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Postoperative administration of ketorolac averts morphine-induced angiogenesis and metastasis in triple-negative breast cancer

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ABSTRACT

Aims: Opioids (i.e. morphine) were found to induce triple negative breast cancer (TNBC) metastasis while nonsteroidal anti-inflammatory drugs (i.e. ketolorac) were associated with decreased metastasis in TNBC. These contradictory findings demand clarification on the effect of postoperative morphine and ketorolac on TNBC metastasis.

Materials and methods: TNBC xenograft mice were established using MDA-MB-231 cells. When tumors reached ~100 mm^3, the primary tumor was resected. Mice were then randomly assigned to four groups ($n = 14$): (i) saline, (ii) morphine (10 mg kg^{-1}) (iii) morphine + ketorolac (10 mg kg^{-1} of morphine and 20 mg kg^{-1} of ketorolac) (iv) ketorolac (20 mg kg^{-1}); administrated for three consecutive days after resection. Three weeks after resection, the number of lung metastases was measured. Microvessel density, thrombospondin-1 (TSP-1) and c-Myc expression in recurrent tumors were determined. To elucidate the above phenomenon in vitro, MDA-MB-231 cells were treated according to the regiment above; with or without supplementation of an AKT inhibitor to determine the activation of PI3K/AKT/c-Myc pathway.

Key findings: In mice, morphine promoted TNBC metastasis and angiogenesis, decreased TSP-1 expression and increased c-Myc expression, while co-administration of ketorolac significantly reversed the phenotypes above ($p < .05$). Mechanistically, morphine inhibited TSP-1 secretion by activating PI3K/AKT/c-Myc pathway ($p < .05$), while ketorolac promoted TSP-1 secretion ($p < .05$) by suppressing PI3K/AKT/c-Myc pathway.

Significance: Our study indicated that morphine enhanced TNBC metastasis and angiogenesis while ketorolac suppressed this effect. Mechanistically, this may be related to the enhancement of TSP-1 synthesis after ketorolac administration which further de-activated PI3K/AKT/c-Myc pathway.

1. Introduction

Triple-negative breast cancer (TNBC), a subtype of breast cancer that lacks estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (HER2) amplification, is highly aggressive and chemoresistant [1,2]. Surgical resection remains the first line of treatment. In surgery, the most abundantly used analgesic is opioids (i.e. morphine) while ketorolac is the most common choice of NSAIDs [3,4]. In most cases, both of these are used simultaneously after resection of primary tumors.

Clinical reports suggested that postoperatively administered opioids may be related to poor prognosis in patients with lung cancer [5]. Even though there is clinical evidences that perioperative opioids may induce immune suppression and metastasis after breast cancer mastectomy [6,7], no consensus has been reached regarding the influence of postoperative opioid administration on breast cancer metastasis [8]. Preclinical studies had shown that morphine improved TNBC progression and angiogenesis in mice models [9–11]. Nevertheless, the effect of morphine administrated after breast cancer resection on TNBC is still uncertain.

Perioperative administration of ketorolac was shown to improve prognosis of breast cancer patients [12,13]. NSAIDs (i.e. celecoxib) were also found to inhibit breast cancer proliferation and angiogenesis induced by morphine [14]. These studies provide a theoretical possibility of the interaction of postoperatively administered morphine and ketorolac on TNBC, but warrant further clarification.

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Microvascular niche formation is crucial for angiogenesis and closely associated with metastasis. Thrombospondin-1 (TSP-1), an angiogenesis suppressor deposited around the endothelium, was found to prevent microvascular niche formation and breast cancer metastasis in recent years [15,16]. Previous reports further indicated that TSP-1 suppressed VEGF-induced angiogenesis mechanistically [17,18], and that the expression of TSP-1 could be regulated by PI3K/AKT/c-Myc signal pathway [19,20]. In previous studies, by co-administrating with methylnaltrexone (μ-receptor agonist), morphine could promote VEGF-induced angiogenesis [21]. This effect was also shown to be related with PI3K/AKT/c-Myc signal pathway activation [22]. Furthermore, it is reported that morphine could down-regulate TSP-1 expression in astrocytes [23]. Holmes et al. recently reported that TSP-1 expression was upregulated after NSAIDs (aspirin) administration in breast cancer patients [24]. Collectively, these studies presented a possibility that TSP-1 expression may be involved in the interaction of other NSAIDs such as ketorolac and morphine on TNBC angiogenesis and progression.

Although these results partially clarify on the link between analgesic agents (morphine and ketorolac) and breast cancer metastasis, ample evidence is needed to validate the influence and mechanism of postoperatively administered morphine and ketorolac on TNBC metastasis and angiogenesis. Herein, we aimed to investigate the influence of postoperative co-administration of morphine and ketorolac on TNBC metastasis and angiogenesis, and to elucidate the underlying mechanism of TSP-1 expression in postoperative TNBC metastasis.

2. Materials and methods

2.1. Ethical approval

This study has obtained the approval from the Animal Ethical and Welfare Committee of SYSU, Guangzhou, China on December 25, 2014 (IACUC-2014-1101).

