RETRACTED ARTICLE: The circular RNA circ-ITCH acts as a tumour suppressor in osteosarcoma via regulating miR-22

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ABSTRACT

Background: Osteosarcoma (OS) is the most prevailing primary bone tumour and the third prevalent tumour in children and adolescents. Despite advanced treatments, the survival rate of OS has not been effectively improved. Here, we intended to investigate the functional impacts of circ-ITCH on OS.

Methods: Circ-ITCH expression in OS tissues and cells was evaluated utilizing qRT-PCR. Viability and proliferation of MG63 and Saos-2 cells were determined by utilizing CCK-8 assay and BrdU assay. Transwell assay was utilized to investigate migration and invasion. Western blot was utilized to distinguish apoptosis and metastasis-related proteins expression. Sequentially, the above-mentioned parameters were reassessed when up-regulating miR-22.

Results: Circ-ITCH was low expressed in OS tissues and cells. Overexpressing circ-ITCH facilitated apoptosis and repressed viability, proliferation, migration and invasion in MG63 and Saos-2 cells. MiR-22 expression was reduced by overexpressing circ-ITCH. The decline of viability, proliferation, migration and invasion made by overexpressing circ-ITCH was alleviated by up-regulating miR-22. Conclusively, circ-ITCH suppressed PTEN/PI3K/AKT and SP-1 pathways via down-regulating miR-22.

Conclusion: Circ-ITCH takes effect on apoptosis, viability, proliferation, migration and invasion through restraining PTEN/PI3K/AKT and SP-1 pathways via down-regulating miR-22. Circ-ITCH was low expressed in OS tissues and cells. Overexpressing circ-ITCH facilitated apoptosis and repressed viability, proliferation, migration and invasion in MG63 and Saos-2 cells.

HIGHLIGHTS

1. Low expression of circ-ITCH is observed in osteosarcoma tissues and cell lines;
2. Overexpression circ-ITCH suppresses miR-22 expression;
3. Circ-ITCH promotes proliferation and represses apoptosis by up-regulating miR-22;
4. Circ-ITCH promotes migration and invasion by up-regulating miR-22;
5. Circ-ITCH activates PTEN/PI3K/AKT and SP-1 pathways by up-regulating miR-22.

Introduction

Osteosarcoma (OS) is the most prevailing primary bone tumour and the third prevalent tumour in children and adolescents [1]. It is characterized by rapid development, high metastatic potentiality and poor clinical prognosis [2]. Although advanced strategies are used in the clinic such as surgery, adjuvant chemotherapy and radiation therapy, the prognosis of OS is still poor [3]. In general, the survival rate of OS has been insufficiently raised with the aforementioned treatment means. Worse still, the morbidity is rising by 1.4% per annum [4]. Moreover, the survival of patients with OS has entered a period of stasis [5]. Therefore, identifying new molecules involved in tumour progression is critical to reducing the morbidity and mortality of this destructive disease.

Circular RNAs (circRNAs) are innovative races of RNAs belonging to non-coding RNA (ncRNA) [6], that have been widely found in many species by high throughput sequencing in recent years [7]. CircRNAs are constituted of covalently closed-loop structures with neither 5’ to 3’ polarity nor polyadenylated tail [8]. CircRNAs have been widely informed to play critical roles in multifarious human cancer cells and regulating multiple cellular mechanisms [9]. Moreover, compared with linear RNA, circRNAs have a closed-loop structure to confer their higher stability and tolerance to RNA enzyme. There are plenty of studies reporting that circRNAs play vital roles in squamous cell carcinoma, gastric cancer and so forth [10,11]. Besides, accumulating evidence demonstrated that circRNAs had a critical effect on OS. For instance, circRNA_100876 had an impact in regulating apoptosis and proliferation of OS cancer cells [12]. And circ_HIPK3 was proved to repress migration and invasion in OS [13]. Furthermore, circ-Itchy E3 ubiquitin-protein ligase (ITCH) has also begun to be studied recently. Luo et al. indicated that circ-ITCH inhibited proliferation and facilitated apoptosis in SKOV3 cells [14]. Nonetheless, the function of circ-ITCH on OS remains unclear.

