Lidocaine induces epithelial-mesenchymal transition and aggravates cancer behaviors in non-small cell lung cancer A549 cells

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Abstract. The effects of clinically relevant concentrations of lidocaine on epithelial-mesenchymal transition (EMT) and associated lung cancer behaviors have rarely been investigated. The aim of the present study was to assess the impact of lidocaine on EMT and its related phenomena, including chemoresistance. Lung cancer cell lines (A549 and LLC.LG) were incubated with various concentrations of lidocaine, 5-fluorouracil (5-FU) or both to test their effects on cell viability. Subsequently, the effects of lidocaine on various cell behaviors were assessed *in vitro* and *in vivo* using Transwell migration, colony-formation and anoikis-resistant cell aggregation assays, and human tumor cell metastasis in a chorioallantoic membrane (CAM) model quantitated by PCR analysis. Prototypical EMT markers and their molecular switch were analyzed using western blotting. In addition, a conditioned metastasis pathway was generated through Ingenuity Pathway Analysis. Based on these measured proteins (slug, vimentin and E-cadherin), the molecules involved and the alteration of genes associated with metastasis were predicted. Of note, clinically relevant concentrations of lidocaine did not affect lung cancer cell viability or alter the effects of 5-FU on cell survival; however, at this dose range, lidocaine attenuated the 5-FU-induced inhibitory effect on cell migration and promoted EMT. The expression levels of vimentin and Slug were upregulated, whereas the expression of E-cadherin was downregulated. EMT-associated anoikis resistance was also induced by lidocaine administration. In addition, portions of the lower CAM with a dense distribution of blood vessels

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exhibited markedly increased *Alu* expression 24 h following the inoculation of lidocaine-treated A549 cells on the upper CAM. Thus, at clinically relevant concentrations, lidocaine has the potential to aggravate cancer behaviors in non-small cell lung cancer cells. The phenomena accompanying lidocaine-aggravated migration and metastasis included altered prototypical EMT markers, anoikis-resistant cell aggregation and attenuation of the 5-FU-induced inhibitory effect on cell migration.

Introduction

Lung cancer is a leading cause of cancer-related death worldwide (1,2). Of note, ~85% of lung cancer cases have been classified as non-small-cell lung cancer (NSCLC) (3), for which surgery and chemotherapy are the primary treatment strategies. 5-Fluorouracil (5-FU) is one of the most commonly used chemotherapeutic agents for patients with NSCLC (4). Therapies affecting cancer behaviors are important for patient outcomes, since the 5-year relative survival rate is as low as 6% for patients with metastatic NSCLC (5). Furthermore, the development of chemoresistance represents a major challenge for the treatment of NSCLC (6,7).

Previous studies have suggested that local anesthetics administered perioperatively can affect the outcome of oncological surgeries (8,9). Lidocaine is a common local anesthetic for regional nerve block, which has been reported to block exogenous tumor necrosis factor (TNF)-α-induced increases in lung cancer cell invasion (10,11) because of its anti-inflammatory properties. In addition to its usage as a conventional antiarrhythmic agent (12), intravenous lidocaine can also be used to treat various types of chronic pain (13) and other specific conditions (14,15), such as refractory chronic daily headaches (16). Furthermore, lidocaine has been recommended as one of the main modalities in enhanced recovery after surgery (ERAS) protocols, as it can block the priming of polymorphonuclear granulocytes (17). However, at the serum concentrations achieved by intravenous infusion, whether lidocaine can affect epithelial-mesenchymal transition (EMT) and its accompanying phenomena remain unclear.

Although high-dose lidocaine (2-8 mM) has been validated to enhance cancer cell apoptosis, and to inhibit the mitogen-activated protein pathway in the growth, migration and invasion of lung cancer (18,19), different phenomena can be observed in response to different scales of lidocaine concentrations. 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 proliferation of lung cancer cells. These render the clinical effects of lidocaine on lung cancer questionable. 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 (5 μg/ml)] to be significant in translational medicine.

The present study used the A549 cell line as a common model of NSCLC (20,21) to investigate whether clinically relevant concentrations of lidocaine could influence EMT and any associated phenomena, including its impact on the effect of 5-FU in lung cancer cells.

Materials and methods

Cell culture and drug treatment. Human NSCLC A549 cells (CCL-185) were purchased from the American Type Culture Collection (ATCC). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Corning, Inc.), and 1% penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2. For experimental purposes, cells cultured to the exponential growth phase (~70% confluence) were used without serum starvation. With the same culture conditions, the mouse lung cancer cell line LLC.LG was obtained from the Agriculture Biotechnology Research Center (Academia Sinica). A combination of 5-FU (MilliporeSigma) and lidocaine (AstraZeneca) was used to determine whether these drugs have synergistic or additive effects on the cells. The concentration of 5-FU was the same as the one used to exert a 30% inhibitory effect in the pilot study.

Cell viability and colony formation assays. A sulforhodamine B (SRB) assay was used to measure drug-induced cytotoxicity (22) and cell proliferation (23). In brief, A549 and LCC.LG cells were seeded in 96-well plates ($7x10^3$/well) and incubated for 24 h. Subsequently, 5-FU and lidocaine were each diluted in DMEM and cells were incubated at 37˚C for 24 h. Following treatment with SRB, cell viability was evaluated by measuring the absorbance at 570 nm in a 96-well flat-bottomed plate reader. By comparing the absorbance in the experimental wells with that in the control well, the percentage of viable cells was determined.

For the colony formation assay, A549 cells were seeded in 6-well plates ($4x10^2$/well) at 37˚C in an atmosphere containing 5% CO_2. The cells were treated with lidocaine (final concentrations: 10 and 20 μM), 5-FU (3.125 μM) or with their combinations. The colony-forming potential of A549 cells after treatment was assessed on day 15. The cells were fixed with 4% paraformaldehyde at room temperate for 30 min and then stained with crystal violet (0.005%) for 30 min at room temperate, followed by manually counting the number of colonies.

Migration assay. A migration assay was performed in a Transwell chamber with a pore size of 8.0 μm in a 24-well plate (Corning, Inc.). A549 and LLC.LG cell density was adjusted to $1x10^6$ cells/ml. With 100 μl cell suspension in serum-free medium, cells were treated with different concentrations of lidocaine (0, 1, 5 and 10 μM) with or without 5FU (0.0375, 3.125 μM) and placed in the upper chamber of the Transwell inserts. Medium containing 10% FBS (500 μl) was added to the lower chamber. After incubation at 37˚C for 16 h, cell migration was evaluated by counting the number of cells that penetrated the membrane. The cells on the lower surface of the chamber membrane were fixed in 95% ethanol for 10 min at room temperature and the cells in the internal compartment were removed with a cotton swab. After the chamber was air-dried, the cells were stained with DAPI (1 μg/ml) for 10 min at room temperature and five randomly selected fields of view (magnification, x100) were captured using a fluorescence microscope for cell counting.

Anoikis-resistant cell aggregation assay. A549 cells ($2x10^3$ cells/well) were exposed to 10 μM lidocaine or an equal volume of DMEM on 6-well plates coated with poly-2-hydroxyethyl methacrylate (poly-HEMA; MilliporeSigma). Images of cell clusters growing in 6-well poly-HEMA plates were captured on day 5. Thereafter, the medium was removed from each well and the cells were harvested 1 day later (on day 6). After anchoring the cell clusters on the 6-well plate without poly-HEMA layer, the detached cells were removed. Before being fixed with 4% formalin solution for 30 sec at room temperature for counting, the cell clusters were stained with 0.01% (w/v) crystal violet at room temperature for 60 min. The result of the crystal violet staining for determining the viability of cultured cells was used to estimate cell survival (24). The cells were visualized and counted using an inverted microscope (CKX31; Olympus Corporation). The number of cell clusters was scored using ImageJ software (version 1.48v; National Institutes of Health).

Western blot analysis. A549 cells were seeded in a 10 cm^2 dish and incubated in culture medium for 24 h. Thereafter, the cells were treated with lidocaine (10 μM) or incubated with 20 ng/ml TGF-β (R&D Systems, Inc.) at 37˚C for 48 h to induce EMT. The use of human TGF-β to elicit EMT in a mouse lung cancer cell line is also feasible as previously described (25). Subsequently, cells were cultured to logarithmic growth phase, and were collected and lysed in RIPA Lysis and Extraction Buffer (cat. no. R0278-50ML; Sigma-Aldrich; Merck KGaA). The protein concentration of total cell lysates was accessed using the bicinchoninic acid method. A total of 30 μg total proteins/lane were separated by SDS-PAGE on 10% gels (Bio-Rad Laboratories, Inc.), then transferred to a PVDF membrane. The membrane was blocked for 1 h at room temperature in TBST (10 mM Tris, pH 7.5; 150 mM NaCl; 0.1% Tween 20) with 5% skim milk. After incubation with the following primary antibodies for 1 h at room temperature: E-cadherin (1:1,000 dilution; cat. no. 3195), vimentin (1:1,000; cat. no. 5741), Slug (1:1,000; cat. no. 9585) and GAPDH (1:1,000; cat. no. 2118) (all from Cell Signaling Technology, Inc.), the membrane was washed three times in TBST (10 min/wash) and then incubated with secondary antibodies, including anti-rabbit (1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.) and anti-mouse (1:5,000; cat no. 7076; Cell Signaling Technology, Inc.,) in TBST at room temperature for 1 h. The membrane was subsequently washed three times with TBST (10 min/wash) and the bands were visualized using enhanced electrochemiluminescence (ECL pierce kit; cat. no. 32109; Thermo Fisher Scientific, Inc.) to analyze the expression of target proteins. ImageJ software (version 1.48v) was used for semi-quantification of the western blots and all measurements were normalized against the GAPDH loading control.

