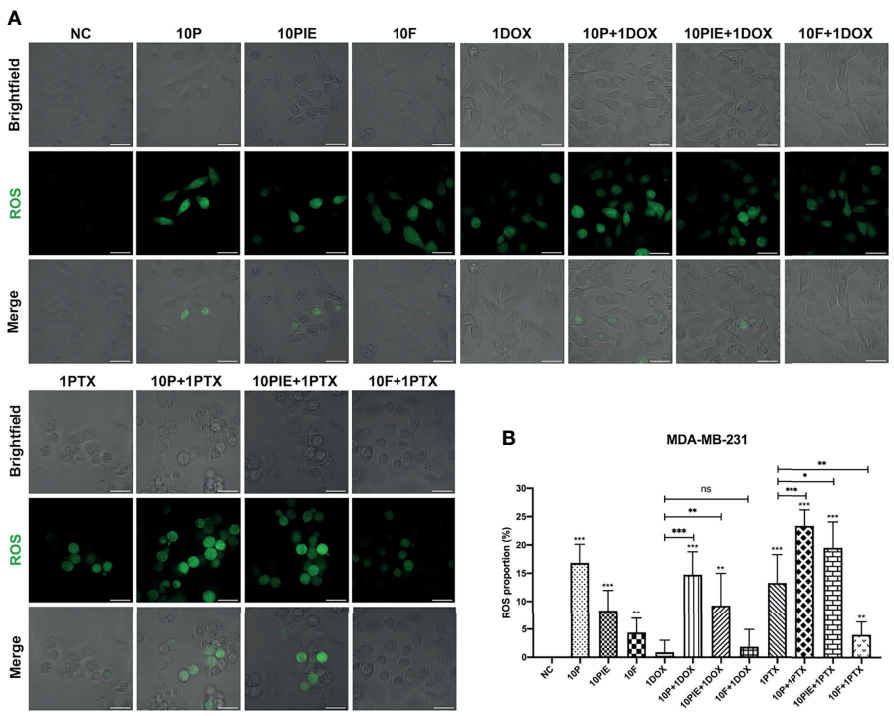

FIGURE 3 | Intracellular ROS level of MDA-MB-231 cells treated with propofol/PIE/fospropofol disodium with or without doxorubicin/paclitaxel. **(A)** Intracellular ROS level of MDA-MB-231 cells treated with different drugs. ROS positive cells were determined using laser scanning confocal microscopy and indicated as green spots. Bar = 34 μm. **(B)** Statistical analysis of ROS proportion in MDA-MB-231 cells under different treatments. ns, no significant difference. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Propofol/PIE/Fospropofol Disodium With or Without Doxorubicin/Paclitaxel Induced Ferroptosis-Related Morphological Changes in MDA-MB-231 Cells

Shrunken mitochondria, which appeared smaller than normal, with increased membrane density or decreased mitochondrial cristae, have been reported as the distinctive morphological features of ferroptotic cells (18). In the present study, we distinguished these morphological changes using TEM to determine the effects of propofol/PIE/fospropofol disodium with or without chemotherapeutics on MDA-MB-231 cells (**Figure 4A**). As shown in **Figure 4B**, shrunken mitochondria were significantly increased in MDA-MB-231 cells treated with propofol/PIE/fospropofol disodium (10 μg/ml) alone, compared with control (shrunken mitochondria percentage, 24.39 ± 6.17% vs. 16.22 ± 2.17%, $P < 0.05$; 25.18 ± 4.50% vs. 16.22 ± 2.17%, $P<0.01$; 21.06 ± 3.08% vs. 16.22 ± 2.17%, $P < 0.05$, respectively). Similarly, 1 μg/ml paclitaxel was also associated with a significant increase in shrunken mitochondria compared with that in the control (shrunken mitochondria percentage, 25.65 ± 2.91% vs. 16.22 ± 2.17%, $P < 0.001$). When propofol/PIE/fospropofol disodium (10 μg/ml) was combined with doxorubicin (1 μg/ml), there were more shrunken mitochondria in MDA-MB-231 cells than doxorubicin (1 μg/ml) only (shrunken mitochondria percentage, 23.57 ± 3.64% vs. 18.78 ± 2.76%, $P < 0.05$; 26.98 ± 5.52% vs. 18.78 ± 2.76%, $P < 0.05$; 30.55 ± 4.81% vs. 18.78 ± 2.76%, $P < 0.01$, respectively). Similar results were obtained from propofol/PIE/fospropofol disodium (10 μg/ml) plus paclitaxel (1 μg/ml) (shrunken mitochondria percentage, 31.54 ± 3.20% vs. 25.65 ± 2.91%, $P < 0.05$; 33.32 ± 6.12% vs. 25.65 ± 2.91%, $P < 0.05$; 30.88 ± 5.93% vs. 25.65 ± 2.91%, $P < 0.05$, respectively).

Propofol/PIE/Fospropofol Disodium With or Without Doxorubicin/Paclitaxel Induced Intracellular Iron Accumulation in MDA-MB-231 Cells

Iron overload is also one of the most important characteristics of cell ferroptosis (18). To assess intracellular iron levels, we determined the MFI of Fe^{2+} in MDA-MB-231 cells using laser scanning confocal microscopy. The fluorescent images of intracellular Fe^{2+} are shown in **Figure 5A**. Statistical analyses indicated that cells exposed to propofol/PIE/fospropofol disodium (10 μg/ml) alone were associated with a higher intracellular iron level than those in the control (relative MFI, 1.08 ± 0.03 vs. 1.00 ± 0.03, $P < 0.001$; 1.16 ± 0.04 vs. 1.00 ± 0.03, $P < 0.001$; 1.07 ± 0.05 vs. 1.00 ± 0.03, $P < 0.05$, respectively; **Figure 5B**). Following treatment with 1 μg/ml doxorubicin alone, ferrous ions accumulated more in MDA-MB-231 cells than in the control (relative MFI, 1.08 ± 0.03) vs. 1.00 ± 0.03, $P < 0.001$, **Figure 5B**). Compared with doxorubicin (1 μg/ml) alone, the relative MFI of Fe^{2+} significantly increased after exposure to a combination of propofol/PIE/fospropofol disodium (10 μg/ml) and doxorubicin (1 μg/ml) (relative MFI, 1.32 ± 0.06 vs. 1.08 ± 0.03, $P < 0.001$; 1.28 ± 0.03 vs. 1.08 ± 0.03, $P < 0.001$; 1.26 ± 0.06 vs. 1.08 ± 0.03, $P < 0.001$, respectively; **Figure 5B**), similar to the results of the paclitaxel groups (relative MFI, 1.12 ± 0.07 vs. 1.03 ± 0.06, $P < 0.05$; 1.15 ± 0.06 vs. 1.03 ± 0.06, $P < 0.01$; 1.17 ± 0.07 vs. 1.03 ± 0.06, $P < 0.01$, respectively; **Figure 5B**).

Propofol May Promote Ferroptosis of MDA-MB-231 Cells *via* the p53-SLC7A11-GPX4 Pathway

The SLC7A11-GPX4 axis and ubiquinone-FSP1-ubiquinol axis were examined to explore the potential mechanisms of propofol-induced ferroptosis-related changes in MDA-MB-231 cells. The locations of p53, SLC7A11, GPX4, ubiquinone, FSP1 and ubiquinol in MDA-MB-231 cells were determined using mIHC and are shown in **Figures 6A, F**, from where it can be observed that p53 is localized in the cytoblast, SLC7A11 mainly on the membrane, and GPX4, ubiquinone, FSP1, and ubiquinol in both mitochondria and cytoplasm. Western blotting was used for quantitative analyses. As shown in **Figures 6B–E**, compared with the control group, after exposure to propofol/PIE/fospropofol disodium (10 μg/ml) alone, the expression levels of GPX4 were significantly decreased. In both doxorubicin- and paclitaxel-related groups, p53 was apparently upregulated, and GPX4 was downregulated compared with the control and other singe propofol-treated groups. SLC7A11 was downregulated in the propofol/PIE/fospropofol disodium plus doxorubicin treatment group compared with doxorubicin alone group. However, although the expression level of FSP1 in cells treated with PIE/fospropofol disodium (10 μg/ml) plus paclitaxel (1 μg/ml) was lower than other groups, there was no significant difference in the expression of ubiquinone and ubiquinol among all groups (**Figures 6G-J**). These results demonstrated that propofol/PIE/fospropofol might promote ferroptosis through regulating the activation of p53-SLC7A11-GPX4 pathway. The schematic diagram of potential mechanisms in this study is shown in **Figure 7**.

DISCUSSION

Propofol has been commonly used in clinical practice for decades. It was first introduced into clinical treatment in 1986 and soon became one of the most extensively used intravenous general anesthetics to produce sedative and anesthetic effects during surgery (4). At present, surgery is the primary treatment for most tumors, which may cause a large number of cancer cells to be released and reduce the activity of T, B and NK lymphocytes, leading to tumor progression however (27). As a general anesthetic, propofol may play an essential role in tumor relapse and metastasis during surgery process (28). Recent investigations have reported that propofol showed tumor inhibitory effect through promoting cell apoptosis *via* regulating multiple signaling pathways, downstream molecules, microRNAs, and long non-coding RNAs in various tumors, including breast cancer (6–11).

Consistent with previous research, results in this study suggested that propofol could inhibit the proliferation of MDA-MB-231 cells and increase their sensitivity to doxorubicin and paclitaxel. Further apoptotic analyses

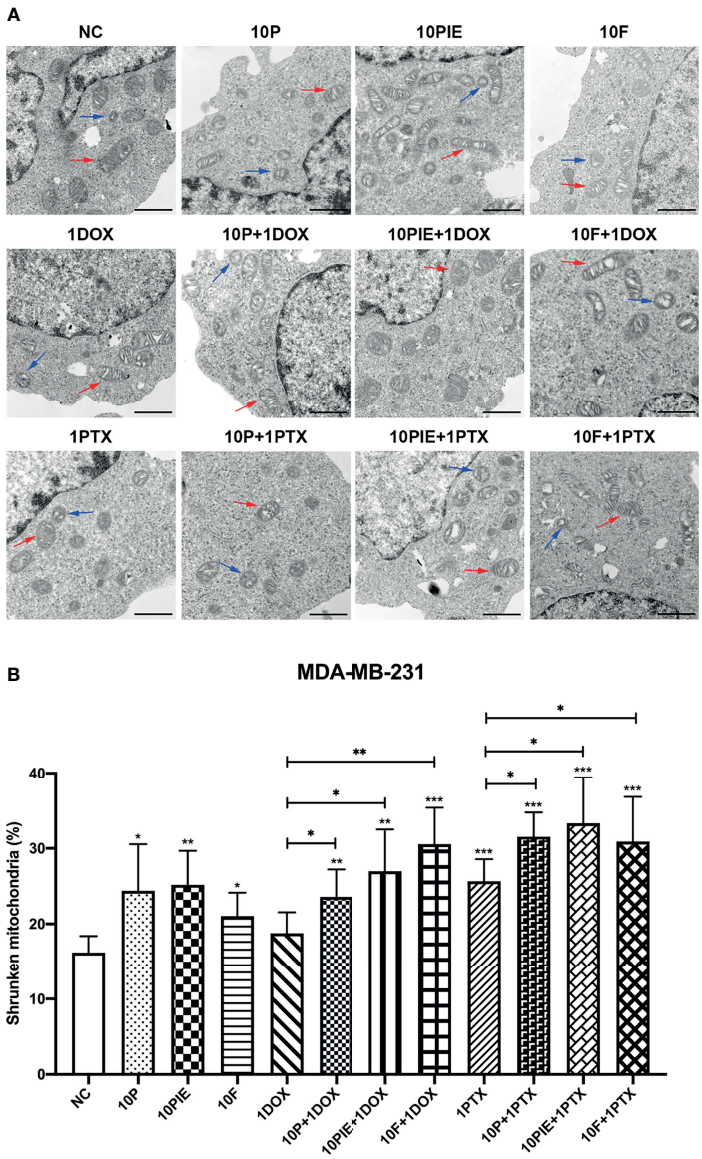

FIGURE 4 | Ferroptosis-related mitochondria morphological changes in MDA-MB-231 cells administered propofol/PIE/fospropofol disodium with or without doxorubicin/paclitaxel. **(A)** Ferroptosis-related mitochondria morphological changes in MDA-MB-231 cells treated with different drugs were determined using TEM. Red arrows: normal mitochondria. Blue arrows: shrunken mitochondria. Bar = 1 μm. **(B)** Statistical analysis of shrunken mitochondria percentage in MDA-MB-231 cells under different treatments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

indicated that these effects of propofol may be mediated through inducing cell apoptosis, with obvious apoptotic morphological changes and higher apoptotic cell proportions in drug treatment groups. Moreover, the expression levels of Bcl-2 and caspase-3 also decreased. Next, intracellular ROS levels of MDA-MB-231 cells were examined and found to be significantly higher in the propofol and PIE treatment groups, with or without chemotherapeutics. Intracellular ROS is closely related to cell death and plays a critical role in both apoptosis and ferroptosis. It could not only stimulate the activation of both intrinsic and extrinsic apoptotic signaling pathways, but also induce cell ferroptosis (26). Accordingly, we speculated that, besides inducing cell apoptosis, propofol might inhibit the proliferation of MDA-MB-231 cells and enhance the anti-tumor effects of doxorubicin and paclitaxel also by promoting ferroptosis.

Ferroptosis, a novel form of regulated cell death characterized by mitochondrial shrinkage and the accumulation of intracellular iron and lipid ROS (18, 29), has been found to play an increasingly important role in an increasing number of diseases since its discovery and definition in 2012 (20, 21). Inducing ferroptosis may be an effective tumor treatment

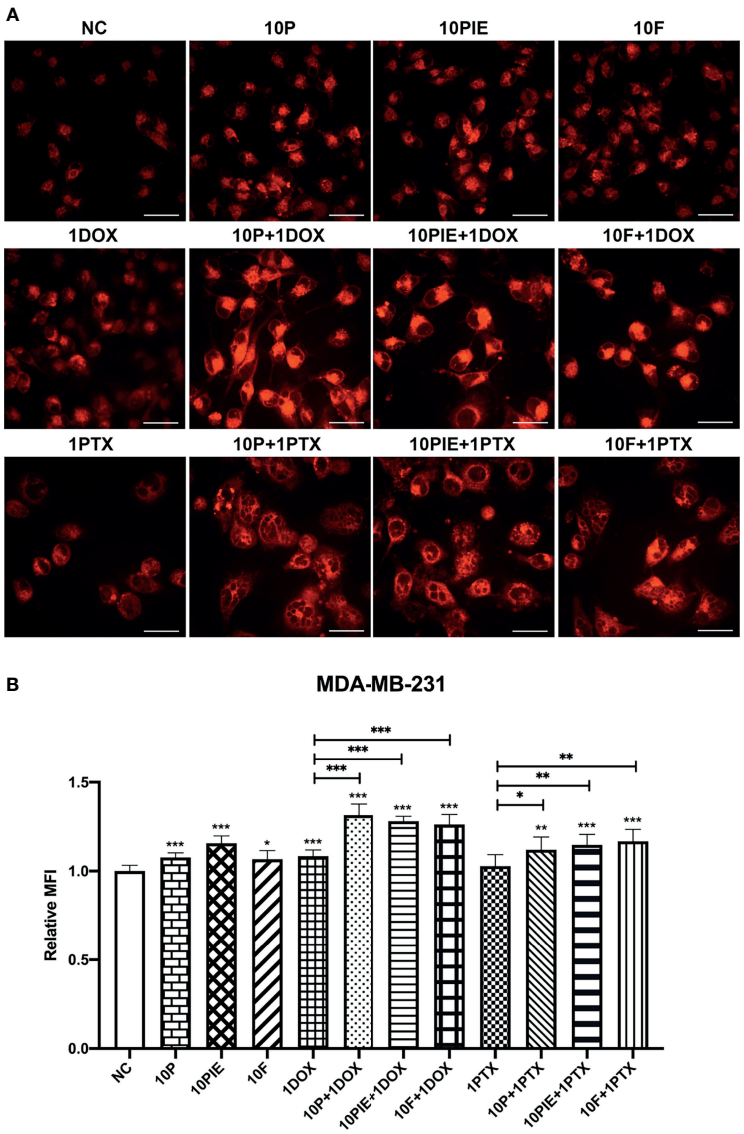

FIGURE 5 | Intracellular iron levels of MDA-MB-231 cells treated with propofol/PIE/fospropofol disodium with or without doxorubicin/paclitaxel. **(A)** Intracellular ferrous ion levels in MDA-MB-231 cells treated with different drugs were determined using laser scanning confocal microscopy. Bar = 34 μm. **(B)** Statistical analysis of relative MFI of intracellular ferrous ions in MDA-MB-231 cells under different treatments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

strategy (19). In our study, in addition to ROS levels, we also examined mitochondrial morphological changes, intracellular iron levels, and protein expression in two key ferroptosis regulation pathways to find the evidence of ferroptosis in MDA-MB-231 cells. Our results revealed that propofol induced ferroptosis-related mitochondrial morphological changes and enhanced intracellular iron accumulation in MDA-MB-231 cells, which indicated propofol might augment MDA-MB-231 cell ferroptosis, thereby inhibiting cell proliferation. Moreover, this effect may be achieved by regulating the activation of p53-SLC7A11-GPX4 pathway.

