



# Thymoquinone Suppresses Migration of Human Renal Carcinoma Caki-1 Cells through Inhibition of the PGE<sub>2</sub>-Mediated Activation of the EP2 Receptor Pathway

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## Abstract

Renal cell carcinoma (RCC) is likely to metastasize to other organs, and is often resistant to conventional chemotherapies. Thymoquinone (TQ), a phytochemical derived from the seeds of *Nigella sativa*, has been shown to inhibit migration and metastasis in various cancers. In this study, we assessed the effect of TQ on the migratory activity of human RCC Caki-1 cells. We found that treatment with TQ reduced the proteolytic activity of matrix metalloproteinase-9 (MMP-9) in Caki-1 cells. TQ significantly repressed prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production, its EP2 receptor expression as well as the activation of Akt and p38, the well-known upstream signal proteins of MMP-9. In addition, treatment with butaprost, a PGE<sub>2</sub> agonist, also induced MMP-9 activity and migration/invasion in Caki-1 cells. Moreover, pharmacological inhibitors of PI3K/Akt and p38 remarkably attenuated butaprost-induced Caki-1 cell migration and invasion, implying that activation of PI3K/Akt and p38 is a bridge between the PGE<sub>2</sub>-EP2 axis and MMP-9-dependent migration and invasion. Taken together, these data suggest that TQ is a promising anti-metastatic drug to treat advanced and metastatic RCC.

**Key Words:** Thymoquinone, Renal cell carcinoma (RCC), Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), EP2 receptor, MMP-9

## INTRODUCTION

Renal cell carcinoma (RCC) is the most common type of kidney cancer and accounts for approximately 3% of total malignancies (Cairns, 2010). If RCC is detected at the initial stage, it can be successfully removed by surgery, with a 5-year survival rate of nearly 70-80%. However, the early stage of RCC is usually asymptomatic, which makes it difficult to diagnose early. Thus, by the time it is discovered, RCC patients often present with metastasis to other organs, such as to the lymph, lung, and bone, which significantly lowers the survival rate (Bianchi *et al.*, 2012). Even worse, advanced and metastatic RCC is particularly resistant to conventional chemotherapy and radiotherapy (Grimm *et al.*, 2010). Therefore, it is crucial to inhibit and limit the metastatic activity of RCC for effective treatment.

Inflammation is a strong driving force for tumor metastasis (Wu and Zhou, 2009). During inflammatory responses, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is synthesized from arachidonic acids by cyclooxygenase-2 (COX-2) (Nakanishi and Rosenberg, 2013). PGE<sub>2</sub> then binds to its four G protein-coupled receptors, EP1-EP4, stimulating cancer cell growth, invasion, and metastasis (Sugimoto and Narumiya, 2007). In addition to overproduction of PGE<sub>2</sub>, the aberrant expression of its receptors can also amplify PGE<sub>2</sub> signaling, which can facilitate cancer promotion and metastasis. Previously, we found that EP2 and EP4 receptors were responsible for PGE<sub>2</sub>-dependent migration in a human RCC Caki-1 cell line (Woo *et al.*, 2015). These results suggested that the PGE<sub>2</sub>-EP2/EP4 axis is a possible drug target for metastatic RCC.

The PGE<sub>2</sub>-EP2/EP4 axis can recruit matrix metalloproteinases (MMPs), which are pivotal for cancer metastasis (Yen *et*

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*al.*, 2008; Jana *et al.*, 2016). MMPs catalyze the proteolytic degradation of the extracellular matrix (ECM) in the tumor microenvironment, which is a physical barrier for cancer cell migration and invasion, thus leading to metastasis (Nabeshima *et al.*, 2002). Consistent with this process, MMPs are overexpressed in various types of metastatic cancers, and serve as chemotherapeutic targets (Bergers *et al.*, 2000; Stamenkovic, 2000).

Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone; TQ) is a monoterpene present in the seeds of black cummin, *Nigella sativa* (Kundu *et al.*, 2014). TQ has been reported to exert various physiological functions, including anti-inflammatory, antiproliferative, and anticancer activities (Kundu *et al.*, 2014). Hsu *et al.* (2017) have demonstrated that TQ inhibits human colon cancer cell migration by blocking the PGE<sub>2</sub>-EP2/EP4 axis. Moreover, TQ can inhibit metastasis through reduction of MMP-2 and MMP-9 in human and mouse brain cancers (Kolli-Bouhafs *et al.*, 2012; Arumugam *et al.*, 2016). TQ is believed to be nontoxic because it has long been used as a food additive, indicating that TQ can be a potential candidate for a nontoxic anti-metastatic drug to treat metastatic RCC. In the present study, we therefore investigated the effect of TQ on the metastatic properties of a human RCC Caki-1 cell line, and identified its underlying molecular mechanisms, particularly in the context of PGE<sub>2</sub> signaling and MMP-9.

## MATERIALS AND METHODS

### Materials

TQ (purity 99%), gelatin, eosin Y, hematoxylin, and  $\beta$ -actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). EP2 antibody (#101750), PGE<sub>2</sub>, butaprost, and were purchased from Cayman Chemical (Ann Arbor, MI, USA). SB203580 was obtained from Calbiochem (San Diego, CA, USA). LY294002, Akt (#9272), p-Akt (#9271), p38 (#9212), and anti-rabbit IgG horseradish peroxidase-conjugated antibodies were obtained from Cell Signaling Technologies (Beverly, MA, USA). MMP-2 (ab92536) and MMP-9 (ab76003) antibodies were purchased from Abcam (Cambridge, UK). The p-p38 antibody (#sc-17852) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

### Cell culture

Human renal cancer Caki-1 cells were provided by Dr. T. K. Kwon (Keimyung University, Daegu, Korea) and were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin G and 100  $\mu$ g/mL streptomycin) at 37°C under a humidified 95% air/5% CO<sub>2</sub> mixture (v/v).