2.2. Mouse models

(i) Establishment of TNBC metastatic model

The mammalian fat pads (MFPs) of 5–7 week-old female BALB/c nude mice (Sun Yat-sen University, Guangzhou, Guangdong, China) were injected with MDA-MB-231 cells (2.5×10^7 ml^{-1}). After 3 weeks, breast cancer tissues were resected under Pentobarbital sodium anesthesia.

(ii) Treatment schedule

In the preliminary study, the mice were randomly assigned to four groups ($n = 5$) after resection and received intraperitoneal (*i.p.*) administration of either (i) saline (ii) morphine (10 mg kg^{-1}), (iii) morphine + ketorolac (10 mg kg^{-1} of morphine and 20 mg kg^{-1} of ketorolac; Selleck Company, TX, USA) or (iv) ketorolac (20 mg kg^{-1}) daily for three consecutive days. The mice were then housed in a specific pathogen-free (SPF) environment for 3 weeks before euthanasia by cervical dislocation and their lungs were collected. Lung metastasis was measured for simple size calculation. After the preliminary study, the mice were randomly assigned to four groups ($n = 14$) as mentioned above and received the same treatment after resection with preliminary study. After resection and treatment, the mice were housed in SPF environment for 3 weeks before euthanasia by cervical dislocation; their lungs and recurrent tumors (tumors that grew at the primary site after resection) were collected.

2.3. In vitro experiments

(i) Cell line

MDA-MB-231 cells were purchased from (ATCC, VA, USA) and maintained in DMEM (Thermo Fisher, MA, USA) supplemented with 10% foetal bovine serum (FBS) (Thermo Fisher) and 1% antibiotic solution (penicillin/streptomycin) (Thermo Fisher) at 37 °C with 5% CO_2.

(ii) Treatment

MDA-MB-231 cells were divided into four groups as noted above. Cells were treated with either (i) DMEM only (negative control group), (ii) morphine (10 μM), (iii) morphine + ketorolac (10 μM morphine and 3.5 μM ketorolac), ketorolac (3.5 μM).

For the pathway analysis experiment, MDA-MB-231 cells were treated with either (i) DMEM only (negative control group), (ii) morphine (10 μM), (iii) morphine + MK2206 (morphine 10 μM + 10 nM MK2206; Selleck Company) or (iv) MK2206 inhibitor (10 nM).

2.4. Haematoxylin and eosin (H&E) staining and immunohistochemistry (IHC)

Collected lung and recurrent tumor tissues were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin and sectioned for H&E staining or IHC analysis. Mouse lungs were serially sectioned at 4 μm, and H&E-stained sections (5 mm apart) and were microscopically analyzed for lung metastasis quantification by scanning at 40× and 400× magnification with an upright microscope (Leica Microsystems Inc., IL, USA), and the total number of lung metastases per mouse was calculated. For IHC analyzes, 5-μm-thick paraffin sections of tumor tissues were cut and deparaffinised. After 10 min of antigen fixation, the sections were incubated overnight with an anti-CD31 antibody (ab28364; Abcam, Cambridge, MA, USA) at a concentration of 1:100 at 4 °C. The sections were then washed with 0.1% Tween-20 in PBS and were incubated with a secondary antibody for 30 min. The nuclei were then counterstained with haematoxylin. Immunostaining results were assessed by two independent researchers who were blinded to the experiment. Five fields were randomly selected from each slide, and the microvascular number was evaluated at 200× magnification.

2.5. Western blot analysis

Recurrent tumor tissues or breast cancer cell lysates containing 30 μg of protein were resolved on 8–15% gradient SDS-PAGE gels and transferred to PVDF membranes (Millipore Sigma, MO, USA). The membranes were blocked in 5% non-fat dry milk in TBST (Tris-buffer saline with 0.1% Tween 20) for 1 h at room temperature and incubated with a 1:1000 dilution of anti-c-Myc antibody (Cell Signaling Technology #13987, Danvers, MA, USA), anti-p-AKT antibody (Cell Signaling Technology #4060), anti-p-AKT antibody (Cell Signaling Technology #4060), anti-TSP-1 antibody (Cell Signaling Technology# 14,778) or a 1:4000 dilution of anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Cell Signaling Technology #5174) at 4 °C overnight. After the membranes were washed, they were incubated with a 1:10000 dilution of species-specific secondary antibodies (Jackson ImmunoResearch Laboratories, PA, USA) for 45 min at room temperature. The proteins were visualized with an Electro-Chemi-Luminescence (ECL) Western Blot System (Syngene, Cambridge, United Kingdom).

