Original Paper

Upregulation of miR-184 Enhances the Malignant Biological Behavior of Human Glioma Cell Line A172 by Targeting FIH-1

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Key Words
FIH-1 • Human glioma cancer • miR-184 • Malignant biological behavior

Abstract

Background: In recent years, miRNAs have been suggested to play key roles in the formation and development of human glioma. The aim of this study is to investigate the effect and mechanism of miR-184 expression on the malignant behavior of human glioma cells.

Methods: The relative quantity of miR-184 was determined in human glioma cell lines, and the expression of hypoxia-inducible factor-1 alpha (HIF-1\textalpha) was explored using western blotting. The effects of miR-184 inhibition on cell viability and apoptosis were explored, and the miR-184 target gene was determined using a luciferase assay and western blotting. Flow cytometry and Hoechst staining were used to evaluate cell growth and apoptosis. Matrigel invasion and scratch assays were performed to measure the ability of cell invasion and migration.

Results: miR-184 and HIF-1\textalpha protein levels were significantly upregulated in human glioma cells. Downregulation of miR-184 inhibited cell viability and increased the HEB cell apoptotic rate. Luciferase and western blot assays verified that FIH-1 was the target gene of miR-184 and negatively controlled the protein level of HIF-1\textalpha. Inhibition of HIF-1\textalpha by siRNA facilitated the apoptosis of HEB cells and suppressed A172 cell invasion and migration.

Conclusion: miR-184 upregulation enhanced the malignant phenotype of human glioma cancer cells by reducing FIH-1 protein expression.

Introduction

MicroRNA (miRNA) is a type of endogenous RNA of approximately 20-24 nucleotides. Recently, microRNAs have been widely indicated to play key roles in cell growth and apoptosis [1]. Increasing evidences suggest that approximately 70% of mammalian miRNA

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genes are located in transcription micro RNA units (TUs), most of which are located in intron regions [1]. After transcription and processing, an miRNA can bind to the complementary site of its target gene to repress gene expression by inhibiting protein translation or affecting mRNA stability [2]. In the past years, the involvement of miRNAs in cancer pathogenesis has been well established, since they can behave as oncogenes or tumor suppressor genes depending on the cellular function of their targets [3]. Recent research using high-throughput detection technology indicates that human tumor tissues have significantly different miRNA expression spectra and that the abundance of miRNAs in different cancer tissues also has obvious differences [4, 5].

Glioma is the most common primary brain tumor and is produced by brain and spinal cord glial cells. The annual incidence rate of glioma is approximately 3-8 people per 10 million people [6]. Similar to other tumors, glioma mainly results from genetic risk factors and environmental carcinogenic factors. Some genetic diseases, such as neurofibromatosis and tuberous sclerosis disease, are predisposing factors for glioma, and the chances of glioma are much higher in patients with these diseases than in the general population [7]. In addition, some environmental carcinogenic factors may also be associated with the occurrence of glioma. Research has shown that electromagnetic radiation, such as mobile phone use, may be related to glioma [8]. In addition, most glioblastoma patients also have cytomegalovirus infection, and most glioblastoma specimens were found to contain virally infected macrophages [9]. miRNAs have emerged as key factors involved in several biological processes, including development, differentiation, cell proliferation, and tumorigenesis [10, 11]. The dysregulation of miRNAs in cancer has been repeatedly described, for example, in prostate, bladder, and kidney cancer [12], breast cancer [13], and colon cancer [14]. Thus, it is possible that microRNA (miRNA), a highly conserved gene regulatory factor, may shed light on the potential mechanism of glioma [9].

In hypoxia, HIF contributes highly to malignant tumor survival and proliferation. HIF mainly functions as a transcriptional activator and significantly induces the expression of target genes, such as proteins inducing angiogenesis and hypoxia tolerance and inhibiting apoptosis. Thus, HIF plays key roles in tumor formation, growth, and malignant progression [15, 16]. HIF is a heterodimer consisting of two subunits: a constitutively expressed β subunit and an oxygen-regulated α subunit. To date, three α subunits have been identified. The HIF-1α and 2α subunits belong to the same helix-loop-helix (HLH) Per/Arnt/Sim (PAS) (bHLH-PAS) family. The molecular structures of HIF-1α and 2α subunits include bHLH-PAS (PAS mediates their combination with β subunit to form HIF-1 and HIF-2), oxygen-dependent degradation (mediates aerobic degradation), and transcriptional activity (responsible for transcriptional activation) domains [17-19]. In comparison, HIF-3α only contains the bHLH-PAS domain and is deficient in the oxygen-dependent domain. Previous studies have found that HIF-1α is significantly upregulated under aerobic conditions in various cancer types [20, 21]. Under hypoxic conditions, HIF-1α is not degraded and is available to dimerize with constitutively present HIF-1β and interact with p300 in the nucleus, leading to the transcriptional activation of genes, such as those for encoding for erythropoietin and vascular endothelial growth factor [10]. Recently, HIF-1α has been shown to be repressed by factor inhibiting HIF-1 (FIH-1), a protein first identified through its association with HIF-1α in yeast two-hybrid assays [17]. FIH-1 is well known as an asparagine hydroxylase that hydroxylates HIF, thereby inhibiting its association with p300 [19]. In other words, FIH-1 mainly functions as an inhibitor molecule necessary for HIF function. Thus, reduced FIH-1 protein levels may account for increased HIF activity in various cancers.