### Cell viability assay

Caki-1 cells were seeded in 96-well plates at a density of  $2 \times 10^3$  cells/well and were treated with 1, 5, and 10  $\mu$ M TQ for 24, 48, and 72 h. Following treatment, the cells were incubated with fresh medium containing 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; AMRESCO, Solon, OH, USA) and incubated at 37°C for an additional 4 h. The medium was removed, and formazan was dissolved in 100  $\mu$ L of dimethyl sulfoxide. The plates were then shaken for 5-10 min, and the absorbance at 570 nm was measured using a microplate reader (Tecan Trading, Mannedorf, Switzerland).

The relative cell viability (%) was expressed as a percentage relative to the vehicle treated group.

### Western blot analysis

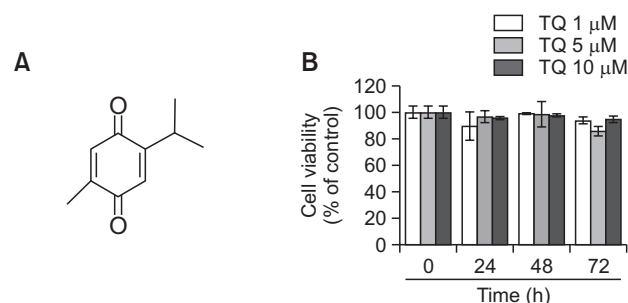
The cells were harvested and lysed with RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) and the resulting protein samples were quantified using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of protein extracts were denatured by boiling at 100°C for 5 min in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA). The proteins were separated by 8-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 buffer (TBS-T) (10 mM Tris, 150 mM NaCl, pH 7.5, and 0.1% Tween 20) for 1 h at room temperature, and incubated with primary antibodies (diluted 1:1,000) overnight at 4°C. The membranes were then washed three times for 10 min each with TBS-T buffer and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies. The membranes were then washed three times for 10 min each with TBS-T buffer, and incubated with Super-signal pico-chemiluminescent substrate or dura-luminol substrate (Thermo Fisher Scientific) according to the manufacturer's instructions, and visualized using an ImageQuant LAS 4000 system (Fujifilm Life Science, Tokyo, Japan).

### Wound-healing migration assay

The wound healing assay was conducted to determine the migratory ability of cells. Briefly, the cells were cultured at a concentration of  $5 \times 10^5$  cells/mL in 6-well plates, and incubated until the cell density reached 90%. The cell monolayers were wounded by scratching with a 10- $\mu$ L pipette tip, washed with phosphate-buffered saline (PBS) to remove detached cells, and then incubated in DMEM supplemented with 5% FBS containing appropriate reagents according to the experimental design. After 24 h at 37°C, the medium was replaced with PBS and washed twice. The gap-closure was then examined using a microscope (Olympus, Tokyo, Japan).

### Cell invasion assay

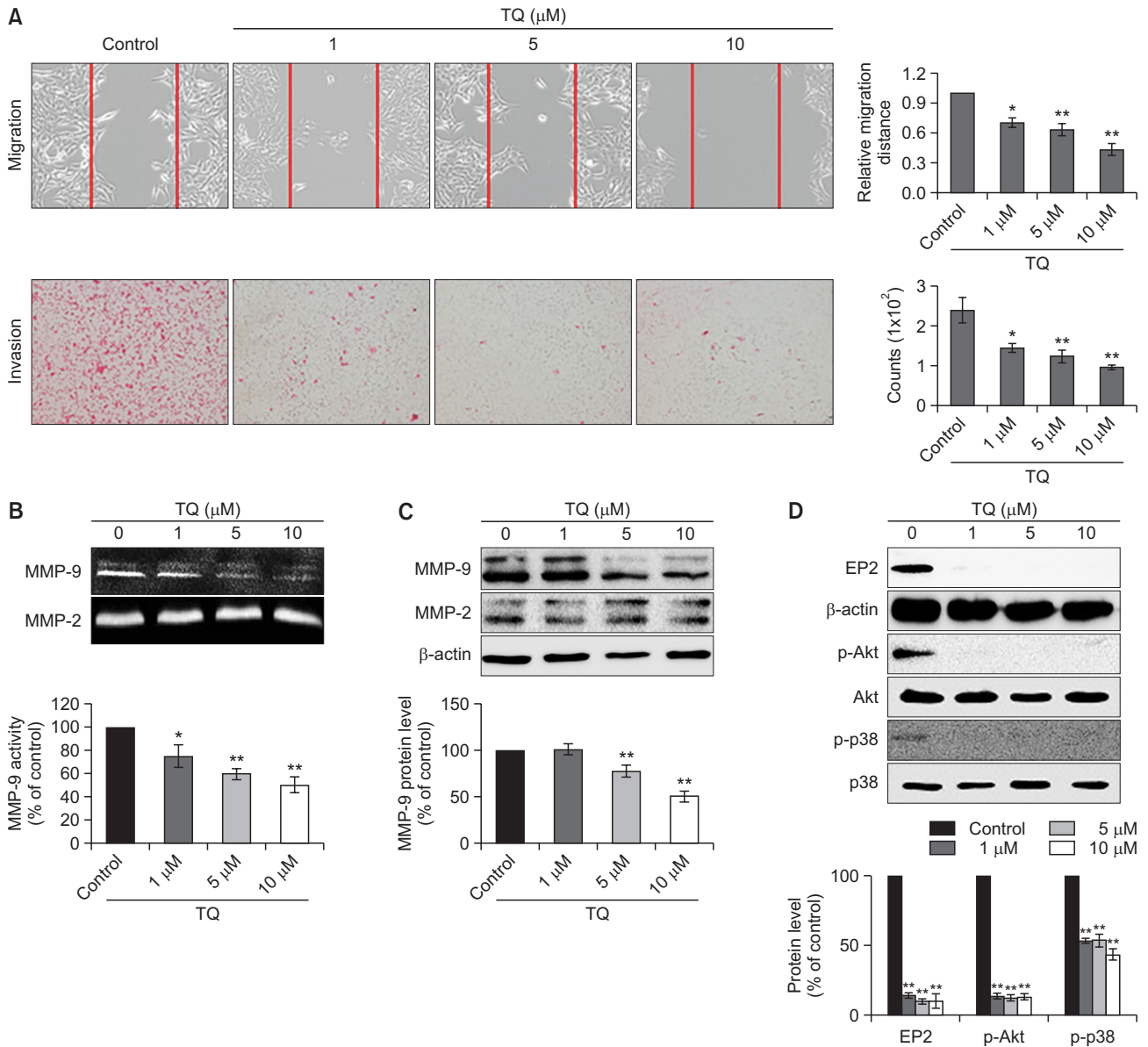
The invasion capacity of Caki-1 cells was determined *in vi-*



