Original Paper

LncRNA HOTAIR is a Prognostic Biomarker for the Proliferation and Chemoresistance of Colorectal Cancer via MiR-203a-3p-Mediated Wnt/ β -Catenin Signaling Pathway

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Key Words

Colorectal cancer (CRC) • HOX transcript antisense RNA (HOTAIR) • Proliferation • Chemoresistance • Signaling pathway

Abstract

Background/Aims: HOX transcript antisense RNA (HOTAIR) plays a vital role in carcinogenesis. However, its functional and regulatory roles remain unclear. In this study, we aimed to investigate its biological function and clinical significance in human colorectal cancer (CRC).

Methods: We examined the expression levels of lncRNA HOTAIR and miR-203a-3p in CRC tissues and CRC cell lines by qRT-PCR. Gain and loss-of-function assays were performed to examine the effects of HOTAIR and miR-203a-3p on the proliferation and chemoresistance of CRC cells. The possible mechanisms of HOTAIR were also explored by fluorescence reporter assay and Western blot. **Results:** The expressions of HOTAIR were upregulated in CRC tissue tissues compared to adjacent control tissues. We also found HOTAIR was downregulated by miR-203a-3p in CRC cell lines. Both HOTAIR knockdown and miR-203a-3p overexpression in CRC cell lines led to inhibited cell proliferation and reduced chemoresistance. We also determined that β -catenin and GRS5 were inhibitory targets of miR-203a-3p, and that Wnt/ β -catenin signaling was inhibited by both HOTAIR knockdown and miR-203a-3p overexpression. Significantly, we found that increased expression of miR-203a-3p is essential for cell proliferation repression, chemoresistance reduction, and Wnt/ β -catenin signaling inhibition induced by HOTAIR knockdown. **Conclusions:** Our study demonstrated that the lncRNA HOTAIR could regulate the progression and chemoresistance of CRC via modulating the expression levels of miR-203a-3p and the activity of Wnt/ β -catenin signaling pathway.

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Introduction

Colorectal cancer (CRC) is the third most common cancer and the leading cause of cancer mortality [1]. According to cancer statistics in 2015, there were 3,763,000 newly diagnosed cases of CRC in China, and 191,000 patients died from CRC [2]. A recent epidemiological study has shown that the morbidity rate of CRC in patients under 50 years old has continued to rise over the past few decades [3]. However, the pathological features and prognosis of young patients with CRC are still controversial. Recent studies have shown that non-coding RNA may be involved in the development of CRC.

The length of long non-coding RNA (lncRNA) is greater than 200 nucleotides [4-6]. Because of its role in gene transcription, epigenetic, and post transcriptional regulation, lncRNA received more and more concerned by researchers [7, 8]. Multiple evidences indicate that lncRNA may participate in a variety of cellular processes, including cell cycle, differentiation, proliferation, growth and apoptosis [9-12]. lncRNA HOTAIR is identified as an oncogene which is involved in a variety of cancers, including breast cancer [13], esophageal squamous cell carcinoma [14], and lung cancer [15]. HOTAIR acts mainly through polycomb repressive complex 2 (PRC2) (EZH2, RbAp46, RbAp48, and SUZ12) [16]. EZH2, which is the core catalytic component of the PRC2, can affect cancer progression by altering H3K27 trimethylation and silencing transcription [17]. Some preliminary evidences demonstrate that HOTAIR interacts with chromatin modifying complexes to affect the epigenetic regulation of gene expression [18], but the mechanism of HOTAIR, especially its role in the development of CRC, is unclear.

MicroRNAs (miRNAs) are a family of small and evolutionarily conserved non-coding RNAs with the length of 21-23 nt [19]. miRNAs have been considered as oncogenes or tumor suppressors by regulating cell proliferation and differentiation [20]. The crosstalk between lncRNAs and miRNAs has been reported to control cancer progression [21]. miR-203a has been found to be a crucial regulator involved in various cancers, such as gastric cancer [22], nasopharyngeal carcinoma [23], breast cancer [24], lung cancer [25], liver cancer [26], and CRC [27]. However, the potential involvement of miR-203a in the chemoresistance of CRC is not well known.

