Up-regulation of DRAM2 promotes tolerance of bladder transitional cell carcinoma to gemcitabine

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Abstract

Introduction: Bladder transitional cell carcinoma (BTCC) is one of the most prevalent human malignant diseases. Gemcitabine is commonly applied in the treatment of BTCC while acquired gemcitabine resistance has caused a severe impediment to recovery. This study aimed to investigate the function of DRAM2 in regulating gemcitabine resistance of BTCC.

Material and methods: GSE77883 was introduced to screen out the differentially expressed autophagy-related genes in T24 cells and gemcitabine-resistant T24-GEM cells. After establishing T24-GEM cells ourselves, aberrant expression of DRAM2 was detected by qRT-PCR and Western blot. After stably manipulating the expression of DRAM2 in T24 and T24-GEM cells, the changes of cell biological functions under gemcitabine treatment were compared, including cell viability, apoptosis and autophagy, using colony formation, flow cytometry and electron microscopy respectively.

Results: DRAM2 was up-regulated in gemcitabine-resistant T24-GEM cells. Silencing of DRAM2 in T24-GEM cells inhibited the cell autophagy induced by treatment with gemcitabine and contributed to attenuated gemcitabine resistance. Also, overexpression of DRAM2 in T24 cells enhanced the autophagy, strengthened the chemoresistance and decreased the cell apoptosis rate under the treatment with gemcitabine.

Conclusions: Our data suggested that downregulation of DRAM2 rescued the sensitivity of T24-GEM cells to gemcitabine, providing an appropriate therapeutic target for BTCC treatment.

Key words: bladder cancer, DRAM2, autophagy, gemcitabine.

Introduction

Bladder cancer, as one of the most prevalent genitourinary malignancies, is characterized by its high recurrence and mortality rate [1]. Transitional cell carcinoma (TCC) represented 90% of all the bladder cancer cases diagnosed in the USA [2]. Although some achievements in chemotherapy regimens improved the therapies of bladder cancer, the 5-year survival rate of bladder transitional cell carcinoma (BTCC) patients still ranges from 48 to 66% [3]. Therefore, the development of new therapies is still desirable. Gemcitabine, a nucleoside antimetabolite that inhibits DNA synthesis, is one of the most often used anticancer drugs in the treatment of BTCC [4, 5]. However, the development of chemoresistance for gemcitabine has become one of the most important reasons for treatment failure and has resulted in relapse, metastasis or patient death [6]. In order to improve...
the therapeutic efficacy of BTCC, it is urgent to advance our understanding of mechanisms for gemcitabine-induced resistance and increase tumor cell sensitivity to gemcitabine [7].

The gene DRAM2 (DNA-damage-regulated autophagy modulator protein 2), also known as TMEM77 (transmembrane protein 77), encodes a 266-amino acid protein with six putative transmembrane domains [8]. Localized to lysosomal membranes, DRAM2 plays a role in autophagy induction via promoting the conversion of endogenous LC3-I (microtubule-associated protein light chain 3) to the general autophagosome marker protein LC3-II (LC3-I/ phosphatidylethanolamine conjugate) [9]. LC3 is required for the elongation of autophagosomes, which has two forms: LC3-I and LC3-II [10]. LC3-II, as the most reliable marker for quantification of cell autophagy, is up-regulated when LC3-I converts to LC3-II during autophagy [10].

Autophagy is a highly conserved process. The function of autophagy is to sequester parts of the cytoplasm, including damaged, superfluous organelles or long-lived proteins, into autophagosomes, which are double-membrane vesicles [7]. Autophagy serves an essential role in maintaining tissue homeostasis to support cell growth and survival [4], such as inflammatory bowel disease, neuronal degeneration, aging and cancer. On the other hand, many studies have reported that autophagy is a significant mechanism in chemoresistance, and inhibition of autophagy may enhance the sensitivity of cancer cells to chemotherapy [11], such as breast cancer, non-small cell lung cancer cells [12] and colorectal cancers [13].

