Curcumin inhibits cell proliferation and motility via suppression of TROP2 in bladder cancer cells

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Abstract. Bladder cancer (BC) has become a serious health problem and represents the second most commonly diagnosed urological tumor. Curcumin is a principal active natural component of turmeric and has long been used in Asia as a traditional herbal medicine. Curcumin suppresses cell growth in various types of cancer, including BC, by regulating numerous molecular signaling pathways. The human trophoblast cell surface antigen 2 (Trop2) belongs to the tumor-associated calcium signal transducer gene family. Trop2 has been described as a cancer driver and is deregulated in various types of cancer. However, whether Trop2 is involved in curcumin-induced BC cell inhibition remains to be elucidated. The present study hypothesized that Trop2 may be a promising target of curcumin in BC cells. It was found that Trop2 was closely involved in curcumin-induced cell proliferation suppression, mobility inhibition, apoptosis, and cell cycle arrest in BC cells. Curcumin decreased the expression of Trop2 and its downstream target cyclin E1, and increased the level of p27. The overexpression of Trop2 enhanced the oncogenic activity of BC cells, whereas downregulation of the expression of Trop2 suppressed cell proliferation and mobility, increased apoptosis, and sensitized BC cells to curcumin treatment. Therefore, Trop2 may be a promising target of curcumin in BC cells and the inhibition of Trop2 may be an important method for the therapeutic management of patients with BC.

Introduction

Malignant cells accumulating in the tissues of the bladder form bladder cancer (BC) (1,2). BC is one of several types of cancer developing from the epithelial cells protecting the innermost tissue surface layer of the urinary bladder, termed urothelial carcinoma. BC has become a serious health problem and is the ninth most common type of cancer worldwide (3). In 2012, ~430,000 new cases were diagnosed in patients, with an associated mortality rate of ~165,000 worldwide (4). Men are more often affected by BC than women, which represent 75% of all BC cases (3). Those cases of BC confined to the mucosa and submucosa, designated as non-muscle invasive BC (NMIBC), represent 75% of new BC cases (5). However, in ~80% of patients with NMIBC, disease recurs following initial treatment within 5 years (5). The high rates of relapse and progression of BC have attracted global attention to investigate the molecular mechanisms underlying its carcinogenesis and progression.

The human trophoblast cell surface antigen 2 (Trop2), also termed GA733-1, EGP-1 or M1S1, is a cell-surface glycoprotein (6). Trop2 is a typical member of the tumor-associated calcium signal transducer gene family and is identified as an epithelial adhesion molecule (7). Trop2 was found to be a biological marker of aggressive human trophoblast cells (8), originating from the outer layer of the blastosphere and contributing to implantation. Trop2 also has multifaceted roles in development and tumorigenesis by regulating cell growth, migration and self-renewal (6). Trop2 was first identified as a tumor-associated antigen when GA733 antibody immunoprecipitated with Trop2 not only in gastrointestinal tumors, but also in bladder, lung and cervical cancer (9). Cancer is a complex disorder during development, which is always accompanied by the deregulation of molecular events (10). Trop2 was confirmed to trigger tumor proliferation, thus acting as a cancer driver (11). The overexpression of Trop2 has been found in the majority of types of human cancer, including esophageal (12), colorectal (13), oral (14), pancreatic (15), breast (16), glioma (17), uterine (18), ovarian (19) and prostate (20) cancer. The overexpression of Trop2 in human tumors promotes tumor aggressiveness and metastasis, resulting in reduced survival rates (7). Although studies have investigated the correlation between the expression of Trop2 and poor survival rates in various cancer patients, the biological significance of Trop2 in BC remains to be fully elucidated.