2.6. Real-time PCR (qRT-PCR)

Total RNA was isolated from breast cancer cells using TRIzol reagent (Thermo Fisher), and 500 ng of total RNA was reverse transcribed using the PrimeScript™ RT Reagent Kit (Takara, Shiga, Japan). After reverse transcription, real-time PCR was performed using SYBR Premix Ex Taq II (Takara). The PCR and mRNA expression analysis were performed on the Gene Amp PCR System 970 (Thermo Fisher) according to the manufacturer's protocol. Relative expression levels were calculated

using the $2^{-\Delta\Delta Ct}$ method. The sequences of primers homologous to the coding region of human TSP-1 were F: 5′-AGACTCCGCATCGCAA AGG-3′ and R: 5′-TCACCACGTTGTTGTCAAGGG-3′. The sequences for human c-Myc were F: 5′-GGCCCCCAAGGTAGTTATCC-3′ and R: 5′-CGTTTCCGCAACAAGTCCTC-3′. The sequences for human GAPDH were F: 5′-GAGCCAAAAGGGTCATCATCTC-3′ and R: 5′-GGTCATGAG TCCTTCCACGATAC-3′. The reactions were run as follows: at 95 °C for 3 min, followed by 40 amplification cycles (95 °C for 15 s, 60 °C for 20 s, 72 °C for 20 s, and 78 °C for 20 s), and then ramped from 72 °C to 99 °C to obtain the melting curve.

2.7. Enzyme-linked ImmunosSorbent assay (ELISA)

TSP-1 secretion was quantified using a commercially available Human Thrombospondin-1 DuoSet ELISA kit (R&D systems, MN, USA). TSP-1 capture antibody diluted to 5 ng ml^{-1} was used to coat a 96-well plate overnight at 4 °C. Recombinant TSP-1 standards and cell culture supernatant were then added and incubated for 2 h at room temperature. Detection Antibody, Streptavidin-HRP, Substrate Solution and Stop Solution were added successively according to the manufacturer's protocol. Absorbance was measured at 450 and 540 nm on a microplate reader (Thermo Fisher). Concentration of TSP-1 in cell culture supernatants was determined by interpolation of the standard curve using logistic curve fitting. No cross-reactivity or interference with TSP-2 or TSP-4 was observed.

2.8. Statistical analysis

The simple size for number of mice needed for each group was calculated based on our preliminary data with a power of 0.8 and an alpha error of 0.05. Continuous data were analyzed by one-way analysis of variance (ANOVA). Inter-group comparisons were performed by Tukey's post-hoc tests or Dunnett's comparison. All data analyzes were performed with GraphPad Prism version 6.02 (GraphPad Software Inc., San Diego, CA, USA). $p < .05$ were considered statistically significant. The data was presented as the mean ± standard deviation (S.D.).

3. Results

3.1. TNBC lung metastasis was positively regulated by postoperative administration of morphine and negatively regulated by ketorolac

In the preliminary study (data is shown in the supplementary material), we recruited 5 mice for each group and lung metastases in each group were measured for sample size calculation. We calculated that $n = 12$ for each group was required to detect significant differences with a power of 0.8 and an alpha error of 0.05, and $n = 14$ for each group was set in our study.

To investigate the influence of postoperatively administered morphine and ketorolac on TNBC metastasis, we collected lung tissues from postoperative breast cancer metastasis mouse models 3 weeks after breast cancer resection. As seen in Fig. 1, H&E staining of lung tissues showed significantly more lung metastases in the morphine group than in the saline group (14.670 ± 5.086 vs. 8.000 ± 2.500, $p = .008$). However, significantly fewer lung metastases were found in the morphine + ketorolac group as compared to metastases in the morphine group (8.667 ± 2.944 vs. 14.670 ± 5.086, $p = .034$) In addition, the numbers of lung metastases in the morphine + ketorolac group and the ketorolac group were not significantly different from that of the saline group ($p > .05$).

3.2. Postoperative co-administration of morphine and ketorolac downregulated neovascularization and prevent angiogenesis in TNBC metastasis

The microvasculature of recurrent tumors was detected by IHC staining of CD31 (Fig. 2), which revealed that the microvasculature in the morphine group was denser than that of the saline group (12.470 ± 1.332 vs. 7.400 ± 1.908, $p = .020$). However, co-administration of morphine and ketorolac significantly reduced the density of the microvasculature compared to the morphine-treated group (5.700 ± 3.535 vs. 12.470 ± 1.332, $p = .024$). This difference was not significant in comparison with the saline or ketorolac group ($p > .05$).

3.3. Postoperative co-administration of morphine and ketorolac downregulated TNBC metastasis via TSP-1 overexpression and c-Myc downregulation

Western blotting was performed to further investigate the possible mechanism by which morphine and ketorolac influence angiogenesis in TNBC metastasis. As seen in Fig. 2, compared to the saline-treated group, the morphine-treated group exhibited significantly lower expression of TSP-1 (0.346 ± 0.042 vs. 1.000 ± 0, $p < .001$). Furthermore, the expression of c-Myc was significantly increased in morphine-treated group compared with saline treated group (1.267 ± 0.014 vs. 1.000 ± 0, $p = .042$). Co-administration of morphine and ketorolac inhibited the influence of morphine on TSP-1 and c-Myc expression (TSP-1 expression in the morphine + ketorolac group vs. morphine group: 0.731 ± 0.347 vs. 0.346 ± 0.042, $p = .027$; c-Myc expression in the morphine + ketorolac group vs. morphine group: 0.592 ± 0.115 vs. 1.267 ± 0.014, $p < .001$) while the expression levels of these two proteins were not significantly different from those in the saline or ketorolac group ($p > .05$).