Given these findings, we hypothesized that DRAM2 mediates chemoresistance in bladder cancer cells. Therefore, we set out to test this hypothesis by investigating the relationship between DRAM2 and autophagy in gemcitabine sensitive/resistance BTCC cells. The results determined the relationship between expression of DRAM2 and autophagy, suggesting a promising new combination in the treatment of bladder transitional cell carcinoma.

Material and methods

Integrated analysis of microarray datasets

The microarray data in the GEO database (accession number GSE77883) at the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under GPL19117 were used to analyze the six samples (three T24 cells and three T24-GEM cells). Differentially expressed mRNAs related to autophagy were identified based on the criteria of over 2-fold expression change within different groups and a p-value less than 0.05.

Cell culture

Bladder transitional cell carcinoma cell lines T24 (TCHu 55) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 (Roswell Park Memorial Institute, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 ug/ml streptomycin. Gemcitabine-resistant BTCC cells (T24-GEM) were derived by treating T24 cells with 5 μg/ml gemcitabine and maintained in the same condition for more than 6 months. The T24-GEM cells were cultured in complete medium supplemented with 12% FBS, 5 μg/ml gemcitabine, 100 U/ml streptomycin and 100 U/ml penicillin. Cells were incubated at 37°C with 5% CO2 in an incubator.

Real-time PCR analysis

Total RNA was prepared from T24/T24-GEM cells transfected with DRAM2/si-DRAM2 according to the manufacturer’s instructions and the concentration of total RNA was determined. The RNA was then reverse transcribed in a 20 μl reaction mixture according to the instructions of the RevertAid First strand cDNA synthesis Kit (Fermentas, China). The PCR program for DRAM2 and GAPDH (Santa Cruz Biotechnology, CA, USA) included one cycle at 95°C for 2 min, 39 cycles at 95°C for 20 s, 65°C for 20 s, 72°C for 30 s, and finally extension at 72°C for 10 min. The PCR products of the DRAM2 gene and internal controls were detected by electrophoresis in agarose gel. All DNA fragments and resulting clones were verified by sequencing.

Western blot

Cells and tissues were lysed with ice-cold RIPA buffer (Beyotime, Shanghai, China) supplemented with phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Roche). Cell protein lysates were subjected in 10% sodium dodecyl sulfate-polyacrylamide gels, electrophoretically transferred to polyvinylidene difluoride membranes (Roche). Total protein concentration was measured using a BCA protein assay kit before loading for SDS-PAGE. The membranes were incubated with 5% skim milk, washed and then incubated with the rabbit anti-human DRAM2 (1 : 500 dilution) or GAPDH (1 : 1000 dilution) overnight at 4°C, followed by incubated with goat anti-human horseradish peroxidase antibody for 1 h at 37°C. All antibodies were purchased from Abcam (Abcam, USA). The proteins were perceived by the enhanced chemiluminescence kit (Invitrogen) and exposed to X-ray film. Protein expression levels were normalized to GAPDH.
Cell transfection

Human DRAM2 cDNA and si-DRAM2 were chemically synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The oligonucleotides were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. Cells were subcultured 1 day before the transfection and were inoculated into 6-well plates. After cell confluence reached 90–95%, 50 μl of serum-free medium was used to dilute 1.0 μg of DNA, and then another 50 μl of serum-free medium was used to dilute 1 μl of Lipofectamine 3000 transfection reagent. Subsequently, the solution was mixed with the diluted DNA at room temperature for 20 min and added to each well. Cells were generally assigned to different groups as follows: (1) NC group (transfected with Lipofectamine 2000), siRNA control group and si-DRAM2 group (T24-GEM cells); (2) NC group (transfected with Lipofectamine 2000), pcDNA3.1 control group, pcDNA3.1-DRAM2 group (T24 cells). The transfection efficiency was observed under qRT-PCR and western blot, and siRNA sequences are listed in Table I.