Chick chorioallantoic membrane (CAM) assay. A total of 9 fertilized chicken eggs were obtained from the Animal Health Research Institute, Council of Agriculture, Executive Yuan, and were incubated at 37˚C in an atmosphere containing 80% relative humidity (26). All methods were carried out in accordance with the relevant guidelines and regulations (The AVMA Guidelines for the Euthanasia of Animals: 2013 Edition-September 19, 2013). A small window was made in the shell on day 7 of chick embryo development under aseptic conditions. The eggs were returned to the incubator immediately after resealing the window on day 7. After 2 days, the eggs in the incubator were taken out for A549 administration into the upper CAM. Briefly, A549 suspensions ($1x10^6$) were mixed with hydrogel (10 mg/ml) at a total volume of 20 μl. Lidocaine (10 μM), 5-FU (3.125 μM) or both were mixed together with the A549 cells and hydrogel. Hydrogel grafts were placed on top of the CAM and eggs were resealed and returned to the incubator for 24 h until day 10 (3 chicken embryos per group). On day 10, the eggshell was cut and the lower CAM tissue was harvested for DNA extraction (DNA extraction kit; cat. no. TX-CD001; TOOLS) and human *Alu* sequences were quantified using quantitative PCR (qPCR). Total RNA was extracted using an RNeasy Mini Kit and treated with RNase-free DNase I set (Qiagen GmbH) according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed using oligo (dT) primers and a reverse transcription system (Promega Corp.). Reactions were carried out using Fast SYBR® Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on the Step One Plus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) by denaturation at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 40 sec. Melting curve analyses were performed to verify the amplification specificity. Relative quantification of gene expression was performed according to the $2^{-\Delta\Delta Cq}$ method using StepOne Software 2.0 (Applied Biosystems; Thermo Fisher Scientific, Inc.) (27). The detection of human tumor cells is based on the quantitative detection of human *Alu* sequences present in chick DNA extracts, and is a modification of the method developed by Kim *et al* (28). The design of the *Alu* primers was performed as described in a previous study (29). To detect human cells, primers specific for the human *Alu* sequences (sense: 5'-ACGCCTGTAATCCCAGCACTT-3'; and antisense: 5'-TCGCCCAGGCTGGAGTGCA-3') were used to amplify the human *Alu* repeats present in genomic DNA (28). A quantitative measure of amplifiable chick DNA was obtained through amplification of the chick GAPDH (chGAPDH) fragment with chGAPDH primers (sense: 5'-GAGGAAAGGTCGCCTGGTGGATCG-3'; antisense: 5'-GGTGAGGACAAGCAGTGAGGAACG-3') using the same PCR conditions as described for *Alu*. On day 10, freezing of the whole egg was applied to end the experiments.

Ingenuity pathway analysis (IPA). To build the conditioned metastasis pathway and to systematically investigate the impact of lidocaine-altered EMT proteins on all other related genes, the relevant networks were generated using IPA (version 68752261; Qiagen GmbH). Focusing on the most extensive pathway regarding metastasis in the 'Diseases and Functions' of the IPA system, the pathway 'Metastasis' with 3,521 associated molecules was then restricted to 'Human', 'Genes, RNAs and Proteins', 'downregulation and upregulation', and filtered on 'non-small cell lung carcinoma' by inclusion ('AND') using the BioProfiler function. The IPA overlay function within this conditioned metastasis pathway further allowed the selection of 'Regulation of the Epithelial Mesenchymal Transition by Growth Factors Pathway' as the canonical pathway to be displayed. A total of 30 molecules

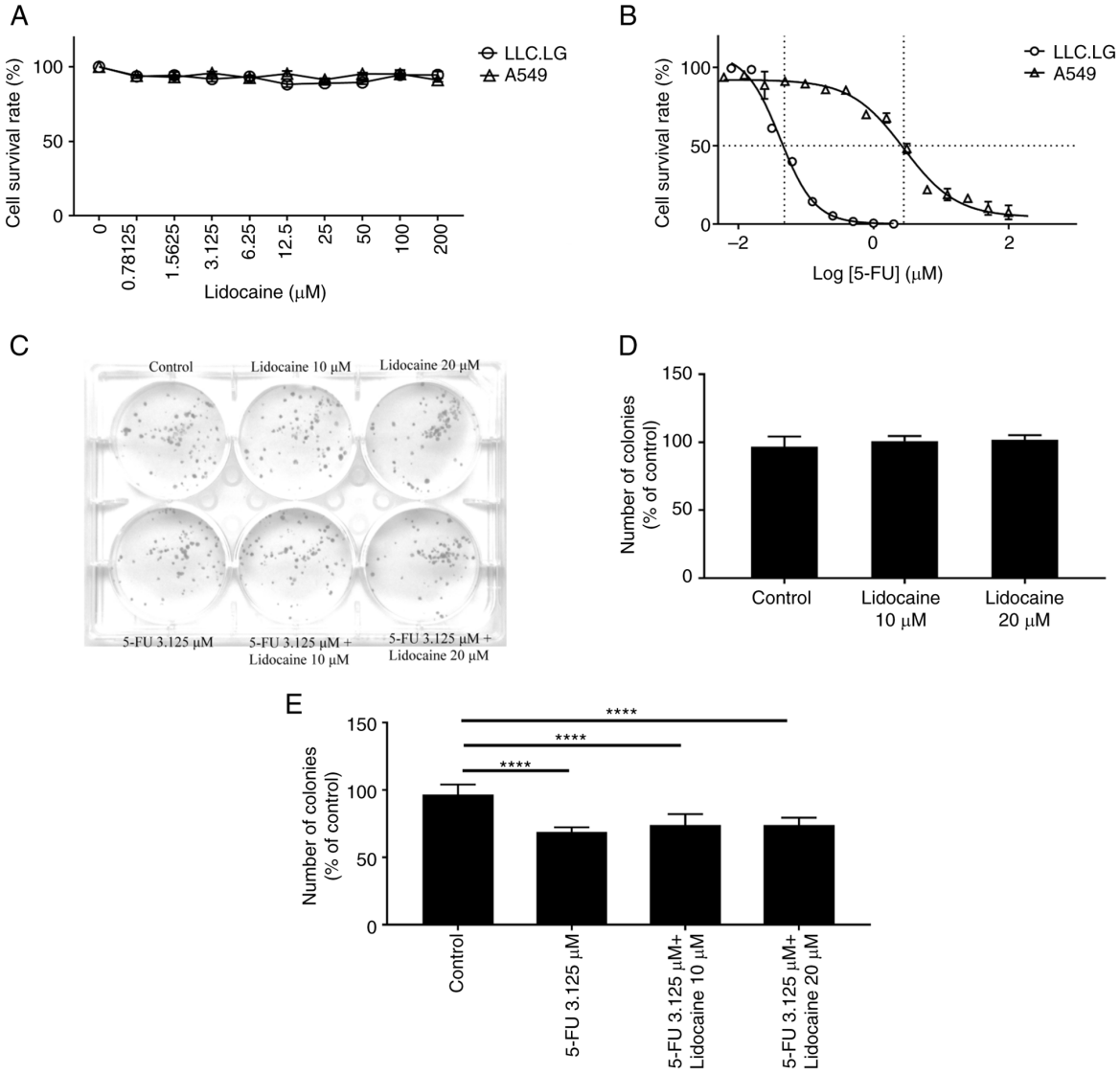

Figure 1. Effect of lidocaine and 5-FU on the survival of lung cancer cells. (A) Non-toxic effect of lidocaine on lung cancer cells (0.78125-200 μM). (B) Inhibitory effect of 5-FU on A549 and LLC.LG cells. Values are expressed as the mean ± SD (n=3). (C) Colony formation assay in A549 cells. (D) Semi-quantification of colony numbers with or without lidocaine treatment in A549 cells. (E) Semi-quantification of colony numbers with or without 5-FU (3.125 μM) or lidocaine (10 and 20 μM) treatment in A549 cells. ****P<0.0001. Values are expressed as the mean ± SD (n=6). 5-FU, 5-fluorouracil.

were revealed to be involved in the final conditioned pathway. To discover possible gene alterations involved in NSCLC metastasis in terms of EMT, the path explorer tool of the IPA system was used to identify all possible relationships between the measured proteins (vimentin, Slug and E-cadherin) and the remaining 27 molecules in this conditioned metastasis pathway. The molecule activity predictor tool was then utilized to predict the impact of the three measured proteins altered by lidocaine.