3.4. In vitro analysis

3.4.1. Morphine and ketorolac regulate TSP-1 synthesis and secretion in vitro

RT-PCR and ELISA were performed to investigate the effect of morphine and ketorolac on TSP-1 synthesis and secretion in MDA-MB-231 cells. As seen in Fig. 3, RT-PCR indicated that the expression of TSP-1 mRNA was significantly decreased during morphine treatment (morphine group vs control group: $7.070 \times 10^{-3} \pm 0.516 \times 10^{-3}$ vs. $13.290 \times 10^{-3} \pm 2.058 \times 10^{-3}$, $p = .002$). ELISA also showed that morphine markedly downregulated the expression of TSP-1 protein (morphine group vs control group: 85.680 ± 17.320 vs. 129.700 ± 7.691 ng ml^{-1}, $p = .018$). However, co-administration of ketorolac with morphine dramatically upregulated the secretion of TSP-1 (morphine + ketorolac group vs morphine group: 202.400 ± 6.657 vs. 85.680 ± 17.320 ng ml^{-1}, $p < .001$); while the effects of morphine on TSP-1 synthesis were also significantly inhibited by ketorolac (morphine + ketorolac group vs. morphine group: $11.690 \times 10^{-3} \pm 2.285 \times 10^{-3}$ vs. $7.070 \times 10^{-3} \pm 0.516 \times 10^{-3}$, $p = .018$) (Fig. 3). The synthesis of TSP-1 was not significantly different from that of the negative control and ketorolac groups ($p > .05$).

3.4.2. The PI3K/AKT/c-Myc signaling pathway played an important role in TSP-1 synthesis and secretion in vitro

To further explore the possible molecular mechanism by which morphine and ketorolac influence TSP-1 synthesis and secretion, Western blotting was utilized to measure c-Myc expression and AKT phosphorylation. Western blots indicated that morphine significantly increased c-Myc expression and AKT phosphorylation compared with the corresponding levels in the control group (c-Myc expression1.544 ± 0.350 vs. 1.000 ± 0, $p = .012$; AKT phosphorylation 1.727 ± 0.375 vs. 1.000 ± 0, $p = .003$). However, co-administration of ketorolac dramatically suppressed the effects of morphine on c-Myc expression and AKT phosphorylation as compared to the morphine-treated group (c-Myc expression: 1.060 ± 0.359 vs. 1.544 ± 0.350, $p = .028$; AKT phosphorylation: 1.181 ± 0.177 vs. 1.727 ± 0.375,

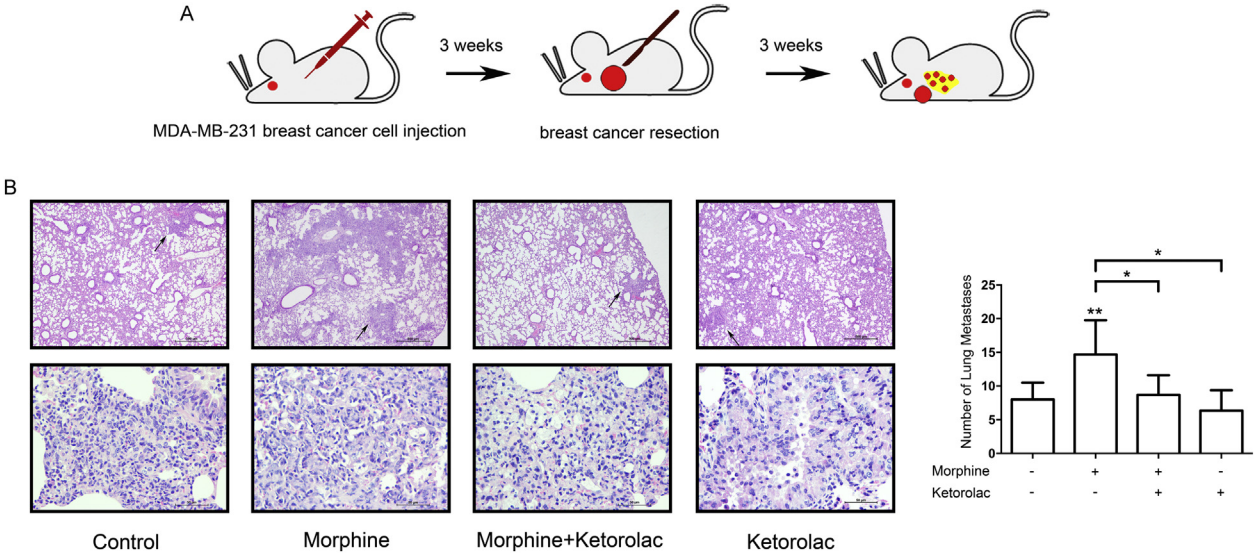

Fig. 1. Postoperative lung metastasis was influenced by postoperatively administered morphine and ketorolac in mice. (A) Establishment of postoperative breast cancer metastasis mouse model. Breast cancer tissues of BALB/c nude mice were resected 3 weeks after injection of MDA-MB-231 cells. Lungs and recurrent tumors were collected 3 weeks after resection. (B) H&E staining was performed on paraffin-embedded lung tissues and captured at 40× and 400× magnification. Lung metastases per mouse were calculated and data showed that lung metastases were significantly increased by postoperatively administered morphine. Co-administration of ketorolac and morphine reduced lung metastasis. The data was presented as the mean ± SD. $^{*}p < .05$, $^{**}p < .01$.