**Fig. 1.** Effect of thymoquinone (TQ) on Caki-1 cell growth. (A) The chemical structure of TQ. (B) Caki-1 cells were treated with the indicated concentrations (1, 5, and 10  $\mu$ M) of TQ for 24, 48, and 72 h. The cell viability was determined using the MTT assay as described in the Materials and Methods. The MTT formazan product was detected using a microplate reader. Data represent the mean  $\pm$  standard deviation of three independent experiments (n=3).

tro using Boyden Chambers (6.5-mm diameter filters, 8-mm pore size; Merck, Darmstadt, Germany). Briefly, Caki-1 cells ( $1 \times 10^5$  cells/200  $\mu$ L serum-reduced medium) were placed in the upper chamber of Boyden Chambers (Merck). The appropriate reagents were added to the lower chamber (200  $\mu$ L).

The chambers were assembled and kept in an incubator for 24 h. At the desired time points, cells from the upper surface of the membranes (Millipore Sigma, Burlington, MA, USA) were removed with gentle swabbing and the migrating cells on the lower surface of membranes were fixed and stained with crys-



**Fig. 2.** The effect of thymoquinone (TQ) on Caki-1 cell migration. (A) Wound healing (upper panel) and invasion (bottom panel) assays were performed to determine the effect of TQ on the migration of Caki-1 cells. For the wound healing assay (upper panel), the cells were treated with dimethyl sulfoxide (control) or TQ (1, 5, and 10  $\mu$ M) for 24 h after wounding. The gap closure was detected using a microscope (Olympus, Tokyo, Japan). The wound sizes were measured using a microscope (Olympus) and expressed as the mean of triplicates  $\pm$  standard deviation (SD). \* $p < 0.05$ , \*\* $p < 0.005$ . For the migration assay (bottom panel), Caki-1 cells were seeded in the upper chamber of a Transwell and treated with TQ (1, 5, and 10  $\mu$ M) for 24 h. The migrated cells were then stained and observed using a microscope (Olympus). The migrating cells per microscopic field were counted and represented on a bar graph as the mean of triplicates  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.005$ . (B) The proteolytic activities of MMP-9 and MMP-2 were determined in Caki-1 cells treated with 1, 5, and 10  $\mu$ M of TQ for 24 h using the gelatin zymography assay. The proteolytic activity of MMP-9 is represented on a bar graph as the mean of triplicates  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.005$ . (C, D) Western blot analysis of the protein expression of MMP-9 and MMP-2 (C), EP2 and phosphorylated/non-phosphorylated forms of Akt and p38 (D) in Caki-1 cells treated with TQ (1, 5, and 10  $\mu$ M) for 24 h. The  $\beta$ -actin was the protein loading control. The protein expression levels of EP2, p-Akt, and pp38 were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA, <https://imagej.nih.gov/ij/>) and then normalized to actin or the respective total forms. A bar graph represents the mean of triplicates  $\pm$  SD. \*\* $p < 0.005$ .

tal violet. The membranes were then washed and mounted on glass slides, and the stained migrating cells were visualized using a microscope (Olympus).

### Gelatin zymography assay

The gelatin zymography assay was performed to assess the proteolytic activities of MMPs by measuring their capability to degrade gelatin. To assess the enzymatic activity of MMPs, culture supernatants were collected, mixed with non-reducing sample buffer, and subjected to 10% SDS-PAGE containing 0.25% gelatin. The gel was washed three times with wash buffer (2.5% Triton X-100), and then incubated in incubating buffer (1 M Tris-HCl at pH 7.5, 5% Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub> and 1 M CaCl<sub>2</sub>) overnight at 37°C. The gel was then stained with Coomassie Blue (Bio-Rad) and the MMP-9 enzymatic activity was quantified using the LAS 4000 imaging system (Fujifilm Life Science, Tokyo, Japan).