Curcumin is an active natural component of turmeric derived from Curcuma longa. It has long been used across Asia, particularly in India, as a food condiment and a traditional herbal medicine. Curcumin exhibits anti-inflammatory, anti-oxidative, antiproliferative, apoptosis-inducing, antitumor and chemopreventive activities (21-23). Accumulating data have...
showed that curcumin offers therapeutic potential in a variety of cancer types (24, 25). Mechanistically, curcumin exhibits its therapeutic properties through regulating multiple targets, including nuclear factor (NF)-κB, Notch, S-phase kinase-associated protein 2 (Skp2), and multiple microRNAs (22, 25–29). Gao et al. found that curcumin promotes Krüppel-like factor 5 (KLF5) proteasome-dependent degradation by regulating Yes-associated protein (YAP)/transcriptional coactivator with PDZ- binding motif (TAZ) in BC cells (30). In T24 and 5637 cells, curcumin was identified to decrease cell growth and migration, and to trigger apoptosis via suppressing matrix metalloproteinase (MMP)-2 and MMP-9 signaling pathways in vitro (31). However, whether curcumin affects Trop2 in BC remains to be elucidated. Therefore, in the present study, using a series of in vitro assays, the toxicity of curcumin towards T24 and RT4 BC cell lines was examined, to reveal whether Trop2 was a target of curcumin. In addition, whether Trop2 was associated with the antiproliferative property of curcumin treatment was examined.

Materials and methods

Reagents and cell culture. The T24 and RT4 human BC cell lines were obtained from the Chinese Academy of Science (Shanghai, China). The BC cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; cat. no. MGC803; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% FBS and 100 U/ml penicillin/streptomycin (HyClone™; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C in a humidified atmosphere containing 5% CO2. Curcumin (Sigma-Aldrich; EMD Millipore, Billerica, MA, USA) was dissolved in dimethylsulfoxide (DMSO) and stored at -20°C. 3-4,5-dimethyl-2-thiazolyl-2,5-diphenyl-2H-tetrazolium bromide (MTT; CAS no. 57360-69-7) was obtained from Sigma-Aldrich; EMD Millipore. Lipofectamine™ 2000 was purchased from Invitrogen; Thermo Fisher Scientific, Inc. Primary antibodies targeting Trop2 (#90540), p27 (#2552), and cyclin E1 (#4129), and monoclonal anti-β-actin (#3700) were purchased from GenePharma (Shanghai, China). Secondary antibodies (#A-11031 and #A-11034) were obtained from Thermo Fisher Scientific, Inc.

Cell proliferation assays. The T24 and RT4 cells were plated in 96-well plates (5x103 cells/well) and cultured for ~24 h. The T24 and RT4 cells were plated in 6-well plates and treated with curcumin for 48 h. The cells were collected by trypsinization and washed with PBS and then suspended with pre-cooling alcohol (70%). Following fixing overnight, the cells were precipitated by centrifugation for 5 min at 200 x g and pelleted cells were washed three times with 10 ml PBS. The cells were then incubated with RNase and PI. Cell cycle distributions were detected using a flow cytometer (BD Biosciences).

Wound-healing assay. The BC cells were plated into a 6-well plate and incubated until the cells grew to ~90% confluence. The confluent monolayer was injured with a sterile 100-μl tip and a rectangular wound was created. Cell debris was carefully removed, and the cells were cultured for ~16 h. The cells migrated into the wound were visualized using an inverted microscope. The wound size was scored by measuring the lesion border and comparing with the size of the initial wound.

Cell invasion assay. Transwell chambers (8-μm pore size, Corning Incorporated, Corning, NY, USA) were used to assess the invasive ability of the BC cells. Following culture for 24 h at 37°C, the cells were suspended in the FBS-free DMEM. The cells (5x104 cells; 200 μl) were placed in the Matrigel (BD Biosciences) pre-coated upper chamber, following which 600 μl of complete DMEM was added to the lower chamber. Following incubation for 48 h, the remaining cells were removed with cotton wool. Cells invading through the filters were stained with Calcein-AM for 10 min, followed by rinsing the filters with water. The stained cells were observed under a light microscope.

Transient transfection of plasmids and small interfering RNAs (siRNAs). The BC cells were transfected with pcDNA3.1-Trop2- or Trop2-targeting siRNAs with Lipofectamine™ 2000 transfection reagent, following the manufacturer's protocol. The siRNA sequences (sense, 5'-ACA CTT GGA GGT TTT GGC CAC TGA CTG ACT CAA AGT TCA ACA-3'; antisense, 3'-CCT GTT GAG CAG CAG ACT TGG AGG TCA GTC ATG CAG TGG CCA AAA CCT CCA AGT GTC TGC TGC TCA AC-5') targeting human Trop2 were purchased from GenePharma (Shanghai, China).