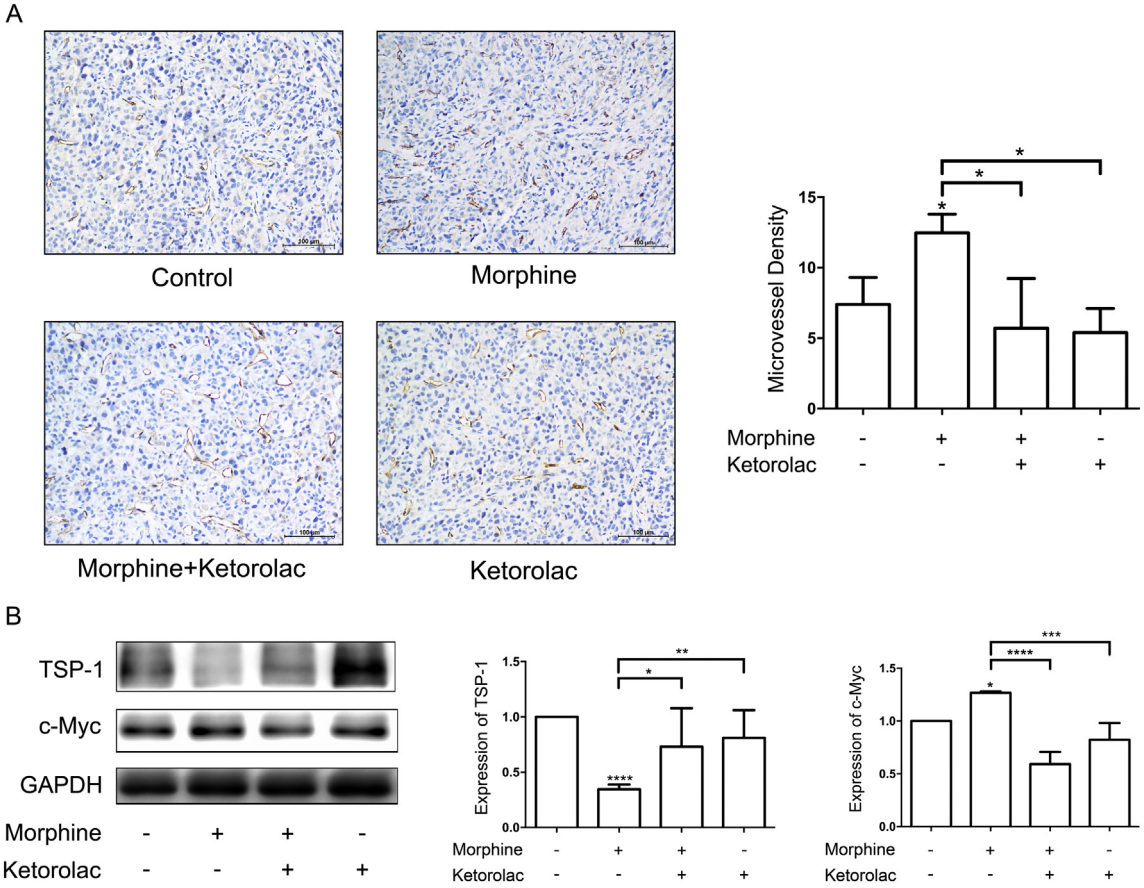

Fig. 2. Morphine and ketorolac influenced breast cancer angiogenesis by altering the expression of c-Myc and TSP-1. (A) IHC staining showed that postoperatively administered morphine promoted angiogenesis in recurrent breast tumors, while ketorolac prevented breast cancer angiogenesis induced by morphine. (B) Western blotting showed that morphine reduced breast cancer cell TSP-1 expression and increased c-Myc expression. Ketorolac reversed the morphine-induced changes in TSP-1 and c-Myc expression. The data was presented as the mean ± SD. $^{*}p < .05$, $^{**}p < .01$.