In our study, lncRNA HOTAIR expression in CRC tissues and its interaction with miR-203a were analyzed. The alterations of proliferation and drug resistance of CRC cell lines after HOTAIR knockdown were evaluated, and the underlying signaling pathways were also investigated.

Materials and Methods

Clinical samples

This study design was approved by the medical ethics board of Xiangya Hospital, Central South University. Total 104 participants from Xiangya Hospital signed written informed consents. Fresh CRC tissues and adjacent para-carcinoma tissues were collected from surgery. All the participants neither receive chemotherapy nor radiotherapy before surgery. The pathological diagnosis of CRC was made by three pathological physicians. The pathological features of CRC patients were shown in Table 1.

Bioinformatics analysis

miRcode online website (<http://www.mircode.org/index.php>) and LncBase v2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2/index) to search the potential miRNAs of HOTAIR. miRcode online website was also used for searching potential targets of miR-203a. Correlation analysis was performed by using Pearson product-moment correlation coefficient.

Cell culture and treatment

CRC cell lines CCD18Co, HCT15, CoLo205, HT29, DLD-1, and SW620 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM contained 10% fetal

bovine serum (FBS; Gibco, Carlsbad, CA, USA) and 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in humidified 5% CO₂. LncRNA HOTAIR siRNA, control siRNA, miR-203a-3p mimics and miR-203a-3p inhibitors were purchased from GenePharma (Carpinteria, CA, USA). Cells were treated with Cisplatin (CDDP) (Taj Pharmaceuticals Ltd, Maharashtra, India) and Paclitaxel (PTX) (Bristol-Myers Squibb Company, Melbourne, Australia). Cell transfection was performed using Lipofectamine™ 2000 reagents (Abcam, Cambridge, UK) according to the instructions. After transfection, cells were treated with different concentrations of CDDP (0, 1, 1.5, 2, 2.5, 3, 3.5 μM) or PTX (0, 0.3, 1, 3, 10, 15, 20, 30 μM).

Table 1. Correlation between HOTAIR expression and clinical clinicopathological parameters of CRC. *P<0.05, **P<0.01

Parameters	n	HOTAIR expression [n(%)]		P
		Low	High	
Age (years)				0.204
<60	37	24(64.9%)	13(35.1%)	
≥60	67	28(41.8%)	39(58.2%)	
Gender				0.176
Male	63	26(41.3%)	37(58.7%)	
Female	41	26(63.4%)	15(36.6%)	
Tumor location				0.815
Colon	50	23(46.0%)	27(54.0%)	
Rectum	54	29(53.7%)	25(46.3%)	
Tumor size (cm)				0.003**
<5	47	34(72.3%)	13(27.1%)	
≥5	57	18(31.6%)	39(68.4%)	
Histologic grade				0.001**
G1+G2	83	50(60.2%)	33(39.8%)	
G3+G4	21	2(9.5%)	19(90.5%)	
Lymph node metastasis				0.016*
Positive	60	21(35.0%)	39(65.0%)	
Negative	44	31(70.5%)	13(29.5%)	
Distant metastasis				0.832
M0	85	44(51.8%)	41(48.2%)	
M1	19	8(42.1%)	11(57.9%)	

qRT-PCR

Total RNA extraction and reverse transcription were carried out. qPCR was done with Power SYBR Green RT-PCR reagent (Applied Biosystems, Waltham, MA, USA) and Applied Biosystems 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The expression of target gene or miRNA was standardized by GAPDH or U6 snRNA. Relative expression level of gene or miRNA was calculated by 2^{-ΔΔCt}.

Cell proliferation and colony formation assay

Cell proliferation rate and inhibition rate were detected by MTT assay. CRC cells were harvested and seeded at a density of 1×10³ cells/ml per well in 96-well plate and cultured at 37°C. After treatment, the cells were washed with PBS twice. Then, 10 μL MTT (5 mg/mL) was added into the wells at different time points. After incubation for 4 hrs, 100 μL DMSO was added to each well to dissolve the formazan crystal, and the absorbance was detected at 590 nm wavelength using a microplate reader (Bio-Tek, Winooski, VT, USA). In colony formation assay, 0.5×10³ COLO205 and SW620 cells were inoculated into 6-well plates for 10 days. The colonies were fixed with 10% formaldehyde for 10 min and dyed with 0.5% crystal violet for 5 min. The number of colonies was photographed by an Olympus microscope (Tokyo, Japan) and counted.