To further investigate the impact of measured prototypical EMT markers (vimentin and E-cadherin) and their molecular switch (Slug) on EMT (30), NSCLC and apoptosis of NSCLC, the path explorer tool of the IPA system was used to discover the impact of each molecule by limiting the relationships to 'activation', 'causation' and 'inhibition' via the filter function. Based on the derived shortest paths and one more path beyond the shortest path, the impact of the individual measured molecule was predicted via the molecule activity predictor tool.

Statistical analysis. Data are presented as the mean ± SD. All data were analyzed using GraphPad Prism version 7.0 software (Dotmatics). Differences between multiple groups were analyzed using one-way ANOVA for single variable analysis, followed by Tukey's multiple-comparisons post-hoc test. Differences between two groups were analyzed using Student's t-test after passing the Shapiro-Wilk normality test. Mann-Whitney U-test was applied if the data failed the normality test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of lidocaine and 5-FU on cell survival. A549 and LLC.LG cells were treated with lidocaine (0-200 μM) for 24 h. Lidocaine at various concentrations did not reduce cell viability (Fig. 1A). According to the SRB assay, the half-maximal inhibitory concentration (IC_{50}) of 5-FU for

A549 and LLC.LG cells was 2.808 and 0.042 μM, respectively (Fig. 1B). The 5-FU concentration resulting in ~30% A549 and LLC.LG cell viability inhibition in the SRB assay was 3.125 and 0.0375 μM (Fig. S1), respectively. The number of colonies formed by A549 cells did not differ significantly between cells treated with lidocaine and untreated cells; however, it significantly differed between cells treated with 5-FU and untreated cells (Fig. 1C-E). To more easily detect the lidocaine's additive or synergistic effect on 5-FU's inhibition, we chose a concentration of 5-FU that has a 30% inhibitory effect. Although a majority of the range (~70%) was left in the 5-FU-based inhibitory model to observe the expected synergistic or additive effect exerted by lidocaine at moderate (10 μM) or high normal (approaching clinically toxic level; 20 μM) concentrations, it was revealed that lidocaine did not aggravate the cytotoxic effects of 5-FU.

Lidocaine does not influence the effect of 5-FU on cell survival at clinically relevant concentrations. According to the results of the colony formation assay, lidocaine at a high normal concentration (approaching clinically toxic level; 20 μM) did not aggravate the cytotoxic effects of 5-FU. In the subsequent experiment, the present study aimed to verify whether lidocaine at a range of clinically safe concentrations (<20 μM) could influence the effect of 5-FU on cell survival and to assess whether it had a dose-dependent effect. Therefore, lidocaine concentrations at 1, 5 and 10 μM were selected. The LLC.LG cells exposed to 5-FU at 0-0.375 μM for 24 h showed a dose-dependent reduction in viability (Fig. 2A). There was a positive association between the increase in 5-FU dosage and decrease in LLC.LG cell viability; however, the addition of lidocaine did not further affect cell viability. Treatment of A549 cells with 5-FU at 0-31.25 μM for 24 h also resulted in a dose-dependent reduction in cell viability (Fig. 2B). No dose-dependency was found regarding the effect of lidocaine (Fig. 2C and D). These findings indicated that 5-FU directly reduced LLC.LG and A549 cell viability, whereas treatment with lidocaine at clinically relevant concentrations did not alter the effect of 5-FU on the viability of either of the cell lines.

Lidocaine attenuates the 5-FU-induced inhibitory effect on cell migration and promotes EMT at clinically relevant concentrations. The results of the cell migration assay showed that the number of migrated LLC.LG cells treated with 5-FU was lower than that in the control group. However, 5-FU combined with 1 μM lidocaine resulted in cell migration similar to that in the control group (Fig. 3A), indicating that lidocaine reversed the inhibitory effect exerted by 5-FU on cell migration. Similar results were obtained in A549 cells (Fig. 3B). To further study the effects of lidocaine on A549 cell migration, the epithelial marker E-cadherin and the mesenchymal marker vimentin were assessed using western blotting. Lidocaine and TGF-β (positive control) significantly upregulated the expression levels of vimentin, and downregulated the expression levels of E-cadherin, indicating that EMT was induced (Fig. 3C and D). The absence of an additive or synergistic effect from the combination of lidocaine and TGF-β indicated that there may be a negative feedback loop triggered by the combination of lidocaine and TGF-β, or a negative interaction between their downstream effector pathways. The expression of Slug, the molecular switch immediately

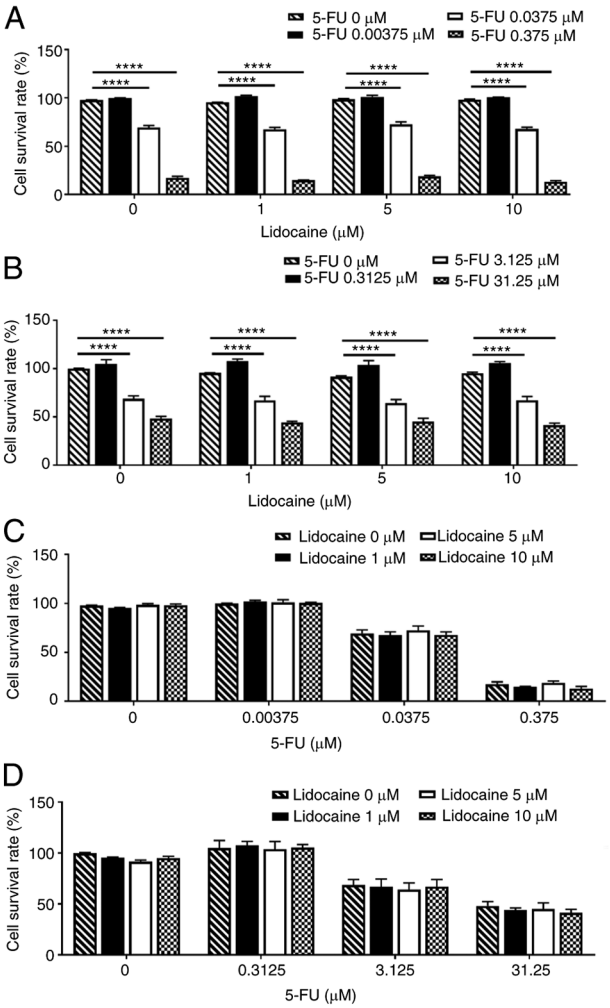

Figure 2. Combination effect of lidocaine and 5-FU on cell viability. Dose-dependent effects of 5-FU under various lidocaine concentrations in (A) LLC.LG and (B) A549 cells. Effects of lidocaine under various 5-FU concentrations in (C) LLC.LG and (D) A549 cells. Values are expressed as the mean ± SD (n=3). ****P<0.0001. 5-FU, 5-fluorouracil.

upstream of EMT in lung cancer (30), was also revealed to be upregulated by lidocaine (Fig. 3E). The aforementioned alterations in A549 cells indicated that EMT was induced, at least in part, by lidocaine at clinically relevant concentrations.

Lidocaine induces anoikis resistance by forming cell clusters at clinically relevant concentrations. Besides being associated with cell migration, EMT is also a characteristic of anoikis resistance (31). Therefore, the present study performed an anoikis-resistant cell aggregation assay and revealed that the number of cell clusters was significantly increased with lidocaine administration (Fig. 4). Thus, we hypothesized that A549 cells had the potential to progress towards EMT, a hallmark of cancer stemness. This was, at least in part, supported by the results of western blotting (Fig. 3C-E). Furthermore, the anchoring of the cell clusters on the 6-well plate without poly-HEMA layer confirmed that the number of clusters formed by aggregation associated with the cell survival status determined by the crystal violet assay (Fig. 4). That led to two important assumptions. First, lidocaine at clinically relevant concentrations is not likely a cause for lung cancer cell death. Second, the lung cancer