Western blot analysis. The BC cells were inoculated into the culture plate at a concentration of 1x104 cells/cm2. The cells were then transfected with pcDNA3.1-Trop2- or Trop2-targeting siRNAs or treated with curcumin for a designated time course. The pretreated BC cells were precipitated and lysed in lysis buffer supplemented with protease inhibitor cocktail and PMSF (Roche Diagnostics, Basel, Switzerland). A BCA protein assay was used to quantify protein concentrations. Protein samples (30 μg) were separated by 10% SDS-PAGE. The separated proteins were then transferred onto a nitrocellulose membrane and were blocked with 5% skimmed milk at room temperature for 1 h. The primary antibodies (Trop2, 1:1,500; p27, 1:1,000; cyclin E1, 1:2,000; and anti-β-actin, 1:3,000) were added and incubated at 4°C overnight. After
washing with TBS-Tween-20, a suitable secondary antibody (anti-mouse, 1:2,500; anti-rabbit, 1:2,500) was added and incubated at room temperature for ~1 h. The target proteins were developed onto a film by an ECL imaging system (Pierce; Thermo Fisher Scientific, Inc.). The protein expression levels were semi-quantitated via densitometry using ImageJ software version 1.51 (National Institutes of Health, Bethesda, MD, USA; https://imagej.nih.gov/ij/).

Statistical analysis. Data are presented as the mean ± standard deviation of the mean following analysis with GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance values were evaluated through one-way analysis of variance with a Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Curcumin inhibits BC cell growth. Curcumin has been reported to suppress proliferation in various types of cancer. The present study aimed to determine whether curcumin inhibits T24 and RT4 BC cell growth. The BC cells were treated with curcumin for 48 and 72 h, respectively. An MTT assay was performed and the resulting data showed that curcumin suppressed BC cell proliferation in a time- and dose-dependent manner (Fig. 1A). These data supported the hypothesis that curcumin exerts its antitumor growth property in BC cells.

Curcumin promotes apoptotic death of BC cells. Subsequently, the present study detected whether curcumin affects apoptotic death of BC cells. Annexin V-FITC/PI apoptosis analysis was performed in T24 and RT4 BC cells following curcumin treatment. As shown in Fig. 1B and C, curcumin induced apoptotic death of T24 and RT4 cells in a dose-dependent manner. For example, treatment with 10 and 15 µM curcumin caused increased apoptotic death of RT4 cells from 3.64% in the control group to 19.95 and 26.36% (Fig. 1B and C), respectively. It was found that curcumin mainly induced late apoptosis, increasing from 1.83% in the control group to 8.32 and 13.42% in T24 cells treated with 10 and 15 µM curcumin, respectively (Fig. 1B). A similar induction of late apoptosis was induced by curcumin in RT4 cells (Fig. 1B). These results demonstrated that curcumin promoted significant apoptotic death and may facilitate inhibiting the proliferation of BC cells.
Curcumin induces cell cycle arrest of BC cells. Further detection of the cell cycle distribution of bladder cancer cells was performed following curcumin treatment. Flow cytometry was performed following PI staining. The findings revealed that curcumin treatment caused a significant increase in the numbers of cells in the G2/M phase in a dose-dependent manner, compared with control cells (Fig. 2A and B). For example, treatment with 10 and 15 µM curcumin increased the G2/M cell populations to 14.95 and 23.21% in RT4 cells, from 9.02% in the control (Fig. 2A and B). A similar cell cycle arrest pattern was observed in T24 cells (Fig. 2A and B). These findings confirmed that curcumin induced G2/M cell cycle arrest in the BC cells.

Curcumin suppresses cell migration of BC cells. In order to detect the effects of curcumin on cell motility, wound-healing assays were performed in T24 and RT4 cells, respectively. As shown in Fig. 3A, curcumin treatment significantly inhibited cell migration in the two BC cell lines. These results revealed that curcumin treatment notably inhibited cell migratory activity in the T24 and RT4 cancer cells.