BrdU immunofluorescence assay

CRC cells were seeded on coverslips placed in 6-well plates and incubated with BrdU (Sigma) stock solution for 60 min. After blocking with 10% goat serum in PBS for 1 h, cells were incubated with a primary rabbit antibody against BrdU (1:200, Abcam) overnight at 4°C, and then incubated with the secondary antibody coupled to Cy3 for 2 hrs. After DAPI staining and PBS washing, the coverslips were visualized using a fluorescence microscope (Olympus 600, Tokyo, Japan) and BrdU-positive cells were counted in five fields of each coverslip.

Luciferase assay

Wild-type (WT) HOTAIR containing a potential miR-203a-3p binding site or HOTAIR mutant containing a variant site was established and linked to the luciferase reporter vector pmirGLO (Sigma-Aldrich, Saint Louis, MO, USA). HEK293T cells were inoculated into 24-well plate until the confluence reached 80%. The luciferase reporter plasmid was transfected into the cells with miR-203a-3p or control miRNA. After transfection 48h, the luciferase activity was detected by dual luciferase reporter assay system (Promega).

Pull-down assay

Pull-down assay was carried out as literature [28]. S1-HOTAIR and S1-HOTAIR mutant were established and co-transfected with or without miR-203a-3p inhibitors. After transfection 48h, the cells were collected and washed, cross-linked with 0.37% formaldehyde, and then incubated with frozen lysate. Streptavidin

conjugated magnetic beads were collected and washed with eluent. Magnetic beads were heated at 60°C for 30 min, and the expression of miR-203a-3p was detected by qRT-PCR.

Western blot

According to the literature, β -catenin, Axin2 protein, Cyclin D1 and GRG5 antibody (Abcom, Cambridge, MA, USA) were used in Western blot. GAPDH monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a loading control antibody. Proteins were detected using ECL Plus Western Blotting Detection Reagents (GE Healthcare Life Sciences, Piscataway, NJ, USA). The gray values were analyzed using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis

All statistical analyses were performed using GraphPad software (version 5.0) (GraphPad Software, CA, USA). Data were reported as means $\pm$ SD. Comparison between groups was performed using Student t-test (non-paired, bilateral) or one-way ANOVA. The relationship between miRNA and its target was analyzed by Spearman test. Survival was analyzed using Kaplan-Meier method. P-values less than 0.05 indicated statistically significant differences.

The dataset(s) supporting the findings of this study are included within the article.

Results

Up-regulation of HOTAIR in CRC tissues

The relative expression of HOTAIR in CRC tissues and the matched para-carcinoma tissues among 104 CRC patients were analyzed by qRT-PCR. The results showed that the expression of HOTAIR was higher in cancer tissues than in the para-carcinoma tissues ($P < 0.001$, Fig. 1A). Moreover, the expression level of HOTAIR was up-regulated in CRC cell lines (CCD18Co, HCT15, CoLo205, HT29, DLD-1 and SW620) compared to normal colon cell line FHC (Fig. 1B). The relationship between HOTAIR expression and the survival rate of 104 CRC patients was analyzed by Kaplan-Meier. As shown in Fig. 1C, high expression of HOTAIR was associated with poorer overall survival in CRC patients (HR=2.13, $P = 0.015$).

HOTAIR interacts with miR-203a-3p

To explore the mechanism of action for HOTAIR in CRC development, we used LncBase Predicted v.2 of DIANA software to predict miRNAs that potentially interacted with HOTAIR. We identified miR-203a-3p, an important regulator in cancers [17-22], as a potential target miRNA of HOTAIR. The binding site between miR-203a and HOTAIR is shown in Fig. 2A. To validate the interaction between miR-203a and HOTAIR, luciferase assays were carried

Fig. 1. Upregulation of HOTAIR in CRC tissue. A, HOTAIR expression in 104 clinical CRC samples compared to those in control samples determined by qRT-PCR ($P < 0.001$). B, HOTAIR expression in CRC cell lines compared with normal cell line. * $P < 0.05$ and *** $P < 0.01$. C, Kaplan-Meier analysis of the association between HOTAIR expression and overall survival in 104 CRC patients. $P = 0.015$.