MTT assay

Cell viability was assessed by using 3-(4,5-di-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. 3000 cells in 100 μl of medium per well were seeded in 96-well plates. Cells were cultured for 24 h, 48 h, 72 h, and then incubated with 0.5 mg/ml of MTT at 37°C for 4 h. Medium was replaced with 150 μl DMSO per well to dissolve the precipitates. Colorimetric analysis using a 96-well micro-plate reader (Bio Tek) was performed at wavelength of 490 nm. An effect curve was drawn and the IC50 value of gemcitabine to bladder cancer cells was calculated depending on cell proliferation and drug concentration.

Transmission electron microscopy

Cells were treated with BIX-01294 1 μM and then fixed in Karnovsky’s fixative (2% paraformaldehyde and 5% glutaraldehyde in 0.1 M cacodylate, pH 7.4) followed by osmium tetroxide. Samples were then dehydrated in ethanol, infiltrated and embedded with TAAB Low Viscosity Resin (TLV) mixture at 60°C for 24 h and sectioned to 80 nm in thickness on 300 mesh copper slot grids. Analysis was performed by transmission electron microscopy (JEOL, JEM-1400) at 60 kV.

Apoptosis analysis

Apoptotic cells were stained using Annexin V-FITC/Propidium Iodide (PI) contained in the Apoptosis Kit (KeyGene Biotech, Nanjing, China). After 48 h of transfection, T24/T24-GEM cells were stained with 5 μl Annexin V-FITC and 5 μl of PI and cultured in the dark at room temperature for 15 min. These cells were analyzed by flow cytometry, and results were processed with CellQuest Pro software (BD Biosciences, San Jose, CA, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software v. 6.0 (GraphPad Prism software Inc., La Jolla, CA, USA). Data are present- ed as mean ± SEM (standard error of mean). All experiments were performed in triplicate and repeated at least three times. The unpaired Student’s t-test was used for comparison of two groups, and one-way ANOVA was performed to analyze differences among more than two experimental groups. Statistical significance was labeled as: *p < 0.05, **p < 0.01, ***p < 0.001.

Results

DRAM2 was up-regulated in gemcitabine-resistant cells

Differentially expressed genes in six cell lines are listed in Table II, DRAM2 highly expressed in T24-GEM cells and lowly expressed in T24 cells. The P-value was computed using the t-test, and fold changes of gene expression were calculated by the ratio of the average expression level between drug-resistant and drug-sensitive groups (Table III). The fold change value of DRAM2 between T24 cells and T24-GEM cells was 2.03924, and the p-value was less than 0.05. Information of related genes in the heatmap, including full name, position and ID, is shown in Table IV. Five genes were highly expressed in the T24 cells and lowly

| Gene | Sequences (5’–3’) |
|------|------------------|
| q-DRAM2-F | GTATCTGGTGGAGTAGTGTAAGT |
| q-DRAM2-R | GTACCGGGTTCGTCAT |
| q-GAPDH-F | ACCCCGGCGCTTGAGG |
| q-GAPDH-R | TCTGACGGCAGTCAGGT |
| Si-DRAM2-F | GCAATAATTAGTTAG |
| Si-DRAM2-R | CCGCATTATATCAG |
| Si-Control-F | CTTCCGATAATTAGTC |
| Si-Control-R | GCCAATAATTATCAG |
| cDNA-DRAM2-F | CAACCTGACGGCACCACAT |
| cDNA-DRAM2-R | CCTACAGGTGCTTCTCTCCT |
Table II. Relative expression values of differential genes

| Gene T24_gemcitabine.resistant_1 | T24_gemcitabine.resistant_2 | T24_gemcitabine.resistant_3 | T24_1 | T24_2 | T24_3 |
|----------------------------------|------------------------------|-------------------------------|-------|-------|-------|
| ATG9B                            | -0.58597                     | -0.28833                     | 0.169592 | 0.431298 | 0.64276 |
| ATG12                            | -0.39833                     | -0.39833                     | 0.291868 | 0.054718 | 0.984777 |
| ATG4D                            | -0.21256                     | 0.169592                     | -0.11866 | 0.984777 | 0.984777 |
| ATG13                            | -0.11866                     | -0.25988                     | -0.28833 | 0.64276 | 0.506704 |
| ATG16L1                          | -0.28833                     | -0.21256                     | -0.06436 | 0.506704 | 0.169592 |
| ATG7                             | 0.431298                     | 0.64276                      | -0.28833 | -0.21256 | -0.39833 |
| DRAM1                            | 0.984777                     | 0.343298                     | 0.431298 | -0.11866 | -0.39833 |
| DRAM2                            | 0.64276                      | 0.506704                     | 0.64276 | -0.39833 | -0.28833 |
| ATG10                            | 0.506704                     | 0.984777                     | 0.984777 | -0.58597 | -0.58597 |

Negative values (–) denote low expression and positive ones denote high expression.