Curcumin suppresses cell invasion of BC cells. The present study also measured whether curcumin inhibits cell invasive ability using Transwell chambers. It was found that the number of cells invaded through the pores of Matrigel-coated filters was reduced in the two curcumin-treated BC cell lines in a dose-dependent manner (Fig. 3B). It is important to note that 15 mM curcumin had a cytotoxic effect with ~20% growth inhibition in the T24 and RT4 cells at 468. (Fig. 1A). However, treatment with 15 mM curcumin for 24 h did not cause any cell growth inhibition in the cell lines (data not shown). The invasion and migration were measured in the two cell lines following treatment with 15 mM curcumin for 20 h. Therefore, the effects on migration and invasion by curcumin treatment were not due to cell viability inhibition. The findings demonstrated that curcumin treatment significantly inhibited the cell invasion potential of the T24 and RT4 BC cells.

Curcumin suppresses the expression of Trop2 in BC cells. Accumulating data have characterized Trop2 as a tumor driver in various types of cancer, and pharmacological inhibition of Trop2 may be a promising approach to treating BC. Therefore, the present study further examined whether curcumin treatment modulates the expression of Trop2 in BC cells. Following treatment with curcumin for 468, western blot analysis revealed that Trop2 was markedly reduced in the T24 and RT4 BC cells (Fig. 4A and B). It was also detected that the protein levels of p27 and cyclin E1, two typical cell cycle regulators, were modulated following curcumin treatment (Fig. 4A and B). p27, the cell cycle inhibitor, was significantly induced by curcumin treatment. cyclin E1, which is crucial in promoting cell cycle progression and contributes to tumorigenesis, was markedly suppressed in the presence of curcumin in BC cells. Taken together, these results suggested that curcumin exercised antitumor function in BC cells at least partially by downregulating the expression of Trop2. In addition, curcumin caused
cell-cycle arrest, which may be attributed to the modulated expression of p27 and cyclin E1.

Overexpression of Trop2 promotes cell proliferation. In order to further identify whether Trop2 was involved in the cytotoxic effects of curcumin, the present study induced the overexpression of Trop2 in BC cells via Trop2 cDNA transfection in the presence of curcumin. An empty vector was transfected as a control. The subsequent MTT assays demonstrated that the overexpression of Trop2 triggered BC cell proliferation (Fig. 5A). In addition, the cell growth inhibition induced by curcumin was partly abrogated under Trop2 overexpression (Fig. 5A).
Overexpression of Trop2 promotes cell migration and inhibits cell apoptosis. The cellular invasive and migratory properties were measured to determine whether Trop2 regulates T24 and RT4 cell mobility. Using a wound-healing assay, an increase in the migration of BC cells was found under Trop2 overexpression (Fig. 5B). Consistently, the overexpression of Trop2 eliminated curcumin-induced suppression of cell mobility (Fig. 5B). The Transwell analysis demonstrated that the overexpression of Trop2 markedly increased the number of invasive BC cells (Fig. 5C). The overexpression of Trop2 reversed the curcumin-induced inhibition of cell invasion (Fig. 5C). Furthermore, the effect of the overexpression of Trop2 on apoptosis was detected. The resulting data demonstrated that the overexpression of Trop2 markedly reduced the apoptotic cell percentage in BC cells and abrogated curcumin-induced apoptotic cell death (Fig. 6A and B).

Overexpression of Trop2 modulates cell cycle proteins. The protein levels of p27 and cyclin E1 were measured in BC cells following Trop2 cDNA transfection. It was observed that the overexpression of Trop2 significantly suppressed protein levels of p27 in the T24 and RT4 cells (Fig. 7A and B; P<0.05). When the overexpression of Trop2 was combined with curcumin treatment, the induced expression of p27 was partially abrogated (Fig. 7A and B; P<0.05). Conversely, the protein level of cyclin E1 was significantly induced in the two BC cell lines with
Figure 6. Overexpression of Trop2 inhibits bladder cancer cell apoptosis. (A) Apoptotic cells were assessed by Annexin V/PI staining and flow cytometry following Trop2 overexpression alone or in combination with 15 µM curcumin. (B) Quantitative results are illustrated of flow cytometry. *P<0.05, vs. control; †P<0.05, vs. 15 µM curcumin treatment or Trop2 cDNA transfection. Trop2, trophoblast cell surface antigen 2; Trop2 cDNA, Trop2-expressing vector; PI, propidium iodide.