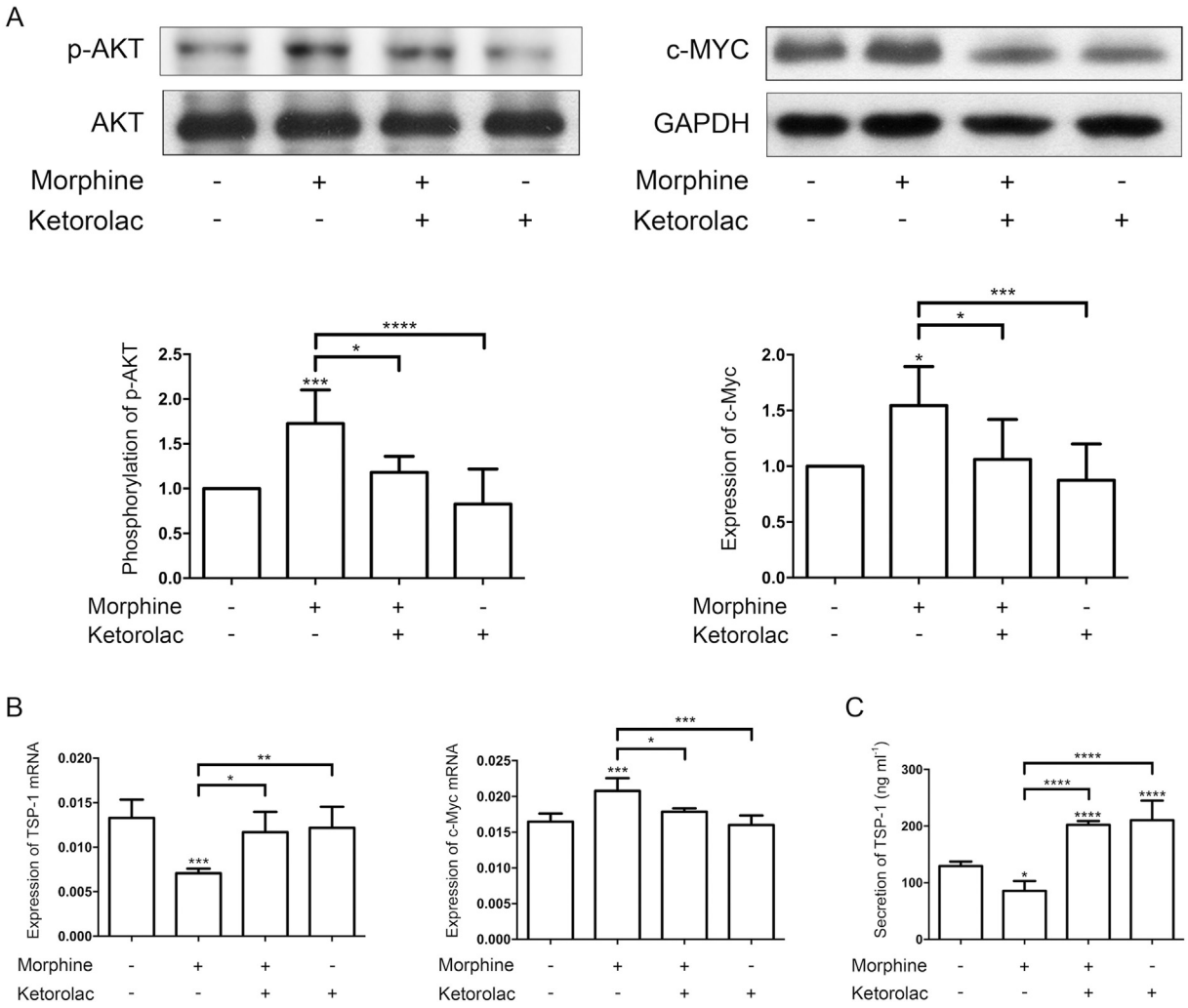

Fig. 3. Morphine and ketorolac influenced TSP-1 synthesis and secretion in MDA-MB-231 cells by regulating PI3K/AKT/c-Myc signaling pathway. (A) Western blotting showed that morphine increased the phosphorylation of AKT and the expression of c-Myc, while ketorolac reversed the influence of morphine on PI3K/AKT/c-Myc pathway activation. RT-PCR (B) and ELISA (C) showed that the synthesis and secretion of TSP-1 was also reduced by morphine, and ketorolac reversed the influence of morphine on TSP-1 synthesis and secretion. The data was presented as the mean ± SD. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .005$, $^{****}p < .001$.

$p = .033$), but ketorolac treatment alone had no significant effect on TSP-1 and c-Myc expression ($p > .05$).

Treatment of MDA-MB-231 cells with the AKT inhibitor MK2206 dramatically upregulated the secretion of TSP-1(MK2206 group vs. control group: 187.300 ± 9.247 vs. 129.700 ± 7.691 ng ml^{-1}, $p = .003$). Co-treatment of morphine with MK2206 also upregulated TSP-1 secretion levels as compared to the morphine group (182.600 ± 31.010 vs. 85.680 ± 17.320 ng ml^{-1}, $p < .001$). Furthermore, the effects of morphine on c-Myc expression and TSP-1 synthesis and secretion were also significantly suppressed by MK2206 (c-Myc expression in the morphine + MK2206 group vs. morphine group: 0.954 ± 0.217 vs. 1.269 ± 0.009, $p = .011$; TSP-1 mRNA synthesis in the morphine + MK2206 group vs. morphine group: $12.470 \times 10^{-3} \pm 1.432 \times 10^{-3}$ vs. $8.570 \times 10^{-3} \pm 1.313 \times 10^{-3}$, $p = .009$; TSP-1 secretion in the morphine + MK2206 group vs. morphine group: 182.600 ± 31.010 vs. 85.680 ± 17.320 ng ml^{-1}, $p < .001$). MK2206 treatment alone had no significant effect on PI3K/AKT/c-Myc signaling pathway and TSP-1 synthesis ($p > .05$) (Fig. 4).