### Statistical analysis

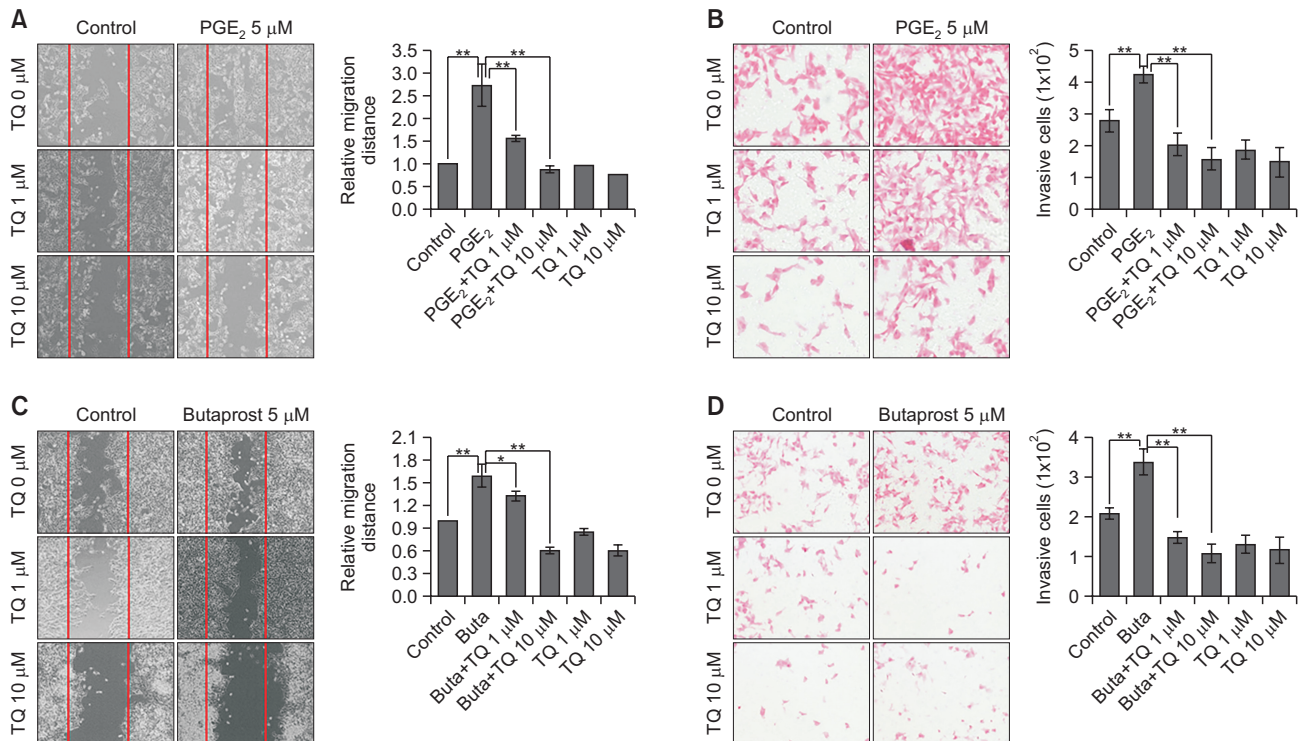
All data were obtained from more than three independent experiments. Statistical analysis was conducted using the paired Student's *t*-test or analysis of variance. A value of *p*<0.05 was considered to be statistically significant.

## RESULTS

### TQ inhibits Caki-1 cell migration through suppression of MMP-9

TQ is a phytochemical extracted from black cumin seeds and classified as a monoterpene based on its chemical structure as shown in Fig. 1A. To evaluate its effect on RCC migration, we utilized a human metastatic RCC Caki-1 cell line. Caki-1 cells were treated with various concentrations (1, 5, and 10 μM) of TQ for 24, 48, and 72 h. The MTT assay showed that TQ did not induce either cell death or cell proliferation, even after 72 h post-treatment (Fig. 1B). All the subsequent experiments were, therefore, performed at 24 h after treatment.

While TQ did not induce cancer cell death in Caki-1 cells, both wound-healing migration (Fig. 2A, upper panel) and invasion (Fig. 2A, bottom panel) assays showed that TQ significantly inhibited the migratory/invasive activity of Caki-1 cells with a high metastatic potential. Thus, we examined the effect of TQ on the activity and expression of MMP-9, a crucial enzyme for wound healing, cell spreading, and migration. Treatment with TQ reduced the proteolytic activity of MMP-9 in a concentration-dependent manner in Caki-1 cells (Fig. 2B). In addition to its activity, TQ also attenuated MMP-9 expression at the protein level (Fig. 2C). However, TQ was not able to modulate either the protein expression or enzymatic activity of MMP-2 (Fig. 2B, 2C). Taken together, these data suggested



**Fig. 3.** The effect of thymoquinone (TQ) on prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)- or butaprost-induced Caki-1 cell migration. (A, C) Caki-1 cells were treated with TQ (1 and 10 μM) in the presence or absence of 5 μM PGE<sub>2</sub> (A) or butaprost (C) after wounding. At 24 h post-treatment, microscopic examination was performed to determine the wound closure. Solid line, the gap space of controls. The wound sizes were measured using a microscope (Olympus, Tokyo, Japan) and expressed as the mean of triplicates ± standard deviation (SD). \**p*<0.05; \*\**p*<0.005. (B, D) Caki-1 cells were seeded in the upper chamber of the Transwell, then incubated in media containing TQ (1 and 10 μM) with or without 5 μM PGE<sub>2</sub> (B) or butaprost (D) for 24 h. Crystal violet was used to stain and detect the migrated cells. The migrating cells per microscopic field were counted and represented on a bar graph as the mean ± SD. \*\**p*<0.005.



that TQ inhibited migration of human metastatic renal carcinoma Caki-1 cells by suppressing MMP-9.

Previously, we reported that the PGE<sub>2</sub>-EP2/EP4 axis was crucial for the migratory activity in Caki-1 cells (Woo *et al.*, 2015). Treatment with TQ significantly repressed the expression of EP2 and activation of Akt and p38, the well-known upstream signals of MMP-9 (Fig. 2D) (Ruhul Amin *et al.*, 2003; Lee *et al.*, 2009). These data indicated the possibility for reduction of the PGE<sub>2</sub>-EP2-MMP9 axis by TQ treatment in Caki-1 cells.

### TQ suppresses the PGE<sub>2</sub>-EP2 axis-mediated migratory activity of Caki-1 cells

To verify the effect of TQ on PGE<sub>2</sub>-EP2 axis-induced migration, Caki-1 cells were co-treated with TQ plus either PGE<sub>2</sub> or butaprost, an EP2 selective agonist. As previously reported, we confirmed that both PGE<sub>2</sub> and butaprost remarkably accelerated wound closure (Fig. 3A, 3C) and invasion (Fig. 3B, 3D) of Caki-1 cells. In the presence of TQ, however, the PGE<sub>2</sub>- or butaprost-induced wound healing and invasion were markedly decreased (Fig. 3). These results showed that TQ suppressed the PGE<sub>2</sub>-EP2 axis-stimulated migration/invasion in Caki-1 cells.