Figure 7. Overexpression of Trop2 abrogates curcumin-mediated inhibition of Trop2. (A) Expression of Trop2, p27 and cyclin E1 was detected in Trop2 cDNA-transfected BC cells. (B) Quantitative results of blots. *P<0.05, vs. control; †P<0.05, vs. 15 µM curcumin treatment or Trop2 cDNA transfection. Trop2, trophoblast cell surface antigen 2; Trop2 cDNA, Trop2-expressing vector.
the overexpression of Trop2 (Fig. 7B; p<0.05). The curcumin-induced suppression of cyclin E1 was partially abrogated when combined with Trop2 cDNA transfection. Taken together, these findings supported the hypothesis that curcumin exhibits anti-tumor activity in BC cells partially via regulating the expression of Trop2 and its downstream targets p27 and cyclin E1.

Downregulation of Trop2 by siRNA transfection sensitizes BC cells to curcumin treatment. In addition, Trop2 siRNA was used to knock down Trop2 in the BC cells and its effects on cell proliferation, mobility, apoptosis, and the expression of p27 and cyclin E1 were examined. Following Trop2 siRNA transfection, the MTT assay results showed that T24 and RT4 cell proliferation was inhibited (Fig. 8A). When Trop2 was downregulated in the presence of curcumin, the suppression of cell proliferation was significantly enhanced compared with that in the cells treated with either curcumin or siRNA transfection alone (Fig. 8A; P<0.05). The downregulation of Trop2 combined with curcumin treatment also suppressed cell invasion and migration (Fig. 8B and C; P<0.05). The sensitivity of BC cells to apoptosis from curcumin was markedly enhanced in the Trop2-silenced cells (Fig. 9A and B). Furthermore, the downregulation of Trop2 altered the levels of p27 and cyclin E1 in the T24 and RT4 cells (Fig. 10A and B). Taken together,
Figure 9. Downregulation of Trop2 triggers bladder cancer cell apoptosis. (A) Apoptotic cells were measured by Annexin V/PI staining and flow cytometry following downregulation of Trop2 alone or in combination with 15 µM curcumin. (B) Quantitative results of flow cytometry. *P<0.05, vs. control; #P<0.05, vs. 15 µM curcumin treatment or Trop2 siRNA transfection. PI, propidium iodide; siRNA, small interfering RNA.

Figure 10. Downregulation of Trop2 enhances curcumin-mediated inhibition of Trop2. (A) Expression of Trop2, p27 and cyclin E1 was detected following Trop2 silencing in bladder cancer cells. (B) Quantitative results of expression. *P<0.05, vs. control; #P<0.05, vs. 15 µM curcumin treatment or Trop2 siRNA transfection. Trop2, trophoblast cell surface antigen 2; siRNA, small interfering RNA.
these findings demonstrated that Trop2 was associated with the curcumin-induced suppression of cell proliferation, increase of apoptosis, and inhibition of invasive and migration abilities in BC cells. Curcumin treatment together with the downregulation of Trop2 enhanced the antineoplastic property of curcumin in BC cells.

Discussion

BC is one of the most commonly diagnosed urological tumors and causes severe tumor-associated mortality worldwide. In China, BC has become the most frequent urological malignancy (32). At initial diagnosis, ~75% of cases are NMIBC. However, disease in ~80% of patients with NMIBC recurs following initial treatment within 5 years (5). Despite surgical techniques and adjuvant chemotherapy having progressed extensively, BC represents a prevalent and life-threatening form of tumor (32,33). In order to improve the poor prognosis of BC, the development of novel treatment methods based on novel molecular networks is urgently required.