The SLC7A11-GPX4 axis is the core signaling pathway in lipid peroxidation and ferroptosis (23). GPX4, glutathione peroxidase 4, uses glutathione to eliminate phospholipid peroxides and prevent ferroptosis. The glutathione is generated from intracellular cysteine, which can be obtained from extracellular cystine through a cystine-glutamate reverse transporter, system x_c^-. SLC7A11 is one of the two subunits comprising system x_c^- and can be inhibited by the tumor suppressor p53 (30). Once SLC7A11 is inhibited, system x_c^- will be inhibited and reduce the exchange of intracellular glutamate and extracellular cystine, then the synthesis of glutathione, the substrate of GPX4, will be impeded, inhibiting the GPX4 function and leading to the ROS accumulation and the further ferroptosis. As shown in our study, GPX4 was significantly downregulated in all drug-treated MDA-MB-231

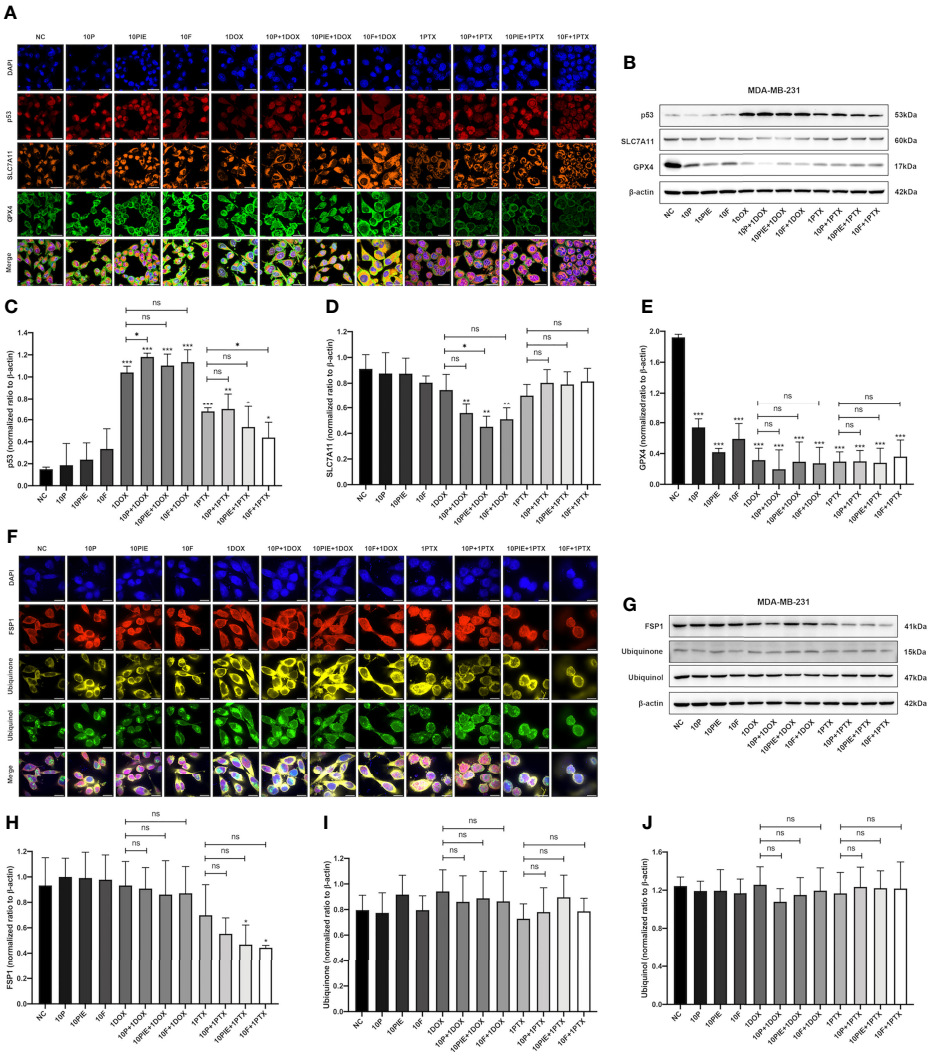

FIGURE 6 | Effects of propofol/PIE/fospropofol disodium with or without doxorubicin/paclitaxel on p53-SLC7A11-GPX4 and ubiquinone-FSP1-ubiquinol signaling pathways in MDA-MB-231 cells. The expression and subcellular localization of p53/SLC7A11/GPX4 **(A)** and FSP1/ubiquinone/ubiquinol **(F)** were determined using mIHC. DAPI was used to stain cell nuclei. Bar = 50 μm **(A)** and 14 μm **(F)**. The expression of p53/SLC7A11/GPX4 **(B)** and FSP1/ubiquinone/ubiquinol **(G)** were determined by western blotting. β-actin was used as the internal reference. The corresponding statistical analyses of western blotting bands of p53 **(C)**, SLC7A11 **(D)**, GPX4 **(E)**, FSP1 **(H)**, ubiquinone **(I)** and ubiquinol **(J)** were conducted and each experiment was repeated at least three times. ns, no significant difference. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cells, with p53 apparently upregulated in chemotherapy-related groups and SLC7A11 obviously downregulated only in the propofol/PIE/fospropofol disodium plus doxorubicin treatment group. Only the expressions of GPX4 were significantly decreased in single propofol-treated groups, while no significant changes were observed in p53 and SLC7A11. From these results we speculated that propofol was more likely to promote ferroptosis by downregulating GPX4. The further mechanisms by which propofol downregulates GPX4 needs to be further elucidated.

The ubiquinone-FSP1-ubiquinol axis is another essential pathway regulating cell ferroptosis independent of the SLC7A11-GPX4 axis (24, 25). FSP1 regenerates ubiquinol from ubiquinone, and suppresses lipid peroxidation and ferroptosis. Western blotting results in our study showed no significant difference in the expression of ubiquinone and ubiquinol among all treatment groups, but FSP1 was evidently downregulated in paclitaxel-treated groups, and its expression was even lower in the propofol/PIE/fospropofol disodium plus paclitaxel treatment group compared with that in paclitaxel alone group. This indicated that, although propofol may not regulate ferroptosis through the ubiquinone-FSP1-ubiquinol pathway, it may promote ferroptosis *via* other mechanisms mediated by FSP1, which need to be conducted in the future.

TNBC accounts for 10-15% of all breast cancers and usually appears in the form of high-grade invasive ductal carcinoma

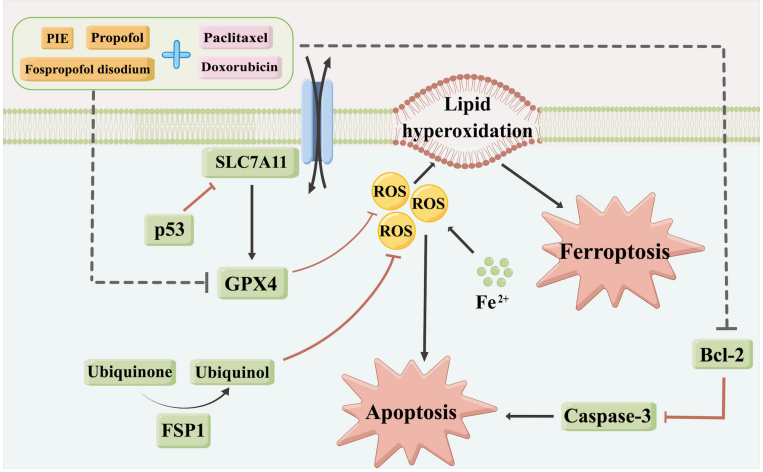

FIGURE 7 | A schematic diagram of potential mechanisms for drug treatment caused apoptosis and ferroptosis in this study (By Figdraw, www.figdraw.com).

(31). The evolution of endocrine therapy and anti-HER2 targeted treatment for other subtypes has significantly improved prognoses in these patients (32, 33), but the clinical outcomes for TNBC remain unsatisfactory, with the median overall survival only approximately 18 months (34). Chemotherapy is the primary adjuvant treatment for patients with TNBC. Considering its poor prognosis after surgery, we intended to explore whether propofol could improve the sensitivity of TNBC cells to chemotherapeutics. In this study, MDA-MB-231 cells, which are TNBC cells, and doxorubicin and paclitaxel, which are chemotherapeutics commonly used for TNBC in clinical practice, were selected for the experiments. Our results suggested that propofol could enhance the inhibitory effects of these two chemotherapeutics against MDA-MB-231 cell proliferation by promoting cell ferroptosis to some extent.

Currently, most *in vitro* studies use propofol only to conduct the investigations. However, PIE is the most common formulation of propofol in clinical practice. Fospropofol disodium is a propofol prodrug with good water solubility, need no lipid emulsion as a drug carrier, metabolized into the active metabolite propofol, and inducing an anesthetic effect (35). In this study, we used these two different propofol formulations and included propofol itself, to investigate whether they are consistent in effects on TNBC cells. Considering the commonly used blood concentration of propofol in clinical practice and the dosage used in previous *in vitro* studies (15, 17), we selected 2, 5, 10 and 20 μg/ml for three different propofol formulations to conduct experiments, and chose the most suitable concentration (10 μg/ml) for combination with chemotherapy according to the results of cell proliferation analyses. As shown in our results, MDA-MB-231 cells exposed to propofol showed a significant dose-dependent decrease in cell proliferation compared with the control, while the inhibition of proliferation when treated with PIE or fospropofol disodium was not significant. When combined with doxorubicin or paclitaxel, all three formulations significantly inhibited cell proliferation compared with doxorubicin or paclitaxel alone. The results of ferroptosis-related morphological changes, intracellular iron levels, and expression of ferroptosis-related proteins were similar among the three propofol formulations. As for the intracellular ROS levels, fospropofol disodium considerably differed from the other two formulations. Although fospropofol disodium alone was associated with higher intracellular ROS levels relative to the control, there was no significant difference between fospropofol disodium plus doxorubicin and doxorubicin alone, and even lower ROS levels in the fospropofol disodium plus paclitaxel group compared with paclitaxel alone. The intracellular ROS levels were similar in the three fospropofol disodium treatment groups. We speculate that PIE and fospropofol disodium may have different anti-proliferation mechanisms because their components are not completely the same as propofol. Furthermore, different water solubility may also lead to different anti-tumor effects. Propofol and PIE are hydrophobic agent, which could be easier taken up into cells through cell membrane and conduct their biological effects, while fospropofol disodium is water-soluble, which may affect its absorption by cells and thus affect its biological effects. Previous research have reported that, the oxidation activity of polyunsaturated fatty acids (PUFA) in membrane phospholipids during cell ferroptosis is competitively influenced by monounsaturated fatty acids (MUFA), indicating that exogenous MUFA may perform ferroptosis resistance (36, 37), which may be another underlying mechanism of different anti-tumor effects between PIE and propofol.

Our study focused on the effects of propofol on breast cancer, and found it could inhibit the proliferation of TNBC cells *in vitro* and enhance their sensitivity to chemotherapeutics. In fact, there are also many studies about the effects of different anesthetics often used in clinical practice on patients under breast cancer surgery. Ecimovic P et al. demonstrated that sevoflurane, one of the most commonly used volatile anesthetics, increased proliferation, migration but not invasion in MDA-MB-231 cells, although observed effect size was small and not dose-dependent (38). Tripolt S et al. found that the common analgesic, opioid,

triggered breast cancer metastasis *via* oncogenic JAK1/2-STAT3 signaling to promote epithelial-mesenchymal transition, emphasizing the importance of selective and restricted opioid use (39). Serum from patients receiving propofol/paravertebral anesthesia for breast cancer surgery inhibited proliferation of MDA-MB-231 cells *in vitro*, to a greater extent than that from patients receiving sevoflurane/opioid anesthesia-analgesia (40). In clinical research, results are quite controversial. Some studies showed using propofol during breast cancer surgery could improve the patients' prognosis compared with using inhalation anesthetics (12), but no significant difference was observed in other studies (13). It is hoped that our results could provide a theoretical support for further clinical research, and assist clinicians in better perioperative management and anesthesia selection for patients with breast cancer.

Our study also had some limitations. We aimed to explore whether propofol could inhibit MDA-MB-231 cell proliferation by affecting cell ferroptosis and analyzed ferroptosis using various methods. Although we also analyzed the expression of two key pathways related to ferroptosis and found a possible regulatory mechanism, we did not test and validate the specific molecules in specific pathways, and did not conducted experiments *in vivo*. Further studies focusing on the specific mechanisms by which propofol regulates cell ferroptosis and including both *in vitro* and *in vivo* experiments should be conducted. On the other hand, as mentioned above, we found the different performances of propofol, PIE and fospropofol disodium in the anti-proliferation effect but did not explore the specific mechanisms between these three drugs. Further research is needed in the future.

CONCLUSIONS

Our study found that propofol showed anti-proliferation effects and could be a potential adjuvant to enhance the chemotherapeutic sensitivity of TNBC cells partly through promoting cell ferroptosis. Future studies are urgently needed to elucidate the potential mechanisms underlying the relationship between propofol and cancer cell ferroptosis.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

CS, PL, LP, and YH designed the study. CS, PL, and MZ performed the experiments and acquired the data under the supervision and guidance of LP. CS performed the statistical analyses and wrote the manuscript. PL and LP revised the manuscript. All authors have read and approved the final version of the manuscript.

FUNDING

This study was supported by the Peking Union Medical College Hospital Precipitation and Integration Foundation (ZC201906511). The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

ACKNOWLEDGEMENTS

We would like to thank Dr. Chao Ma and Joint Laboratory of Anesthesia and Pain, Peking Union Medical College, Chinese Academy of Medical Sciences, for the great support to our study. We would also like to thank Editage (www.editage.cn) for English language editing.

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frontiers | Frontiers in Pharmacology

ORIGINAL RESEARCH
published: 20 April 2022
doi: 10.3389/fphar.2022.816432

Propofol Augments Paclitaxel-Induced Cervical Cancer Cell Ferroptosis *In Vitro*

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Introduction: Cervical cancer is common in women. The present standardized therapies including surgery, chemotherapy, and radiotherapy are still not enough for treatment. Propofol is the most commonly used intravenous anesthetic agent for induction and maintenance of anesthesia and has been shown to exert anti-malignancy effects on cancer cells, inducing oxidative stress and apoptosis. However, the biological effects of propofol have not yet been systematically assessed. In this study, we examined the ferroptosis-related changes caused by propofol and the chemotherapeutic agent paclitaxel besides apoptosis *in vitro*.

Methods: Cervical cancer cell lines (C-33A and HeLa) were treated with propofol alone (1, 2, 5, 10, and 20 μg/ml) or in combination with paclitaxel (0.5, 1, and 5 μg/ml). The viability was assessed using cell counting kit-8 (CCK8), apoptosis was detected by flow cytometry, morphological changes of mitochondria were examined using transmission electron microscope (TEM), cellular reactive oxygen species (ROS), and intracellular ferrous ions were determined by fluorescence microscope or confocal microscopy. The expression and cellular localization of apoptosis and ferroptosis-related molecules were detected by Western blot and multiplex immunohistochemistry (mIHC), respectively. Calcusyn software was used to determine whether propofol has a synergistic effect with paclitaxel.

Results: Propofol and paclitaxel inhibited C-33A and HeLa cell viability. There were also synergistic effects when propofol and paclitaxel were used in combination at certain concentrations. In addition, propofol promoted paclitaxel-induced cervical cancer cell death via apoptosis. ROS level and Fe^{2+} concentrations were also influenced by different drug treatments. Furthermore, propofol, propofol injectable emulsion, and paclitaxel induced ferroptosis-related morphological changes of mitochondria in C-33A and HeLa cells. Ferroptosis-related signaling pathways including SLC7A11/GPX4, ubiquinol/CoQ10/FSP1, and YAP/ACSL4/TFRC were found to be changed under drug treatments.

Conclusion: Propofol showed synergistic anticancer effects with paclitaxel in cervical cancer cells. Propofol and paclitaxel may induce ferroptosis of cervical cancer cells besides apoptosis.

Keywords: propofol, paclitaxel, cervical cancer, ferroptosis, apoptosis

OPEN ACCESS

Edited by:
Daqing Ma,
Imperial College London,
United Kingdom

Reviewed by:
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Pharmacology

Received: *16 November 2021*
Accepted: *11 March 2022*
Published: *20 April 2022*

Citation:
Zhao M-Y, Liu P, Sun C, Pei L-J and Huang Y-G (2022) Propofol Augments Paclitaxel-Induced Cervical Cancer Cell Ferroptosis In Vitro. Front. Pharmacol. 13:816432. doi: 10.3389/fphar.2022.816432

INTRODUCTION

Cervical cancer is a kind of common cancer in women (Bray et al., 2018). Standardized therapies involve surgical resection for early-stage cervical cancer and systemic chemotherapy in combination with radiotherapy for progressive cervical cancer (Olusola et al., 2019). However, even after curative primary resection, metastasis and recurrence are reported (Freeman et al., 2016). For anesthesiologists, it is of great interest to improve anesthetic management to reduce the cancer burden. The ways in which anesthetic management is conducted and the type of anesthetic drug chosen during the few hours of the anesthetic management period may influence cancer recurrence months or years later (Sessler and Riedel, 2019). To explore the potential effects of anesthetic drugs on cervical cancer cells is crucial.

Propofol (2,6-diisopropylphenol) is the most frequently used intravenous sedative–hypnotic agent for both induction and maintenance of anesthesia. Animal experiments and *in vitro* cell experiments consistently show that propofol possesses the potential to inhibit the malignancy of primary cancer (Sessler and Riedel, 2019). Several literature discussing relationships among propofol, chemotherapeutic agents, and cancer cell *in vitro* have focused on cell apoptosis (Li et al., 2017; Li et al., 2021; Shen et al., 2021). Cell apoptosis caused by propofol and chemotherapeutic drugs has been widely reported. To the best of our knowledge, none of the previous studies have reported the effects of propofol on cancer cell ferroptosis.

Ferroptosis is a unique form of regulated cell death (RCD), distinguishing itself from apoptosis, autophagy, and necrosis by characteristic iron-dependent accumulation of lipid hydroperoxides to lethal levels. Glutathione peroxidase 4 (GPX4) is one of the central regulating enzymes that protects cells from ferroptosis by neutralizing lipid peroxides, which are the byproducts of cellular metabolism (Stockwell et al., 2017; Wu et al., 2019). Ferroptosis can be initiated through two major pathways: the extrinsic pathway by inhibition of cell membrane transporters such as the cystine/glutamate transporter and the intrinsic pathway by blocking of intracellular antioxidant enzymes such as GPX4 (Chen et al., 2021). SLC7A11 is the cystine/glutamate transporter which acts as a suppressor for ferroptosis (Koppula et al., 2021). FSP1/ubiquinone (Coenzyme Q10, or CoQ10) is a pathway against ferroptosis independent of GPX4. FSP1 functions as a suppressor of ferroptosis through CoQ10, and CoQ10 is an antioxidant that protects cells from lipid peroxidation. Ubiquinol, the reduced form of CoQ10, traps lipid peroxyl radicals that mediate lipid peroxidation (Doll et al., 2019; Tsui et al., 2019). Furthermore, the intracellular YAP/ACSL4/TFRC signaling also regulates the activities of ferroptosis (Wu et al., 2019).

Our study investigated whether propofol or PIE in combination with or without paclitaxel at clinical concentrations exerts effects on apoptosis and ferroptosis of C-33A and HeLa cells and also how different drug treatment regimens regulate pathways involved in ferroptosis including SLC7A11/GPX4, ubiquinol/CoQ10/FSP1, and YAP/ACSL4/TFRC. The apoptosis of cancer cells caused by anesthetic drugs and chemotherapeutic drugs has been widely reported. Partial common characteristics can be found in both apoptosis and ferroptosis, such as ROS accumulation (Su et al., 2019), but whether these drugs could cause ferroptosis in a certain portion of cancer cells besides apoptosis is still elusive, so we examined the ferroptosis-related features and found that drug treatment could cause ferroptosis-related features and treatment of anesthetic drugs in combination with chemotherapeutic drug-enhanced ferroptosis-related features of cervical cancer cells.