### The EP2 agonist, butaprost, activates MMP-9, possibly via the Akt and p38 signaling pathways in Caki-1 cells

As shown in Fig. 2, TQ inhibited migration and MMP-9 activity, simultaneously suppressing the expression of EP2, phosphorylation of Akt, p38, and the MMP-9 upstream regulator. We thus assumed that Akt and p38 might be responsible for the PGE<sub>2</sub>/EP2 axis-induced MMP-9 activation and subsequent migration in Caki-1 cells. To test this hypothesis, we examined whether the EP2 selective agonist, butaprost, regulated the Akt and p38 signaling pathways in Caki-1 cells. Butaprost induced phosphorylation of both Akt and p38 in Caki-1 cells (Fig. 4A). Moreover, butaprost increased MMP-9 proteolytic activity (Fig. 4B) as well as protein expression levels (Fig. 4C) in a concentration-dependent manner. These data indicated that the PGE<sub>2</sub>-EP2 axis may stimulate migration through activation of Akt and p38 in Caki-1 cells.

### The PI3K/Akt inhibitor, LY294002, reduces the migratory activity of Caki-1 cells

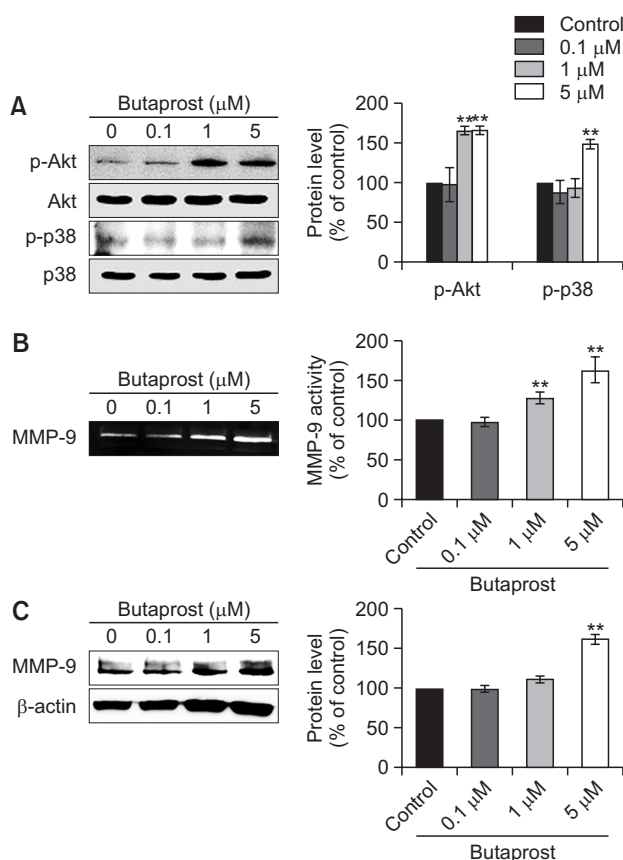
To verify the involvement of the Akt and p38 signaling pathways, pharmacological inhibitors of Akt and p38, LY294002 and SB203580, respectively, were selected for subsequent studies. First, the effect of the PI3K/Akt inhibitor LY294002 on butaprost-induced Caki-1 cell migration was examined. The wound healing assay showed that treatment with LY294002 significantly reduced closure of open scratches stimulated by butaprost in Caki-1 cells (Fig. 5A, upper panel). In addition, LY294002 attenuated butaprost-induced invasion (Fig. 5A, bottom panel), further supporting the involvement of PI3K/Akt signaling in the PGE<sub>2</sub>-EP2 axis-promoted migration. Western blot analysis confirmed the inhibitory effect of LY294002 on Akt activation in Caki-1 cells (Fig. 5B). Together, the results showed that inhibition of the PI3K/Akt pathway by LY294002 treatment blocked butaprost-induced MMP-9 expression (Fig. 5B) as well as its enzymatic activation (Fig. 5C).

### Inhibition of p38 inhibition by treatment with SB203580 suppresses Caki-1 cell migration

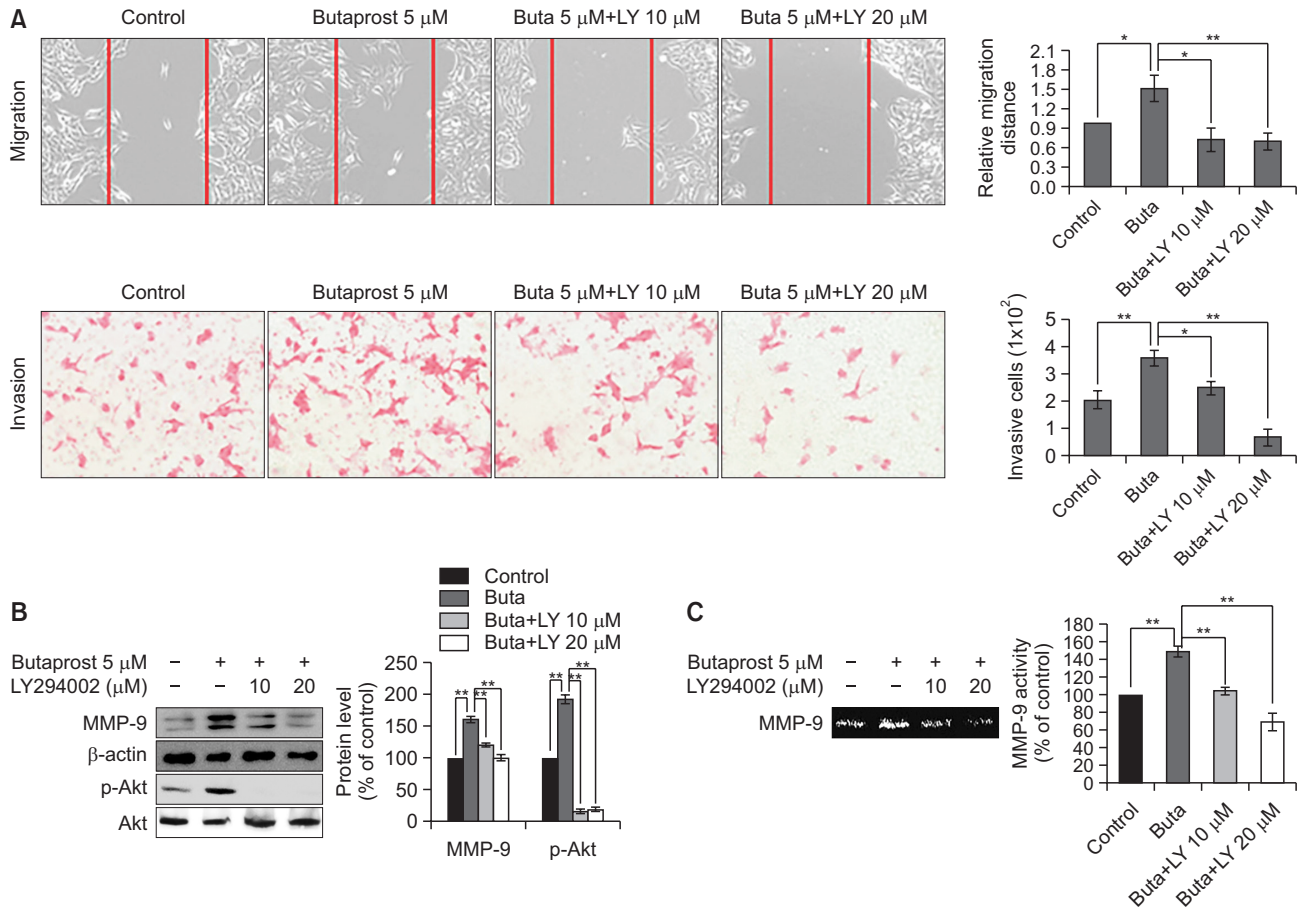
We also investigated the effects of the p38 inhibitor, SB203580, in the same manner as shown in Fig. 5. SB203580 also had similar inhibitory effects on butaprost-induced Caki-1 cell migration/invasion (Fig. 6A). SB203580 treatment inactivated p38 in a concentration-dependent manner, which led to the attenuation of MMP-9 induction by butaprost (Fig. 6B, 6C).