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MicroRNAs (miRNAs) are small ncRNAs molecules that control gene expression level after transcription [15]. Accumulating evidence shows that miRNAs represent abnormal expression in many human tumours, such as OS [16], gastric tumour [17] and mucoepidermoid carcinoma [18]. Moreover, miRNAs participate in numerous biological, pathological proceedings, physiological perturbations and disease conditions. For instance, miR-181a facilitated apoptosis in granulosa cells [19]. In addition, Wang’s report demonstrated that miR-30b and miR-340 took part in proliferation, invasion and migration in gallbladder carcinoma [20]. Moreover, many studies revealed that miRNAs played vital roles in OS. For example, miR-486 suppressed proliferation and migration of OS [21]. And miR-504 facilitated growth and migration in OS [22]. Furthermore, miR-22 played diverse roles in different diseases and cells. As an illustration, miR-22 inhibited migration and invasion in hepatocellular carcinoma cells [23]. Additionally, miR-22 protected cardiomyocytes against autophagy and apoptosis [24]. Here, we aimed to investigate circ-ITCH and miR-22 impact and interaction mechanism in MG63 and Saos-2 cells.

Materials and methods

Clinical specimens

Clinical human OS tissues and para-OS tissues (n = 22) were obtained from The Affiliated Hospital of Qingdao University (Qingdao, China). All patients accepted no preoperative treatment before surgery. We informed each patient and obtained their consent. The present research was allowed by the Medical Ethics Committee of The Affiliated Hospital of Qingdao University.

Cell culture

MG63 (ATCC® CRL-1427), U2OS (ATCC® HTB-96), Saos-2 (ATCC® HTB-85) and human osteoblast cell line hFOB1.19 (ATCC® CRL-11372) were acquired from ATCC (Manassas, VA, USA). OS732 (Y-01689) were purchased from FuHeng Biology (Shanghai, China), and all cells were hatched in high glucose Dulbecco’s modified Eagle medium (DMEM, GIBCO, Grand Island, NY, USA) consisting of 10% fetal bovine serum (FBS, GIBCO) in an incubator that contained 95% air and 5% CO2 at 37°C.

Cell counting kit-8 (CCK-8) assay

Viability was ascertained utilizing CCK-8 (Dojindo Laboratories, Tokyo, Japan). H9c2 cells were inoculated in a 96-well plate at the density of 5 × 10³ cells/well. When the treatments were completed, the used cell culture medium was substituted with fresh cell culture media containing 10μL CCK-8, and then the cultures were hatched for 1 h. The absorbance value at 450 nm was determined by utilizing a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Apoptosis assay

Experimental cells were inoculated in a 6-well plate. After cells had been subjected to the treatments as described above, treated cells were rinsed twice gently with cold phosphate-buffered saline (PBS, Thermo Scientific, Waltham, MA, USA) and re-suspended in the buffer. Apoptotic cells were evaluated utilizing flow cytometry (Beckman Coulter, Atlanta, GA, USA) following Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China) protocols.

Proliferation assay

Bromodeoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO, USA) was augmented at a concentration of 1 mg/mL to the experimental cells. At least 1000 cells were counted in each condition. Each condition contained at least five replicates.

Migration and invasion assay

Transwell chambers (Millipore) were placed in 24-well plates in the transwell assay. To assess migration capacity, experimental cells were inoculated in the upper chambers. As for invasion assay, cells were inoculated in the upper chamber preprocessing with Matrigel (Corning, Corning, NY, USA). Cells in the upper chamber were suspended in 200 μL serum-free culture media. The lower chamber was added with 600 μL complete media containing 10% FBS. After cultivated for 48 h, the experimental cells which were lacking in migration of invasion capacity were maintained in the upper chambers and they were transferred gently with a cotton swab. The migrated or invaded experimental cells were immobilized with 4% paraformaldehyde (Beyotime) for 30 min and then dyed utilizing crystal violet solution for 15 min. The cells were calculated using a microscope. And statistics were illustrated as the mean value of experimental cells adhered to the underside of the chamber of the five stochastic selected fields for each chamber.