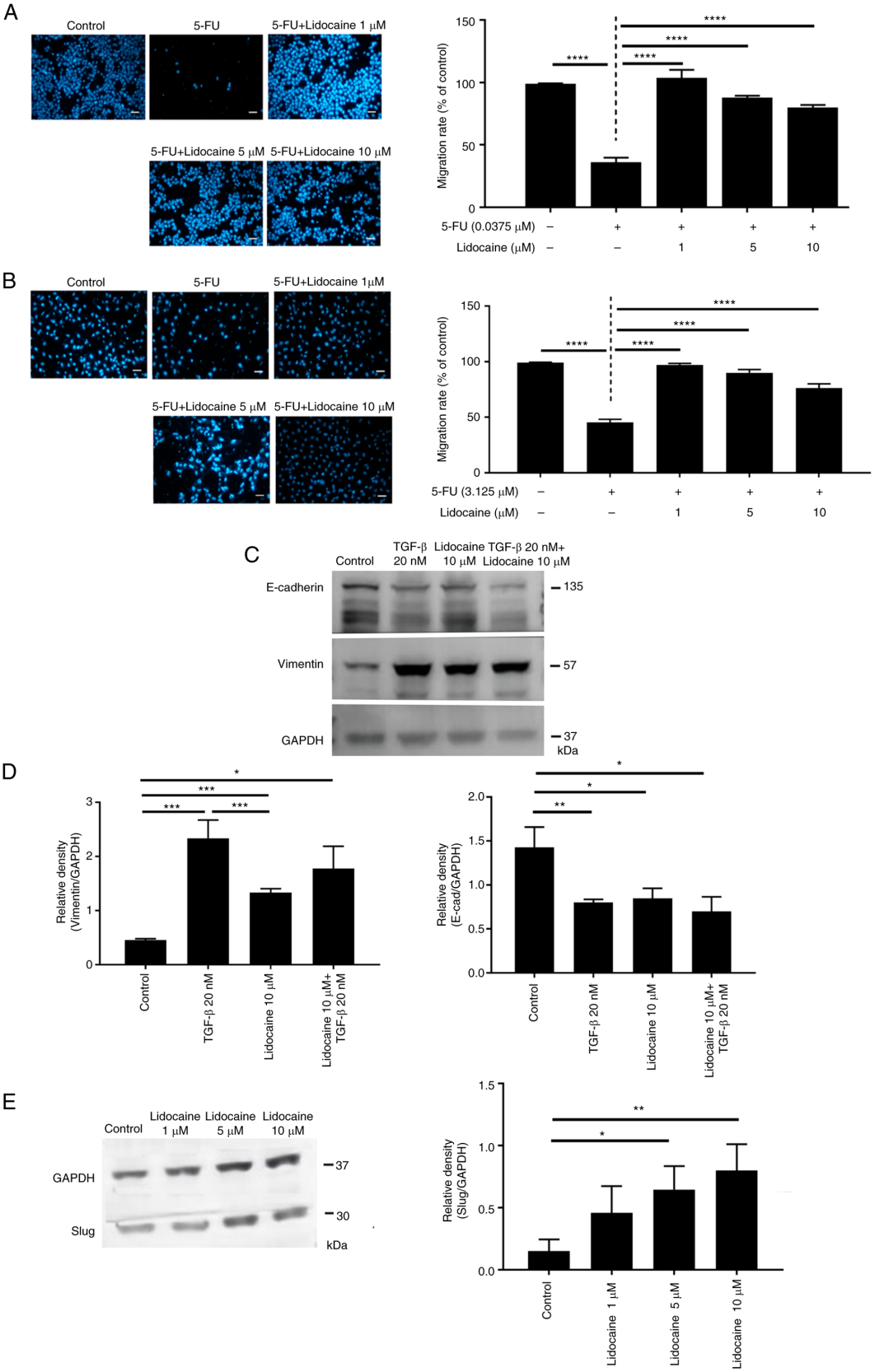

Figure 3. Attenuation of the inhibitory effects of 5-FU on cell migration by lidocaine. Combination effect in (A) LLC.LG (n=6 for 5-FU 0.0375 μM group, n=7 for other groups) and (B) A549 (n=4) cells. Cell migration is indicated by the number of DAPI-stained nuclei. Medium group served as the control. Values are expressed as the mean ± SD. ****P<0.0001. Scale bar, 100 μm. (C) E-cadherin and vimentin expression. A549 cells were treated with 20 nM TGF-β and lidocaine for 48 h, and the expression levels of epithelial and mesenchymal markers were determined using western blotting. Naive group served as the control. (D) Semi-quantification of vimentin and E-cadherin (n=3). Naive group served as the control. (E) Upregulation of Slug expression with lidocaine treatment (n=4). A549 cells were treated with different concentrations of lidocaine for 48 h and the expression levels of Slug, the molecular switch upstream of prototypical epithelial-mesenchymal transition markers, were determined using western blot analysis. The cropped blots for E-cadherin, vimentin, Slug and GAPDH were grouped from different parts of the same gel and the grouping was made explicit by using (C) the white spaces or (E) cutting line. Values are expressed as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. 5-FU, 5-fluorouracil.

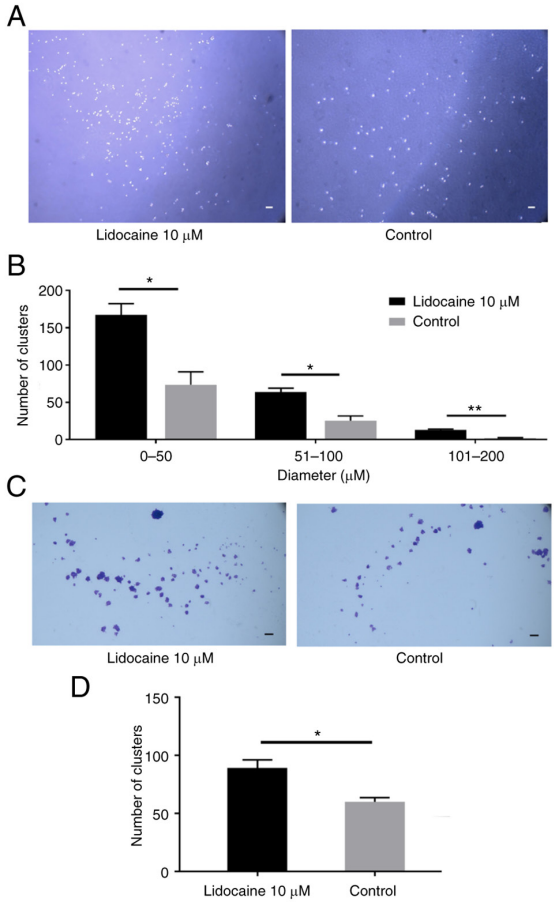

Figure 4. Effects of lidocaine on the aggregation of A549 lung cancer cells. (A) Images of the cell aggregation assay were captured under a dissecting microscope on day 5 (scale bar, 100 μm). (B) Number of clusters per well according to cluster size. Number of colonies per well was counted. (C) Crystal violet staining was captured under a dissecting microscope on day 6. (D) Semi-quantification of the colonies after crystal violet staining. Untreated cells served as the control. *P<0.05, **P<0.01. Values are expressed as the mean ± SD (n=3).

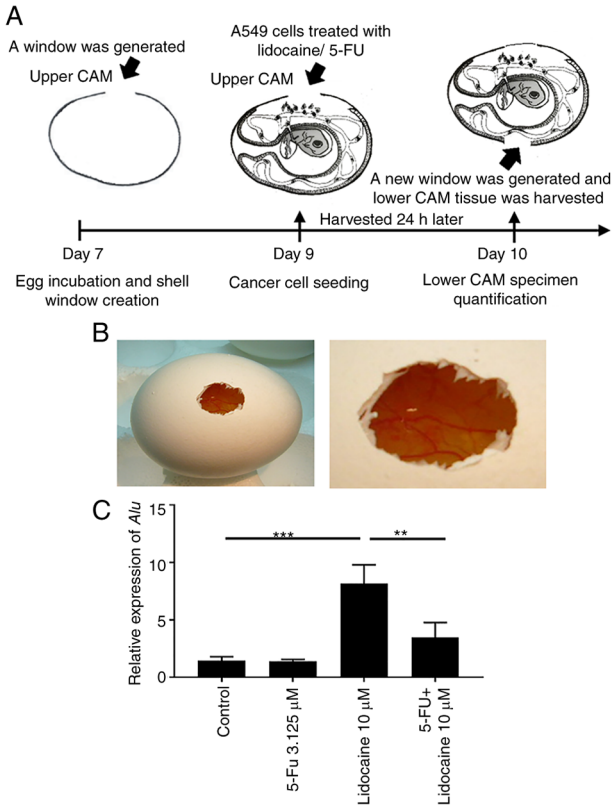

Figure 5. Metastatic trend mediated by lidocaine and 5-FU in the chicken embryo CAM model. (A) Illustration of the CAM model. A window was made in the eggshell of a 7-day-old chicken embryo. After 2 days, A549 cells treated with the indicated drugs were inoculated on the upper CAM. A total of 24 h after inoculation, portions of the lower CAM with a dense distribution of blood vessels were collected to analyze *Alu* content. (B) Image of upper CAM tissues. The picture on the left shows a panoramic view and on the right is a close-up view. (C) Quantitative PCR analysis of *Alu* in the lower CAM. Values are expressed as the mean ± SD (n=3). **P<0.01, ***P<0.001. 5-FU, 5-fluorouracil; CAM, chorioallantoic membrane.

cells did not dissolve into a single cell state when placed on the plate again after lidocaine treatment, indicating a trend towards sphere formation, which is characteristic of stemness.