Table III. Results of fold-change and p-value of differential genes

| Gene symbol | logFC  | P-value               | adj. P-value    |
|-------------|--------|-----------------------|-----------------|
| ATG9B       | -0.901304 | 0.000252951          | 0.001011803    |
| ATG12       | -0.842114 | 0.001995655          | 0.004561498    |
| ATG4D       | -0.728634 | 0.009445382          | 0.01679179     |
| ATG13       | -0.702731 | 0.001070063          | 0.002853502    |
| ATG16L1     | -0.528279 | 0.00794332           | 0.01588664     |
| ATG7        | 0.82666  | 0.000201318          | 0.001011803    |
| DRAM1       | 0.84541  | 0.000836573          | 0.002677033    |
| DRAM2       | 0.9224   | 6.02E-05             | 0.000481658    |
| ATG10       | 1.41139  | 2.45E-06             | 3.91E-05       |

Negative values (–) denote low expression and positive denote high expression. logFC represents the gene expression fold change of T24 cells compared with T24 gemcitabine resistant cells. P-value and adj. p-value refer to statistical values, p < 0.05 indicates statistical significance.

Table IV. Detailed information of the differential genes

| Genes     | Full name                          | Position        | ID     |
|-----------|------------------------------------|-----------------|--------|
| ATG9B     | ATG9 autophagy related 9 homolog B | chr7:150720259-150720200 | NM_173681 |
| ATG12     | ATG12 autophagy related 12 homolog | chr5:115167432-115167373 | NM_004707 |
| ATG4D     | ATG4 autophagy related 4 homolog D | chr9:10663725-10663798 | NM_032885 |
| ATG13     | ATG13 autophagy related 13 homolog | chr11:46690061-46690120 | NM_001205119 |
| ATG16L1   | ATG16 autophagy related 16-like 1  | chr2:234202948-234203007 | NM_030803 |
| ATG7      | ATG7 autophagy related 7 homolog   | chr3:11468321-11468380 | NM_006395 |
| DRAM1     | DNA-damage regulated autophagy modulator 1 | chr12:102317112-102317171 | NM_018370.2 |
| DRAM2     | DNA-damage regulated autophagy modulator 2 | chr1:111662564-111662505 | NM_178454.5 |
| ATG10     | ATG10 autophagy related 10 homolog | chr5:81550614-81550673 | NM_001131028 |

expressed in T24-GEM cells, and four genes were lowly expressed in T24 cells and highly expressed in T24-GEM cells, including DRAM2 (Figure 1 A, p < 0.05). Due to three other genes being expressed both in humans and yeast, the DRAM2 gene was selected as the target gene for further
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study. The IC50 value of T24-GEM cells (9.953 μg/ml) was significantly higher than that of T24 cells (2.366 μg/ml), and the cell viability of T24-GEM cells was significantly higher than that of T24 cells after treatment with gemcitabine (Figure 1 B, $p < 0.01$). Also, protein expression of DRAM2 in T24-GEM cells was higher than that of T24 cells, which was consistent with the results of microarray analyses (Figure 1 C).