## DISCUSSION

Although cancer treatment strategies have been evolving rapidly, including cancer patient-derived xenograft and immunotherapies, the treatment of metastatic RCC using cancer therapies has not undergone similar advances. At the time of diagnosis,



**Fig. 4.** The effect of butaprost on the activation and expression of MMP-9 in Caki-1 cells. (A) Western blot analysis of the phosphorylated levels of Akt and p38 in Caki-1 cells treated with butaprost (0.1, 1, and 5 μM) for 24 h. The protein expression levels were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA) and then normalized to the respective total forms. A bar graph represents the mean of triplicates ± standard deviation (SD). \*\*\**p*<0.005. (B, C) Under the same condition as in (A), the effects of butaprost on the proteolytic activity (B) and protein expression (C) of MMP-9 were determined by gelatin zymography (B) and western blotting (C), respectively. β-actin was used as a protein loading control for western blotting. Densitometry was performed using Image J software (National Institutes of Health). A bar graph represents the mean of triplicates ± SD. \*\*\**p*<0.005.



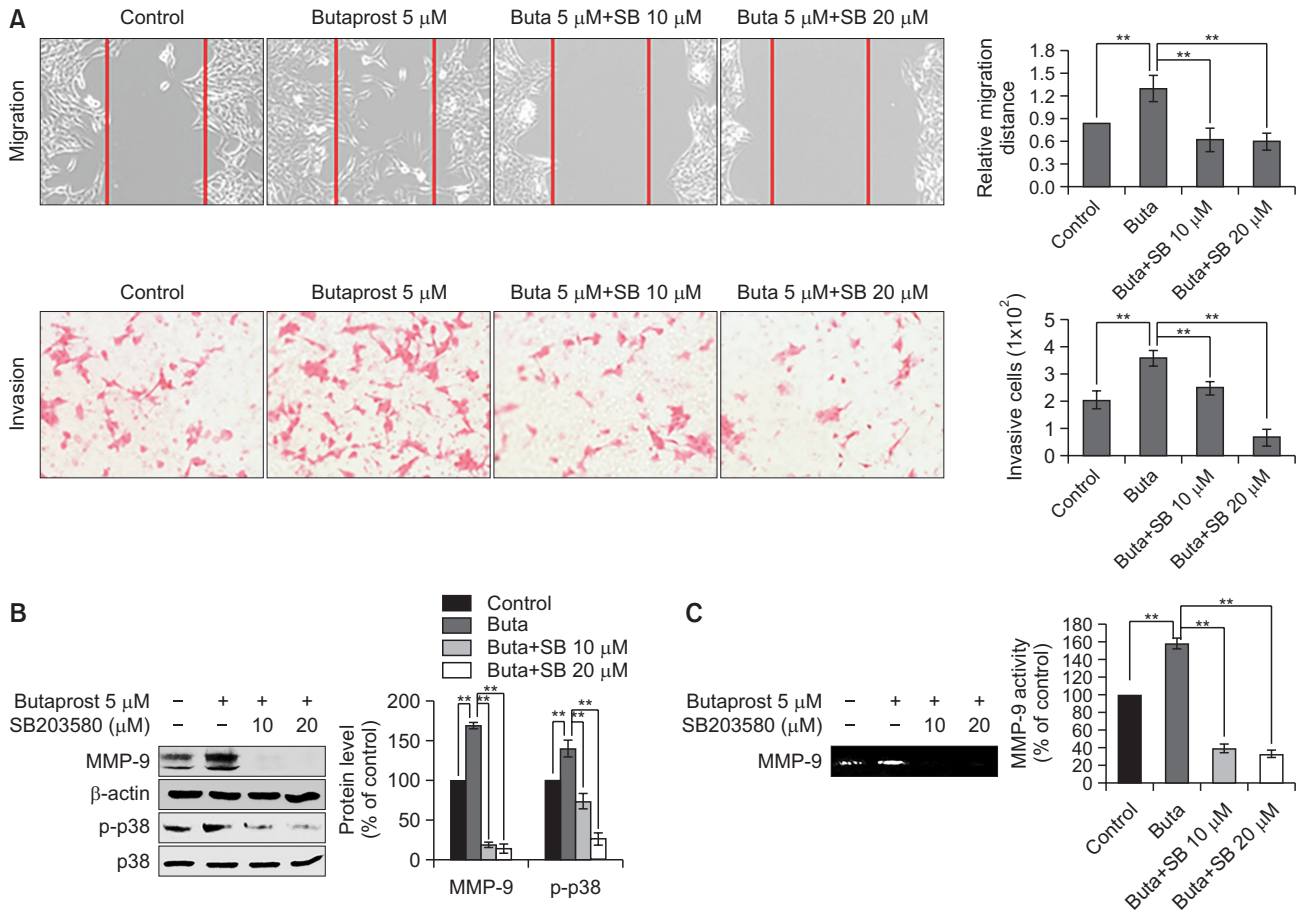
**Fig. 5.** The effect of the PI3K inhibitor, LY294002, on cell migration and MMP-9 expression in Caki-1 cells. (A) Wound healing (upper panel) and invasion (bottom panel) assays were conducted to assess the effect of LY294002 (LY) on butaprost-induced cell migration. Caki-1 cells were treated with butaprost (5  $\mu$ M) plus or minus LY294002 (10 and 20  $\mu$ M) for 24 h, followed by obtaining microphotographs of the gap closure (for the wound healing assay, upper panel) and Crystal violet-stained migrated cells (for the migration assay, bottom panel). Bar graphs represent the relative gap distance (upper panel) and the migrating cells per field (lower panel); \* $p$ <0.05; \*\* $p$ <0.005. (B, C) Caki-1 cells were treated in the same manner as described in (A). At 24 h after treatment, western blotting analyzed Akt phosphorylation and MMP-9 expression (B), and gelatin zymography revealed MMP-9 proteolytic activity (C).  $\beta$ -actin was used as a protein loading control for western blotting. Densitometry was performed using Image J software (National Institutes of Health, Bethesda, MD, USA). A bar graph represents the mean of triplicates  $\pm$  standard deviation. \*\* $p$ <0.005.