Effect of lidocaine on cancer metastasis. Compared with the control group, *Alu* expression was significantly increased upon treatment with 10 μM lidocaine (Fig. 5), which corresponds with the finding that lidocaine reduces the 5-FU-induced inhibitory effects on cell migration, induces EMT and increases anoikis-resistant cell aggregation. Notably, Alu expression was increased by lidocaine treatment when compared with that induced by 5-FU alone and the control. The addition of 5-FU to lidocaine reduced Alu expression when compared with lidocaine alone, while there was no statistically significant difference compared with the control (Fig. 5C).

Impact of the measured EMT proteins on the conditioned metastasis pathway and NSCLC

Effect of measured EMT proteins on the conditioned metastasis pathway. Regarding the relationship between the measured molecules (increased expression of Slug and vimentin, and decreased expression of E-cadherin) and the remaining 27 molecules in this conditioned metastasis pathway, 24 relationships were observed. Of the affected molecules, those that were predicted to be activated included SNAI1, MMP1, MMP2, MMP9, TGFB1 and MET, and those that were predicted to be inhibited included Akt, ERBB2, EGFR, MTOR and miR-8 (Fig. 6). The main results of the present study are schematically presented in Fig. 7, which integrated the results of lung cancer behaviors and prediction of associated gene expressions according to measured EMT protein alterations in response to lidocaine and 5-FU treatment in A549 cells.

Effect of individual measured proteins on NSCLC, EMT and apoptosis of NSCLC. Upregulation of Slug expression strongly promoted EMT but did not affect NSCLC and its apoptosis (Fig. S2). In addition, the upregulation of vimentin expression strongly promoted NSCLC and EMT, but did not affect the apoptosis of NSCLC (Fig. S3). Downregulation of E-cadherin expression also strongly promoted NSCLC and EMT, despite possible activation of NSCLC apoptosis (Fig. S4).

Discussion

The present study revealed that lidocaine, at clinically relevant concentrations (1-10 μM), could reduce the inhibitory effect

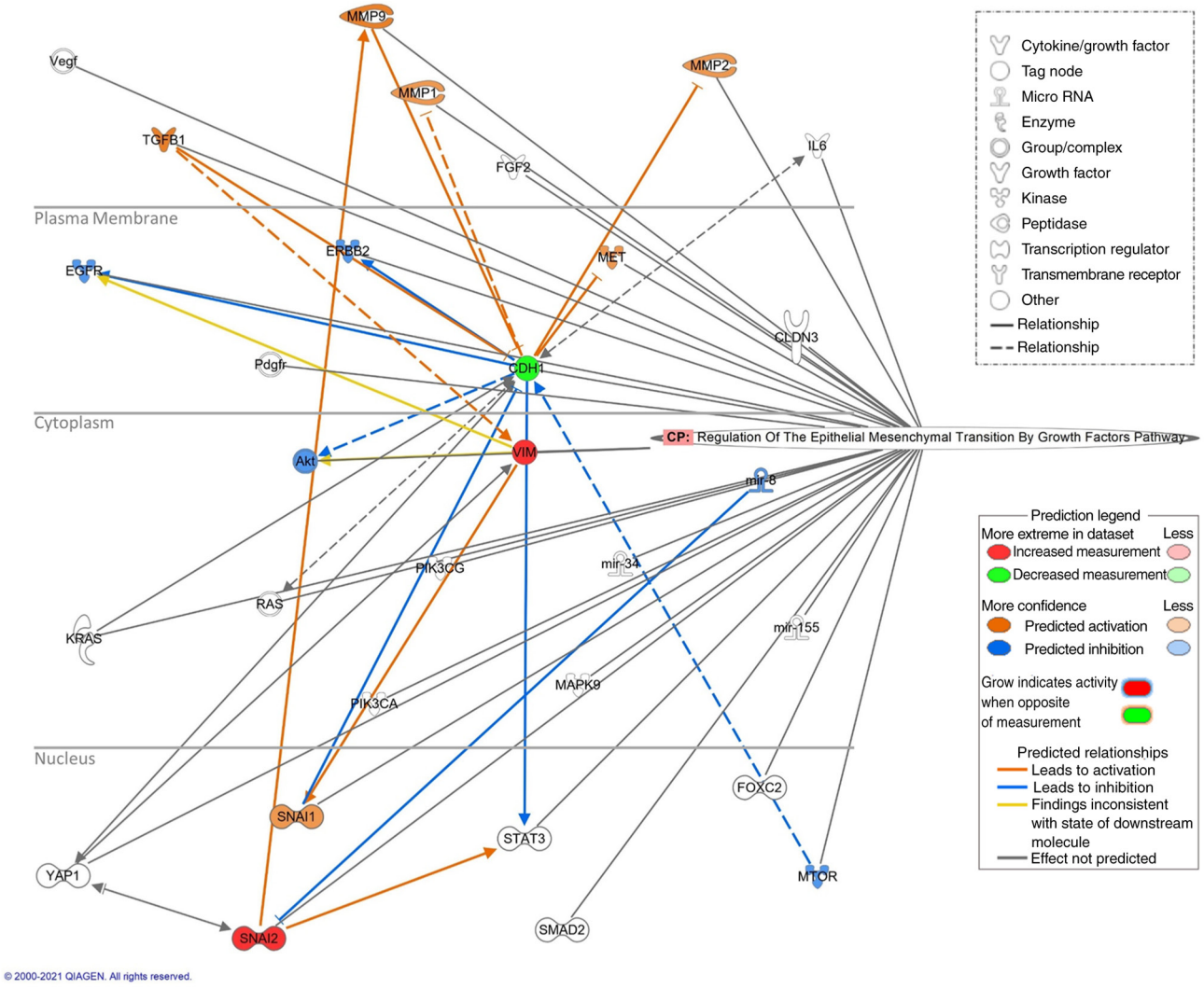

Figure 6. Predicted gene expression in the conditioned metastasis pathway affected by lidocaine-induced epithelial-mesenchymal transition. The symbols in the Ingenuity Pathway Analysis system for VIM, E-cadherin and Slug are VIM, CDH1 and SNAI2, respectively. The molecules involved are presented in a subcellular layout. VIM, vimentin; CDH1, E-cadherin; SNAI2, slug.

induced by 5-FU on the migration of lung cancer cells, whereas the survival of lung cancer cells was not affected. In addition to inducing EMT by altering migration-related EMT markers (slug, vimentin and E-cadherin), anoikis-resistant cell aggregation characteristic of EMT was also increased with lidocaine treatment in this dosing range. Furthermore, the potential of lidocaine-induced lung cancer metastasis was evidenced using a CAM model. IPA analysis based on the results of measured prototypical EMT proteins and their molecular switch yielded the predicted gene expression map. This predicted gene expression map revealed the effect of measured EMT proteins on the conditioned metastasis pathway. The possible relationships between the measured proteins (vimentin, E-cadherin and Slug) affected by lidocaine and the molecules in this conditioned metastasis pathway showed those that were predicted to be activated included SNAI1, MMP1, MMP2, MMP9, TGFB1 and MET, and those that were predicted to be inhibited included Akt, ERBB2, EGFR, MTOR and miR-8. Based on these phenomena, it was proposed that at clinically relevant concentrations, lidocaine may cause potential negative therapeutic effects on lung cancer.

To the best of our knowledge, the present study is the first to report the effect of clinically relevant concentrations of lidocaine on EMT, EMT-related cancer behaviors and chemoresistance in NSCLC. Although the involvement of EMT has been studied in epithelial cancer stem cells (CSCs) in various tumors, data are currently limited for NSCLC (32). Furthermore, EMT has long been linked to drug resistance in NSCLC; however, but the mechanisms underlying EMT-related resistance are still far from being fully explored (33). The benefit of the present study lies in the discovery of the effect of clinically relevant concentrations of lidocaine on EMT and various cancer behaviors in NSCLC, both *in vivo* and *in vitro*. In a recent review that focused on the impact of EMT on NSCLC, vimentin and E-cadherin were regarded as the most relevant EMT markers to be examined in routine practice. This was mainly because higher vimentin expression in tumor cells has been proposed as a predictor of metastasis and both markers have been shown to be independent predictors of cancer mortality (34). Tumor cells utilize EMT as a strategy to acquire CSC-like properties and achieve resistance to anti-tumor drugs (33). The results of the present study validated