MATERIALS AND METHODS

Cell Culture

Human cervical cancer cell line C-33A and HeLa were obtained from the Chinese National Infrastructure of Cell Line Resource (Beijing, China). C-33A and HeLa cells were cultured with Minimum Essential Medium (MEM) (11095080, Gibco, Grand Island, NY, United States) containing 1% nonessential amino acids (NEAA) (11140050, Gibco, Grand Island, NY, United States), and Roswell Park Memorial Institute (RPMI) 1640 medium (61870036, Gibco, Grand Island, NY, United States), respectively, supplemented with 10% fetal bovine serum (FBS) (AQmv09900, Analysis Quiz, Uruguay, South America) and 1% penicillin–streptomycin (15140122, Gibco, ThermoFisher Scientific) in an incubator with 5% CO_2 at 37°C.

Reagents

Propofol was purchased from Sigma-Aldrich (1572503, St. Louis, MO, United States). Propofol injectable emulsion (PIE) was obtained from AstraZeneca Co., United Kingdom. Paclitaxel was from Apexbio (A4393, Houston, TX, United States). All the reagents were dissolved according to the manufacturer's instructions. Stock solutions of propofol and paclitaxel were stored in −20°C, while PIE was stored in 4°C.

Cell Viability Assay

Before cell viability detection, propofol and PIE were administered to cells at the concentrations of 1, 2, 5, 10, and 20 μg/ml for 24 h. Paclitaxel was given at the concentrations of 0.5, 1, and 5 μg/ml for 24 h. The viability of C-33A and HeLa cells were detected by Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan). Briefly, the cells were seeded into 96-well plates (Costar, Corning, NY, United States) at 5×10^3 cells per well. The cells were cultured overnight and then replenished with fresh medium containing drugs at indicated concentrations for 24 h. Before detection, the plates were replenished with fresh medium containing 10 μl of CCK-8 for each well and incubated for 2 h. The optical density (OD) was measured at 450 nm on an Epoch Microplate Reader (BioTek, Winooski, VT, United States). The viability of cells was calculated as cell viability (%) = (OD of treatment − OD of blank control)/(OD of control − OD of blank control) × 100%.

Analysis of Cytotoxic Synergy

The combination index (CI) values were calculated using Calcusyn software to determine whether propofol or PIE has a

synergistic effect with paclitaxel. The CI values were calculated as the following equation: CI = (D)1/(Dx)1 + (D)2/(Dx)2 + (D)1(D)2/(Dx)1(Dx)2, where (Dx)1 or (Dx)2 indicates the dosage for x% inhibition by drug 1 or drug 2 alone, (D)1 or (D)2 indicates the dosage in combination that inhibits cell growth by x%. A CI value of 1 suggests additive effects of the two drugs, while a CI value greater than 1 suggests antagonism effects, and a CI value less than 1 indicates synergism effects.

Flow Cytometry

To detect the apoptosis of C-33A and HeLa cells, flow cytometry experiments were conducted using apoptosis detection kit (Dojindo Laboratories, Japan). After treatment with drugs for 24 h, C-33A and HeLa cells were digested with 0.05% trypsin (Gibco, ThermoFisher Scientific), and then collected and washed with ice cold PBS two times; 1× Annexin V Binding Solution was added to make a cell suspension with a concentration of 1×10^6 cells/ml. The cells were then stained with Annexin V, fluorescein isothiocyanate (FITC), and propidium iodide (PI) for 15 min at room temperature (RT) before 400 μl 1× Annexin V Binding Solution was added. The cells were then loaded to a flow cytometer (Accuri C6 Plus, BD BioSciences, United States) within 1 h. Results were analyzed using BD Accuri C6 Plus software.

Transmission Electron Microscope (TEM)

TEM was used to examine the mitochondrial morphological changes of C-33A and HeLa cells after drug treatment at indicated concentrations. C-33A and HeLa cells were trypsinized, harvested, and fixed in 2.5% glutaraldehyde (EM Grade) at 4°C overnight. After fixation in 1% osmium acid, cell samples were subsequently dehydrated, and placed in embedding molds in a standard fashion. Appropriate areas were selected and ultrathin sections of 0.08 μm were stained with lead citrate and uranyl acetate. Those sections were then examined using a transmission electron microscope (JEM-1400Plus, JEOL, Ltd., Tokyo, Japan).

Cellular ROS Assay

C-33A and HeLa cells were treated with indicated concentrations. Cells were then washed two times with 1× buffer and stained by diluted DCFDA Solution (Abcam Plc., Cambridge, United Kingdom) for 45 min in an incubator. Then, the cells were washed two times with 1× buffer. The cells were observed using a fluorescent microscopy under low light conditions. Cellular ROS proportion were analyzed with Image J software and compared between the negative control (NC) and drug treated groups.

FerroOrange Iron Assay

C-33A and HeLa cells were seeded into 8-well plates and incubated overnight. The cells were then replenished with fresh medium containing drugs at indicated concentrations and incubated for 24 h. Then, the cells were washed with serum-free medium 3 times; 1 μmol/l of FerroOrange working solution (Dojindo Laboratories, Japan) was added into each well and incubated for 30 min. The cells were imaged with a laser scanning confocal microscope (UltraVIEW VOX, PerkinElmer, Inc., Waltham, MA, United States). Relative mean fluorescence intensity (MFI) of ferrous ions was calculated using ImageJ software.

Western Blot

C-33A and HeLa cells were treated with drugs at indicated concentrations for 24 h, followed by lysis in RIPA buffer containing protease inhibitor. Protein concentrations were determined using bicinchoninic acid assay system (Beyotime, Shanghai, China). Protein samples were added 20 μg per lane and separated by SDS-PAGE gel and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was then blocked with 5% skimmed milk for 2 h at RT. Primary antibodies including anti-Bcl-2 (Abcam, ab182858), anti-caspase-3 (Abcam, ab32351), anti-SLC7A11 (Abcam, ab175186), anti-GPX4 (Abcam, ab125066), anti-ubiquinol-cytochrome C reductase core (Abcam, ab110252), anti-CoQ10A (Proteintech, 17812-1-AP), anti-FSP1 (Proteintech, 20886-1-AP), anti-YAP (CST, 14074S), anti-ACSL4 (Abcam, ab155282), and anti-TFRC (Abcam, ab84036) antibodies were diluted in primary antibody dilution buffer (NCM, WB100D) and incubated with PVDF membrane at 4°C overnight. Next, the membrane was incubated with goat anti-mouse/anti-rabbit secondary antibodies (Proteintech, SA00001-1, SA00001-2) at RT for 2 h, followed by detection via an enhanced chemiluminescence detection kit (New Cell and Molecular, P10100). β-Actin (Proteintech, 66009-1-Ig) was used as the internal control. Images were captured via a chemiluminescence imaging system (Tanon 5800, Shanghai, China).

Multiplex Immunohistochemistry (mIHC)

Multiplex immunohistochemistry modified for adherent C-33A and HeLa cells was performed to identify the protein expression and localization using the PANO Multiplex IHC Kit (0001100100, Panovue, Beijing, China).

Statistical Analysis

Statistical analysis was conducted by performing one-way ANOVA followed with Tukey's post hoc analysis using GraphPad Prism 8.1.1 software to compare the differences between two groups, and data were presented as mean ± standard deviation. A p value of <0.05 was considered to be statistically significant.

RESULTS

Propofol/PIE Inhibits Cell Viability and Enhances Sensitivity of Paclitaxel for Cervical Cancer Cell *In Vitro*

Cervical cancer cell lines C-33A and HeLa cells were treated with 1–20 μg/ml propofol or PIE, 0.5–5 μg/ml paclitaxel, or 10 μg/ml propofol or PIE combined with 0.5–5 μg/ml paclitaxel for 24 h. In C-33A cells, exposure to 2, 5, 10, or 20 μg/ml propofol for 24 h resulted in significant decrease of cell viability compared with NC

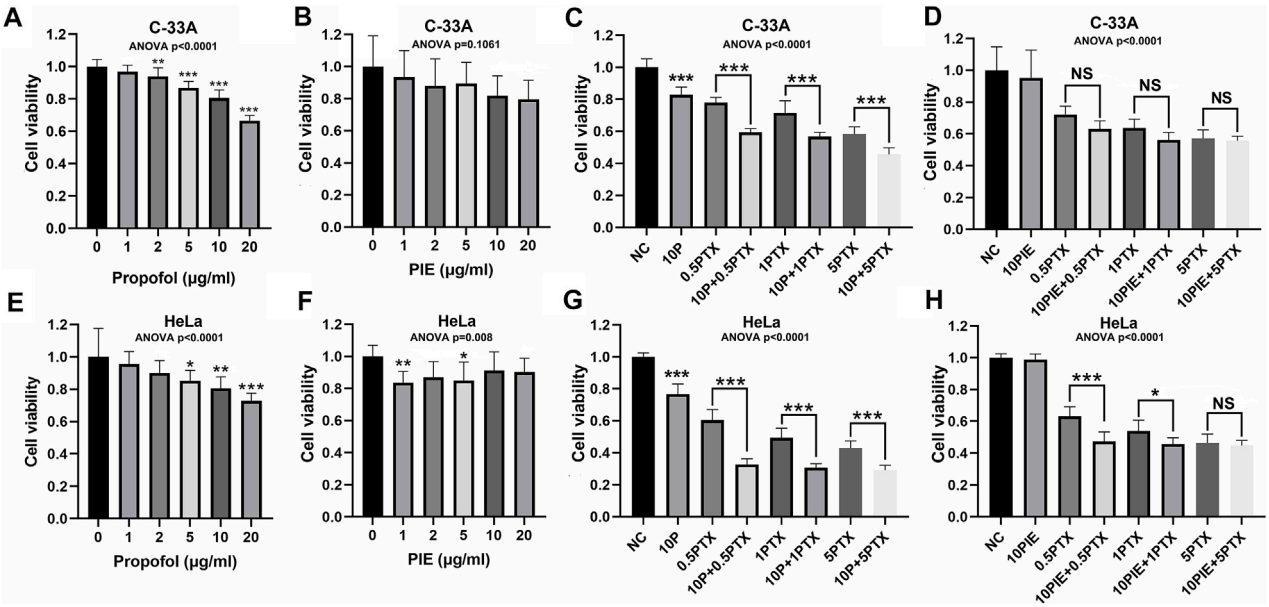

FIGURE 1 | Effects of propofol or PIE with or without paclitaxel on cell viability of C-33A and HeLa cells. **(A,B)** Effects of propofol/PIE on cell viability of C-33A cells. **(C,D)** Combination effects of propofol/PIE with paclitaxel on cell viability of C-33A cells. **(E,F)** Effects of propofol/PIE on cell viability of HeLa cells. **(G,H)** Combination effects of propofol/PIE with paclitaxel on cell viability of HeLa cells. NS, no significant difference. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 10P: 10 μg/ml propofol; 0.5PTX: 0.5 μg/ml paclitaxel; 10P+0.5PTX: 10 μg/ml propofol plus 0.5 μg/ml paclitaxel; 1PTX: 1 μg/ml paclitaxel; 10P+1PTX: 10 μg/ml propofol plus 1 μg/ml paclitaxel; 5PTX: 5 μg/ml paclitaxel; 10P+5PTX: 10 μg/ml propofol plus 5 μg/ml paclitaxel; 10PIE: 10 μg/ml PIE; 10PIE+0.5PTX: 10 μg/ml PIE plus 0.5 μg/ml paclitaxel; 10PIE+1PTX: 10 μg/ml PIE plus 1 μg/ml paclitaxel; 10PIE+5PTX: 10 μg/ml PIE plus 5 μg/ml paclitaxel.

($p < 0.01$), however, PIE did not significantly reduce C-33A cell viability compared to NC (**Figures 1A,B**). In combination treatment groups of C-33A cells, exposure to 10 μg/ml propofol combined with paclitaxel for 24 h resulted in significant decrease of cell viability compared with treatment of paclitaxel alone ($p < 0.001$); however, 10 μg/ml PIE combined with paclitaxel did not significantly inhibit C-33A cell viability compared to paclitaxel treatment alone (**Figures 1C,D**). Propofol at 10 μg/ml significantly reduced the C-33A cell viability by 19.44% ($p < 0.001$), while 10 μg/ml of PIE did not significantly decrease the C-33A cell viability. Propofol at 10 μg/ml, when added to 5 μg/ml of paclitaxel, produced a further significant reduction in the C-33A cell viability of 12.79% ($p < 0.001$), whereas PIE at 10 μg/ml added to a similar concentration of paclitaxel had no significant effect. The CI values of the co-administration of propofol with paclitaxel in C-33A cells were 0.460, 0.484, and 0.465 corresponding to 10P+0.5PTX, 10P+1PTX, and 10P+5PTX groups. The CI values were 0.235, 0.197, and 0.803 when PIE and paclitaxel were co-administered in C-33A cells corresponding to the groups of 10PIE+0.5PTX, 10PIE+1PTX, and 10PIE+5PTX, respectively. Thus, both propofol and PIE in combination of paclitaxel can exert cytotoxic synergistic effects to inhibit C-33A cell viability.

In HeLa cells, exposure to 5, 10, or 20 μg/ml propofol, or 1 or 5 μg/ml PIE for 24 h significantly reduced cell viability compared with NC ($p < 0.05$, **Figures 1E,F**). For combination treatment, 10 μg/ml propofol or PIE combined with 0.5 or 1 μg/ml paclitaxel for 24 h significantly decreased HeLa cell viability compared with the same concentration of paclitaxel alone ($p < 0.05$, **Figures 1G,H**). Propofol at 10 μg/ml significantly inhibited HeLa cell viability by 19.40% ($p < 0.01$), while 10 μg/ml of PIE did not significantly decrease HeLa cell viability. Propofol at 10 μg/ml, when added to 5 μg/ml of paclitaxel, produced a further significant reduction in the HeLa cell viability of 13.66% ($p < 0.001$), whereas PIE at 10 μg/ml added to a similar concentration of paclitaxel showed no significant effect. In HeLa cells, when propofol and paclitaxel were administered to the groups of 10P+0.5PTX, 10P+1PTX, and 10P+5PTX; the CI values were 0.065, 0.065, and 0.168, respectively. When PIE and paclitaxel were co-administered in HeLa cells to the groups of 10PIE+0.5PTX, 10PIE+1PTX, and 10PIE+5PTX; the CI values were all greater than 1, which means antagonistic effects of PIE with paclitaxel. Thus, for HeLa cells, propofol but not PIE has a synergistic effect on the inhibition of cell viability when combined with paclitaxel.

As above, our data suggest that propofol is a potential adjuvant to augment the inhibitory effects of paclitaxel on viability of cervical cancer cells. However, PIE showed different properties compared with propofol.

Propofol/PIE Induces Apoptosis and Enhances Sensitivity of Paclitaxel-Induced Apoptosis for Cervical Cancer Cell *In Vitro*

To assess whether the effects of propofol or PIE on the viability of cervical cancer cells were correlated with cell apoptosis, we conducted flow cytometry experiment for apoptosis analysis (**Figures 2A–C**). Western blot analysis and mIHC of adherent

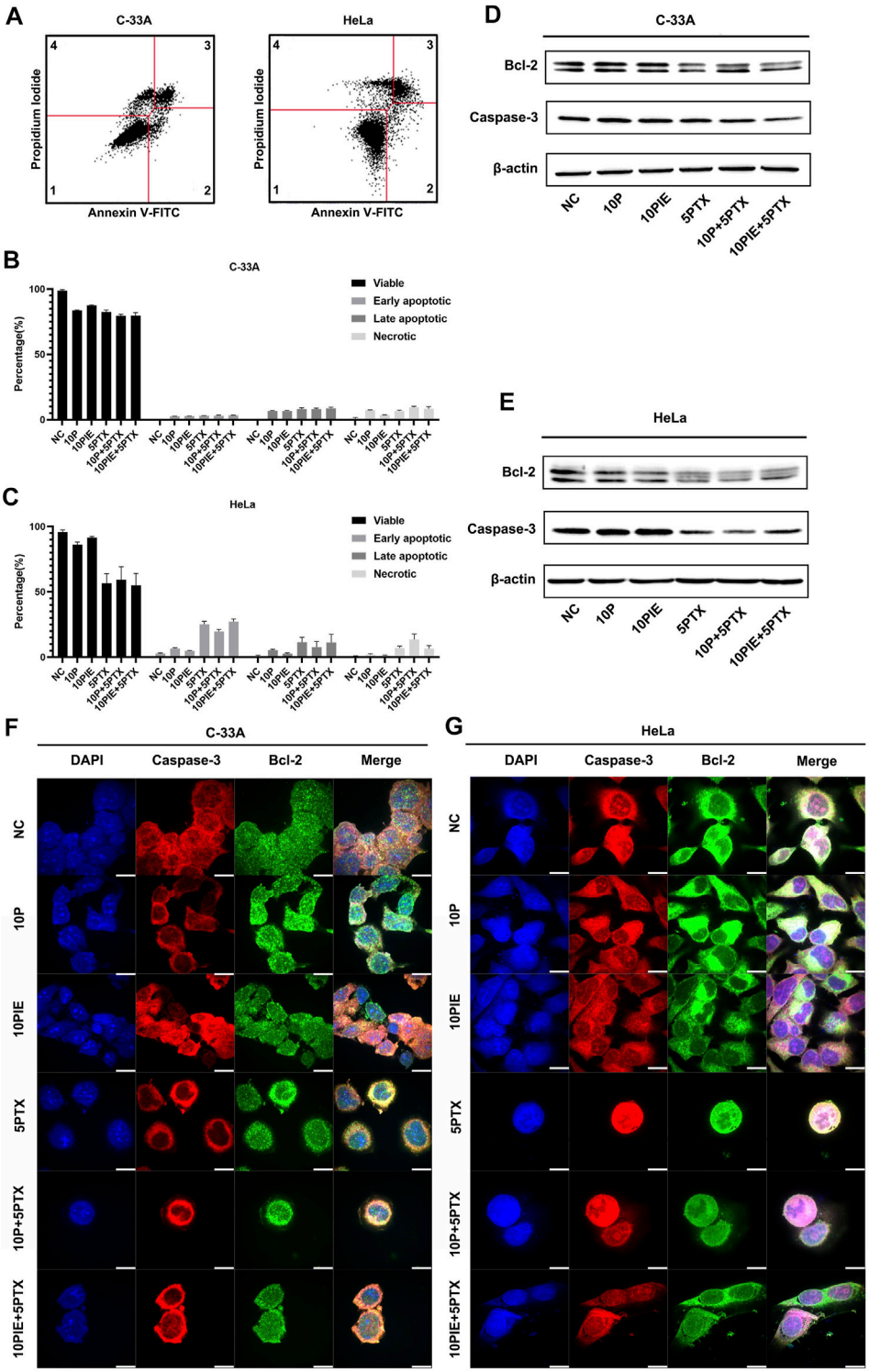

FIGURE 2 | Effects of propofol or PIE with or without paclitaxel on cervical cancer cell apoptosis. **(A)** Gating strategy for cell apoptosis assessment. Representative plots were obtained from 5 μg/ml paclitaxel-treated C-33A cells and 5 μg/ml paclitaxel-treated HeLa cells. **(B,C)** Percentage of viable, early apoptotic, late apoptotic, and necrotic C-33A and HeLa cells treated with propofol/PIE with or without paclitaxel. **(D,E)** The expression level of Bcl-2 and caspase-3 in C-33A and HeLa cells under treatment of different drug combinations. β-Actin was used as the internal reference. **(F,G)** The expression and subcellular localization of Bcl-2 and caspase-3 in C-33A and HeLa cells after treatment with different drug combinations were indicated by mIHC. DAPI was used to stain the nuclei of the cells. All the bars indicated 10 μm.

