BTG2 as a tumor target for the treatment of luminal A breast cancer

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Abstract. As one of the most common breast cancer subtypes, luminal A breast cancer is sensitive to endocrine-based therapy and insensitive to chemotherapy. Patients with luminal A subtype of breast cancer have a relatively good prognosis compared with that of patients with other subtypes of breast cancer. However, with the increased incidence in endocrine resistance and severe side effects, simple endocrine therapy has become unsuitable for the treatment of luminal A breast cancer. Therefore, identifying novel therapeutic targets for luminal A breast cancer may accelerate the development of an effective therapeutic strategy. The bioinformatical analysis of the current study, which included KEGG and GO analyses of the GSE20437 dataset containing 24 healthy and 18 breast cancer tissue samples, identified key target genes associated with breast cancer. Moreover, survival analysis results revealed that a low expression of BTG2 was significantly associated with the low survival rate of patients with breast cancer, indicated that B‑cell translocation gene 2 (BTG2) may be a potential target in breast cancer. However, BTG2 may be cancer type-dependent, as overexpression of BTG2 has been demonstrated to suppress the proliferation of pancreatic and lung cancer cells, but promote the proliferation of bladder cancer cells. Since the association between BTG2 and luminal A‑subtype breast cancer remains unclear, it is important to understand the biological function of BTG2 in luminal A breast cancer. Based on the expression levels of estrogen receptor, progesterone receptor and human epidermal growth factor receptor, MCF‑7 cells were selected in the present study as a luminal A breast cancer cell type. MTT, Transwell invasion and wound healing assays revealed that overexpression of BTG2 suppressed the levels of MCF-7 cell proliferation, migration and invasion. In addition, the downregulation of BTG2 at the mRNA and protein level was also confirmed in luminal A breast tumor tissue, which was consistent with the results in vitro. These results indicated that BTG2 may act as an effective target for the treatment of luminal A breast cancer.

Introduction

Breast cancer was the second leading cause of cancer-related mortality (17-20%) in women worldwide in 2019 (1). Based on the sensitivity to different treatments, prognosis and clinicopathological characteristics, breast cancer can be divided into several subtypes (2,3). According to the expression levels of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER) 2 (HER2) and Ki67 (a proliferation index marker), various molecular subtypes of breast cancer have been identified, including luminal A-like, luminal B-like, HER2-positive, basal-like (mainly triple-negative) and normal breast-like (4,5). Among these subtypes, luminal A tumors are defined as ER-positive, PR >20%, HER2-negative and Ki67 <14% (6,7).

Previous epidemiological studies reported that luminal A breast cancer accounted for >50% of all new diagnosed cases of breast cancer (8-12). Endocrine therapy (ET) is the main treatment for almost all luminal A‑subtype breast tumors, unless endocrine resistance occurs (12,13). In recent years, known ETs and novel targeted drugs have been combined to reduce tumor resistance to hormonal therapy (14). These targeted drugs are divided into two main categories: i) Specific rapamycin (mTOR)/phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α inhibitors and ii) cyclin-dependent kinase 4/6 (CDK4/6) inhibitors. However, numerous challenges prevent the identification of effective treatment for metastatic luminal A breast cancer. For example, drug resistance can occur with combinations of CDK4/6 inhibitors and ETs (15). Therefore, there is an urgent need for identifying effective therapeutic targets for luminal A-subtype breast cancer.