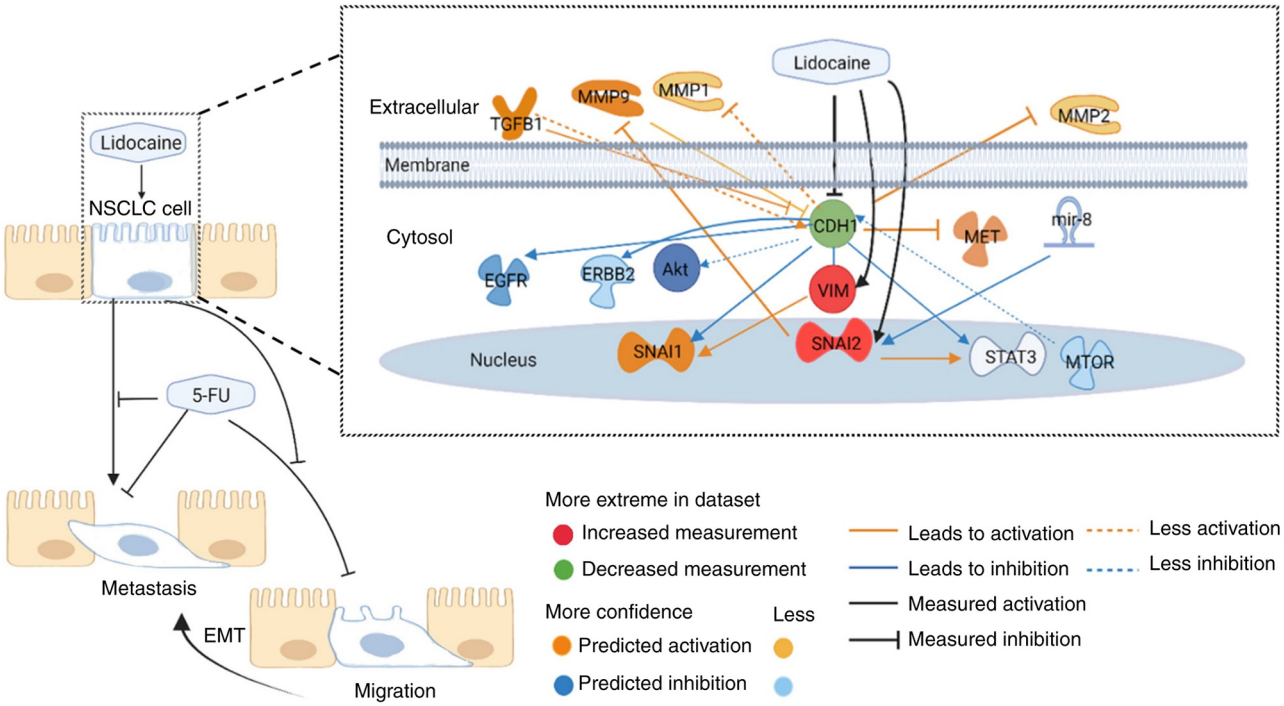

Figure 7. Schematic illustration of the findings integrating measured protein expression, predicted gene expression and behavioral changes related to lidocaine treatment at clinically relevant concentrations. 5-FU, 5-fluorouracil; NSCLC, non-small cell lung cancer; EMT, epithelial-mesenchymal transition; CDH1, E-cadherin. SNAI2, slug.

that lidocaine attenuated the inhibitory effect of 5-FU on cell migration, while promoting EMT and the associated anoikis resistance, indicating that clinically relevant concentrations of lidocaine may contribute to antitumor drug resistance. Furthermore, as a key molecule in EMT-induced cell migration and an invariably expressed protein on EMT-transformed CSCs, vimentin is central to EMT-mediated metastasis (31). Therefore, lidocaine-induced vimentin upregulation has the potential, at least in part, to be associated with attenuation of 5-FU-induced migratory inhibition, metastasis and cancer stemness (such as anoikis resistance) as indicated in the present results.

Because the EMT-governing mechanisms are complex non-linear networks (35), the IPA-based network analyses applied in the present study may be a practical tool to systematically predict gene alteration based on web bench results, such as altered EMT markers. The predicted gene expression profile could foster further research. Being the only local anesthetic agent allowed for intravenous administration, lidocaine has been recommended as part of the protocol for ERAS during the perioperative period to facilitate postoperative recovery in some surgical procedures with variable evidence levels, including thoracic surgery with a moderate evidence level (17). However, it has been confirmed that even when continuous intravenous infusion is performed at a 2 mg/min or 1.33 mg/kg/h, which is a dosage higher than that recommended by the ERAS protocol (17) (0.5-1 mg/min) for prolonged periods (4 or 24 h) after colorectal resection, the concentration does not exceed the generally recognized toxic concentration of ~20 μM (5.0 μg/ml) (36,37). The safety of prolonged lidocaine infusion at this higher rate (2 mg/min) was further evidenced by the fact that the first clinical signs of toxicity were not achieved when lidocaine was administered intravenously over 14 days in cases of severe migraine (16). As for regional blocks, the plasma lidocaine concentrations achieved during epidural administration was ~1 μM. Therefore, it is reasonable to assume that clinically relevant concentrations of lidocaine achieved through either the epidural or intravenous route during the perioperative period or using higher doses for longer duration during migraine treatment were <20 μM in terms of clinical signs of toxicity.

Numerous studies have indicated that lidocaine can inhibit tumor invasion and metastasis (38,39). There is also evidence that tumor cell proliferation causes increased activity of voltage-gated sodium channels (VGSCs) and blocking these channels with local anesthetics may potentially inhibit tumor progression (40,41). However, in the present study, the clinical concentrations of lidocaine did not affect cell viability but they did promote migration, thus indicating the importance of pathways other than VGSCs. Data from previous research have shown that the effect of lidocaine on lung cancer cell behavior and associated gene expression depends on the range of concentrations that the cells are exposed to and varies with the investigation protocols. Some studies have demonstrated that 8 mM lidocaine can inhibit the viability, migration and invasion of the A549 lung cancer cells, and can induce apoptosis (18,42). However, patients have not been exposed to 8 mM lidocaine in clinical practice. Although lidocaine at high concentrations can result in inhibition in a number of aspects, clinically relevant low concentrations have seldom been explored in clinical setups. As a biphasic effect of drugs is not uncommon in clinical practice from our previous experience (43), it may be premature to deny the possibility that clinically relevant concentrations of lidocaine (<20 μM) could

exert opposite effects when compared with high concentrations in the millimolar scale.

Previous studies have reported that exposure to 8 μM lidocaine can reduce the barrier property of A549 cells using electric cell-substrate impedance sensing (ECIS) technology (44). However, to the best of our knowledge, the effect of 8 μM lidocaine on cell migration has yet to be clarified. The ECIS findings indirectly support the current result that clinically relevant concentrations of lidocaine can suppress the inhibitory effect of 5-FU on migration, because the attenuation of barrier function involves the first step in cancer cell dissemination, including migration. Another related study reported that 10 μM lidocaine inhibits the invasion and migration of lung cancer cells induced by TNFα (10). However, the living environment of the cells, as stimulated by TNFα, is no longer physiological while the cells are exposed to a clinical concentration of lidocaine. The present results varied from these previous findings due to a different protocol setting that was without potent inflammatory stimuli, which proved the negative effects of clinically relevant concentrations of lidocaine through both *in vitro* and *in vivo* studies. The negative therapeutic effects of lidocaine on lung cancer cell behavior were further exemplified by analyzing anoikis-resistant cell aggregation. Furthermore, the status of crystal violet staining to confirm cell survival after reattachment was similar to the status when the attached cells were forced to be suspended from the plate to show how lidocaine induces the anoikis-resistant ability in lung cancer. Cell reattachment also occurred irreversibly in the form of cell clusters because reattached cell clumps did not disperse into single cells, indicating the progression towards sphere formation. As a stem cell characteristic, the tendency of sphere formation hints at the possibility of stemness transformation induced by clinically relevant concentrations of lidocaine in A549 cells, a phenomenon that requires further verification.

When translating the findings to clinical practice for patients with lung cancer, especially those using 5-FU as an adjuvant therapy, considering the possible negative effects of intravenous lidocaine infusion should not be neglected. Analgesic methods other than intravenous lidocaine infusion should be chosen to treat refractory headaches in such patients. When an epidural block is required, local anesthetics other than lidocaine should be considered. Based on the present findings, before implementing intravenous lidocaine infusion as a part of the ERAS protocol for lung cancer surgery, the risk-benefit ratio should be re-calculated because the ERAS lidocaine concentration (<20 μM) tends to increase lung cancer migration and metastasis according to the findings of this study. Additionally, the CAM model was applied to simulate and evaluate *in vivo* tumor cell growth (intravasation) and metastasis (45). The CAM model is a well-established *in vivo* system used to study the cancer behaviors of various tumors. It has also been proven to be a highly efficient *in vivo* approach to evaluate compounds with cancer-modulating activities (46). The tumor cells can break down the extracellular matrix in tissues and then penetrate, migrate and infiltrate into chick embryo blood vessels for circulation. By assessing whether the lower CAM contains DNA components of the injected tumor cells, it is possible to determine whether lidocaine at such low concentrations enhances the overall ability of tumor cells in terms of moving into chick embryo blood vessels. Additionally, the effect of lidocaine on the metastatic tendency of lung cancer can be explored (26). Notably, CAM is a relatively simple, fast and low-cost model, and the method has been widely used to study the effects of different drugs on cell behaviors (47,48). The present study elucidated the effects of clinically relevant concentrations of lidocaine on the metastatic behavior of human lung cancer cells. However, the number of cells inoculated in these *in vivo* experiments is considerably higher than that of the circulating tumor cells in patients with lung cancer.