metastasis is found in more than half of RCC patients, who often cannot be treated using conventional chemotherapy or radiotherapy (Grimm *et al.*, 2010). Thus, the most efficient strategy to effectively treat metastatic RCC is to prevent metastatic spread from the primary tumor site.

The metastatic process can be initiated by cancer cell migration, requiring proteolytic degradation of the ECM that acts as a physical barrier against cell migration (Nabeshima *et al.*, 2002). MMPs, the main ECM enzymes, can cleave the connection between ECM components, leading to the disruption of cellular physical scaffolds as well as facilitation of cell invasion and migration. MMP-2 and MMP-9 have been reported to play major roles in the tumor microenvironment. Wang *et al.* (2010) have shown that MMP-9 gene silencing significantly inhibits metastasis in a tail vein injection model. In addition, inhibition of either MMP-2 or MMP-9 by using small molecules can reduce the metastatic potential of retinoblastoma, as determined by wound healing and Boyden chamber assays (Webb *et al.*, 2017). MMP-9 is strongly associated with tumor

invasion and metastasis in RCC (Sun *et al.*, 2014). Moreover, reduction of TIMP1 expression induces the activity of MMP-9, and promotes migration and invasion of RCC cells (Guo *et al.*, 2019). Notably, consistent with our results, silencing of MMP-9, but not MMP-2, abolished migration induced by the treatment of the G-protein coupled estrogen receptor agonist in RCC cells (Sun *et al.*, 2014). In the present study, we tested whether MMP-2 and MMP-9 served as crucial molecular targets for metastatic RCC treatment by using TQ.

TQ is a phytochemical derived from the seeds of *Nigella sativa*, classified as a monoterpene based on its chemical structure (Kundu *et al.*, 2014). Numerous investigators have reported that TQ exerts various beneficial effects, such as antitumorigenic, antioxidative, and anti-inflammatory activities (Kundu *et al.*, 2014). In addition, clinical interventions also recommend consumption of TQ or *Nigella sativa* to improve the health or prevent and/or treat clinical symptoms without severe adverse effects in patients (Tavakkoli *et al.*, 2017; Gholamnezhad *et al.*, 2019; Yimer *et al.*, 2019). We thus aimed to



**Fig. 6.** The effect of the p38 inhibitor, SB203580, on cell migration and MMP-9 expression in Caki-1 cells. (A) Wound healing (upper panel) and invasion (bottom panel) assays were performed to assess the effect of SB203580 (SB) on butaprost-induced cell migration. Caki-1 cells were treated with butaprost (5  $\mu$ M) plus or minus SB (10 and 20  $\mu$ M) for 24 h, followed by obtaining microphotographs of the gap closure (for the wound healing assay, upper panel) and Crystal violet-stained migrated cells (for the migration assay, bottom panel). For the control and butaprost-treated groups, the same representative images were used as shown in Fig. 5A, because the experiments for Fig. 5A and 6A were conducted simultaneously. Bar graphs represent the relative gap distance (upper panel) and the migrating cells per field (lower panel).  $**p < 0.005$ . (B, C) Caki-1 cells were treated in the same manner as described in (A). At 24 h after treatment, western blotting determined p38 phosphorylation and MMP-9 expression (B) and gelatin zymography revealed the MMP-9 proteolytic activity (C).  $\beta$ -actin was used as a protein loading control for western blotting. Densitometry was performed using Image J software (National Institutes of Health, Bethesda, MD, USA). A bar graph represents the mean of triplicates  $\pm$  standard deviation.  $**p < 0.005$ .