The accumulation of multiple mutations results in tumorogenesis, including tumor suppressor gene inactivation and oncogene activation (16). The inactivation of tumor suppressor genes is considered to play an important role in the occurrence
of cancer. As the first member of the BTG/transducer of ERBB2 gene family, BTG2 has two highly conserved domains (BTG boxes A and B), which are separated by 20-25 non-conserved amino acids (17-19). As a novel tumor suppressor in malignancies, BTG2 is associated with numerous cellular functions, such as cell proliferation, apoptosis and DNA damage repair (20-23). In pancreatic cancer, microRNA (miR)-27a silencing has been indicated to inhibit cell proliferation and invasion, and promote apoptosis through the elevation of BTG2 (24). In non-small-cell lung cancer, the downregulation of nucleolar and spindle-associated protein 1 or LINC01234 inhibits cell growth, migration and invasion by increasing the expression of BTG2 (25,26). In human muscle-invasive bladder cancers, BTG2 also suppresses muscle invasion via inhibition of DNA methyltransferase 1 (27). In ER-positive breast cancer, downregulation of BTG2 is associated with overexpression of cyclin D1 protein and with increased tumor grade and size (28). In addition, BTG2 inhibits the expression of HER ligands and serves an essential role in the endocrine (tamoxifen) resistance of ER-positive tumors (29). Thus, the suppression or absence of BTG2 promotes the progression of triple-negative breast cancer. However, to the best of our knowledge, studies on the function of BTG2 in luminal A-subtype breast cancer and its association with these cell processes have not been conducted to date.

The present study aimed to investigate the function of BTG2 in luminal A-subtype breast cancer using the MCF-7 cell line due to its positive expression of ER and PR and negative expression of HER (30,31). MTT, Transwell invasion and wound healing assays were applied to investigate the function of BTG2 on MCF-7 cell proliferation, migration and invasion. Finally, the mRNA and protein level of BTG2 was also confirmed in luminal A breast tumor tissue. Materials and methods

Bioinformatics prediction based on Gene Expression Omnibus (GEO) database. The expression profile dataset GSE20437 was obtained from the GEO database (http://www.ncbi.nlm.nih.gov/geo/), which is a public and free database for gene expression data (32,33). GSE20437 includes 24 healthy and 18 breast cancer tissue samples (34). GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/) was used to identify differentially expressed genes (DEGs) between healthy and breast cancer tissue. P<0.05 and |log fold-change|>1 were considered the criteria to classify significant DEGs between healthy and breast cancer tissue samples.

Protein-protein interaction (PPI) network construction. A PPI network was constructed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; https://string-db.org/) and Cytoscape software 3.6.1 (www.cytoscape.org). Hub genes were identified from the PPI network.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. To understand the biological significance of DEGs, GO enrichment (http://geneontology.org/) and KEGG analyses (https://www.genome.jp/kegg/) were conducted using Database For Annotation, Visualization And Integrated Discovery (DAVID; http://david.ncifcrf.gov) (35), which is a free analysis online tool that provides a convenient method for identifying the biological role of DEGs.

Survival analysis. The OncoLnc (http://www.oncolnc.org) database was used to perform survival analysis based on DEGs (36). OncoLnc contains clinical data of 8,647 patients from 21 studies on cancer and precomputed survival analyses for users to explore survival associations in cancer. The difference in the expression level of BTG2 between healthy and breast cancer tissues was further analyzed with the Oncomine database (www.oncomine.org) (37). BTG2 expression was assessed in breast cancer tissues relative to that in healthy tissues. UALCAN (http://ualcan.path.uab.edu), which is an open web-portal for cancer subgroup gene expression, was then used to perform survival analysis of BTG2 in different cancer subgroups based on race, menopause status and cancer type (38).

Tissue samples. The luminal A breast cancer and paracarcinoma tissues (collected >5 mm from the tumor border) were collected from 8 patients with luminal A breast cancer at the affiliated Weihai Second Municipal Hospital of Qingdao University (Weihai, China) between July 2019 and November 2019. All patients were female and aged between 18 and 60 years with ER-positive, PR-positive (>20%), HER2-negative and Ki67 (<30%). Patients were excluded if they had received chemotherapy or radiotherapy prior to surgical resection. The patients signed an informed consent form prior to study commencement, and the study was approved by the Ethics committee of Clinical Trails of the affiliated Weihai Second Municipal Hospital of Qingdao University (Weihai, China; approval no. 2019-ER-04).