4. Discussion

Opioids (i.e. morphine) and NSAIDs (i.e. ketorolac) are the most widely used postoperative analgesic agents for breast cancer mastectomy. Even though limited evidence has been found to clarify the association between postoperative analgesia (opioids and NSAIDs) and TNBC progression, the present study indicated that postoperative morphine promoted TNBC metastasis and angiogenesis and up-regulated c-Myc and down-regulated TSP-1 expression in mice, while co-administration of ketorolac with morphine could prevent all these effects induced by morphine alone on TNBC. In the in vitro study, morphine down-regulated TSP-1 secretion and activated PI3K/AKT/c-Myc pathway in MDA-MB-231 cells, while co-administration of ketorolac with morphine inhibited activation of PI3K/AKT/c-Myc pathway and up-regulated TSP-1 secretion.

By administrating morphine and ketorolac in a clinically relevant dose for 3 days after breast cancer resection in TNBC xenograft mice, we found that postoperative morphine promoted TNBC metastasis and angiogenesis; and, this effect was averted by ketorolac. The effects of morphine on breast cancer progression and angiogenesis in preclinical studies remain controversial [25,26]. Morphine was reported to promote TNBC proliferation and progression in both xenograft mouse models and in vitro studies [9–11]. The present study further validated that postoperative morphine may also promote TNBC metastasis and angiogenesis, similar to the effects presented by previous studies on TNBC metastasis and angiogenesis. However, Doornebal et al. found that morphine had no effect on breast cancer progression in preclinical

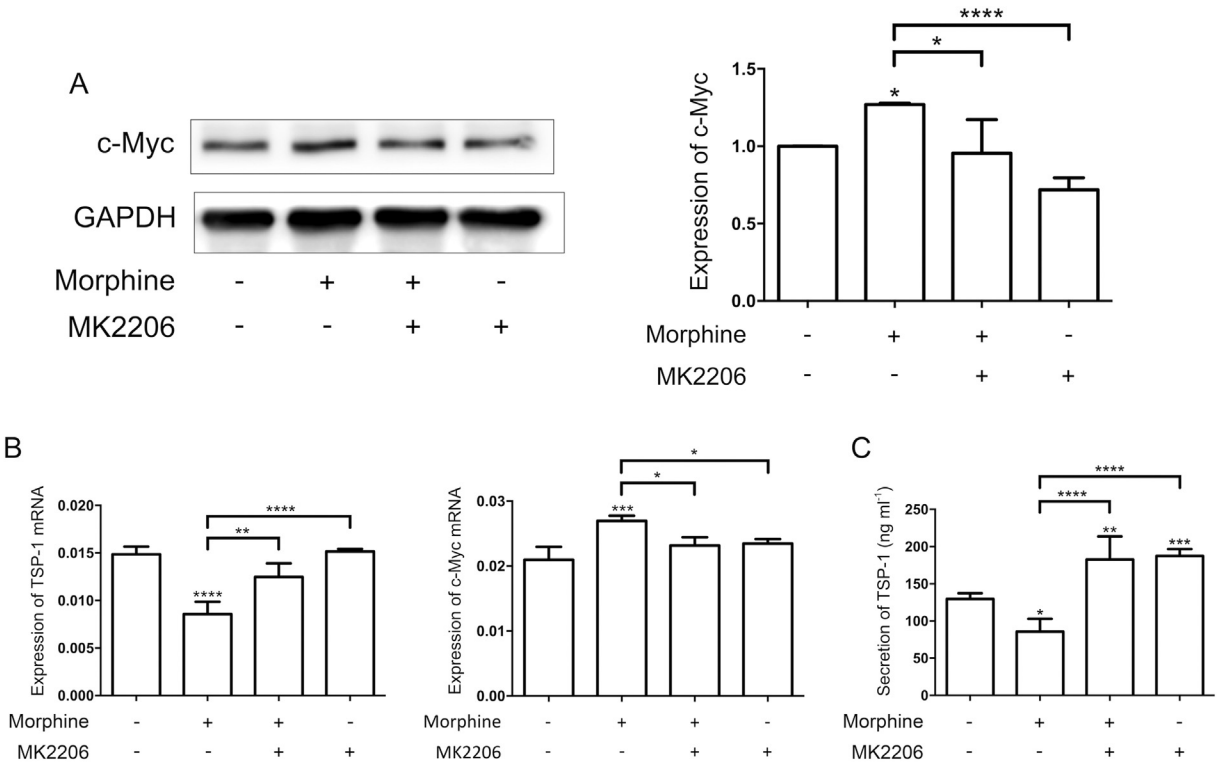

Fig. 4. Activation of PI3K/AKT/c-Myc signaling pathway played a crucial role in the reduction of TSP-1 synthesis and secretion by morphine in MDA-MB-231 cells. (A) Western blotting showed that the AKT inhibitor dramatically reduced c-Myc expression compared with morphine. RT-PCR (B) and ELISA (C) showed that inhibiting AKT phosphorylation reversed the morphine-induced changes in c-Myc synthesis and TSP-1 synthesis and secretion. The data was presented as the mean ± SD. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .005$, $^{****}p < .001$.

models of metastatic invasive lobular and HER2+ breast cancer, even after surgical stimulation [27]. One explanation of the controversy might be the difference of pathological subtypes, for it has been reported that different subtypes of breast cancer differ in their expression of opioid receptors in addition to their differences in the expression of ER, PR and HER-2; therefore, their reactions to morphine could differ [11,28]. Consequently, effort is still needed to clarify the effects of postoperative morphine on different subtypes of breast cancer.