Fig. 2. HOTAIR was a target of miR-203a-3p. A, Mutation of HOTAIR at potential miR-203a-3p binding sequence. B, luciferase assays in 293 T cells transfected WT or MUT HOTAIR with miR-203a-3p (* P <0.05). C, miR-203a-3p expression in CRC and control tissues from 104 CRC patients determined by qRT-PCR (P <0.001). D-E, Expression levels of HOTAIR and miR-203a-3p in Colo205 and SW620 cells transfected with siR-HOTAIR or siR-control (* P <0.05, *** P <0.001). F-G, Expression levels of HOTAIR and miR-203a-3p in Colo205 and SW620 cells transfected with WT or MUT HOTAIR (*** P <0.001). H, pull-down assays were performed in HEK293T after transfection (* P <0.05). I, Correlation of the HOTAIR expression and miR-203a-3p in CRC tissues (104 cases) (r = -0.643, P <0.001).

out. We constructed luciferase reporter plasmids for HOTAIR containing predicted WT and mutant-binding sites for miR-203a-3p and found that miR-203a-3p reduced the luciferase activity of the wild-type plasmid but not that of the mutant plasmid (Fig. 2B), indicating that miR-203a-3p could bind to HOTAIR transcript. Notably, the expression level of miR-203a-3p was significantly down-regulated in cancer tissues from 104 CRC patients (Fig. 2C). To further determine whether HOTAIR regulates miR-203a-3p expression, we knocked down the expression of HOTAIR in Colo205 and SW620 cells (Fig. 2D). The expression of miR-203a-3p significantly increased in the two CRC cell lines after HOTAIR knockdown (Fig. 2E). Subsequently, we increased WT and mutant HOTAIR expression in CRC cell lines (Fig. 2F) and the results showed that the WT HOTAIR but not the mutant HOTAIR significantly inhibited the expression levels of miR-203a-3p (Fig. 2G). Moreover, we found that the binding of HOTAIR with miR-203a-3p was inhibited by miR-203a-3p inhibitors (Fig. 2H). HOTAIR mutation inhibited miR-203a-3p precipitation with HOTAIR (Fig. 2H). We also found that the expressions of HOTAIR and miR-203a-3p were negatively correlated (Fig. 2I, r = -0.643, P <0.001). Taken together, the results showed that miR-203a-3p was an inhibitory target of HOTAIR in both clinical tissues and CRC cell lines.

HOTAIR promotes the proliferation and drug resistance of CRC cells

To investigate the role of HOTAIR in the proliferation of CRC cells, we knocked down the expressions of HOTAIR in Colo205 and SW620 cells. The results showed that HOTAIR knockdown significantly inhibited the proliferation of CRC cells (Fig. 3A). Overexpression of miR-203a-3p could recapitulate the repressive effect on the proliferation of Colo205 and SW620 cells conferred by HOTAIR knockdown (Fig. 3B). In colony formation assay, the proliferation of CRC cells was inhibited after HOTAIR knockdown (Fig. 4A) or miR-203a-3p overexpression (Fig. 4B). Consistently, results from the Brdu assay also demonstrated that the proliferation was obviously decreased after knockdown of HOTAIR (Fig. 4C) or

Fig. 3. Proliferation assays in Colo205 and SW620 cells. A, MTT assays in Colo205 and SW620 cells transfected with siR-HOTAIR or siR-control. B, Colony formation assays in Colo205 and SW620 cells after transfection with miR-203a-3p or miR-control. * $P < 0.05$.

Fig. 4. HOTAIR knockdown and miR-203a-3p overexpression inhibit CRC cell proliferation. A, BrdU assay in Colo205 and SW620 cells after HOTAIR knockdown. B, BrdU assay in Colo205 and SW620 cells after miR-203a-3p transfection. C, Colony formation assay in Colo205 and SW620 cells after HOTAIR knockdown. D, Colony formation assay in Colo205 and SW620 cells after miR-203a-3p transfection.

overexpression of miR-203a-3p (Fig. 4D). These results suggested that HOTAIR promoted the proliferation of CRC cells.