Cell culture and transfection. The MCF-7 breast cancer cell line representing luminal A cancer was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C under 5% CO2 (27,28). BTG2 overexpression (OE-BTG2) and empty (OE-NC) vectors were designed and synthesized by Wanleibio Co., Ltd. Prior to transfection, cells with 70-90% density were washed twice with serum-free Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.). OE-BTG2 and OE-NC vectors (50 nM) were subsequently transfected into MCF-7 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 4 h according to the manufacturer’s protocol. The cells were cultured at 37˚C for 24 h and then collected for further study.

Reverse transcription-quantitative (RT-q)PCR. TRIZol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from MCF-7 cells or breast cancer/paracarcinoma tissues, according to the manufacturer’s protocol. The extracted RNA was reverse transcribed into cDNA using a PrimeScript RT Reagent Kit with gDNA Eraser (cat. no. RR047A; Takara Biotechnology Co., Ltd.) according to the manufacturer’s protocol. Subsequently, SYBR Green PCR Master Mix kit (Toyobo Life Science) was used for PCR-mediated amplification. Relative mRNA expression
was calculated using the $2^{-\Delta\Delta C_{q}}$ method (39). The primer sequences used for qPCR were as follows: BTG2-forward (F), 5'-CATCATCAGCGGGTGC-3'; BTG2-reverse (R), 5'-CCAATGCAGGACACC-3'; β-actin-F, 5'-CTAATGCGTTACACCTTCTTG-3'; and β-actin-R, 5'-CTGTCACCTACGTTCAGTTT-3'. The reactions were performed using the following thermocycling conditions: Initial denaturation at 95°C for 5 min, followed by 32 cycles of 95°C for 30 sec, 56°C for 40 sec and 72°C for 40 sec. All quantifications were normalized to the internal reference gene β-actin.

Western blotting. Total protein was extracted from MCF-7 cells or breast cancer/paracarcinoma tissues using RIPA lysis buffer (Thermo Fisher Scientific, Inc.), and quantified using Pierce BCA Protein Assay Kit (cat. no. 23225; Thermo Fisher Scientific, Inc.). Total protein (40 µg per lane) was separated by 7.5-15% SDS-PAGE and transferred to a 0.45-µm PVDF membrane (ABclonal Biotech Co., Ltd.). The membrane was pre-blocked with 5% non-fat dry milk for 1 h at room temperature before incubation with the corresponding primary antibodies, including anti-BTG2 (cat. no. ab197362; 1:1,000; Abcam) and anti-β-actin (cat. no. ab8227; 1:2,000; Abcam) overnight at 4°C. After washing the membranes three times with TBS-0.1%Tween-20, the membranes were incubated with HRP-conjugated goat anti-rabbit (cat. no. ab205718; 1:5,000; Abcam) at 37°C for 1 h. Protein bands were visualized with an enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.) using the ChemiDoc™ XRS+ imaging system (Image Lab 4.0; Bio-Rad Laboratories, Inc.).

Proliferation curve of MCF-7 cells. Non-transfected, OE-NC or OE-BTG2 transfected MCF-7 cells (~8,000/well) were seeded in a 96-well plate in 100 µl DMEM supplemented with 10% FBS and incubated for 24, 48, 72 or 96 h at 37°C in the presence of 5% CO$_2$. Subsequently, a total of 10 µl MTT (Sigma-Aldrich; Merck KGaA) was added into the 96-well plate, which was placed in an incubator at 37°C in the presence of 5% CO$_2$ for 4 h. After removing the medium, 100 µl DMSO was added to each well to dissolve formazan crystals. Finally, the plate was placed in a microplate reader (BioTek Instruments, Inc.) for measurement of the absorbance at 570 nm.