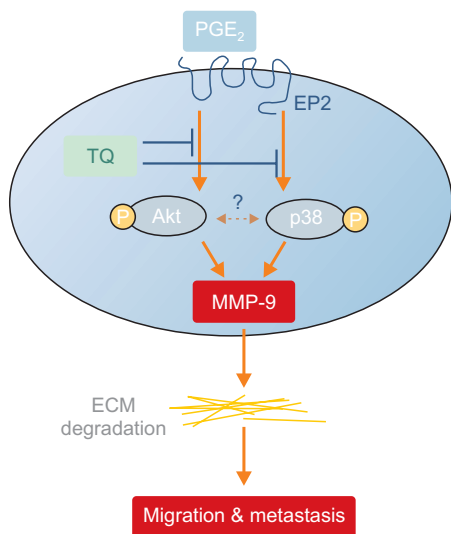
validate TQ as a potential candidate for metastatic RCC treatment. In the present study, we found that TQ treatment significantly inhibited Caki-1 cell migration. Moreover, TQ reduced MMP-9 protein expression, as well as its proteolytic activity, in Caki-1 cells. However, as shown in the Fig. 2B and 2C, TQ was unable to modulate both expression and enzymatic activity of MMP-2 (Fig. 2B, 2C), suggesting that the inhibitory effect of TQ on Caki-1 cell migration specifically inactivated MMP-9. Consistent with our results, multiple studies have reported that MMPs play important roles in cellular interactions with the matrix in different organ systems (Johnson and Galis, 2004; Giannandrea and Parks, 2014). While MMP-2 is constitutively expressed, MMP-9 levels are usually low and enzyme expression is induced by various cytokines (Sternlicht and Werb, 2001; Lynch and Matrisian, 2002). Because the matrix-degrading activity of MMP-9 is stronger than that of MMP-2, MMP-9 may be a more important target for metastasis in carcinomas (Yamagata *et al.*, 1989; Yasumitsu *et al.*,

1992). Furthermore, MMP-9 expression has been found to be significantly correlated with poor prognoses in RCC patients (Kallakury *et al.*, 2001).

In response to inflammatory stimuli, COX-2 induces eicosanoid synthesis from arachidonic acids, which can modulate cell proliferation, inflammation, and immune responses (Nakanishi and Rosenberg, 2013). Among the eicosanoids produced by COX-2 activation, PGE<sub>2</sub> is the most potent pro-tumorigenic eicosanoid that promotes cell proliferation, pro-inflammatory cytokine stimulation, and cancer cell migration (Greenhough *et al.*, 2009). PGE<sub>2</sub> exerts its cellular functions via binding to its G protein-coupled receptors, EP1-EP4. EP2 is mostly responsible for the protumorigenic effects of PGE<sub>2</sub>. EP2 knockout mice showed reduced tumorigenesis in a carcinogen-induced skin cancer model, while EP2 overexpressing transgenic mice exhibited increased numbers of tumors when compared with wild-type mice (Sung *et al.*, 2006; Asting *et al.*, 2017). Moreover, the PGE<sub>2</sub>-EP2/EP4 signaling axis can

induce MMP-9 expression as well as its activation, resulting in the invasion and migration of human endometrial epithelial and stromal cells (Lee *et al.*, 2011). Importantly, increased levels of PGE<sub>2</sub> in RCC indicate poor prognoses and a reduced survival time (Wang and Dubois, 2010). In agreement with our findings, Li *et al.* (2013) reported that PGE<sub>2</sub> treatment promoted EP2-mediated Akt activation in human renal cell carcinoma SN12C cells. In a similar manner, we have previously shown that the PGE<sub>2</sub>-EP2 signaling is crucial for Caki-1 cell migration (Woo *et al.*, 2015). It is thus worthwhile identifying the function of EP2 signaling that regulates the migration and/or invasion in RCC. In the present study, TQ treatment simultaneously inhibited EP2 expression and MMP-9 activation, which strongly suggested that TQ inhibited Caki-1 cell migration via suppression of the PGE<sub>2</sub>-EP2-MMP-9 axis. Previously, we have reported that activation of the EP4 receptor induces Caki-1 cell migration (Woo *et al.*, 2015). Given all these results, we assumed that Caki-1 cells utilize either EP2 or EP4 as a driving force for migration, depending on their surrounding microenvironment. This will provide a distinct direction for further investigation of regulatory mechanisms, to identify the antimigratory and antimetastatic effects of TQ in metastatic RCCs.

Here, we found that activation of PI3K/Akt and p38 was a missing link between the PGE<sub>2</sub>-EP2 axis and MMP-9 in Caki-1 cells. It has been reported that proinflammatory cytokines, such as IL-1 $\beta$  and TNF $\alpha$ , promote the secretion of MMP-9 via upregulation of PI3K/Akt and mitogen-activated protein kinases (Ruhul Amin *et al.*, 2003; Lee *et al.*, 2009). Inflammation is a pivotal driving force for MMP-9-dependent metastasis because it produces either PGE<sub>2</sub> or cytokines. Thus, inflammation could also facilitate combination of the regulatory circuits, such as EP2 activation, Akt and p38 phosphorylation, and MMP-9 induction. In a previous report, Chun *et al.* showed that EP2 overexpression led to Src and  $\beta$ -arrestin binding, and induced the phosphorylation/activation of p38 in the human



**Fig. 7.** A proposed mechanism underlying the inhibition of EP2-Akt/p38-MMP-9 by thymoquinone (TQ) in Caki-1 cells. TQ inhibits renal carcinoma cell migration via the inactivation of Akt and p38 as well as decreasing MMP-9 activity by decreasing the expression of prostaglandin E<sub>2</sub>-EP2.

embryonic kidney HEK293 cells (Chun and Shim, 2015). The elucidation of the relationship between EP2 and  $\beta$ -arrestin in Caki-1 cells, together with TQ, would, therefore, be an important objective for future studies.

As described in Fig. 7, TQ inhibits the migration of RCC cells via inactivation of Akt and p38, as well as decreased MMP-9 activity, by decreasing PGE<sub>2</sub>-EP2 expression in RCC cells. However, more investigations are needed to determine whether Akt and p38 occur continuously or independently in regulating the activity of MMP-9 in RCC. Taken together, these findings suggested that TQ is a promising antimetastatic drug to treat human RCCs. The phytochemical TQ has long been used as a food additive, ensuring its nontoxicity, which should benefit patients during long-term therapy. Moreover, it can be utilized as an adjuvant for combination therapy or immunotherapy, which would be helpful, at least in part, in the establishment of the most effective treatment strategy in clinical settings.

## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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