To analyze whether HOTAIR and miR-203a-3p could regulate drug-resistance of CRC cells, we treated CRC cells with CDDP after HOTAIR knockdown or miR-203a-3p overexpression. As expected, CDDP inhibited cell proliferation in a dose-dependent manner. HOTAIR knockdown or miR-203a-3p overexpression increased the sensitivity of CRC cells to CDDP (Fig. 5A, B). We confirmed these results with another chemotherapy drug, PTX. HOTAIR knockdown and miR-203a-3p overexpression increased the sensitivity of CRC cells to PTX (Fig. 5C, D). More importantly, we found that the expression of HOTAIR was significantly

Fig. 5. HOTAIR knockdown and miR-203a-3p overexpression inhibit the chemoresistance of CRC cells. A, Inhibitory rates of Colo205 and SW620 cells after si-HOTAIR transfection under different concentrations of CDDP detected by MTT assay. B, Inhibitory rates of Colo205 and SW620 cells after miR-203a-3p transfection under different concentrations of CDDP detected by MTT assay. C, Inhibitory rates of Colo205 and SW620 cells after si-HOTAIR transfection under different concentrations of PTX detected by MTT assay. D, Inhibitory rates of Colo205 and SW620 cells after miR-203a-3p transfection under different concentrations of PTX detected by MTT assay. * $P < 0.05$.

Fig. 6. miR-203a-3p targets β -catenin/GRG5 and inhibits Wnt/ β -catenin signaling. A, The protein expression levels of β -catenin and GRG5 in Colo205 and SW620 cells transfected with miR-203a-3p detected by Western blot. B, Correlation of miR-203a-3p expression with β -catenin ($r = -0.574$, $P < 0.001$), and GRG5 ($r = -0.533$, $P < 0.001$) in clinical CRC tissues from 104 CRC patients. C, TOP/FOP luciferase activity in Colo205 and SW620 cells after miR-203a-3p transfection. D, The protein expression levels of Cyclin D1 and Axin2 in Colo205 and SW620 cells after miR-203a-3p transfection. * $P < 0.05$.

up-regulated in drug-resistant cancer cells (Fig. 5E). Therefore, these data suggested that HOTAIR could enhance the drug resistance of CRC cells.

MiR-203a-3p inhibits Wnt/ β -catenin pathway through targeting β -catenin/GRG5

Furthermore, we analyzed the potential targets of miR-203a-3p. Overexpression of miR-203a-3p could reduce the expression of β -catenin and GRG5 in CRC cells (Fig. 6A). The inhibitory effect of miR-203a-3p on β -catenin and GRG5 was also confirmed in 104 cancer tissue samples. The expression of miR-203a-3p and β -catenin were negatively correlated ($r = -0.574$, $P < 0.001$), and it also be negative correlation between miR-203a-3p and GRG5 ($r = -0.533$, $P < 0.001$, Fig. 6B). TOP/FOP luciferase activity assay showed that overexpression of miR-203a-3p significantly inhibited the activity of Wnt/ β -catenin signaling (Fig. 6C). Finally, overexpression of miR-203a-3p in CRC cells significantly reduced the expression of downstream genes in the Wnt/ β -catenin signaling pathway (Fig. 6D).

HOTAIR promotes the proliferation and chemoresistance of CRC cells through targeting miR-203a-3p-Wnt/ β -catenin signaling pathway

After HOTAIR knockdown, we further knocked-down the expression of miR-203a-3p in CRC cells. The inhibitory effect of CRC cell proliferation by HOTAIR knockdown was

Fig. 7. HOTAIR regulates the proliferation and chemoresistance of CRC cells through miR-203a-3p. A, Cell proliferation ability detected by MTT assay. B, Cell proliferation ability detected by colony formation assay, C-D, Cell inhibitory rate after CDDP and PTX treatment detected by MTT assay, E, Protein expression levels of β -catenin and GRG5 detected by Western blot. F, TOP/FOP luciferase activity assay in HCT116 cells in different transfection-groups. * $P < 0.05$.