Silencing of DRAM2 increased the sensitivity of T24-GEM cells to gemcitabine and inhibited autophagy

To determine whether DRAM2 plays a role in the regulation of cellular autophagy and apoptosis in response to gemcitabine, siRNA against DRAM2 and control siRNA were transfected into T24-GEM cells. We observed a decrease in the expression of mRNA and protein after knockdown of DRAM2 (Figure 2 A, B, $p < 0.05$). In the si-DRAM2 group, T24-GEM cells became more sensitive to gemcitabine-induced damage with the IC50 level decreasing from 9.953 μg/ml to 2.266 μg/ml (Figure 2 C, $p < 0.01$). We also investigated the viability and apoptosis of these cells using MTT and flow cytometry. The results showed that under the treatment of 0.5 μg/ml gemcitabine, DRAM2-knockdown cells were less active compared with si-Con cells (Figure 2 D, $p < 0.01$), and the number of apoptotic cells increased (Figure 2 E, $p < 0.01$). Also, the number of autophagosome decreased (Figure 2 F). LC3 I level increased while LC3 II level decreased (Figure 2 G). These results suggested that silencing of DRAM2 decreased T24-GEM cells’ sensitivity to gemcitabine and inhibited autophagy.

Over-expression of DRAM2 decreased the sensitivity of T24 cells to gemcitabine and promoted autophagy

Plasmids of pcDNA 3.1 and pcDNA 3.1-DRAM2 were transfected into T24 cells.

Figure 1. DRAM2 was up-regulated in gemcitabine-resistant cells. A – The heat map showed that DRAM2 was up-regulated in gemcitabine-resistant cells. B – The IC50 of T24-GEM cells (9.953 μg/ml) was significantly higher than that of T24 cells (2.366 μg/ml), and the cell viability of T24-GEM cells was higher compared with T24 cells. C – The expression level of DRAM2 in T24-GEM cells was remarkably higher than that in T24 cells as examined by western blot. **$p < 0.01$ compared with T24 cells.
Figure 2. Knockdown of DRAM2 increased the sensitivity of T24-GEM cells to chemotherapy and inhibited autophagy. A – The expression of DRAM2 in T24-GEM cells significantly decreased after transfection with si-DRAM2 as measured by qRT-PCR. B – The expression of DRAM2 in T24-GEM cells significantly decreased after transfection with si-DRAM2 as measured by western blot. C – T24-GEM cells became more sensitive to gemcitabine-induced damage with the IC50 level decreasing from 9.953 µg/ml to 2.266 µg/ml after transfection with si-DRAM2. D – The cell growth of T24-GEM cells transfected with si-DRAM2 was suppressed significantly confirmed by MTT assay. E – The apoptosis rate of T24-GEM cells in si-DRAM2 groups significantly increased in comparison with the si-Con group as detected by flow cytometry.
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Discussion

Drug resistance is the major cause of the failure of clinical effectiveness of chemotherapy for bladder cancers. For example, cisplatin is one of the most effective bladder cancer agents, while inherent and acquired resistance limits its application [14]. The pyrimidine analog gemcitabine, alone or in combination with other drugs, is the current standard of care for advanced TCC. However, the response to gemcitabine in patients is very poor, with no drastic reduction in metastasis or increase in the survival rate of patients [15]. Multiple molecular mechanisms of gemcitabine resistance encompassing different pathways have been suggested. Recently, Geller et al. hypothesized that intratumor bacteria might contribute to drug resistance of pancreatic ductal adenocarcinoma (PDAC) [16]. Amantini et al. demonstrated a mechanism of chemoresistance by which cancer cells increase intracellular cytidine pools that can in turn render gemcitabine ineffective by molecular competition [17]. Many studies have documented genetic aberrations in the development of BTCC, including point mutations of the TP53 [18], FHIT [19], KAI1 [20], BCL-2 and BCL-X genes [21]. Here, by screening the differentially expressed genes between T24 cells and T24-gemcitabine-resistant cells, DRAM2, DNA-damage regulated autophagy modulator 2, was found to be up-regulated in gemcitabine-resistant cells in our study. DRAM2, a critical regulator of autophagy, encodes a transmembrane lysosomal protein thought to play a role in the initiation of autophagy [8]. It has been reported that DRAM2 expression is frequently dysregulated in cancers and that over-expression of DRAM2 induces autophagy [22]. DRAM2 was more highly expressed in gastric cancer (GC) tissues than in matched normal tissues [23]. Importantly, the knockdown phenotypes for DRAM2 inhibited autophagy and arrested myeloid cell differentiation in an acute promyelocytic leukemia (APL) mouse model [24].
Figure 3. Over-expression of DRAM2 decreased the sensitivity of T24 cells to chemotherapy and promoted autophagy. 