Transfection

For circ-ITCH overexpression, the circ-ITCH sequence was cloned into PLCDH-circ-vector (Ribobio, Guangzhou, China) for producing lentivirus. MiR-22 mimic and negative control (NC) mimic were incorporated (Life Technologies, Carlsbad, MD, USA) and cells were cultured in 6-well plates. Experimental cells were transfected with circ-ITCH expressing vector, miR-22 mimic and NC mimic for 48 h. All transfections were completed utilizing Lipofectamine 3000 reagent (Invitrogen, San Diego, CA, USA) in line with the manufacturer’s specifications. Experimental cells were harvested after 48 h for the examinations listed in the following sections.

Quantitative reverse transcription PCR (qRT-PCR)

RNA from transfected cells was separated utilizing Trizol reagent (Invitrogen) as stated in the manufacturer’s protocols. The RNA concentration and purity were measured by UV
spectrophotometer at 260 nm and 280 nm. MiRNA reverse transcription was done utilizing the MultiscribeRTkit (Biosystems, Barcelona, Spain). The PCR was utilized of the SYBR® Green Master Mix (TaKaRa, Tokyo, Japan) following the protocols. The expression levels were computed utilizing the $2^{-\Delta\Delta CT}$ method, all experiments were performed thrice.

**Western blot assay**

Proteins of experimental cells were separated utilizing RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) fortified with protease-inhibitor (Roche, Basel, Switzerland). An equal amount of extracted protein was evaluated utilizing the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). Each protein bands were detached by 10% SDS-PAGE and then transferred onto a nitrocellulose membrane (Millipore, Boston, MA, USA). Afterwards, the appropriate primary antibodies were diluted and conducted with the membranes at 4°C overnight. Primary antibodies were listed as shown below: cleaved-Caspase-3 (No. ab2302, Abcam, Cambridge, MA, USA), cleaved-Caspase-9 (No. ab2324, Abcam), CyclinD1 (No. ab16663, Abcam), PTEN (No. ab32199, Abcam), t-PI3K (No. ab151549, Abcam), p-PI3K (No. ab138364, Abcam), t-SP1 (No. ab13370, Abcam), p-SP1 (No. ab59257, Abcam), t-AKT (No. 4685, Cell Signalling, Boston, MA, USA), p-AKT (No. sc-271966, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (No. sc-47778, Santa Cruz Biotechnology) was utilized as an endogenous protein for normalization. After the treated membranes were rinsed, they were conjunct with suitable secondary antibodies marked with horseradish peroxidase for 1 h at room temperature. Ultimately, the membranes with protein bands were removed into the Bio-Rad ChemiDoc™ XRS system. Finally, each area of the protein band was detected and assessed by Image Lab™ Software (Bio-Rad). Each test was performed in triplicates.

**Statistical analysis**

Each analysis was replied triple times at lowest. The evidence of various experimentations was performed as the mean ± SD and measured utilizing SPSS 19.0 statistical software (SPSS, Chicago, IL, USA). In addition, the $p$-values were determined to utilize a one-way analysis of variance (ANOVA) or Student’s t-test followed by the post hoc Tukey’s test. A $p$-value of < .05 was statistically significant.

**Results**

**Circ-ITCH was low expressed in the tissues and cell lines of OS**

To investigate whether circ-ITCH expression was changed in OS tissues and cell lines, qRT-PCR was utilized to assess circ-ITCH expression. As the data showed in Figure 1(A), circ-ITCH expression was remarkably declined in OS tissues compared with para-OS tissues ($p < .01$). Furthermore, similar results were observed in OS cell lines. Circ-ITCH expression was all diminished in MG63, U2OS, OS732 and Saos-2 cells compared with hFOB1.19 cells ($p < .05$ or $p < .01$, Figure 1(B)). And the change was most evident in MG63 and Saos-2 cells. Thus, we chose MG63 and Saos-2 cells for follow-up experiments.

**Circ-ITCH was elevated by vector transfection in MG63 and Saos-2 cells**

In order to explore the function of circ-ITCH, circ-ITCH was elevated by vector transfection in MG63 and Saos-2 cells. qRT-PCR was utilized to detect whether circ-ITCH was transfected into experimental cells successfully. The data indicated the circ-ITCH expression was strongly raised in MG63 and Saos-2 cells ($p < .01$, Figure 2).