C-33A and HeLa cells were also performed to identify the potential molecular mechanisms. We found that the drug treatments caused apoptosis of cervical cancer cells, which could explain the inhibition of cell viability caused by drug treatments from a certain perspective.

In C-33A cells, the percentage of early apoptotic cells significantly increased after single drug treatment with 10 μg/ml propofol [(2.60 ± 0.10%) vs. (0.50 ± 0.20%), $p < 0.001$] or PIE [(2.67 ± 0.15%) vs. (0.50 ± 0.20%), $p < 0.001$], or 5 μg/ml paclitaxel [(3.03 ± 0.06%) vs. (0.50 ± 0.20%), $p < 0.001$] compared to NC. Combined treatment of 10 μg/ml propofol or PIE with 5 μg/ml paclitaxel did not significantly increase early apoptotic proportion of C-33A cells compared with paclitaxel treatment alone [(3.13 ± 0.38%) vs. (3.03 ± 0.06%), $p = 0.99$; (3.37 ± 0.12%) vs. (3.03 ± 0.06%), $p = 0.36$; respectively]. As for late apoptosis in C-33A cells, 10 μg/ml propofol [(6.57 ± 0.35%) vs. (0.00 ± 0.00%), $p < 0.001$] or PIE [(6.50 ± 0.53%) vs. (0.00 ± 0.00%), $p < 0.001$], or 5 μg/ml paclitaxel [(8.10 ± 1.06%) vs. (0.00 ± 0.00%), $p < 0.001$] could significantly increase late apoptotic percentage compared to NC. However, 10 μg/ml propofol [(8.07 ± 0.70%) vs. (8.10 ± 1.06%), $p > 0.99$] or PIE [(8.67 ± 0.86%) vs. (8.10 ± 1.06%), $p = 0.90$] combined with 5 μg/ml paclitaxel did not significantly increase late apoptotic C-33A cell proportion compared with paclitaxel treatment alone. Notably, 10 μg/ml propofol [(7.23 ± 0.21%) vs. (0.77 ± 0.93%), $p < 0.001$], 10 μg/ml PIE [(3.50 ± 0.26%) vs. (0.77 ± 0.93%), $p < 0.05$], or 5 μg/ml paclitaxel [(6.57 ± 0.64%) vs. (0.77 ± 0.93%), $p < 0.001$] also significantly increased the necrotic percentage of C-33A cells compared to NC; 10 μg/ml propofol plus 5 μg/ml paclitaxel [(9.47 ± 0.70%) vs. (6.57 ± 0.64%), $p < 0.05$] significantly increased the necrotic proportion of C-33A cells compared to 5 μg/ml paclitaxel treatment, while 10 μg/ml PIE plus 5 μg/ml paclitaxel did not show this significant enhancement [(8.47 ± 1.48%) vs. (6.57 ± 0.64%), $p = 0.12$] (**Figure 2B**).

For HeLa cells, the proportion of early apoptotic cells significantly increased after single drug treatment with 5 μg/ml paclitaxel [(25.07 ± 2.29%) vs. (3.33 ± 1.46%), $p < 0.001$]. However, treatment of 10 μg/ml propofol [(6.63 ± 0.51%) vs. (3.33 ± 1.46%), $p = 0.16$], or 10 μg/ml PIE [(4.70 ± 0.46%) vs. (3.33 ± 1.46%), $p = 0.88$] did not significantly increase early apoptotic HeLa cell proportion. Combined treatment of 10 μg/ml propofol with 5 μg/ml paclitaxel significantly decreased early apoptotic HeLa cell percentage compared with paclitaxel treatment alone [(19.63 ± 1.57%) vs. (25.07 ± 2.29%), $p < 0.01$]; 10 μg/ml PIE plus 5 μg/ml paclitaxel did not significantly affect the early apoptotic percentage compared to paclitaxel alone [(27.23 ± 1.99%) vs. (25.07 ± 2.29%), $p = 0.54$]. Late apoptotic proportion significantly elevated after 5 μg/ml paclitaxel [(11.40 ± 3.72%) vs. (0.07 ± 0.06%), $p < 0.05$] treatment. However, treatment of 10 μg/ml propofol [(5.40 ± 0.66%) vs. (0.07 ± 0.06%), $p = 0.46$], or 10 μg/ml PIE [(2.77 ± 0.49%) vs. (0.07 ± 0.06%), $p = 0.92$] did not significantly increase late apoptotic HeLa cell percentage.10 μg/ml propofol [(7.63 ± 4.39%) vs. (11.40 ± 3.72%), $p = 0.76$] or PIE [(11.30 ± 6.20%) vs. (11.40 ± 3.72%), $p > 0.99$] plus 5 μg/ml paclitaxel did not show significant effect on the late apoptotic proportion of HeLa cells compared with paclitaxel alone. For cell necrosis, 10 μg/ml propofol [(1.83 ± 0.83%) vs. (0.83 ± 0.15%), $p > 0.99$], or 10 μg/ml PIE [(1.10 ± 0.44%) vs. (0.83 ± 0.15%), $p > 0.99$] did not significantly increase necrotic HeLa cell proportion; 10 μg/ml propofol [(13.57 ± 7.60%) vs. (7.00 ± 1.35%), $p = 0.22$] or 10 μg/ml PIE [(6.57 ± 2.34%) vs. (7.00 ± 1.35%), $p > 0.99$] plus 5 μg/ml paclitaxel did not significantly increase necrotic proportion compared to paclitaxel alone (**Figure 2C**).

In C-33A cells, the anti-apoptotic protein Bcl-2 decreased in the groups of 5PTX, 10P+5PTX, and 10PIE+5PTX. Full-length caspase-3 also decreased notably in the groups of 10P+5PTX, 10PIE+5PTX (**Figure 2D**). In HeLa cells, the anti-apoptotic protein Bcl-2 decreased after drug treatment compared with NC, and caspase-3 decreased in groups involving paclitaxel treatment (**Figure 2E**). The expression and localization of caspase-3 and Bcl-2 in C-33A and HeLa cells were indicated in mIHC pictures (**Figures 2F,G**). Caspase-3 was mainly expressed in cytoplasm as indicated. Bcl-2 has a wider distribution range as indicated in **Figures 2F,G**.

Propofol/PIE and Paclitaxel Induces Ferroptosis-Related Morphological Changes in C-33A and HeLa Cells

Through TEM examination, treatment of either 10 μg/ml propofol or PIE alone, or combined treatment of 10 μg/ml propofol/PIE with 5 μg/ml paclitaxel, increased the characteristic morphological changes of ferroptosis in C-33A and HeLa cells (**Figure 3A**). Shrunken mitochondria (smaller volume, thicker membrane, lesser mitochondrial cristae, etc.) was reported to be the morphological features of ferroptosis (Dixon et al., 2012), and we detected these features in this study to determine the effects of drugs on cervical cancer cells. The quantification of shrunken mitochondria was performed manually by counting shrunken and normal mitochondria in TEM images and calculating their proportion. Propofol or PIE at 10 μg/ml significantly increased shrunken mitochondria percentage in C-33A cells compared with NC [(0.20 ± 0.06) vs. (0.08 ± 0.04), $p < 0.05$; (0.20 ± 0.06) vs. (0.08 ± 0.04), $p < 0.05$; respectively; **Figure 3B**]; 5 μg/ml paclitaxel also significantly increased shrunken mitochondria percentage in C-33A cells compared to NC [(0.29 ± 0.08) vs. (0.08 ± 0.04), $p < 0.001$, **Figure 3B**]. In C-33A cells, 10 μg/ml propofol or PIE plus 5 μg/ml paclitaxel did not significantly increase ferroptosis-related morphological changes compared with the treatment of paclitaxel alone [(0.27 ± 0.09) vs. (0.29 ± 0.08), $p > 0.99$; (0.30 ± 0.13) vs. (0.29 ± 0.08), $p > 0.99$; respectively; **Figure 3B**]. In HeLa cells, neither 10 μg/ml propofol nor PIE caused any significant increase in the shrunken mitochondria percentage compared to NC [(0.15 ± 0.06) vs. (0.11 ± 0.06), $p = 0.85$; (0.15 ± 0.08) vs. (0.11 ± 0.06), $p = 0.82$; respectively; **Figure 3C**]; 5 μg/ml paclitaxel either did not significantly increase the percentage of shrunken mitochondria compared to NC [(0.21 ± 0.10) vs. (0.11 ± 0.06), $p = 0.10$, **Figure 3C**]. For combined treatment in HeLa cells, 10 μg/ml propofol plus 5 μg/ml paclitaxel significantly increased ferroptosis-related morphological changes of mitochondria compared with 5 μg/ml paclitaxel alone [(0.32 ± 0.09) vs. (0.21 ± 0.10), $p < 0.05$,

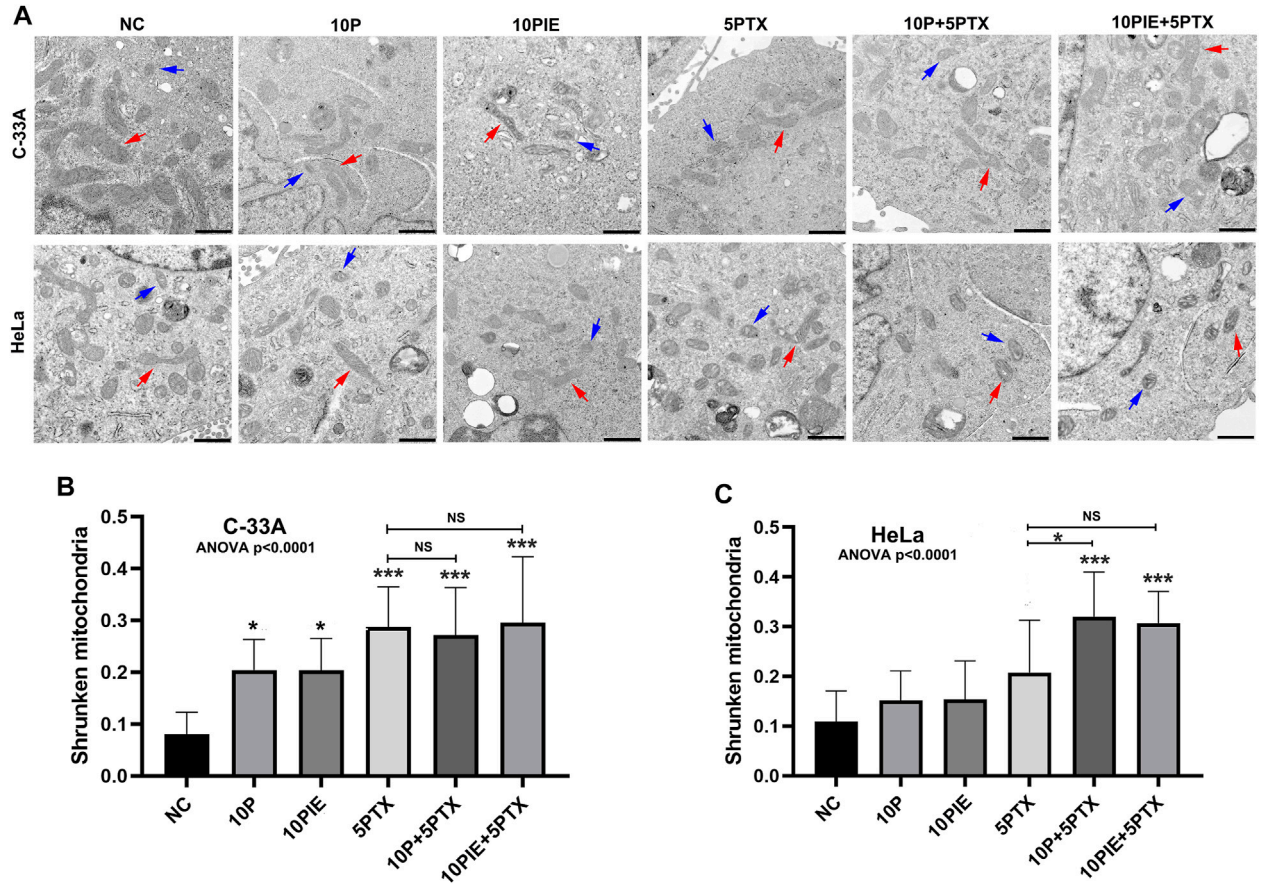

FIGURE 3 | Propofol/PIE induced and enhanced paclitaxel-induced ferroptosis-related mitochondrial morphological changes of C-33A and HeLa cells. **(A)** TEM assay was conducted to determine mitochondrial morphological changes of ferroptosis in C-33A and HeLa cells. Red arrows: typical mitochondria morphology. Blue arrows: shrunken mitochondria morphology. All the bars indicated 1 μm. **(B,C)** Statistical analysis of shrunken mitochondria percentage in C-33A and HeLa cells under different drug treatments. NS, no significant difference. *$p < 0.05$, ***$p < 0.001$.

Figure 3C], whereas 10 μg/ml PIE plus 5 μg/ml paclitaxel did not significantly increase shrunken mitochondria percentage compared to treatment of 5 μg/ml paclitaxel alone [(0.31 ± 0.06) vs. (0.21 ± 0.10), $p = 0.09$, **Figure 3C**].

These data demonstrate that propofol or PIE could induce ferroptosis-related morphological changes and increase paclitaxel-triggered intracellular ferroptosis-related morphological changes of cervical cancer cells *in vitro*.

Propofol/PIE Plus Paclitaxel Combination Treatment Increases Cellular ROS Proportion of Cervical Cancer Cell

To identify the intracellular ROS level, fluorescent assay was adapted. As shown in **Figure 4A**, intracellular ROS-positive cells were increased after treatment of 10 μg/ml propofol, 10 μg/ml PIE, 5 μg/ml paclitaxel, 10 μg/ml propofol or PIE plus 5 μg/ml paclitaxel for 24 h.

In C-33A cells, the cellular ROS proportion (**Figure 4B**) was significantly increased after the combined treatment of 10 μg/ml propofol or PIE plus 5 μg/ml paclitaxel [(14.97 ± 7.80%) vs. (0.29 ± 0.32%), $p < 0.001$; (9.54 ± 3.69%) vs. (0.29 ± 0.32%), $p < 0.05$; respectively] compared to NC; 10 μg/ml propofol or PIE alone did not significantly enhance the cellular ROS proportion of C-33A cells [(2.75 ± 1.69%)vs. (0.29 ± 0.32%), $p = 0.95$; (1.04 ± 0.88%) vs. (0.29 ± 0.32%), $p > 0.99$; respectively] compared to NC. The combination drug treatment of 10 μg/ml propofol or PIE plus 5 μg/ml paclitaxel did not significantly enhance cellular ROS proportion in C-33A cells compared to 5 μg/ml paclitaxel [(14.97 ± 7.80%) vs. (11.22 ± 6.22%), $p = 0.76$; (9.54 ± 3.69%) vs. (11.22 ± 6.22%), $p = 0.99$; respectively].

For HeLa cells, the cellular ROS proportion (**Figure 4C**) significantly increased after the combined treatment of 10 μg/ml propofol or PIE plus 5 μg/ml paclitaxel [(14.31 ± 8.51%) vs. (0.25 ± 0.21%), $p < 0.001$; (13.35 ± 5.87%) vs. (0.25 ± 0.21%), $p < 0.01$; respectively] compared to NC. However, 10 μg/ml propofol [(4.43 ± 1.24%) vs. (0.25 ± 0.21%), $p = 0.71$], or 10 μg/ml PIE [(2.62 ± 1.91%) vs. (0.25 ± 0.21%), $p = 0.96$] did not significantly increase the cellular ROS proportion of HeLa cells compared to NC. Combined treatment of either 10 μg/ml propofol [(14.31 ± 8.51%) vs. (11.22 ± 4.18%), $p = 0.89$] or 10 μg/ml PIE [(13.35 ± 5.87%) vs. (11.22 ± 4.18%), $p = 0.98$] plus 5 μg/ml paclitaxel did not significantly increase the cellular ROS proportion compared to 5 μg/ml paclitaxel alone.