During the invasion-metastasis cascade, tumor cells exit their primary sites of growth (local invasion, intravasation), translocate systemically (survival in the circulation, arrest at a distant organ site, extravasation), and adapt to survive and thrive in the foreign microenvironments of distant tissues (micrometastasis formation, metastatic colonization). Angiogenesis is a part of the invasion process (49). Like any of the mechanisms during metastasis, angiogenesis is considered one of the critical steps to support cancer metastasis. However, the cancer-causing or cancer-promoting substance does not necessarily potentiate every step related to tumor metastasis, and recent evidence has shown that tumors can grow without angiogenesis (50). Furthermore, considering the fact that non-angiogenic tumors have also been described in histopathology studies of NSCLC and carcinoma metastases in the lung (50), the notion that NSCLC is not necessarily angiogenesis-dependent may not be biased. Therefore, although the CAM model can be used to assess angiogenesis, the most appropriate endpoint to be measured should be the metastasis itself, rather than any other step during metastasis. For this reason, metastasis was examined in the CAM model in the present study.

The reason why a higher concentration (3.125 μM) of 5-FU only reduced cell survival by 30% when compared with its predicted IC_{50} value (2.808 μM) may be described as follows. The IC_{50} value was merely first approximated by GraphPad Prism software, and a dose-response curve was depicted with IC_{50} predicted from the non-linear correlation equation. With the predicted value of IC_{50} (2.808 μM) narrowed down from a broader range of concentrations, the serially diluted concentrations (2.34-4.68 μM) near the predicted IC_{50} (2.808 μM) were validated in a further SRB assay. Because the difference in biological response between concentrations of 3.125 and 2.34 μM was not significant, 3.125 μM was used as the experimental concentration in the subsequent study. To summarize, 3.125 μM was the concentration that was chosen near the 'predicted' IC_{50} value (2.808 μM). Therefore, 30% inhibition by 3.125 μM is possible because 2.808 μM is just a preliminary predicted value from a non-linear equation for IC_{50} performed using GraphPad.

The present study has a few limitations. First, the simulated laboratory conditions on lidocaine are limited, and ultimate studies involving human trials with clinically relevant lidocaine concentrations are recommended. Second, physiological mechanisms are very complex, especially regarding cancer physiology. Therefore, surgical techniques and anesthesia methods need to be considered more comprehensively in clinical practice. Third, only two cancer cell lines were studied whose cell characteristics are not completely representative of cancer cells that are directly

cultured from clinical tissues. Thus, there is a need to establish a model for studying the effects of lidocaine on lung cancer primary culture. The present study directly stimulated lung cancer cell lines with lidocaine using a range of clinical concentrations without the support of the tumor microenvironment. Besides, there is always a high level of circulating inflammatory cytokines in cancer patients undergoing surgeries. However, the present study used the CAM model that simulated an *in vivo* tumor environment wherein residual tumor cells are ready to metastasize to the circulatory system for distant metastasis. Fourth, as for the effects of lidocaine on metastasis-related gene expression, it would not be possible to measure the expression of all genes responsible for metastasis. Nonetheless, to address this issue and systemically discover the essential genes responsible for metastasis in our study setting, the present study generated relevant networks using IPA, and performed prediction analysis according to the results of the measured prototypical EMT markers (vimentin and E-cadherin) and their molecular switch (Slug). Unfortunately, the results from IPA predictions were not experimentally validated. The trend that cell migration decreased with an increase in lidocaine concentration when combined with 5-FU suggested that complex interactions between these two drugs may exist in terms of migration. It would not be possible to explain this trend with the current data and further investigation is warranted in this regard. To reveal the versatility of lidocaine, the effects of lidocaine were evaluated in both mouse and human cell lines (LLC.LG and A549) only in the first half of the study. In the future, we hope to perform mouse experiments based on the results of the mouse cell lines (LLC.LG) to develop drugs against lung cancer.

In conclusion, the present findings revealed that clinically relevant concentrations of lidocaine may lead to enhanced migratory and metastatic effects in human lung cancer cells. The phenomena accompanying lidocaine-aggravated migration and metastasis included the altered expression of prototypical EMT markers and their molecular switch, anoikis-resistant cell aggregation characteristic of EMT, and attenuation of the 5-FU-induced inhibitory effect on cell migration. The findings also indicated that at clinically relevant concentrations, lidocaine may contribute to resistance toward antitumor drugs. Based on these findings, caution should be exercised before administering intravenous/epidural lidocaine to reach clinically relevant concentrations (1-20 μM), either as a part of the ERAS protocol or as a treatment option for patients with lung cancer that have migraines. Additionally, relevant samples should be collected from ongoing clinical studies to establish the association between lidocaine and the clinical outcomes of lung cancer surgery using primary cultures produced under the ERAS protocol or epidural infusion, elucidate relevant mechanisms, and validate the impact of intravenous lidocaine on tumor progression and cancer stemness.

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Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Authors' contributions

JAL conceptualized the study. JAL, BYH and WHH designed the study. WHH, SWL, SMC and JDH performed the main experiments and prepared the first draft of the manuscript. WHH acquired and analyzed the data. WHH and SYW interpreted the data. BYH, KYC and YTT reviewed the draft of the manuscript. SWL, HWF, BYH, WHH and CYF performed the CAM model. SMC, JAL, SYC, KYC and YTT performed IPA analysis and produced the figure. JDH and WHH carried out the Transwell migration study. JDH and JAL revised the manuscript and acquired the funding. WHH and JAL confirmed the authenticity of all the raw data and performed the statistical analysis. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Dela Cruz CS, Tanoue LT and Matthay RA: Lung cancer: Epidemiology, etiology, and prevention. Clin Chest Med 32: 605-644, 2011.
2. Mountain CF: The international system for staging lung cancer. Semin Surg Oncol 18: 106-115, 2000.
3. Chen R, Manochakian R, James L, Azzouqa AG, Shi H, Zhang Y, Zhao Y, Zhou K and Lou Y: Emerging therapeutic agents for advanced non-small cell lung cancer. J Hematol Oncol 13: 58, 2020.
4. Noro R, Miyanaga A, Minegishi Y, Okano T, Seike M, Soeno C, Kataoka K, Matsuda K, Yoshimura A and Gemma A: Histone deacetylase inhibitor enhances sensitivity of non-small-cell lung cancer cells to 5-FU/S-1 via down-regulation of thymidylate synthase expression and up-regulation of p21(waf1/cip1) expression. Cancer Sci 101: 1424-1430, 2010.
5. Ettinger DS, Wood DE, Aggarwal C, Aisner DL, Akerley W, Bauman JR, Bharat A, Bruno DS, Chang JY, Chirieac LR, *et al*: NCCN guidelines insights: Non-small cell lung cancer, version 1.2020. J Natl Compr Canc Netw 17: 1464-1472, 2019.
6. Tan X, Zhang C, Gao W, Sun B, Jiang B and Song P: Overexpression of microRNA-124-5p sensitizes non-small cell lung cancer cells to treatment with 5-fluorouracil via AEG-1 regulation. Oncol Lett 21: 5, 2021.
7. Chang A: Chemotherapy, chemoresistance and the changing treatment landscape for NSCLC. Lung Cancer 71: 3-10, 2011.
8. Tedore T: Regional anaesthesia and analgesia: Relationship to cancer recurrence and survival. Br J Anaesth 115 (Suppl 2): ii34-ii45, 2015.