In the present study, ketorolac was proved to avert the promotion of TNBC metastasis and angiogenesis induced by morphine, showing a similar trend with clinical observations [12,13]. To date, in preclinical experiments, only Cox-2 inhibitor (celecoxib) was found to prevent morphine induced angiogenesis and progression of breast cancer [14]. By co-administrating clinically relevant doses of ketorolac with morphine, our study indicated that ketorolac shares similar effects with celecoxib on breast cancer progression.

The present study found that promotion of angiogenesis induced by morphine may be related to the down-regulation of TSP-1 secretion and activation of PI3K/AKT/c-Myc pathway. TSP-1 is known to act as an angiogenesis suppressor by inhibiting VEGF-induced angiogenesis [16]. When studying the micro-metastasis niche, Ghajar et al. found that the down-regulation of TSP-1 expression was essential for triggering distant metastasis [19]. Morphine was reported to improve angiogenesis in breast cancer, and co-administration of methylnaltrexone (an opioid receptor antagonist) with morphine was observed to avert VEGF-induced angiogenesis [9,22]. In both in vitro and in vivo experiments, we further discovered that morphine could directly down-regulate TSP-1 expression. This finding indicated that in addition to improving vascular endothelial growth factor receptor (VEGFR) and platelet derived growth factor receptor (PDGFR) expression [29,30], morphine may promote angiogenesis by reducing TSP-1 expression. Besides, promotion of breast cancer progression and angiogenesis induced by morphine has been found to be related to the activation of survival promoting pathways (i.e. PI3K/AKT/c-Myc pathway) [21]. By co-administrating morphine with AKT inhibitor, activation of PI3K/AKT/c-Myc signal pathway was found to be associated with the down-regulation of TSP-1 secretion induced by morphine. All these provide possible novel mechanisms of morphine induced promotion of angiogenesis and TNBC metastasis.

Farooqui M et al. have illustrated that NSAIDs (i.e. Celecoxib) could prevent breast cancer metastasis and angiogenesis induced by morphine [14], and that the mechanism may lie in the inhibition of cyclooxygenase-2 (COX-2) and prostaglandins (PGs) [31,32]. Interestingly, the present study found that ketorolac prevented angiogenesis induced by morphine through silencing the PI3K/AKT/c-Myc signal pathway and up-regulating TSP-1 secretion, which to the best of our knowledge, has yet to be reported. Therefore, this study offers a new understanding of how NSAIDs might circumvent the angiogenic effect of opioids in TNBC metastasis.

However, we do acknowledge the limitations of this study. Firstly, the xenograft mouse models utilized in the present study could have suffered from confounding factors of graft versus host immune interaction. Secondly, the correlation between TNBC angiogenesis and distant metastasis still needs further clarification. Therefore, further preclinical and clinical studies are needed to identify the effect of perioperatively administered analgesics on TNBC progression.

5. Conclusion

Postoperative administration of morphine and ketorolac influenced TNBC distant metastasis and angiogenesis in our TNBC mouse models, which was related to their effects on TSP-1 secretion and PI3K/AKT/c-Myc activation. Our data provided preclinical evidence for postoperative analgesic selection and possible targets to prevent the effects of postoperative analgesic agents on breast cancer.

Supplementary data to this article can be found online at https://

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Abbreviations

NSAIDs	nonsteroidal anti-inflammatory drugs
TNBC	triple-negative breast cancer
TSP-1	thrombospondin-1
HER2	human epidermal growth factor receptor 2
MFPs	mammalian fatty pads
SPF	specific pathogen-free
H&E	haematoxylin and eosin
IHC	immunohistochemistry
ECL	electro-chemi-luminescence
ANOVA	one-way analysis of variance
NO	nitric oxide
VEGFR	vascular endothelial growth factor receptor
PDGFR	platelet derived growth factor receptor
COX-2	cyclooxygenase-2
PGs	prostaglandins

Ethics approval

Ethical approval for this study (IACUC-2014-1101) was provided by the Animal Ethical and Welfare Committee of SYSU, Guangzhou, China.

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CRediT authorship contribution statement

Zhongqi Liu:Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Writing - original draft.**Shi Cheng**:Methodology, Formal analysis, Project administration, Resources, Visualization, Writing - original draft.**Ganglan Fu**:Data curation, Formal analysis, Validation, Writing - review & editing.**Fengtao Ji**:Data curation, Investigation, Supervision, Validation, Writing - review & editing.**Chengli Wang**:Formal analysis, Writing - original draft.**Minghui Cao**:Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing - review & editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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