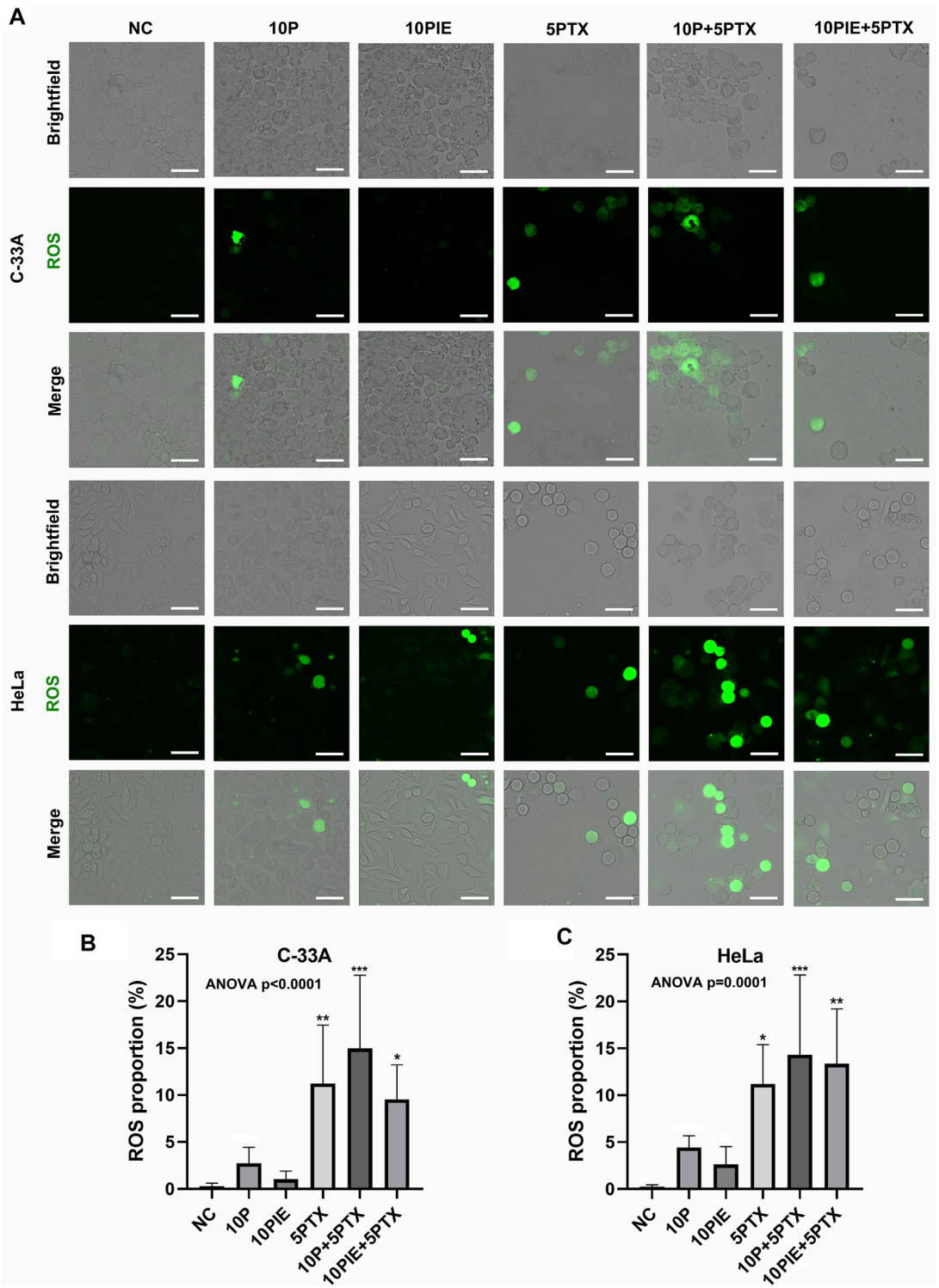

FIGURE 4 | Propofol/PIE plus paclitaxel enhanced cellular ROS in C-33A and HeLa cells. **(A)** Intracellular ROS was detected via fluorescent microscopy and indicated as green spots. All the bars indicated 30 μm. **(B,C)** Statistical analysis of cellular ROS proportion in C-33A and HeLa cells after different combinations of drug treatment. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ represent statistical significance.

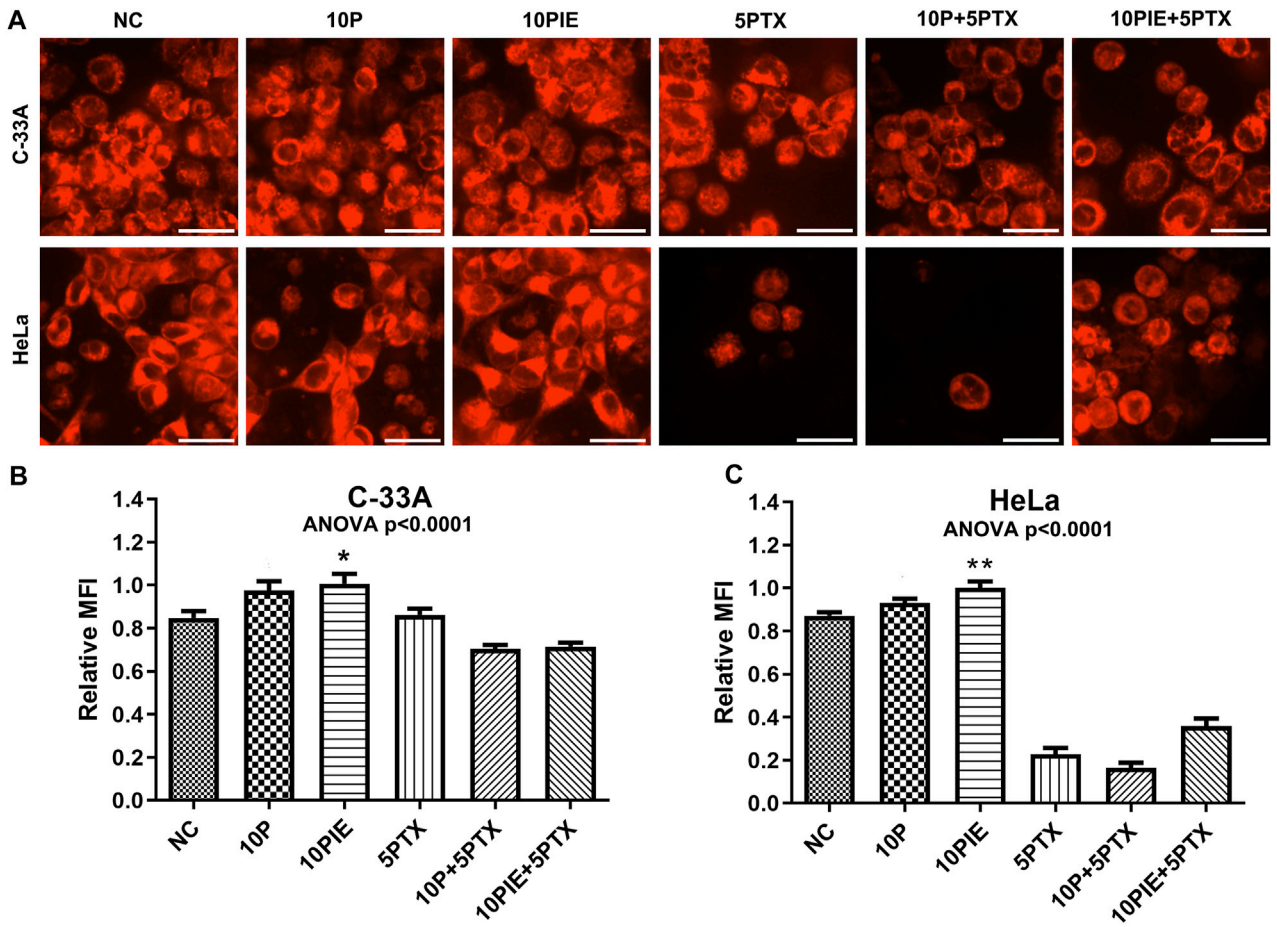

FIGURE 5 | PIE increased intracellular relative MFI of ferrous ions in C-33A and HeLa cells. **(A)** Intracellular ferrous ions were detected via laser scanning confocal microscopy. All the bars indicated 20 μm. **(B,C)** Statistical analysis of intracellular MFI in C-33A and HeLa cells under different drug treatments. *$p < 0.05$, **$p < 0.01$.

These findings indicate that drug treatment could enhance ROS accumulation in cervical cancer cells. Since ROS level was associated with both cell apoptosis and ferroptosis (Srinivas et al., 2019; Battaglia et al., 2020), we next examined the level of intracellular Fe^{2+} concentrations.

PIE Increases Intracellular Fe^{2+} MFI in C-33A and HeLa Cells

To assess Fe^{2+} concentrations in C-33A and HeLa cells, we detected the intracellular MFI of ferrous ions via laser scanning confocal microscopy. Fluorescent images of intracellular Fe^{2+} in C33-A and HeLa cells treated with indicated drugs were shown in **Figure 5A**.

Relative MFI of ferrous ions in C-33A cells (**Figure 5B**) was significantly increased after treatment with 10 μg/ml PIE [(1.01 ± 0.12) vs. (0.85 ± 0.08), $p < 0.05$] compared to NC, however, 10 μg/ml propofol did not significantly increase the relative MFI compared to NC [(0.97 ± 0.12) vs. (0.85 ± 0.08), $p = 0.10$]. In the three groups involving treatment of 5 μg/ml paclitaxel, intracellular relative MFI of ferrous ions was decreased possibly due to the Fe^{2+} leakage, since apoptosis caused by paclitaxel treatment could disrupt cell membrane integrity.

Similarly, relative MFI of Fe^{2+} in HeLa cells (**Figure 5C**) was significantly elevated after treatment with 10 μg/ml PIE [(1.00 ± 0.10) vs. (0.87 ± 0.05), $p < 0.01$] compared to NC, however, 10 μg/ml propofol did not exert significant influence on relative MFI compared to NC [(0.93 ± 0.06) vs. (0.87 ± 0.05), $p = 0.49$]. In the three groups of treatment involving paclitaxel, intracellular relative MFI of ferrous ions was also significantly reduced.

These findings reveal that PIE treatment could influence intracellular Fe^{2+} accumulation.

Propofol Augments Paclitaxel-Initiated Cell Ferroptosis by Regulating SLC7A11/GPX4 Pathway

To explore possible mechanisms for propofol/PIE individually or in combination with paclitaxel triggering ferroptosis-related changes in C-33A and HeLa cells, a key pathway involved in ferroptosis were examined firstly.

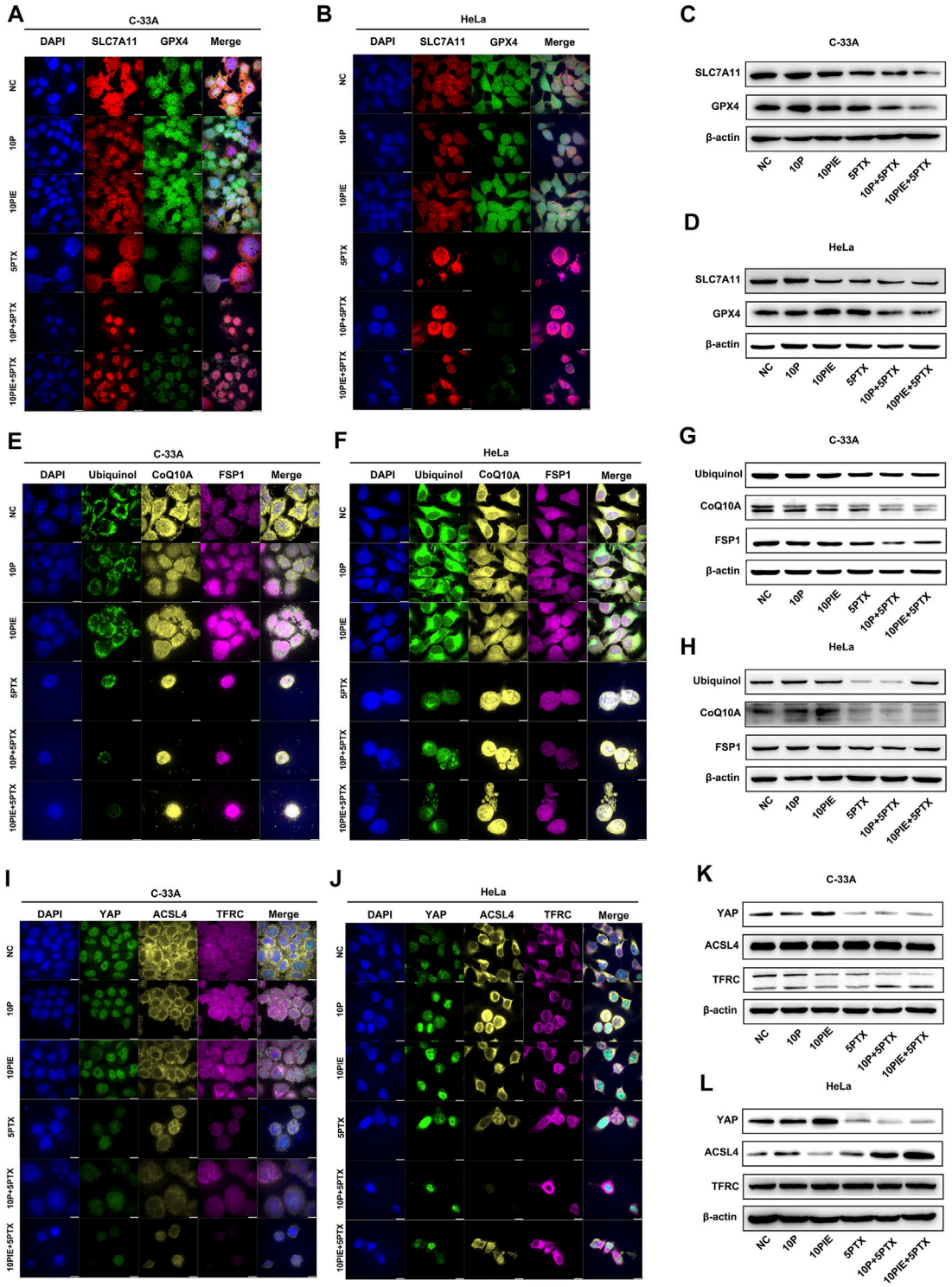

FIGURE 6 | Effects of propofol or PIE with or without paclitaxel on SLC7A11/GPX4, ubiquinol/CoQ10A/FSP1, and YAP/ACSL4/TFRC signaling pathways. The expression and subcellular localization of SLC7A11/GPX4 **(A,B)**, ubiquinol/CoQ10A/FSP1 **(E,F)**, and YAP/ACSL4/TFRC **(I,J)** in C-33A and HeLa cells were detected by mIHC. The expression of SLC7A11/GPX4 **(C,D)**, ubiquinol/CoQ10A/FSP1 **(G,H)**, and YAP/ACSL4/TFRC **(K,L)** in C-33A and HeLa cells were determined by WB. All the bars indicated 10 μm.

As shown in **Figures 6A,B**, in C-33A and HeLa cells, SLC7A11 was mainly localized on the membrane. While GPX4, another essential ferroptosis inhibitor, exerts its effect by catalyzing the reduction of lipid peroxides, was expressed both in mitochondrion and cytoplasm as indicated.

As shown in **Figure 6C**, the expression level of SLC7A11 in C-33A cells was notably diminished after propofol plus paclitaxel, or PIE plus paclitaxel treatment compared to that of paclitaxel single treatment. The expression of GPX4 in C-33A cells was distinctly reduced after propofol plus paclitaxel or PIE plus paclitaxel treatment compared to that of paclitaxel treatment alone.

Comparably, in HeLa cells (**Figure 6D**), the expression level of SLC7A11 was largely decreased by PIE, paclitaxel, propofol plus paclitaxel, or PIE plus paclitaxel treatment. The expression of GPX4 in HeLa cells was exceedingly diminished by propofol plus paclitaxel, or PIE plus paclitaxel treatment compared to that of paclitaxel treatment alone.

Thus, we found that propofol/PIE alone or in combination with paclitaxel could regulate the SLC7A11/GPX4 pathway, which is reported to be correlated with ferroptosis.

These results indicate that propofol or PIE may enhance the anti-tumor effect of paclitaxel by inducing ferroptosis through the SLC7A11/GPX4 pathway.

Propofol Enhances Paclitaxel-Initiated Cell Ferroptosis by Regulating Ubiquinol/CoQ10/FSP1 Pathway

Ubiquinol/CoQ10/FSP1 is another essential pathway regulating cell ferroptosis independent of SLC7A11/GPX4 (Doll et al., 2019; Tsui et al., 2019).

In C-33A and HeLa cells, CoQ10A, FSP1, and ubiquinol were mainly expressed in the mitochondrion and cytoplasm (**Figures 6E,F**).

In C-33A cells, the expression level of ubiquinol was decreased after the treatment of paclitaxel, propofol plus paclitaxel, or PIE plus paclitaxel for 24 h compared to NC. The expression of CoQ10A in C-33A cells was apparently reduced in propofol, PIE, or paclitaxel groups compared with NC. Propofol or PIE plus paclitaxel treatment also diminished the CoQ10A level compared to that of paclitaxel alone. FSP1 was dramatically downregulated in groups of propofol/PIE plus paclitaxel treatment compared to the group of paclitaxel single treatment (**Figure 6G**).

While in HeLa cells (**Figure 6H**), the expression level of ubiquinol and FSP1 was distinctly diminished by paclitaxel or propofol plus paclitaxel treatment compared to NC. The expression level of CoQ10A was evidently downregulated in groups involving treatment of paclitaxel in HeLa cells.

In summary, we found that propofol or PIE may induce or enhance paclitaxel-triggered ferroptosis in C-33A and HeLa cells via inhibiting the ubiquinol/CoQ10/FSP1 pathway.

Propofol Augments Paclitaxel-Initiated Cell Ferroptosis by Regulating YAP/ACSL4/TFRC Pathway

The proto-oncogenic transcriptional co-activator YAP is activated by antagonizing E-cadherin-regulated intracellular Merlin–Hippo signaling, promoting ferroptosis by upregulating the expression of ferroptosis modulators ACSL4, TFRC, etc. (Wu et al., 2019).

In C-33A and HeLa cells, YAP was mainly localized in the nucleus, ACSL4 was expressed in the cytoplasm, while TFRC was localized in both cytoplasm and cell membrane as shown in **Figures 6I,J**.

In C-33A cells, the expression level of YAP was incredibly increased after the treatment of PIE for 24 h compared to NC. The three groups involving treatment of paclitaxel presented low expression level of YAP. The expression level of ACSL4 or TFRC showed no evident difference among the six groups in C-33A cells (**Figure 6K**).

The expression level of YAP showed comparable increase after PIE treatment, and also drastically decline in groups containing paclitaxel in HeLa cells. ACSL4 was notably upregulated by propofol compared to NC, and by propofol/PIE plus paclitaxel compared to paclitaxel alone. The expression level of TFRC showed no difference among six treatment groups in HeLa cells (**Figure 6L**).

In summary, we found that propofol or PIE may also have an effect on the intracellular YAP/ACSL4/TFRC signaling to promote or enhance paclitaxel-induced ferroptosis in C-33A and HeLa cells (**Figure 7**).