**Circ-ITCH overexpression restrained proliferation and promoted apoptosis in MG63 and Saos-2 cells**

As displayed in Figure 3(A), viability was declined in both MG63 and Saos-2 cells when circ-ITCH was overexpressed ($p < .05$). In the meantime, proliferation was measured utilizing BrdU assay. Similar to the results in Figure 3(A), proliferation in experimental cells was markedly reduced ($p < .01$, Figure 3(B)). On the contrary, apoptosis was obviously escalated in MG63 and Saos-2 cells ($p < .01$ or $p < .001$, Figure 3(C)). Sequentially, proliferation-associated and
apoptosis-associated proteins were evaluated utilizing Western blot. CyclinD1 expression was notably declined in MG63 and Saos-2 cells (p < .01, Figure 3(D–F)). Besides, cleaved-Caspase-3 and cleaved-Caspase-9 expressions were elevated when overexpressed circ-ITCH (p < .001, Figure 3(G–I)).

**Circ-ITCH overexpression restrained migration and invasion in MG63 and Saos-2 cells**

To further investigate whether circ-ITCH affects migration and invasion in the experimental cells, transwell assay was utilized. The ability of cell migration and invasion was notably declined in MG63 and Saos-2 cells when overexpressed circ-ITCH (p < .05 or p < .01, Figure 4(A,B)).

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**Figure 2.** circ-ITCH expression was elevated by vector transfection in MG63 and Saos-2 cells. When circ-ITCH was transfected into experimental cells successfully, circ-ITCH was overexpressed in cells. **p < .01 compared with marked group.**

**Figure 3.** Proliferation was declined and apoptosis was raised when overexpressed circ-ITCH in MG63 and Saos-2 cells. (A,B) Viability and proliferation were reduced by overexpressing circ-ITCH. (C) Apoptosis was raised by overexpressing circ-ITCH. (D–F) CyclinD1 expression was reduced when circ-ITCH was overexpressed. (G–I) Cleaved-Caspase-3 and cleaved-Caspase-9 expression was elevated when circ-ITCH was overexpressed. *p < .05, **p < .01 or ***p < .001 compared with marked group.

**Figure 4.** Migration and invasion capacity was weakened when overexpressed circ-ITCH in MG63 and Saos-2 cells. After circ-ITCH was overexpressed in experimental cells, migration and invasion were strongly lessened. *p < .05 or **p < .01 compared with marked group.
**Circ-ITCH overexpression suppressed miR-22 expression**

In order to determine the relation between circ-ITCH and miR-22, miR-22 expression was stimulated by circ-ITCH overexpression. MiR-22 expression level was strongly reduced by overexpressing circ-ITCH \((p < .01\), Figure 5).**

**Circ-ITCH overexpression restrained proliferation and promoted apoptosis via down-regulating miR-22**

To identify whether miR-22 was transfected into experimental cells successfully, qRT-PCR was utilized to identify miR-22 expression. MiR-22 expression was remarkably enhanced after transfected with miR-22 mimic in MG63 and Saos-2 cells \((p < .001\), Figure 6(A)). Viability and proliferation of both cells were attenuated when up-regulating miR-22 expression and overexpressing circ-ITCH in the meantime \((p < .05\), Figure 6(B–C)). What’s more, apoptosis was declined when up-regulating miR-22 expression and overexpressing circ-ITCH \((p < .05\), Figure 6(D)). Furthermore, CyclinD1 expression was notably evaluated when up-regulating miR-22 expression and overexpressing circ-ITCH both in MG63 and Saos-2 cells \((p < .05\), Figure 6(E–G)). Inversely, cleaved-Caspase-3 and cleaved-Caspase-9 expressions were reduced \((p < .05\), Figure 6(H–J)).**

**Circ-ITCH overexpression restrained migration and invasion via down-regulating miR-22**

Migration and invasion ability were also evaluated after miR-22 mimic was transfected into MG63 and Saos-2 cells. Migration and invasion ability were raised when up-regulating miR-22 expression and overexpressing circ-ITCH \((p < .05\), Figure 7(A–B)).**