Detection of MCF-7 cell invasion. Cell invasion was calculated based on the number of cells that passed through the polycarbonate membrane that separated the upper and lower chambers of an 8.0-µm Transwell chamber (Corning, Inc.). Briefly, Matrigel was thawed and diluted in serum-free DMEM (1:3) on ice. The Transwell chambers were placed in a 24-well plate, and 40 µl diluted Matrigel was added, followed by incubation at 37°C for 2 h. Subsequently, 2x10$^5$ MCF-7 cells overexpressing BTG2 or transfected with the pcDNA3.1 empty vector (OE)-negative control (NC) or non-transfected cells were suspended in 200 µl serum-free DMEM and added to the upper chamber, while 800 µl DMEM containing 10% FBS was added to the lower chamber. The 24-well plate was placed in an incubator at 37°C in the presence of 5% CO$_2$ for 24 h. Subsequently, the upper chamber was removed while the lower chamber was washed with PBS three times. The cells in the lower chamber surface of the membrane were subsequently stained with 1% crystal violet at 25°C for 10 min and the number of invading cells was counted under a light microscope (magnification, x200; Olympus Corporation).

Scratch test. A total of 5x10$^5$ MCF-7 cells/well were seeded into a six-well plate. Subsequently, a 1-ml sterile pipette tip was used to create a linear scratch in the cell monolayer. Fresh serum-free DMEM was then added to each well, and the cells were incubated at 37°C in a 5% CO$_2$ incubator for 24 h. A light microscope (magnification, x100) was used to observe the progressive change in the scratch width after 24 h. The migration distance was measured using ImageJ software 1.8.0 (National Institutes of Health).

Statistical analysis. Data are presented as the mean ± SEM. All experiments were duplicated and repeated at least three times. Statistical analyses were performed using GraphPad Prism 8.0.
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Unpaired Student's t-tests were used for the comparison between two groups. One-way ANOVA with the post hoc Tukey's test was used for the comparison of the mean values between multiple groups. Multiple regression analysis was used for the survival analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of DEGs and bioinformatics analysis. In the present study, 236 DEGs were identified in dataset GSE20437, which comprised epithelial samples from patients with breast cancer and patients that were cancer-free and receiving prophylactic mastectomy. The up- and downregulated genes are displayed in the volcano plot of Fig. 1. Results of the KEGG analysis demonstrated that the DEGs were enriched in ‘tight junction’, ‘DNA replication’, ‘base excision repair’, ‘pathways in cancer’ and ‘human T-cell leukemia virus type 1 infection’ (Fig. 2).

GO analysis demonstrated that the DEGs were enriched in the category biological process, including ‘response to mechanical stimulus’, ‘response to radiation’, ‘response to estradiol’, ‘ventricular cardiac muscle cell differentiation’ and ‘positive regulation of transcription from RNA polymerase II promoter’ (Fig. 3A). The results of GO analysis in the category cellular component demonstrated that the DEGs were enriched in the ‘integral component of plasma membrane’, ‘d DNA polymerase complex’, ‘nucleolus’, ‘nucleoplasm’ and ‘nucleus’ (Fig. 3B). Furthermore, results of the GO analysis in the category molecular function indicated that the DEGs were enriched in ‘DNA binding’, ‘sequence-specific DNA binding’, ‘transcription factor activity’, ‘transcriptional activator activity’ and ‘protein heterodimerization activity’ (Fig. 3C).

Hub gene analysis. In total, 236 DEGs were uploaded to the STRING online database, and Cytoscape was subsequently used to identify the cluster. Among the DEGs, nuclear receptor subfamily 4 group A member (NR4A1), immediate early response 2, dual-specificity phosphatase 1, activating transcription factor 3, NR4A2, protein FOSB, BTG2, proto-oncogene c-JUN and proto-oncogene c-FOS exhibited the closest

Figure 3. Gene Ontology terms enriched by the differentially expressed genes in breast cancer. (A) Biological process, (B) cellular component and (C) molecular function.

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association. The PPI network of these nine genes is presented in Fig. 4.

**Survival analysis.** To further evaluate the prognostic value of the aforementioned hub genes, a survival analysis was conducted using the OncoLnc database. The results revealed that low expression of BTG2 was significantly associated with low survival rate of patients with breast cancer (Fig. 5A). In contrast, the low expression of other genes, including DUSP1, FOS, FOSB, JUN, MR4A1, MR4A2 and ATF3, was not associated with a low survival rate of patients with breast cancer (Fig. 5B-I). Furthermore, the Oncomine database revealed that the BTG2 expression was lower in breast cancer tissues containing luminal breast cancer compared with that in normal counterparts (Fig. 6). Further survival analysis based on UALCAN revealed that the expression level of BTG2 and menopause status may have an impact on the survival of patients with breast cancer (Fig. 7A and C). In contrast, the expression level of BTG2 and cancer type or race were not significantly associated with the survival of patients with breast cancer (Fig. 7B and D).