9. Xuan W, Hankin J, Zhao H, Yao S and Ma D: The potential benefits of the use of regional anesthesia in cancer patients. Int J Cancer 137: 2774-2784, 2015.
10. Piegeler T, Schläpfer M, Dull RO, Schwartz DE, Borgeat A, Minshall RD and Beck-Schimmer B: Clinically relevant concentrations of lidocaine and ropivacaine inhibit TNFα-induced invasion of lung adenocarcinoma cells in vitro by blocking the activation of Akt and focal adhesion kinase. Br J Anaesth 115: 784-791, 2015.
11. Piegeler T, Votta-Velis EG, Liu G, Place AT, Schwartz DE, Beck-Schimmer B, Minshall RD and Borgeat A: Antimetastatic potential of amide-linked local anesthetics: Inhibition of lung adenocarcinoma cell migration and inflammatory Src signaling independent of sodium channel blockade. Anesthesiology 117: 548-559, 2012.
12. Samarin MJ, Mohrien KM and Oliphant CS: Continuous intravenous antiarrhythmic agents in the intensive care unit: Strategies for safe and effective use of amiodarone, lidocaine, and procainamide. Crit Care Nurs Q 38: 329-344, 2015.
13. Kosharskyy B, Almonte W, Shaparin N, Pappagallo M and Smith H: Intravenous infusions in chronic pain management. Pain Physician 16: 231-249, 2013.
14. Keller D, Seamon J and Jones JS: BET 2: Usefulness of IV lidocaine in the treatment of renal colic. Emerg Med J 33: 825-826, 2016.
15. Nguyen NL, Kome AM, Lowe DK, Coyne P and Hawks KG: Intravenous lidocaine as an adjuvant for pain associated with sickle cell disease. J Pain Palliat Care Pharmacother 29: 359-364, 2015.
16. Williams DR and Stark RJ: Intravenous lignocaine (lidocaine) infusion for the treatment of chronic daily headache with substantial medication overuse. Cephalalgia 23: 963-971, 2003.
17. Dunn LK and Durieux ME: Perioperative use of intravenous lidocaine. Anesthesiology 126: 729-737, 2017.
18. Sun H and Sun Y: Lidocaine inhibits proliferation and metastasis of lung cancer cell via regulation of miR-539/EGFR axis. Artif Cells Nanomed Biotechnol 47: 2866-2874, 2019.
19. Wang HW, Wang LY, Jiang L, Tian SM, Zhong TD and Fang XM: Amide-linked local anesthetics induce apoptosis in human non-small cell lung cancer. J Thorac Dis 8: 2748-2757, 2016.
20. Zhou Y, Du Q, Zhao Q, Zhang M, Qin X, Jiang Y and Luan Y: A heme-regulatable chemodynamic nanodrug harnessing transcription factor Bach1 against lung cancer metastasis. J Colloid Interface Sci 610: 698-708, 2022.
21. Ning J, Cui X, Li N, Li N, Zhao B, Miao J and Lin Z: Activation of GRP78 ATPase suppresses A549 lung cancer cell migration by promoting ITGB4 degradation. Cell Adh Migr 16: 107-114, 2022.
22. Ediriweera MK, Tennekoon KH, Samarakoon SR, Thabrew I and De Silva ED: Induction of apoptosis in MCF-7 breast cancer cells by sri lankan endemic mango (*Mangifera zeylanica*) fruit peel through oxidative stress and analysis of its phytochemical constituents. J Food Biochem 41: e12294, 2017.
23. Orellana EA and Kasinski AL: Sulforhodamine B (SRB) assay in cell culture to investigate cell proliferation. Bio Protoc 6: e1984, 2016.
24. Feoktistova M, Geserick P and Leverkus M: Crystal violet assay for determining viability of cultured cells. Cold Spring Harb Protoc 2016: pdb.prot087379, 2016.
25. Miyashita N, Enokido T, Horie M, Fukuda K, Urushiyama H, Strell C, Brunnström H, Micke P, Saito A and Nagase T: TGF-β-mediated epithelial-mesenchymal transition and tumor-promoting effects in CMT64 cells are reflected in the transcriptomic signature of human lung adenocarcinoma. Sci Rep 11: 22380, 2021.
26. Ho BY, Wu YM, Hsu YW, Hsu LC, Kuo YH, Chang KJ and Pan TM: Effects of Monascus-fermented rice extract on malignant cell-associated neovascularization and intravasation determined using the chicken embryo chorioallantoic membrane model. Integr Cancer Ther 9: 204-212, 2010.
27. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
28. Kim J, Yu W, Kovalski K and Ossowski L: Requirement for specific proteases in cancer cell intravasation as revealed by a novel semiquantitative PCR-based assay. Cell 94: 353-362, 1998.
29. Kariya Y, Kato K, Hayashizaki Y, Himeno S, Tarui S and Matsubara K: Revision of consensus sequence of human Alu repeats-a review. Gene 53: 1-10, 1987.
30. Unahabhokha T, Chanvorachote P, Sritularak B, Kitsongsermthon J and Pongrakhananon V: Gigantol inhibits epithelial to mesenchymal process in human lung cancer cells. Evid Based Complement Alternat Med 2016: 4561674, 2016.
31. Usman S, Waseem NH, Nguyen TKN, Mohsin S, Jamal A, Teh MT and Waseem A: Vimentin is at the heart of epithelial mesenchymal transition (EMT) mediated metastasis. Cancers (Basel) 13: 4985, 2021.
32. Mahmood MQ, Shukla SD, Ward C and Walters EH: The underappreciated role of epithelial mesenchymal transition in chronic obstructive pulmonary disease and its strong link to lung cancer. Biomolecules 11: 1394, 2021.
33. Zhu X, Chen L, Liu L and Niu X: EMT-mediated acquired EGFR-TKI resistance in NSCLC: Mechanisms and strategies. Front Oncol 9: 1044, 2019.
34. Ancel J, Dewolf M, Deslée G, Nawrocky-Raby B, Dalstein V, Gilles C and Polette M: Clinical impact of the epithelial-mesenchymal transition in lung cancer as a biomarker assisting in therapeutic decisions. Cells Tissues Organs 211: 91-109, 2022.
35. Nieto MA, Huang RY, Jackson RA and Thiery JP: Emt: 2016. Cell 166: 21-45, 2016.
36. Herroeder S, Pecher S, Schönherr ME, Kaulitz G, Hahnenkamp K, Friess H, Böttiger BW, Bauer H, Dijkgraaf MG, Durieux ME and Hollmann MW: Systemic lidocaine shortens length of hospital stay after colorectal surgery: A double-blinded, randomized, placebo-controlled trial. Ann Surg 246: 192-200, 2007.
37. Kaba A, Laurent SR, Detroz BJ, Sessler DI, Durieux ME, Lamy ML and Joris JL: Intravenous lidocaine infusion facilitates acute rehabilitation after laparoscopic colectomy. Anesthesiology 106: 11-18, 5-6, 2007.
38. Mammoto T, Higashiyama S, Mukai M, Mammoto A, Ayaki M, Mashimo T, Hayashi Y, Kishi Y, Nakamura H and Akedo H: Infiltration anesthetic lidocaine inhibits cancer cell invasion by modulating ectodomain shedding of heparin-binding epidermal growth factor-like growth factor (HB-EGF). J Cell Physiol 192: 351-358, 2002.
39. Mao L, Lin S and Lin J: The effects of anesthetics on tumor progression. Int J Physiol Pathophysiol Pharmacol 5: 1-10, 2013.
40. Koltai T: Voltage-gated sodium channel as a target for metastatic risk reduction with re-purposed drugs. F1000Res 4: 297, 2015.
41. Elajnaf T, Baptista-Hon DT and Hales TG: Potent inactivation-dependent inhibition of adult and neonatal NaV1.5 channels by lidocaine and levobupivacaine. Anesth Analg 127: 650-660, 2018.
42. Zhang L, Hu R, Cheng Y, Wu X, Xi S, Sun Y and Jiang H: Lidocaine inhibits the proliferation of lung cancer by regulating the expression of GOLT1A. Cell Prolif 50: e12364, 2017.
43. Lin JA, Chen JH, Lee YW, Lin CS, Hsieh MH, Chang CC, Wong CS, Chen JJ, Yeh GC, Lin FY and Chen TL: Biphasic effect of curcumin on morphine tolerance: A preliminary evidence from cytokine/chemokine protein array analysis. Evid Based Complement Alternat Med 2011: 452153, 2011.
44. Chan SM, Lin BF, Wong CS, Chuang WT, Chou YT and Wu ZF: Levobuipivacaine-induced dissemination of A549 lung cancer cells. Sci Rep 7: 8646, 2017.
45. Lokman NA, Elder ASF, Ricciardelli C and Oehler MK: Chick chorioallantoic membrane (CAM) assay as an in vivo model to study the effect of newly identified molecules on ovarian cancer invasion and metastasis. Int J Mol Sci 13: 9959-9970, 2012.
46. Merlos Rodrigo MA, Casar B, Michalkova H, Jimenez Jimenez AM, Heger Z and Adam V: Extending the applicability of in ovo and ex ovo chicken chorioallantoic membrane assays to study cytostatic activity in neuroblastoma cells. Front Oncol 11: 707366, 2021.
47. Agrawal S, Chaugule S, More S, Rane G and Indap M: Methanolic extract of Euchelus asper exhibits in-ovo anti-angiogenic and in vitro anti-proliferative activities. Biol Res 50: 41, 2017.
48. Ribatti D: The chick embryo chorioallantoic membrane (CAM). A multifaceted experimental model. Mech Dev 141: 70-77, 2016.
49. Valastyan S and Weinberg RA: Tumor metastasis: Molecular insights and evolving paradigms. Cell 147: 275-292, 2011.
50. Pezzella F and Gatter KC: Evidence showing that tumors can grow without angiogenesis and can switch between angiogenic and nonangiogenic phenotypes. J Natl Cancer Inst 108: djw032, 2016.