**Circ-ITCH overexpression repressed PTEN/Pi3K/AKT and SP-1 pathways via down-regulating miR-22 in MG63 and Saos-2 cells**

In order for identifying the potential molecular mechanisms of which circ-ITCH and miR-22 effected in MG63 and Saos-2 cells. So far, there are limited literature studies on the PTEN/Pi3K/AKT pathway.
PI3K/AKT pathway in OS. However, PTEN/PI3K/AKT pathway is widely explored in numerous diseases, as its essential role in apoptosis, migration and invasion [25,26]. Furthermore, SP-1 was proved to be highly expressed in OS tissues, and involved in the disease pathogenesis [27]. In our current research, the expression of PTEN, t-PI3K, p-PI3K, t-AKT, p-AKT, t-SP-1 and p-SP-1 was assessed. PTEN expression was conspicuously escalated in experimental cells (p < .01 or p < .001). Meanwhile, the rates of p/t-PI3K and p/t-AKT were remarkably diminished when overexpressing circ-ITCH (p < .05). Besides, SP-1 expression was lessened (p < .05). On the other hand, after miR-22 mimic was transfected into experimental cells, PTEN expression was reduced (p < .05 or p < .01) and the rates of p/t-PI3K, as well as, p/t-AKT were elevated (p < .05). More than that, SP-1 expression was enhanced when up-regulating miR-22 (p < .01, Figure 8(A–H)). The data implied that circ-ITCH repressed PTEN/PI3K/AKT and SP-1 pathways and the suppression was raised by up-regulating miR-22.

**Discussion**

CircRNAs have been found for more than 40 years. However, the functions of circRNAs are recognized in recent years. Accumulating researches illustrated that circRNAs played vital roles in human cancer pathogenesis. For instance, hsa_circ_0010729 was elucidated to be involved in regulating proliferation and apoptosis in vascular endothelial cells [28]. Zhou et al. demonstrated circ_0023642 increased the apoptosis, invasion and migration through controlling EMT of gastric cancer cells [29]. Moreover, hsa_circ_001569 promoted cell growth and metastatic capacity via repressing PI3K/AKT pathway [30]. Circ-ITCH is a newly discovered circRNA that has been explored to participate in the development and progression of diverse tumours, it exerted its function as a tumour suppressor in multiple types of tumours [14]. According to previous research, circ-ITCH was proved to impede proliferation and facilitate apoptosis in SKOV3 cells via modulating miR-10a [14]. Besides, several other studies have indicated circ-ITCH could repress proliferation and develop apoptosis in tumour cells [31]. However, the relationship between circ-ITCH and OS remains widely unknown. Similar to these studies, we also evaluated viability, proliferation, apoptosis, migration and invasion in MG63 and Saos-2 cells. Our results elucidated that overexpressing circ-ITCH promoted apoptosis, as well as repressed viability, proliferation, migration and invasion in experimental cells. More than that, circ-ITCH may function through PTEN/PI3K/AKT and SP-1 pathways.

MiRNAs participate in numerous biological and pathological proceedings, such as proliferation, metastasis, apoptosis and so on [32]. According to Guo’s research, miR-22 impeded proliferation, migration and invasion of OS cells [33]. Similarly, Gai et al. investigated overexpressing miR-22 had an effect on diminishing proliferation and raising apoptosis in MG63 cells [34]. Those researches indicated that miR-22 was a critical factor in tumourgenesis and development. Besides, miR-22 was also associated with cell migration and invasion. Liu et al. demonstrated that miR-22 restrained migration, invasion, apoptosis and viability in Y79 cells [35]. In the current research, we also suggested that miR-22 played a critical role as a suppressive factor in OS. Furthermore, miR-22 could act as a downstream effector of circ-ITCH, as its expression was negatively regulated by circ-ITCH and the effects of circ-ITCH overexpression on OS cell lines were attenuated when miR-22 was up-regulated. Nowadays, accumulating studies indicate circRNAs can exert their function by binding with miRNAs. For instance, circ-ITCH repressed progression on papillary thyroid cancer via sponging miR-22-3p through CBL/β-catenin pathway [36]. In addition, circ-ITCH inhibited proliferation, as well as, stimulated apoptosis in epithelial ovarian cancer cells via regulating miR-10a-α [14]. Moreover, Yang et al. showed that circ-ITCH suppressed the progression of bladder cancer by sponging miR-17/miR-224 [31]. Another study implied that circ-ITCH served as a sponge for miR-154 and circ-ITCH inhibited malignant progression of ovarian carcinoma cells via circ-ITCH-miR-154-3p-RASA1 axis in vitro and in vivo [37].