**BTG2 expression in MCF-7 cells.** Western blotting and RT-qPCR were carried out to identify the expression levels of BTG2 in MCF-7 cells following transfection of BTG2. As presented in Fig. 8, the protein and mRNA expression level of BTG2 was low in MCF-7 cells of the OE-NC and control groups, while high BTG2 expression was detected in BTG2-overexpressing MCF-7 cells. Thus, the transfection of plasmids overexpressing BTG2 in MCF-7 cells was successful, and the transfected cells were used for further experiments.

**BTG2 suppresses the proliferation of MCF-7 cells.** Subsequently, the effect of BTG2 overexpression on MCF-7 cell proliferation was investigated. As demonstrated in Fig. 9, the proliferation of MCF-7 cells in the OE-BTG2 group was significantly lower than that of the OE-NC and control groups. In addition, there
was no significant difference between the OE-NC and control groups. These results demonstrated that overexpression of BTG2 suppressed the proliferation of MCF-7 cells.

**BTG2 suppresses the invasion and migration of MCF-7 cells.** Crystal violet staining demonstrated that the number of MCF-7 cells that crossed the polycarbonate membrane of the Transwell invasion chamber in the OE-BTG2 group was significantly reduced, compared with the empty vector and blank control groups (Fig. 10A and B).

Furthermore, a scratch assay was used to further identify the effect of BTG2 overexpression on the migration of MCF-7 cells. Results displayed in Fig. 10C and D revealed that the wounded scratch area of MCF-7 cells in the OE-BTG2 group was markedly larger than that of the OE-NC and control groups after 24 h. These results suggested that the expression level of BTG2 may be associated with the inhibition of invasion and migration in MCF-7 cells.

**Expression of BTG2 in luminal A breast cancer tissue.** Finally, the protein and mRNA expression of BTG2 in luminal A breast cancer tissue was investigated. As demonstrated in Fig. 11, compared with the paracarcinoma tissues of patients, the expression of BTG2 in luminal A breast tumor tissue was downregulated at the mRNA and protein level, which was consistent with the results in vitro. These results indicated that BTG2 may be a promising target and biomarker for luminal A breast cancer therapy in the future.

**Discussion**

Breast cancer is considered to be a complex disease; based on the expression level of immunohistochemistry markers, such as PR, ER, HER2 and the proliferation index marker Ki67, breast cancer can be molecularly divided into luminal A, luminal B, HER2-enriched, basal and normal...
As the most common subtype, luminal A breast cancer exhibits the following characteristics: ER-positive, PR >20%, HER2-negative and Ki67 <14%. To the best of our knowledge, luminal A tumors are sensitive to ET and insensitive to chemotherapy, and patients with luminal A subtype exhibit a better prognosis. However, ET often causes severe side effects and endocrine resistance, leading to poor prognosis (15,44-46). Therefore, there is an urgent need to identify effective treatment strategies and therapeutic targets for luminal A breast cancer.

The BTG2 gene is widely expressed in numerous organs and tissues, such as the lung, intestines, pancreas and prostate, as well as breast-like subtypes (40-43). As the most common subtype, luminal A breast cancer exhibits the following characteristics: ER-positive, PR >20%, HER2-negative and Ki67 <14%. To the best of our knowledge, luminal A tumors are sensitive to ET and insensitive to chemotherapy, and patients with luminal A subtype exhibit a better prognosis. However, ET often causes severe side effects and endocrine resistance, leading to poor prognosis (15,44-46). Therefore, there is an urgent need to identify effective treatment strategies and therapeutic targets for luminal A breast cancer.