Curcumin has been described to exhibit antineoplastic properties in various types of cancer, including the inhibition of cell growth and metastasis, and promotion of apoptosis, via interacting with numerous cell signaling molecules (28,34). Kamat et al found that curcumin enhanced the antitumor effects of Bacillus Calmette-Guerin on BC by reducing NF-κB and inducing tumor necrosis factor-related apoptosis-inducing ligand receptors (35). Curcumin has been found to inhibit cell proliferation and invasive ability and trigger apoptosis by the suppression of Skp2 and induction of p21 in pancreatic cancer cells (27). Curcumin enhances the effect of 5-fluorouracil by disrupting AMP-activated protein kinase/Unc-51 like autophagy activating kinase-dependent autophagy and inducing apoptotic death in colon cancer cells (36). Curcumin inhibits cell growth through increasing p21 and p27 cyclin-dependent kinase inhibitors and inhibiting cyclin D1 and phosphatidylinositol-3 kinase (PI3K)/Akt signaling (37). YAP/TAZ are markedly suppressed by curcumin treatment, and the expression of Notch-1 is also suppressed (38). Curcumin triggers the degradation of KLF5 by the suppression of YAP/TAZ in BC cells (30). It has been reported that curcumin inhibits the mobility of BC cells through modulating the level of β-catenin and abrogating epithelial-mesenchymal transition (EMT) (39). In the present study, curcumin notably inhibited BC cell growth, invasion and migration, and triggered apoptotic cell death and G2/M phase arrest (Figs. 1 and 2A and B). These findings suggested the therapeutic possibility of curcumin for treating BC. Furthermore, it was found that Trop2 was a target of curcumin in the BC cell lines.

A number of studies have implicated the oncogenic role of Trop2 in tumorigenesis, most likely through triggering cell proliferation. A high expression level of Trop2 has been observed in the majority of types of epithelial cancer. The overexpression of Trop2 was shown to promote cancer cell growth and enhance the tumorigenic potential of cells when injected into mice (11,40,41), supporting Trop2 as a key cancer driver. It was found that murine Trop2 activated the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase pathway through inducing cyclin D1 and cyclin E, and reducing p27. ERK was also activated upon overexpressing Trop2 in human pancreatic cancer and colorectal cancer cells (40). Trop2 has been revealed to enhance invasion of thyroid cancer by increasing MMP-2 (42). In the present study, the overexpression of Trop2 by cDNA transfection increased BC cell growth (Fig. 3A). The invasion and migration abilities were also enhanced (Fig. 3B and C). Noteworthy, the overexpression of Trop2 significantly decreased the apoptotic cell percentage in the two BC cell lines and inhibited curcumin-induced apoptosis (Fig. 4A). Consistent with a previous study, the overexpression of Trop2 significantly suppressed the protein levels of p27 in the T24 and RT4 cells (Fig. 4B and C). When combined with curcumin treatment, the induced expression of p27 was partially abrogated (Fig. 4B and C). The expression of cyclin E1 was upregulated by the overexpression of Trop2 (Fig. 4B and C). By contrast, the depletion of Trop2 through siRNA transfection in colon and breast cancer cells suppresses growth and colony forming abilities (11,41). The depletion of endogenous Trop2 by Trop2-siRNA retroviral infection also inhibits the invasion and migration of thyroid cancer cells (42), and Trop2 deletion in gallbladder cancer cells notably suppressed cell growth, colonies formation, and invasive and migration abilities via modulating PI3K/AKT signaling and EMT characteristics (43). Similarly, the silencing of Trop2 by siRNA transfection in the present study led to a significant suppression of cell proliferation, invasion and migration of BC cells (Fig. 5), whereas apoptotic cell death was markedly increased (Fig. 6A). The silencing of Trop2 sensitized the BC cells to curcumin treatment (Figs. 5 and 6). The expression of p27 and cyclin E1 were also modulated by silencing Trop2 (Fig. 6B and C). Taken together, Trop2 may serve as an attractive therapeutic target for the clinical treatment of patients with BC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LZ, JB, WX and YH were involved in conception and design of the study; LZ, GY and RZ were involved in data collection; LD, JB, WX and HC were involved in data analysis; LZ, GY, RZ, LD, JB, WX and HC conducted investigative experiments; JB, WX and YH were involved in project administration; JB and YH supervised the study; LZ, JB and YH wrote and edited the manuscript. 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.

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