reversed by knockdown of miR-203a-3p, indicating that the increase of miR-203a-3p played an important role for the inhibitory effect of CRC cell proliferation by HOTAIR knockdown (Fig. 7A). Consistent with the results, miR-203a-3p knockdown completely reversed colony formation in CRC cells induced by HOTAIR knockdown (Fig. 7B). As for the regulation of HOTAIR on drug resistance of CRC cells, we found that HOTAIR knockout could enhance the sensitivity of cancer cells to CDDP and PTX (Fig. 7C, D), but this effect was also completely reversed by knockdown of miR-203a-3p, indicating that miR-203a-3p overexpression was essential for the increased sensitivity of cancer cells induced by HOTAIR knockout. Moreover, miR-203a-3p knockdown reversed the inhibition of β -catenin and GRG5 expression (Fig. 7E). In addition, TOP/FOP luciferase assay showed that HOTAIR knockdown inhibited Wnt/ β -catenin pathway activity, but this inhibition could be reversed by knockdown of miR-203a-3p, indicating that HOTAIR regulated Wnt/ β -catenin pathway by targeting the expression of miR-203a-3p in CRC cells (Fig. 7F). Together, these results demonstrated that HOTAIR suppressed the expression of miR-203a-3p, thus inducing Wnt/ β -catenin signaling activation and CRC cell proliferation and chemoresistance enhancements.

Discussion

In this study, we first detected the expression of HOTAIR in the clinical colon cancer tissues and found that the expression of HOTAIR increased in CRC tissue samples. Multiple lines of evidence have demonstrated that the expression of HOTAIR was upregulated in many kinds of cancers [29-32], but the molecular mechanism of HOTAIR on cancer progression remains unclear.

Based on sequence complementarity, we inferred that miR-203a-3p was the target of HOTAIR by bioinformatics prediction. The expression alteration of HOTAIR resulted in the abundance of miR-203a-3p, but the expression change of miR-203a-3p did not affect the expression of HOTAIR, indicating that miR-203a-3p was in the downstream of HOTAIR. In the clinical tissue samples, the expression of HOTAIR was negatively correlated with the expression of miR-203a-3p, which further confirmed the targeting relationship between the two non-coding RNAs. In order to investigate the roles of HOTAIR and miR-203a-3p in progression and drug resistance of CRC, we further studied them in two CRC cell lines. The results showed that HOTAIR knockdown inhibited the proliferation and decreased drug resistance of CRC cells, which was consistent with previous studies on the carcinogenesis of HOTAIR. After knockdown of HOTAIR, the proliferation of CRC cells was inhibited, indicating that the progression of CRC required HOTAIR physiologically. We also found that miR-203a-3p overexpression or HOTAIR knockdown induced the same phenotype, which were consistent with the negative regulation of HOTAIR on miR-203a-3p.

Growing evidences have focused on the role of miR-203a-3p in cancer progression, but the results were controversial. miR-203a-3p in liver cancer and bladder cancer was up-regulated [33, 34], and the expression of other miR-203 family members could be up-regulated in cervical cells [35]. Other studies have shown that the expression of miR-203a-3p was down-regulated in nasopharyngeal carcinoma [23]. Our results suggested that miR-203a-3p was a tumor suppressor in CRC from the point of view of the effects on cell proliferation and drug resistance. We first investigated the mechanism of miR-203a-3p through target prediction, and found that miR-203a-3p suppressed β -catenin and GRG5 and thus thereby inhibited the activity of Wnt/ β -catenin signaling pathway.

Many studies have implicated that Wnt/ β -catenin signaling pathway played a role in regulation of cell proliferation and drug resistance [36, 37]. In this context, it is reasonable for us to conclude that HOTAIR promotes the proliferation of and enhanced the drug resistance of CRC cells through miR-203a-3p mediated Wnt/ β -catenin signaling pathway. Further studies are still needed to elucidate the effect of HOTAIR on CRC metastasis.

Conclusion

In conclusion, lncRNA HOTAIR regulates progression and chemoresistance of CRC by targeting miR-203a-3p and Wnt/ β -catenin signaling pathway. Our research may provide new strategies for the treatment of CRC.

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Disclosure Statement

The authors declare that they have no competing interests.

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