The BTG2 gene is widely expressed in numerous organs and tissues, such as the lung, intestines, pancreas and prostate,
and is involved in various biological activities in cancer cells as a tumor suppressor (47). It has been reported that BTG2 serves an important role in cell proliferation, DNA damage repair and apoptosis. Overexpression of BTG2 inhibits cell proliferation
in pancreatic and lung cancer cells (48). However, overexpression of BTG2 also promotes the migration of bladder cancer cells and causes poor survival rates in patients with bladder cancer, indicating that the biological functions of BTG2 as a tumor suppressor may be cancer type-dependent (49). A previous study on the function of BTG2 on breast cancer mainly focused on triple-negative breast cancer (50), while few studies on BTG2 and luminal A breast cancer have been reported to date, to the best of our knowledge. Therefore, the identification of the biological function of BTG2 in luminal A breast cancer may accelerate the development of effective therapeutic targets for luminal A breast cancer.

The present study used bioinformatics analysis to identify key target genes associated with breast cancer, and it was revealed that low expression of BTG2 was significantly associated with the low survival rate of patients with breast cancer, indicating that BTG2 may serve as a potential biomarker in breast cancer. Considering the potential cancer-type dependent role of BTG2, it is necessary to understand the function of BTG2 in different subtypes of breast cancer, such as luminal A breast cancer. Therefore, MCF-7 cells were used in the present study due to their positive expression of ER and PR and their negative expression of HER, which is similar to the molecular expression profile of luminal A breast cancer.

Initially, the pcDNA3.1-BTG2 vector was constructed and transfected into MCF-7 cells. Western blotting and RT-qPCR were subsequently performed to determine the expression of BTG2 in MCF-7 cells, with or without transfection. Overexpression of BTG2 was confirmed in BTG2-transfected MCF-7 cells, while a low level of BTG2 expression was observed in the OE-NC and control groups. An MTT assay was utilized to determine the effect of BTG2 overexpression on the proliferation of MCF-7 cells. Results of the present study demonstrated that overexpression of BTG2 significantly inhibited the proliferation of MCF-7 cells, compared with that of the OE-NC and control groups. Additionally, the effect of BTG2 overexpression on the migration and invasion of MCF-7 cells was investigated. Transwell invasion and scratch assays revealed that BTG2 overexpression suppressed the migration and invasion of MCF-7 cells.

Results of a previous study highlighted that miR-25-3p was upregulated in the triple-negative breast cancer cell lines MDA-MB-231 and SUM-1315, and miR-25-3p promoted cell proliferation (15,44-46). Moreover, suppression of miR-25-3p induced cell apoptosis. The aforementioned processes were mediated through regulation of BTG2 and the subsequent activation of the AKT and ERK-MAPK signaling pathways (50). In addition, miR-92a-3p expression was elevated in triple-negative breast cancer cell line MDA-MB-231 and luminal cell line MCF, and miR-92a-3p promoted cell proliferation and metastasis via BTG2 downregulation (51). Further investigations into the miR-92a-3p/BTG2 axis may lead to the development of an effective strategy for the treatment of luminal A breast cancer.

In conclusion, the results of the present study demonstrated that BTG2 was a key targeted gene associated with breast cancer, and overexpression of BTG2 may suppress cell proliferation, invasion and migration in luminal A breast cancer. Thus, BTG2 may serve as a novel target for the treatment of luminal A breast cancer; however, further studies are required to fully elucidate the mechanisms underlying its specific function.

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Availability of data and materials

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

Authors' contributions

RuW, JW and RoW were responsible for the conception and design of the present study. RoW, TW and HW carried out administrative support. RuW, JT and JW obtained the study materials. HT, TW and HW were responsible for data acquisition, and JT, HT and RuW were responsible for data analysis and interpretation. RuW wrote the manuscript. RuW and RoW confirm the authenticity of all raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All patients signed an informed consent form, and the experiments were approved by the Affiliated Weihai Second Municipal Hospital of Qingdao University's Ethics Review Board.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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