A – The expression of DRAM2 in T24 cells dramatically increased after transfection with pcDNA3.1-DRAM2 as observed by qRT-PCR.

B – The expression of DRAM2 in pcDNA3.1 group of T24 cells was significantly higher than that in the pcDNA3.1 group as detected by western blot.

C – After over-expression of DRAM2, the IC50 value increased to 3.595 µg/ml, which was much higher than that in the pcDNA3.1 group.

D – MTT assay showed that cell proliferation of T24 cells was significantly enhanced in the DRAM2 overexpression group compared with the pcDNA3.1 group.

E – Compared with the pcDNA3.1 group, the apoptosis rate of T24 cells drastically increased after transfection with pcDNA3.1-DRAM2 as detected by flow cytometer.
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The DRAM2-dependent autophagic cascade affects Hodgkin lymphoma (HL) cell proliferation and tumor growth [24]. Yoon et al. demonstrated that silencing of endogenous DRAM2 interferes with starvation-induced autophagy [22]. Similarly, our results showed that DRAM2 was up-regulated in T24-gemcitabine-resistant cells, which enhanced cell viability and inhibited cell apoptosis in T24-gemcitabine-resistant cells.

Dysregulated autophagy is highly prevalent in many types of cancer including drug-resistant bladder cancer and has been implicated in cytoprotection and tumor death [25–27]. Exploring the mechanism of gemcitabine resistance in bladder cancer from the perspective of autophagy may provide a new insight into solving the problem of drug resistance in bladder cancer. Previous studies have focused on suppressing autophagy-related genes or using autophagy inhibitors to strongly potentiate gemcitabine-induced apoptosis and inhibiting gemcitabine-induced autophagy [28]. Conflicts and controversies are often accompanied by studies of cancer cell autophagy. Some studies have indicated that in cancer autophagy exerts cytoprotective effects [29–31], whereas others suggest that autophagy positively contributes to cell death by enhancing cytotoxicity of anticancer drugs [32–34].

Our study firstly researched the dysregulated expression of the autophagy-related gene DRAM2 in T24 cells and T24-gemcitabine-resistant cells and explored resistance of gemcitabine altered through the regulation of DRAM2. Similar to studies of autophagy, studies on DRAM2 remain disputatious. In contrast to our results, Park et al. pointed out that expression of DRAM or DRAM2 individually did not induce cell death, co-expression of DRAM2 with DRAM significantly induced cell death, while the silencing of endogenous DRAM2 attenuated cell death [35]. These conflicts may be caused by the stage and type of the cancer cells and the ignorance about drug-induced autophagy. Our study investigated the effects of DRAM2 on gemcitabine-resistant bladder cell biology. However, some limitations still exist. For instance, we only examined DRAM2 functions, while other genes and factors are also worth studying, such as micronutrients [36]. Furthermore, the pathway of DRAM2 in gemcitabine-resistant bladder cells needs to be clarified by future work.

In conclusion, overall, the current study demonstrated that DRAM2 was up-regulated in gemcitabine-resistant bladder cancer T24 cells. DRAM2 silence in T24-GEM cells promoted apoptosis whereas it inhibited autophagy and decreased the cell viability and resistance to gemcitabine, while in T24 cells, overexpression of DRAM2 promoted autophagy and cell viability but inhibited cell apoptosis and sensitivity to gemcitabine. These results allowed us to speculate that up-regulation of DRAM2 may confer on gemcitabine-resistant bladder cancer cells an advantage of insensitivity to gemcitabine. Therefore,
the treatment of bladder cancer with gemcitabine in combination with drugs targeting the autophagic gene DRAM2 could represent an interesting new approach in bladder cancer therapy.

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Baihetiya Azhati and Naibijiang Maolakuerban are first co-authors.

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**Conflict of interest**

The authors declare no conflict of interest.

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