DISCUSSION

Ranking as the fourth leading cause of cancer death in women worldwide, cervical cancer has approximately 530,000 new cases and 275,000 deaths every year (Olusola et al., 2019). Surgery is the optimal treatment regimen for early cervical cancer. Systemic chemotherapy and radiotherapy in combination are standardized care for the most progressive cervical cancer (Olusola et al., 2019). However, recurrence rates in cervical cancer are unsatisfying. The rate of recurrence after radical hysterectomy for cervical cancer was 1.4–2.9% (Uppal et al., 2020). For metastatic, recurrent, and persistent cervical cancer, the prognosis is extremely poor, as the progression-free survival is 12.0 and 13.1 months for paclitaxel–cisplatin–ifosfamide-treated patients and paclitaxel–cisplatin–bevacizumab-treated patients, respectively (Choi et al., 2020). Therefore, targeting on new mechanisms that enhance the anticancer effect of traditional chemotherapy is urgently required. Propofol, the most widely used intravenous sedative–hypnotic agent in the operation room, has been reported to exert anticancer effects either as single treatment or as an adjuvant *in vitro*. In cervical cancer cells, propofol is able to induce cisplatin-mediated cellular apoptosis through repression of the EGFR/JAK2/STAT3 pathway (Li et al., 2017). In colorectal cancer, gastric cancer, and renal cell carcinoma, propofol directly inhibits viability, migration, and invasion of cancer cells *in vitro* (Liu et al., 2021; Shi et al., 2021; Zhao and Liu, 2021). To our best knowledge, effects of combination therapy of propofol/PIE with paclitaxel on ferroptosis of cervical cancer cells have not yet been discussed. In the current study, we are the first to report that propofol/PIE exerts synergistic anticancer effects

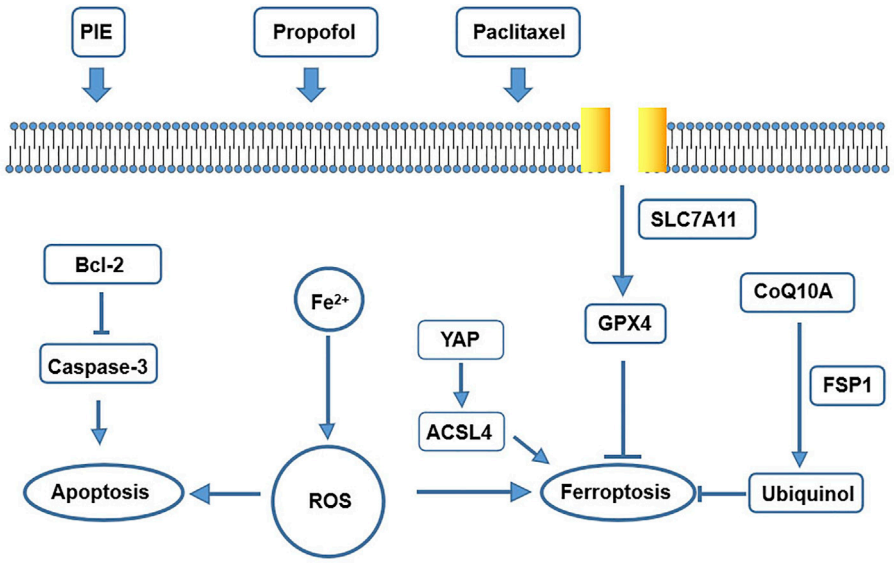

FIGURE 7 | A schematic map of potential mechanisms for drug treatment leading to apoptosis and ferroptosis in our study.

with paclitaxel on C-33A and HeLa cells by promoting not only apoptosis, but also ferroptosis-related changes *in vitro*.

Our study found that propofol/PIE at clinical-relevant concentrations could inhibit cervical cancer cell viability *in vitro* and combination treatment of propofol/PIE with paclitaxel resulted in further suppression of cell viability. In C-33A cells, propofol and paclitaxel, PIE and paclitaxel both showed synergistic effects on the suppression of cell viability. In HeLa cells, propofol and paclitaxel had synergistic effects on inhibition of cell viability, while PIE and paclitaxel did not. Apoptosis is an extensively studied type of regulated cell death in most type of cancer cells. However, the clinical implementation of chemotherapeutic agents targeting apoptosis in oncology can be unsatisfactory due to drug resistance acquired by cancer cells. Therefore, discovering non-apoptotic RCD might provide an alternative anticancer strategy (Chen et al., 2021). The present study proved that propofol/PIE promotes apoptosis of HeLa and C-33A cells, which was consistent with previous studies (Li et al., 2017). In addition, the inhibition effects were influenced not only by apoptosis, but also by other factors such as proliferation ability or other kind of cell death, such as ferroptosis.

Furthermore, TEM tests have shown significantly more ferroptosis-related morphological changes, shrunken mitochondria, after single treatment of PIE or paclitaxel in C-33A cells. Propofol significantly enhanced paclitaxel-induced ferroptosis-related morphological changes of HeLa cells. These findings are novel as no publication has discussed the possible effects of anesthetic propofol/PIE on ferroptosis of C-33A or HeLa cells. Ferroptosis can be inducted by iron accumulation, excessive ROS, or inhibited GPX4 (Chen et al., 2021). Our results showed that PIE increased ferrous ions, propofol/PIE combined with paclitaxel enhanced intracellular ROS, and suppressed GPX4 expression in C-33A and HeLa cells. To elucidate how propofol/PIE exerted effects on ferroptosis-related changes in C-33A and HeLa cells, we detected the changes of the SLC7A11/GPX4 pathway, ubiquinol/CoQ10/FSP1 pathway, and YAP/ACSL4/TFRC pathway. We found the ferroptosis-related pathways were influenced by drug treatments (**Figure 7**).

In conclusion, this *in vitro* cell study suggests that propofol or PIE (the clinical anesthetic containing propofol as a major component) could be a potential adjuvant to augment chemotherapeutic sensitivity of cervical cancer cells via the ferroptosis activities. Future studies are needed to elucidate the potential mechanisms of the relationship between propofol/PIE and cancer cell ferroptosis more thoroughly.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

M-YZ designed the study. PL performed the statistical analysis. M–YZ, PL, and CS performed the experiments and acquired the data under supervision and guidance of L–JP and Y–GH. M–YZ analyzed the data and wrote the manuscript. PL, L–JP, and Y–GH revised the manuscript. All authors have read and approved the final version of the manuscript.

FUNDING

This study was supported by the Peking Union Medical College Hospital Precipitation and Integration Foundation (ZC201906511). The funder was not involved in the design of the study; collection, analysis, and interpretation of data; nor in writing the manuscript.

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frontiers | Frontiers in Pharmacology

ORIGINAL RESEARCH
published: 21 April 2022
doi: 10.3389/fphar.2022.770892

Enhanced Antitumor Activity of Lidocaine Nanoparticles Encapsulated by a Self-Assembling Peptide

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Pharmacology

Received: *05 September 2021*
Accepted: *21 March 2022*
Published: *21 April 2022*

Citation:
Yang Y, Sun J, Peng F, Liu H, Zhao G, Chen J, Zhang W and Qiu F (2022) Enhanced Antitumor Activity of Lidocaine Nanoparticles Encapsulated by a Self-Assembling Peptide. Front. Pharmacol. 13:770892. doi: 10.3389/fphar.2022.770892

Although local anesthetics (LAs) such as lidocaine have been traditionally used for pain relief, their antitumor activity has attracted more and more attentions in recent years. However, since nearly all LAs used in clinic are in their hydrochloride forms with small molecular weight and high water-solubility, their fast absorption and clearance greatly limit their antitumor activity *in vivo*. To better exploit the antitumor activity of LAs, lidocaine nanoparticles (LNPs) are prepared by using a self-assembling peptide to encapsulate the hydrophobic base form of lidocaine. In cultured A375 human melanoma cells, the LNPs show much higher cellular uptake level than the clinic formulation of lidocaine hydrochloride, which leads to enhanced efficacy in inhibiting the proliferation, migration and invasion of the cells, as well as in inducing cell apoptosis. Compared with lidocaine hydrochloride, LNPs can also significantly slow down the release rate of lidocaine. In nude mice, LNPs can effectively inhibit the development of solid tumors from seeded A375 cells and prevent the recurrence of tumors after surgical excision. These results indicate that by using self-assembling peptide to fabricate nanoparticle formulations of local anesthetics, their antitumor activity can be significantly enhanced, suggesting a potential postoperative treatment to prevent tumor recurrence after surgical excision.

Keywords: lidocaine, antitumor activity, self-assembling peptide, local anesthetics, nanoparticles, tumor recurrence

1 INTRODUCTION

Local anesthetics (LAs) are a group of small molecular drugs widely used for anesthesia and analgesia in clinic practice. Compared with traditional pain-relieving opioids, LAs are relatively safe and non-addictive, so that more and more frequently have they become the choice of physicians for the treatment of perioperative and postoperative pain (Farag et al., 2013; Guay et al., 2016; Svirskis et al., 2020).

Abbreviations: AFM, atomic force microscope; DOX, doxorubicin; DLS, dynamic light scattering; FTIR, Fourier transform infrared; GQY, abbreviation for the peptide sequence of GQQQQQY; LAs, local anesthetics; LB, lidocaine base; LH, lidocaine hydrochloride; LNPs, lidocaine nanoparticles; NS, normal saline; OD, optical density; ThT, thioflavin T; TEM, transmission electron microscope.

Surgical excision of solid tumor for curing cancer is one of the most important and common kinds of operations routinely carried out in clinical practice. With the extensive involvement of LAs in cancer-related surgeries, more and more clinical evidences have implied that the use of LAs during or after the surgery was usually associated with improved outcome of patients (Call et al., 2015; Royds et al., 2016; Zhang et al., 2021). It is generally accepted that potential benefits of LAs include relieving the pain, reducing the stress caused by surgery, blunting the inflammatory response, thus improving the overall rehabilitation quality of the patient (Yang et al., 2017; Müller et al., 2021). Nevertheless, some studies have also suggested that LAs, if in direct contact with tumor cells, may also kill them directly (Liu et al., 2020).

Following these findings, more and more studies have been carried out on the direct antitumor activity of LAs. To date, many conventional LAs including lidocaine, bupivacaine, ropivacaine, and mepivacaine have shown antitumor activity against different tumor cells including lung cancer, breast cancer, colon-rectal cancer, melanoma, et al. (Bundscherer et al., 2015; Chamaraux-Tran et al., 2018; Li et al., 2018; Chen et al., 2019; Siekmann et al., 2019). Extensive mechanism studies have indicated that LAs may exert their antitumor activity through various pathways such as ion channel blocking (Baptista-Hon et al., 2014; Fraser et al., 2014), microRNA regulation (Qu et al., 2018; Sui et al., 2019; Xia et al., 2019), inflammation inhibiting (Piegeler et al., 2012; Wall et al., 2019), and so on.

However, on the contrary to the extensively reported antitumor activity of LAs on cultured tumor cell lines *in vitro*, only a few animal studies have been reported to show their antitumor activity *in vivo*. There seems to be a huge gap between the confirmed cytotoxicity of LAs against tumor cells and their actual antitumor activity *in vivo*. One possible reason is that all LAs in their clinical formulations are highly water-soluble small molecules so that they tend be diffused, absorbed, and cleared out very quickly in body before they can accumulate a high enough antitumor effect. Interestingly, in those studies reporting antitumor activity of LAs *in vivo*, the drugs were all encapsulated in nanoparticles based on carrier materials (Gao et al., 2018; Zhang et al., 2020), suggesting that nanoparticle formulations might be a promising strategy to improve the antitumor activity of LAs. However, in these studies LAs were combined with other treatments such as nutrient deprivation and classic antitumor drugs, and LAs seemed to only play a supplementary role. The antitumor activity of LAs alone, if encapsulated as nanoparticles, has not been evaluated yet.

As an emerging category of biomaterials with excellent biocompatibility and controllability, self-assembling peptide nanomaterials have shown great potential as advanced drug carriers (Qiu et al., 2018; Peng et al., 2020). In our previous study, a self-assembling peptide GQY with the sequence of GQQQQQY was designed to form nanoparticles with very high drug-loading capacity (Liu et al., 2021). This biocompatible nanomaterial can readily encapsulate hydrophobic drugs into nanoparticles, providing a simple strategy to fabricate drug nanoparticles. Although LAs currently used in clinic practice are their soluble hydrochlorides, their base forms are hydrophobic molecules with poor water solubility, making it possible to get LA nanoparticles using GQY as the carrier material. To test the feasibility of this strategy, lidocaine as one of the most widely used LAs with well-defined antitumor activity was used as a model drug. In this study, we show how lidocaine base (LB) can be encapsulated with GQY and form lidocaine nanoparticles (LNPs), which show enhanced antitumor activity against human melanoma cell line A375 *in vitro* and effectively inhibit the development and recurrence of solid tumors *in vivo*.

2 MATERIALS AND METHODS

2.1 Sample Preparation

2.1.1 Peptide and Reagents

GQY peptide with purity of 98% was synthesized by Bootech BioScience and Technology Co., Ltd. (Shanghai, China) and provided as lyophilized powder. Lidocaine hydrochloride (LH) (purity ≥99%) and LB (purity ≥99%) were purchased from Aladdin Biochemical Technology Co. Ltd (Shanghai, China). Thioflavin T (ThT) was purchased from Sigma-Aldrich Co. (St Louis, MO, United States).

2.1.2 Preparation of LNP Suspension and LH Solution

GQY was dissolved in sterile 10 mM phosphate buffer with a pH value of 7.8 to obtain a working solution with a peptide concentration of 5 mM. LB powder was added into the GQY solution at a theoretical lidocaine concentration of 100 mM, then the mixture was vigorously stirred at room temperature for 24 h to obtain a stable milky suspension. Transparent LH solution with a concentration of 100 mM was obtained by directly dissolving LH powder in sterile Milli-Q water.

2.2 Characterization of Nanostructures

2.2.1 Transmission Electron Microscope

LNP suspension or GQY solution was diluted ten-fold by Milli-Q water, and each 10 μL of diluted sample was set on the surface of a 400-mesh copper grid [Zhongjingkeyi (Beijing) Film Technology Co., Ltd]. After 2 min of incubation to deposit the sample, a piece of filter paper was used to blot the excess liquid. After that 10 μL of phosphotungstic acid (2%) was dropped onto the grid to stain the sample for 2 min. Finally, the excess staining solution was blotted with filter paper and the grid was air-dried. The grids were then observed and images were collected with a Tecnai G2 F20 transmission electron microscope (FEI, United States).

2.2.2 Atomic Force Microscope

For AFM observation, each 10 μL of LNP or GQY sample diluted in the above section was spread on a freshly cleaved mica surface. After 2 min of incubation to deposit the sample, excess liquid was pipetted away and the mica surface was gently rinsed with 1 mL of Milli-Q water. After that the mica surface was air-dried and scanned with AFM (SPM-9700HT, Shimadzu, Japan) operated in tapping model.

2.2.3 Dynamic Light Scattering

The size distribution and zeta potential of LNP and GQY nanoparticles were measured by DLS using a Zetasizer Nano

ZS90 (Malvern Panalytical, Malvern, United Kingdom). Briefly, LNP suspension or GQY solution was diluted 10-fold with Milli-Q water and added into a disposable size cuvette or a potential cell for measurement. The size distribution plot and zeta potential plot were collected 3–5 times to make sure similar results were obtained.

2.2.4 ThT-Binding Assay

ThT stock solution (1 mM) was prepared by dissolving ThT powder in Milli-Q water and passing through a 0.22 μm filter. To measure the ThT-binding fluorescence, 5 μL of ThT stock solution was mixed with 495 μL of LH, GQY or LNP, and the mixture was incubated at room temperature for 5 min. Fluorescent spectra between 460–600 nm were measured by a Fluorolog spectrofluorometer (Horiba scientific Inc., United States) with an excitation wavelength of 450 nm.

2.2.5 Fourier Transform Infrared Spectrometer

For FTIR assay, dry powder of LB, LH, and GQY was used as received. LNP powder was obtained by lyophilization of the suspension. The physical mixture of LB and GQY (LB + GQY) was also used as a comparison. Each sample was mixed with KBr powder and compressed into a translucent thin film, and the FTIR spectra were collected with an IRTracer-100 spectrometer (Shimadzu, Japan).

2.3 Cell Experiments

2.3.1 Cellular Uptake

A375 cells were purchased from Cell Center of Chinese Academy of Sciences (Shanghai, China) and conventionally maintained in DMEM containing 10% FBS (Thermo Fisher Scientific, MA, United States) and 1% penicillin/streptomycin (Invitrogen, CA, United States). To test the ability of GQY nanoparticles to deliver encapsulated drugs into cultured cells, insoluble doxorubicin (DOX) was prepared as previously described (Peng et al., 2021). Following the protocol described in **Section 2.1.2**, DOX was encapsulated in GQY to obtain a GQY-DOX nanoparticle stock suspension with a DOX concentration of 200 μg/mL. A375 cells suspended in 2 mL of medium were seeded into a glass-bottomed 35-mm dish (Nest Biotechnology, Wuxi, China) at a density of 2.5×10^4 cells/mL and cultured overnight. Then fresh medium containing 10 μg/mL of GQY-DOX was added to the cells, which were kept in incubator for 4 h. The cells were then rinsed three times with PBS and stained with 20 μg/mL Hoechst (Sigma-Aldrich, St Louis, MO) for 20 min, following which the cells were rinsed three times with PBS and 5 μM ThT was used to stain the cells for another 5 min. After another three times of rinse with PBS, cells were imaged using an IXplore SpinSR confocal microscope (Olympus, Tokyo, Japan).

To compare the cellular uptake level of LH and LNP, A375 cells were seeded in 12-well plates at an initial density of 2.5×10^5 cells/well and incubated overnight. Then the medium was replaced by fresh medium containing 6 mM of lidocaine as LH or in LNP and the cells were cultured for 4 h. Then the medium was removed and the cells were gently washed by PBS for 3 times to remove drug not absorbed by the cells. Three rounds of freeze-thaw cycle were then applied to induce cell lysis, and the amount of lidocaine in each well was determined by HPLC method.