In our study, we identified the relationship between circ-ITCH and miR-22; overexpressing circ-ITCH remarkably reduced miR-22 expression both in MG63 and Saos-2 cells. Furthermore, circ-ITCH could affect on viability, proliferation, apoptosis, migration and invasion via modulating miR-22.

PI3K family members are lipid kinases which participated in a variety of cell processes, containing apoptosis, proliferation, migration and survival [38]. In addition, activated AKT could participate in phosphorylating a large number of substrates, controlling nearly all sides of cell physiology and pathology, containing growth metabolism, tumourgenesis and metastasis [39]. And then PI3K/AKT pathway is genetically spotted in more pathway ingredients and in more tumour sorts than other growth factor signalling pathways. Moreover, PI3K/AKT pathway has been deeply explored to be functioned in repressing proliferation, migration and invasion [40]. PTEN is a tumour suppressor gene that controls cell and apoptosis in a variety of cancers [41]. It has been reported...
that PTEN expression was declined in diverse malignant cancers such as breast cancer and prostate cancer [42]. More importantly, PTEN negatively regulates PI3K/AKT pathway [43]. In our study, PTEN expression was significantly escalated when overexpressing circ-ITCH and declined via up-regulating miR-22. Whereas, the changing trend of p/t-PI3K and p/t-AKT

Figure 8. PTEN/PI3K/AKT and SP-1 pathways were repressed when overexpressing circ-ITCH via down-regulating miR-22. (A–D) The expression of PTEN was strongly escalated when overexpressing circ-ITCH. Sequentially, PTEN expression was lessened via up-regulating miR-22. Whereas, the rates of p/t-PI3K and p/t-AKT were observably declined when overexpressing circ-ITCH. Meanwhile, the decline was reversed by up-regulating miR-22. (E–H) SP-1 expression was reduced by overexpressing circ-ITCH. Furthermore, SP-1 expression was remarkably elevated via up-regulating miR-22. *p < .05, **p < .01 or ***p < .001 compared with marked group.
rates was contrary to PTEN. Another factor detected in our assay was SP-1. SP-1 is an extensively expressed transcription factor that acts as a critical part in promoting oncogenes which is necessary for tumour survival, progression and metastasis. For now, SP-1 is exceedingly expressed in various cancer tissues such as colorectal cancer [44]. Our data explored SP-1 expression was declined when overexpressing circ-ITCH and strongly escalated via up-regulating miR-22 in MG63 and Saos-2 cells. Taken together, PTEN/Pi3K/AKT and SP-1 pathways were involved in suppressing proliferation, migration and invasion, as well as, facilitating apoptosis in OS cells.

In conclusion, the current study is the first to explore the function and interaction of circ-ITCH and miR-22 in OS. Circ-ITCH suppressed cell growth, migration and invasion capacity via down-regulation of miR-22. And circ-ITCH may, therefore, act as a biomarker and regulator through modulating miR-22 in OS progression. These findings revealed a novel molecular mechanism of circ-ITCH and miR-22 effect on OS, and provided an innovative target for clinical treatment.

Ethical approval
All procedures performed in studies involving human participants and were in accordance with the ethical standards of the institutional committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Written informed consent was obtained from all subjects before the study. The present research was allowed by the Medical Ethics Committee of The Affiliated Hospital of Qingdao University. Informed consent was obtained from all individual participants included in the study.

Author contributions
Chongmin Ren, Bin Yue conceived and designed the experiments; Chongmin Ren, Jia Liu, Bingxin Zheng, Peng Yan, Yuerong Sun performed the experiments and analyzed the data; Chongmin Ren, Bin Yue wrote the manuscript.

Disclosure statement
The authors declare that they have no competing interests.

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

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