2.3.2 Cytotoxicity Assay

A375 cells were seeded into a 96-well plate at an initial density of 1×10^5 cells/well and incubated overnight. To show the cytotoxicity of LH under short-time incubation, medium containing 1, 5 mM or 10 mM of LH was incubated with the cells for 4 h and then replaced by fresh medium without LH, and the cytotoxicity assay was carried out after another 20 h. Alternatively, medium containing 1, 5 mM or 10 mM of LH was incubated with the cells for 24 h until cytotoxicity assay was carried out. To compare the cytotoxicity of LH and LNP, medium containing 1–10 mM lidocaine as LH or in LNP was prepared by diluted the stock solution/suspension into fresh medium. Drug-containing medium was incubated with A375 cells for 4 h, after which the medium in each well was replaced by fresh medium without drug and the cells were cultured for another 20 h until cytotoxicity assay. After a total of 24 h of incubation, cell viability in each experiment was tested using an Enhanced Cell Counting Kit-8 (Saint-Bio, Shanghai, China) following the manufacture's instruction. In all experiments, cells always incubated with drug-free medium were used as the control group, and medium without cells seeded was used as the blank. The optical density (OD) values were detected at 490 nm by an Eon microplate spectrophotometer (BioTek Instruments Inc., United States). Cell viability was calculated as followed:

$$Cell\ viability\ (\%) = \frac{(OD_{test} - OD_{blank})}{(OD_{control} - OD_{blank})} \times 100\%$$

2.3.3 Cell Migration

Migration of A375 cells under different treatments was assessed using a wound-healing assay as described previously (Li et al., 2018). The cells were cultured in 12-well plates and allowed to grow until reaching 95% confluency. The cell monolayer was scratched using a 200-μL pipet tip to create a "wound." Then the cells were cultured in serum-free medium containing 6 mM of lidocaine as LH or in LNP for 4 h, after which the medium was replaced by serum-free medium without drug and the cells were culture until 24 h. Cells always cultured in serum-free medium containing no drug were used as control. Images of the wounds were taken at 0 and 24 h. The migration distance was analyzed with ImageJ.

2.3.4 Cell Invasion

A375 cells were seeded in 6-well plate at a density of 6×10^5 cells/well and cultured overnight. Then the medium was replaced by serum-free medium containing 6 mM lidocaine as LH or in LNP. Serum-free medium without drug was used as control. After 4 h of incubation, the cells in each well were washed with PBS and collected by trypsinization. Treated cells were suspended in serum-free medium and seeded into the Matrigel-coated upper chambers of transwells at a density of 2×10^4 cells/well. The lower chamber of each well was filled with medium containing 10% FBS. After 24 h of incubation, cells attached on the outside bottom of the upper chambers were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. For each sample, at least five random areas on the bottom were imaged and counted for the cell numbers. All results were normalized to the average number in the control group.

2.3.5 Flow Cytometry

A375 cells were seeded in 6-well plates at an initial density of 5×10^5 cells/well and incubated overnight, following which the medium was replaced by fresh medium containing 10 mM of lidocaine as LH or in LNP. Fresh medium containing no drug was used as the control. The cells were incubated with drugs for 4 h and then collected by trypsinization. Using an Annexin V Apoptosis Detection Kit (Thermo Fisher Scientific) and following the manufacture's introduction, the cells were double-stained by FITC-labeled Annexin V and propidium iodide. Then the stained cells were analyzed by flow cytometry (BD FACSCelesta).

2.4 *In vitro* Drug Release

In vitro drug release profile of LH and LNP was studied using a dialysis method. Briefly, 1 mL of each formulation was placed in a Spectra/Por Float-A-Lyzer G2 dialysis device (8–10 kDa MWCO, Spectrum Labs, United States) and dialyzed against 30 mL of PBS (pH7.4). At each time point between 0.25 and 48 h, 10 mL of PBS containing released lidocaine were taken out and another 10 mL of fresh PBS was added. The amount of released lidocaine was determined by HPLC method using an Agilent Extend C18 column (4.6 mm × 150 mm, 5 μm), solvent A (10 mM ammonium bicarbonate solution) and B (acetonitrile) with an isocratic gradient ratio of 50:50. The peak of lidocaine was detected at the wavelength of 214 nm. Drug release profiles were constructed by plotting the amount of drug released over time.

2.5 Animal Experiments

All animal experiment procedures were approved by the Animal Ethical Committee of West China Hospital, Sichuan University, and conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals by the United States National Institutes of Health. Six-week-old BALB/c-nu nude mice (GemPharmatech Co., Ltd, Chengdu, China) were used to establish tumor models *in vivo*.

2.5.1 Tumor Development Suppression

To compare the efficacy of LH and LNP in suppressing the development of tumor from seeded tumor cells, 1×10^6 A375 cells in 100 μL of serum-free medium were injected subcutaneously into the armpit of the right forelimb of each mouse. The mice were randomly divided into four groups (n = 6) for treatment with different formulations including normal saline (NS), 5 mM GQY, 25 mM LH or 25 mM LNP. On day 3 after cell injection, 50 μL of each formulation was injected into the cell lump of each mouse. Starting from day 5 after cell injection, solid tumors were formed and their size was monitored every 2 days by measuring the longest diameter (a) and the shortest diameter (b). The tumor volume was calculated according to the following equation:

$$V = 0.5 \times a \times b^2$$

On day 15 after cell injection, the mice were euthanized and the tumors in each group were collected and weighed.

2.5.2 Tumor Recurrence Inhibition

To compare the efficacy of LH and LNP in preventing tumor recurrence after surgical excision, a tumor excision model was used as described previously (Gao et al., 2019). Briefly, 1×10^6 A375 cells in 100 μL of serum-free medium were injected subcutaneously into the armpit of the right forelimb of each mouse. On day 10 after cell injection, solid tumors with an average size around 200 mm^3 were formed. For each mouse, an incision was made at the tumor site and 3/4 of the tumor was removed. The mice were randomly divided into four groups (n = 9) for treatment with different formulations including NS, 5 mM GQY, 25 mM LH or 25 mM LNP. To prevent potential drug leaking, each formulation was mixed with 1% hyaluronic acid hydrogel and injected into the tumor site through the incision, after which the incision was sutured carefully. From day 12 to day 40 post-operation, tumor recurrence was monitored by measuring the tumor size every 2 days. Animals were euthanized when tumor volume reached 1,500 mm^3.

3 RESULTS

3.1 Formation and Characterization of LNPs

As shown in **Figure 1A**, LB cannot be fully dispersed in water even after vigorous stirring, which confirms its poor water solubility. While in 5 mM GQY solution, LB could be well dispersed and form a stable milky suspension. TEM and AFM images in **Figure 1B** show that LNPs with a homogenous size smaller than 100 nm were formed, which are morphologically similar to the self-assembling nanoparticles formed by GQY. Furthermore, the particle size distribution shown in **Figure 1C** indicates that the average size of LNPs in the suspension is similar to that of empty GQY nanoparticles, and zeta potential data in **Figure 2D** show that the surface of LNPs carries weak negative charge similar to GQY nanoparticles.

The self-assembling behavior of GQY with or without LB was monitored by a ThT-binding fluorescence assay described in our previous study, which could quantify the peptides' aggregation in a real-time manner (Chen et al., 2018a). As shown in **Figure 1E**, LNP suspension shows an even higher ThT-binding fluorescence compared with GQY solution at the same peptide concentration, suggesting that the self-assembling behavior of peptide was even strengthened by LB incorporated.

As shown in **Figure 1F**, the FTIR spectrum of GQY shows two peaks at 1,660 and 1,627 cm^{-1}, which can be assigned to amide I band from the peptide backbone. Generally, peptides with very strong aggregation behavior would show only one peak at 1,627 cm^{-1}, so that the existence of the peak at 1,660 cm^{-1} suggests that the self-assembly of GQY is not so compact like some other peptides. LH shows two peaks at 1,543 and 1,473 cm^{-1}, which can be assigned to the $N\text{-}H^+$ group of protonated lidocaine, while LB with unprotonated N-H group shows only one peak at 1,496 cm^{-1}. Since LNP also shows only one peak at 1,496 cm^{-1}, it can be confirmed that in the LNP formulation LB kept its base form and didn't get protonated. Furthermore, the FTIR spectrum of LNP seems to be a simple summation of the spectra of LB and GQY, and it was also very similar to that of the physical mixture of LB and GQY.

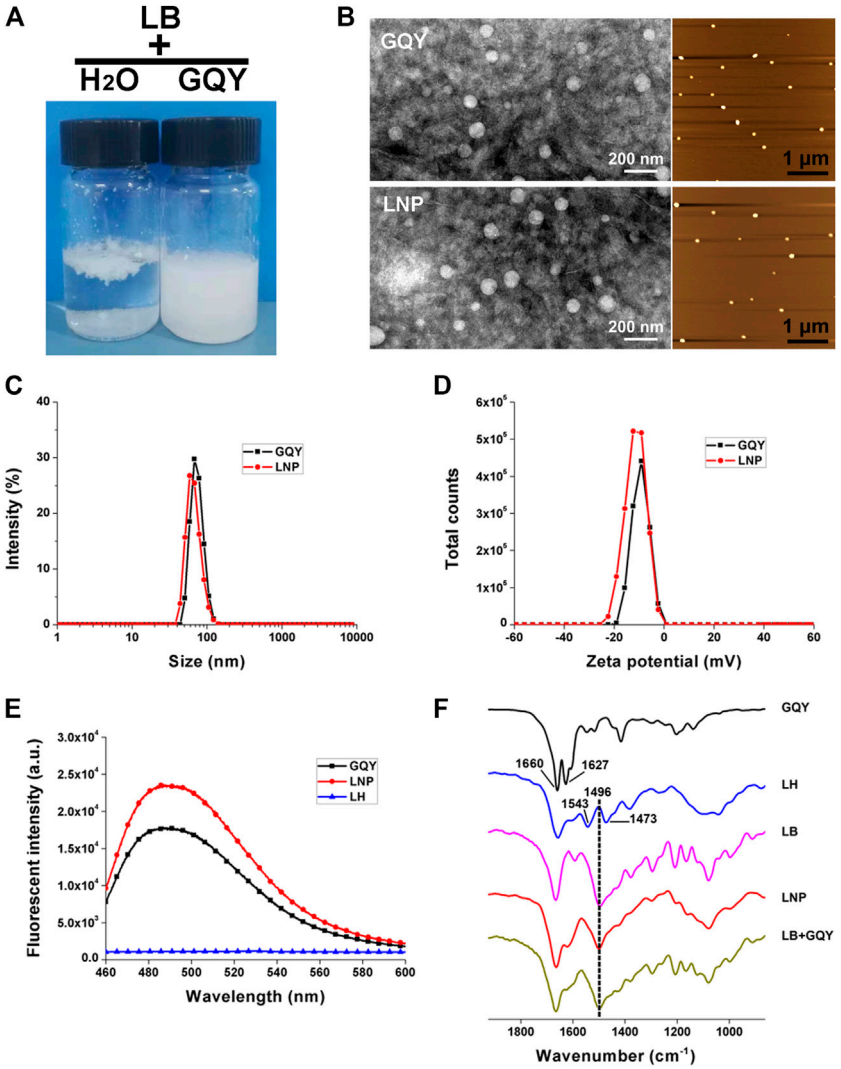

FIGURE 1 | Characterization of LNPs. **(A)** photo pictures of LB dispersed in water (left) or in GQY (right). **(B)** TEM (left) and AFM (right) images of empty GQY nanoparticles and LNPs. **(C)** size distribution of LNPs and empty GQY nanoparticles. **(D)** zeta potential of LNPs and empty GQY nanoparticles. **(E)** ThT-binding fluorescence of GQY, LNPs, and LH. **(F)** FTIR spectra of GQY, LH, LB, LNP, and LB + GQY.

3.2 LNPs Enhanced Cellular Uptake and Cytotoxicity

Due to the very short half-life time of LH *in vivo*, cells in solid tumor tissue are expected to expose to LH for only a very short period of time. For this reason, we investigated the antitumor activity of LH under short-term incubation, which can mimic the short-term contact of drug with cells *in vivo*. As shown in **Figure 2A**, when LH was incubated with A375 cells for 4 h, its cytotoxicity decreased significantly compared with 24-h of incubation.

Using DOX as a fluorescent drug model, it was confirmed that GQY nanoparticles can efficiently deliver hydrophobic drug into the cytosol of cells within 4 h. As shown in **Figure 2B**, fluorescence of ThT-stained GQY nanoparticles fully overlaps with the fluorescence of DOX, suggesting the co-localization of the carriers and the drug. As shown in **Figure 2C**, compared with soluble LH, LNPs exhibit a much higher level of cellular uptake. It should be noted that in this cellular uptake experiment, even only after 4 h of incubation, LNPs have already led to the cytolysis of a considerable number of cells as observed by microscope. Since drugs taken by these cells cannot be measured, the actual cellular uptake level in the LNP group is supposed to be even higher. As a result, in a 4-h incubation treatment, LNPs significantly enhanced the cytotoxicity of lidocaine compared with the soluble LH formulation (**Figure 2D**).

3.3 LNPs Inhibited Cell Migration and Invasion

Migration and invasion of tumor cells are two important features related to their malignance. As shown in **Figure 3**, LNPs

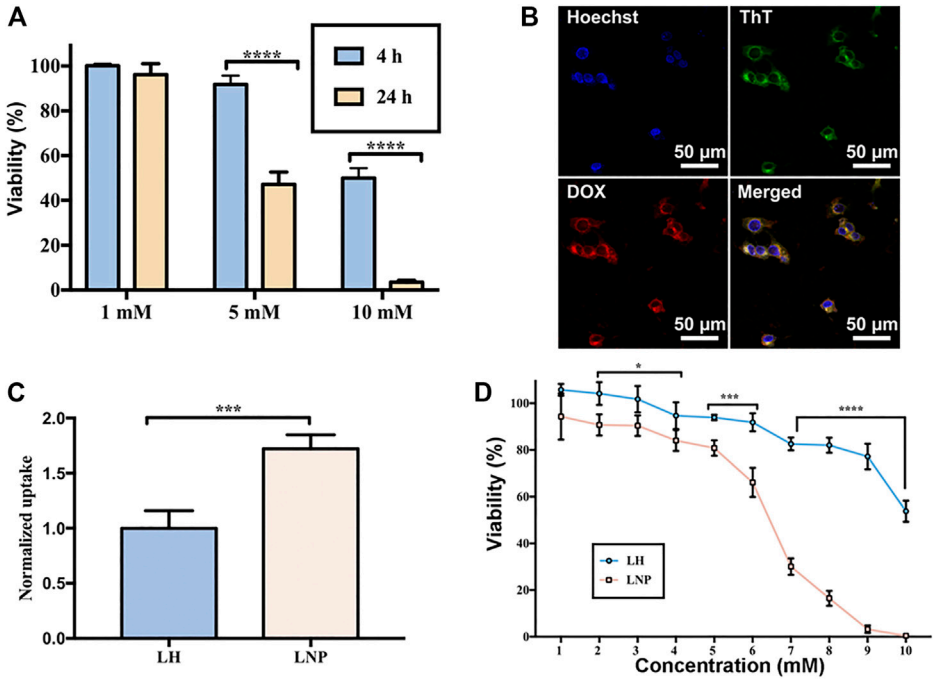

FIGURE 2 | Enhanced cellular uptake and cytotoxicity of LNPs. **(A)** cytotoxicity of LH incubated with A375 cells for 4 or 24 h. **(B)** co-localization of GQY (stained green by ThT) and encapsulated DOX (red) in A375 cells. **(C)** cellular uptake of LH and LNPs. **(D)** cytotoxicity of LH and LNPs with 4-h of incubation. *$p < 0.05$, ***$p < 0.001$,****$p < 0.0001$ by one-way ANOVA with Tamhane's T2.

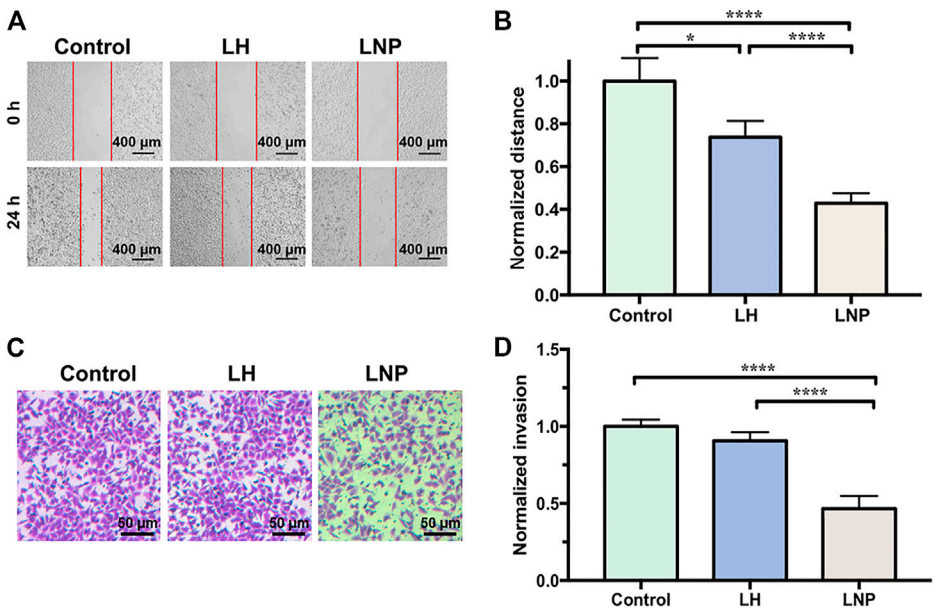

FIGURE 3 | LNPs inhibit migration and invasion of A375 cells. **(A)** representative images of wound healing, with the "wound" area marked by red lines. **(B)** comparison of normalized migration distance between different groups. **(C)** representative images of cells invaded through the membrane of transwell. **(D)** comparison of normalized invasion rate between different groups. *$p < 0.05$, ****$p < 0.0001$ by one-way ANOVA with Tamhane's T2.

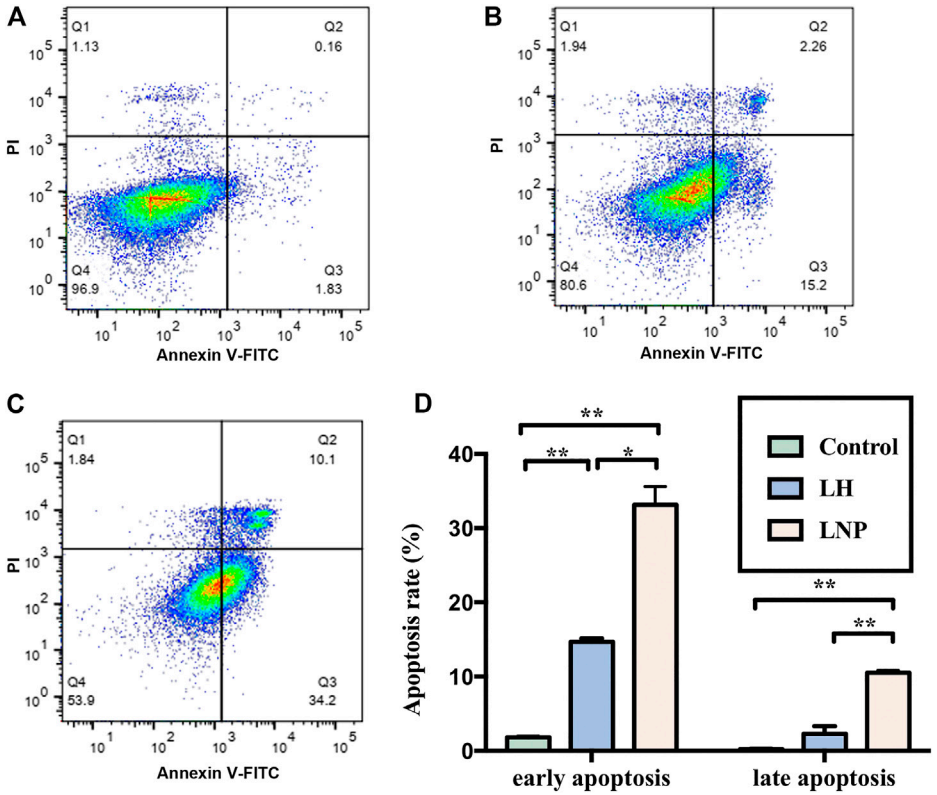

FIGURE 4 | Apoptosis of A375 cells induced by LNPs or LH. Representative flow images were shown for the control group **(A)**, LH group **(B)**, and LNP group **(C)**. Comparison of analyzed apoptosis rate between different groups was shown in **(D)**. *$p < 0.05$, **$p < 0.01$ by one-way ANOVA with Tamhane's T2 post-hoc test.

significantly inhibited the migration and invasion of A375 cells, suggesting a strong antitumor activity. On the contrary, the effect of soluble LH on cell migration and invasion is relatively weak. It should be noted that although the cells were only treated by 4-h of drug incubation, LNPs have already generated a profound effect in inhibiting the cells migration and invasion, which can last till 20 h after drug exposure.

3.4 LNPs Induced Apoptosis of A375 Cells

Although no confirmed mechanism has been established to explain the antitumor activity of lidocaine yet, many studies have suggested that the drug may kill tumor cells by inducing apoptosis (Lu et al., 2011; Wang et al., 2019; Xia et al., 2019). In this study, we incubated A375 cells with LH or LNPs for 4 h to see if they can efficiently induce apoptosis of the cells. As shown in **Figure 4**, LH only induced apoptosis at a relatively low level after 4 h of incubation, likely due to its inefficient cellular uptake. On the other hand, LNPs significantly enhanced the apoptosis rate of A375 cells, suggesting that LNPs could induce the death of tumor cells through the apoptosis pathway rather quickly.

3.5 Slow Drug Release *in vitro*

As shown in **Figure 5**, in the highly water-soluble LH formulation, 89.13% of the drug was released within 2 h, while only 38.43% of the drug was released from LNPs. Almost no drug was left in the LH formulation only after 4 h, while a considerable amount of drug was retained in the LNPs for up to 24 h. These results demonstrate that LNPs can significantly slow down the release of lidocaine, which is expected to be beneficial for

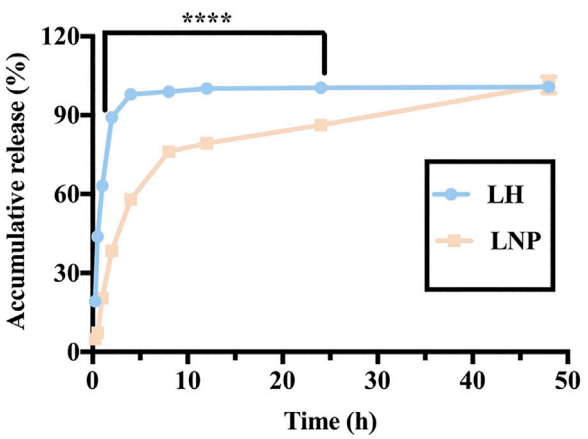

FIGURE 5 | *In vitro* release profile of LH and LNPs. ****$p < 0.0001$ by one-way ANOVA with Tamhane's T2.

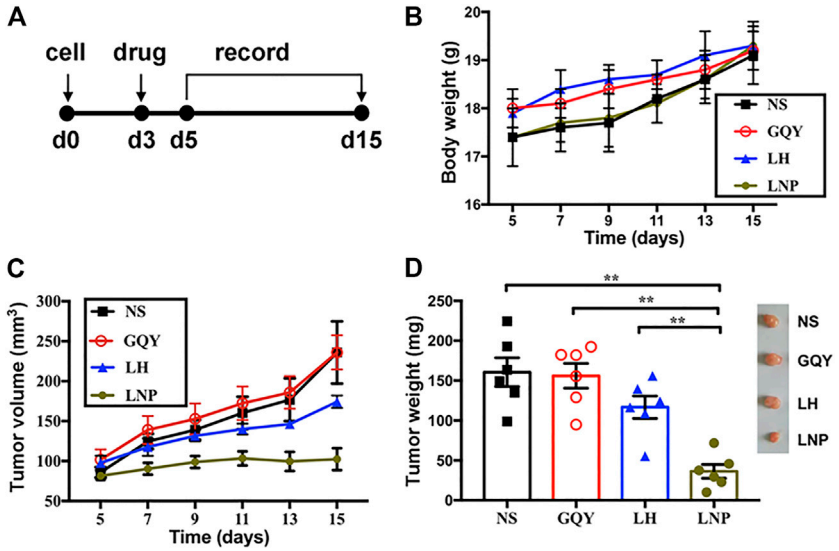

FIGURE 6 | LNPs inhibited tumor development *in vivo*. **(A)** different formulations were injected subcutaneously on day 3 after cell injection and the development of solid tumor was monitored. **(B)** change of body weight of animals during tumor development. **(C)** change of tumor volume measured during tumor development. **(D)** weight comparison and representative pictures (right) of harvested tumors from different groups. $^{**}p < 0.01$ by one-way ANOVA with Tamhane's T2 post-hoc test.

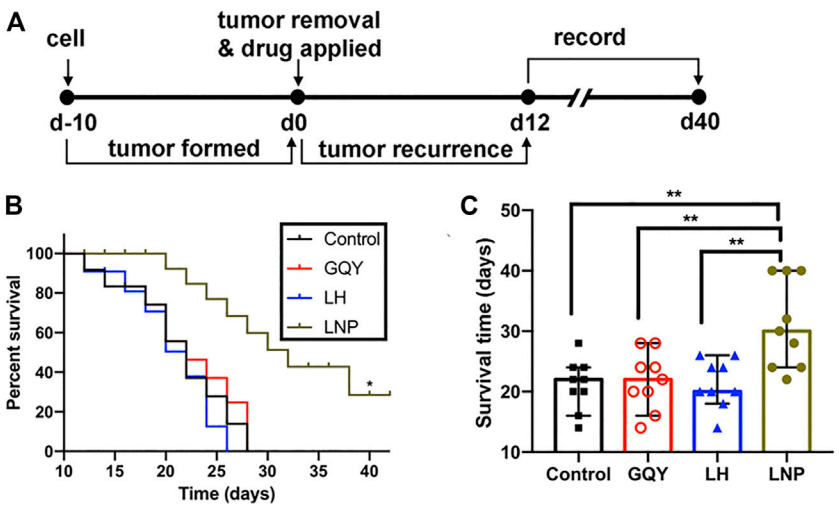

FIGURE 7 | (A) preformed tumors were partially excised and different formulations were injected on day 0, and the recurrence of tumors was monitored from day 12. **(B)** survival curve and **(C)** survival time were compared between different groups, determined based on a tumor volume of 1,500 mm^3. $^{**}p < 0.01$ by one-way ANOVA with Tamhane's T2 post-hoc test.

retaining lidocaine longer at the injection site and enhancing its antitumor activity *in vivo*.

3.6 LNPs Inhibit Tumor Development and Recurrence *in vivo*

To evaluate the antitumor efficacy of LNPs *in vivo*, we investigated their ability to inhibit early tumor development as shown in **Figure 6A**. First of all, the body weight of animals in all groups steadily increased in a similar trend, suggesting that the formulations used in our study were free of severe side effect (**Figure 6B**). As shown in **Figure 6C** a single injection of LNPs significantly inhibited the development of solid A375 tumors in nude mice, while the inhibiting effect of LH is limited and non-significant. As shown in **Figure 6D**, although tumors could still be formed in mice treated with LNPs, they are significantly smaller than the tumors in other groups. On the contrary, LH formulation with the same

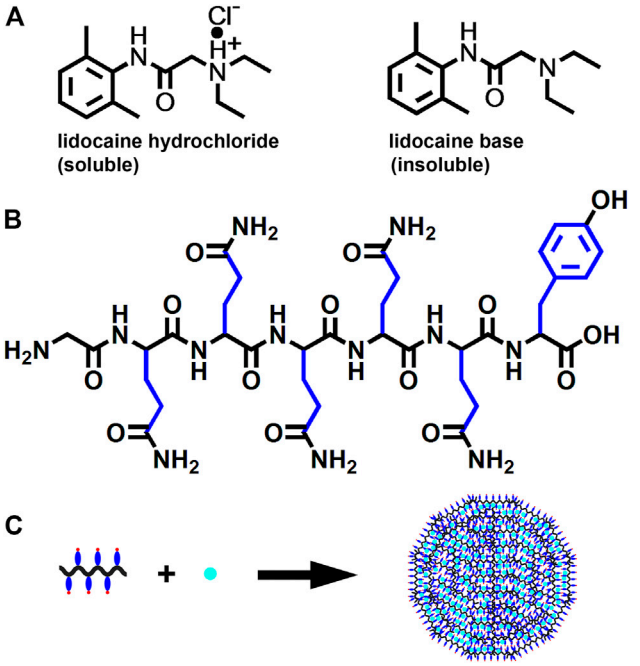

FIGURE 8 | Schematic illustration of LNPs preparation. **(A)** chemical structure of LH and LB. **(B)** chemical structure of GQY peptide with hydrophobic motifs in side-chain shown in blue. **(C)** proposed model for the formation of LNPs.

lidocaine dosage shows a limited effect in suppressing the development of solid tumors.

The efficacy of LNPs in inhibiting the recurrence of tumor from residual tumor tissue after surgical excision was also evaluated (**Figure 7A**). As shown in **Figures 7B,C**, none of the animals in the NS, GQY and LH groups survived for more than 30 days, with an averaged survival time of less than 22 days. On the contrary, animals in the LNP group survived for at least 22 days, with an average survival time of more than 30 days. Additionally, it should be pointed out that the experiment was terminated on day 40 when the last tumor-bearing mouse was euthanized, so that the longest survival time was set to 40 days. Actually, no tumor recurrence was observed for two mice in the LNP group, which means the animals were completely cured.

4 DISCUSSION

For many years, LH has been routinely used in clinic practice for its excellent water-solubility, which comes with the positive charge provided by the additional proton (**Figure 8A**). On the contrary, LB bearing no charge is poorly water soluble, making it a potential candidate for fabricating nanoparticles simply by encapsulating it with carrier materials for hydrophobic compounds. **Figure 8B** shows the chemical structure of GQY, a designer short peptide containing a glycine residue, five glutamine residues and a tyrosine residue. Although glutamine and tyrosine are conventionally regarded as hydrophilic amino acids, their side-chains contain plenty of hydrophobic motifs, endowing GQY with considerable ability to self-assemble and encapsulate hydrophobic drugs (Chen et al., 2018b). As demonstrated in **Figure 8C**, with the extensive crosslink among the brush-like hydrophobic side-chains, GQY could self-assemble into nanoparticles with plenty of hydrophobic cavities, in which hydrophobic LB could be embedded.

This encapsulating mechanism is demonstrated by the characterization results shown in **Figure 1**. The incorporation of LB didn't change the shape, size and surface charge of GQY nanoparticles drastically, indicating that LB was embedded in the inner hydrophobic cavities of GQY nanoparticles. As shown in **Figure 1E**, the self-assembling behavior of the peptide was not disturbed by LB. On the contrary, the incorporation of LB even promoted the peptide's self-assembly, probably by providing hydrophobic cores to induce the aggregation of more GQY molecules at the same peptide concentration.

Additionally, how LB was embedded in the nanoparticles could also be explained by analyzing the FTIR spectra in **Figure 1F**. Firstly, in the spectrum of GQY, the peak at 1,660 cm^{-1} suggests that the self-assembly of GQY is not very compact, leaving possible hollow cavities inside the nanoparticles for drug loading. Secondly, LNP shows a peak at 1,496 cm^{-1} similar to LB, suggesting that rather than dissolving the drug, GQY is encapsulating the drug without changing its chemical property. Lastly, the spectrum of LNP is very similar to that of the physical mixture of LB and GQY, suggesting that LB is simply embedded in the hollow cavities of GQY nanoparticles without forming extra chemical bonds with the peptide material.

Based on this mechanism, milky suspension with a very high LB concentration could be obtained. The milky suspension shown in **Figure 1A** could be caused by the self-aggregation of LNPs, since they bear relatively weak charge on their surface. However, it should be mentioned that this milky suspension is still very stable for at least 1 month and could be easily dispersed well by dilution. On the contrary, LB powder cannot be well dispersed in plain water, so that it is not suitable for further cell or animal studies.

In many *in vitro* studies evaluating the antitumor activity of lidocaine or other LAs, the drug was usually incubated with cultured cells for 24 or even 48 h, where they did exhibit excellent cytotoxicity against tumor cells in a concentration-dependent manner. But such a long period of direct drug exposure cannot be possible for an *in vivo* experiment when LH is locally injected. For this reason, in this study we focused on the cytotoxicity of lidocaine formulations under short-term incubation. As shown in **Figures 2**, **3**, LNPs could inhibit the proliferation, migration and invasion of A375 cells more efficiently, which was achieved by enhanced cellular uptake of lidocaine. Furthermore, the results in **Figure 4** suggested that LNPs may exert their stronger antitumor activity by inducing apoptosis more efficiently.

Nanoparticles containing antitumor drug can not only kill cultured tumor cells more efficiently by promoting cellular uptake, but also can they release the drug slowly and lead to improved antitumor activity *in vivo*. As shown in **Figures 6**, **7**,

LNPs effectively inhibited the development and recurrence of tumors, while the effect of LH formulation was very limited. This is not surprising, since highly soluble LH can be cleared out *in vivo* rather quickly. On the contrary, LNPs as a slow-releasing depot can retain lidocaine at the injection site much longer, leading to a more significant inhibiting effect. Furthermore, it should be noted that these antitumor efficacies were achieved by a single injection of the LNPs formulation. Even better outcome can be expected if the formulation is injected for multiple times.

Although not tested in our current study, it would be interesting to evaluate analgesic effect of the LNPs formulation beside its antitumor activity. Actually, many previous studies have shown that fabricating LA nanoparticles was also an effective strategy to prolong their duration of anesthesia and analgesia effect (Zhan et al., 2018; Li et al., 2019). Since lidocaine as a popular LA has been widely used for perioperative and postoperative analgesia, it would be highly advantageous if its antitumor activity can be exploited and combined with its analgesic effect.

As a biomaterial composed of natural amino acids, GQY didn't show any effect on the tumor development or health condition of animals, indicating that the peptide is non-toxic and safe. For this reason, the LNPs formulation based on this material is promising for clinical application. Moreover, the GQY peptide can be further ameliorated, for example to bear stronger negative charge to improve the formulation's dispersity and stability, or to bearing tumor-targeting motifs to inhibit tumor cells more selectively.

Nevertheless, there're also some limitations for this LNPs formulation due to the nonselective toxicity of lidocaine. Since lidocaine is known to cause severe systemic toxicity if enters the blood stream at high concentration, the LNPs formulation could only be locally applied for better safety. Even though, the concentration of locally injected LNPs should still be controlled to avoid potential reginal toxicity. As a result, the antitumor activity of LNPs within a safe concentration is not strong enough to completely eliminate the development and recurrence of tumor. To solve this problem, a potential direction would be combining LNPs with other antitumor drugs.

There're two important procedures need to be noted in our study. First, for all cell experiments investigating the effect of different formulations on cultured tumor cells, we incubated the formulations with cells for only 4 h. This is important to keep the *in vitro* condition mimicking the *in vivo* condition as close as possible, since small water-soluble drug injected *in vivo* is usually cleared out very quickly. Second, we evaluated the *in vivo* anti-tumor activity with two different models. In the first model inhibiting the development of solid tumors from planted cells, the number of injected cells can be accurately controlled, providing a normalized starting point for different groups. However, this model cannot mimic the clinical situation when a preformed solid tumor is excised. On the contrary, in the second model we partially removed preformed tumors and use different formulations to inhibit the recurrence of residual tumors. Although in this model it is difficult to normalize the initial size of residual tumors in different animals, it can closely mimic the surgical excision of solid tumor in clinic practice. Combining the two models, the *in vivo* effect of different formulations can be well-evaluated.

On the other hand, there're also several important works to be carried out beyond our current study. First, the mechanism of how lidocaine and other LAs kill tumor cells need to be clarified, so that we can ameliorate our LNP formulations accordingly for even better antitumor activity. Second, it would be highly advantageous if we can exploit the prolonged analgesic effect of our LNP formulations, which would be an interesting dual-functional formulation for both pain-relief and recurrence inhibiting after tumor excision. And last, our animal experiments only investigated the inhibiting effect on local tumor recurrence. However, metastasis after tumor excision surgery is also an important issue need to be addressed. It would be interesting to investigate if locally injected LNP formulations can also inhibit tumor metastasis and cure the animals more thoroughly.

CONCLUSION

In this study, we introduced a simple strategy to fabricate LNP formulation, in which hydrophobic LB was encapsulated by a self-assembling peptide GQY. By enhancing the cellular uptake of active lidocaine, the LNPs showed significantly improved antitumor activity *in vitro* as compared with soluble LH. In animal models, the LNPs formulation also significantly inhibited the development and recurrence of solid tumors. Considering the wide application of lidocaine to relieve the pain caused by cancer-relative surgery, this simple LNPs system can be a promising dual-functional formulation for both pain relief and tumor recurrence suppression. Considering the similarity of other LAs with lidocaine, their nanoparticle formulations can also be exploited using similar peptide material. Following these directions, the application of LAs in postoperative treatment of cancer can be further exploited.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethical Committee of West China Hospital, Sichuan University.

AUTHOR CONTRIBUTIONS

Study design: WZ and FQ; Data collection: YY, JS, GZ, and FP; Analysis and interpretation: JS, HL, and FQ; Statistical analysis: YY; Funding: WZ; Drafting article: YY and FQ; Revision article: JS, WZ, and JC; Animal model: JS, FP, and HL. All authors contributed to article revision, read, and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 81973274), Chengdu Science and Technology Bureau (2021-YF05-00917-SN).

ACKNOWLEDGMENTS

Authors are grateful to Qiaorong Huang and Xue Li from the Laboratory of Stem Cell Biology, West China Hospital, Sichuan University for their technical support in cell apoptosis analysis.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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