In this paper, we mainly focus on miR-184 because it has been proven to be dysregulated in various types of tumors [13, 14]. Furthermore, the protein levels of HIF-1α in human glioma cancer cells were found to be significantly upregulated. However, little research has studied the correlation of miR-184 and HIF-1α in human glioma. Thus, this work mainly investigates miR-184 expression patterns and the correlation with HIF-1α in human glioma cancer cells.
Materials and Methods

Cell culture

Human glioma cell lines U251, TJ899, and A172 and human glia HEB cells were purchased from American Type Tissue Culture Collection. TJ861 and TJ905 were gifts from the Department of Pathology, Peking University Health Science Center and were established in our lab and maintained in DMEM supplemented with 10% fetal calf serum at 37°C in 5% CO₂. The U251, TJ899, A172, and HEB cells were cultured in DMEM medium containing 10% (v/v) fetal bovine serum (FBS, HyClone), 100 IU/L penicillin, and 100 mg/L streptomycin at 37°C in a humidified 5% CO₂ atmosphere.

Transient transfections

miR-184 mimics, miR-184 inhibitor, or miR negative control were pre-incubated with HiperFect transfection reagent (QIAGEN) according to the manufacturer’s instructions. The final concentration of microRNA analogs was 100 nmol/L.

Western Blotting Analyses

Cellular proteins were extracted using RIPA buffer (SolarBio, 50 mM Tris/HCl, pH 7.4, 150 mM NaCl 1% (v/v) NP-40, 0.1% (w/v) SDS) containing 1% (v/v) PMSF (SolarBio), 0.3% (v/v) protease inhibitor (Sigma) and 0.1% (v/v) phosphorylated proteinase inhibitor (Sigma). Lysates were centrifuged at 12,000 rpm at 4°C for 15 min and the supernatant was collected for total protein. A BCA protein assay kit (Pierce) was used to determine the protein concentration. Equal amounts of protein (15 μg) was separated on an SDS-PAGE gel (10% (v/v) polyacrylamide) and transferred onto a PVDF membrane. Nonspecific binding was blocked using 8% (w/v) milk in TBS-T for 2 hr at room temperature. The membranes were then incubated with primary antibodies against GAPDH, FIH-1, HIF-1, Bcl-2, and Bax (Cell Signaling) overnight at 4°C. After several washes with TBS-T, the membranes were incubated in HRP-conjugated goat anti-rabbit and anti-mouse IgG or HRP-conjugated mouse anti-goat IgG (Abmart, all at a 1:5000 dilution) for 2 hr at room temperature and then washed. The target proteins were visualized using enhanced chemiluminescence (Millipore) according to the manufacturer’s recommendations, and quantified using density analysis normalized against GAPDH to the manufacturer’s recommendations, and expressed as the fold-change compared to control.

MTT assay

Cell viability was determined using a colorimetric, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. Briefly, HEB cells [5×10⁴ cells per well] were seeded in 96-well tissue culture plates. At 70–80% confluence, the cells were incubated for 16 h in serum-free DMEM medium. After drug treatment, the HEB cells were cultured in fresh medium containing 0.5 mg/ml MTT for a further 4 h. The blue formazan products in the HEB cells were dissolved in DMSO and spectrophotometrically measured at a wavelength of 550 nm.

Hoechst 33258 staining

HEB cells (1×10⁵ cells per well) were cultured in six-well tissue culture plates and at 70–80% confluence, the cells were incubated for 16 h in serum-free DMEM medium. After drug treatment, the medium was removed, and the cells were rinsed once with cold PBS and then fixed with 4% formaldehyde (Zhongshan Technology) in PBS for 15 min (37°C). The cells were washed three times with PBS, and the nuclei were then stained with Hoechst 33258 (10μg/ml) (Sigma) for 5 min before being washed three times with PBS and dried.

RNA isolation and real-time RT-PCR

Total RNA was isolated with RNAiso Plus (Takara Bio) following the manufacturer’s instruction. For the quantitation of miRNA, 10 ng of the total RNA were reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) with specific primers for miR-184 and U6, and subsequently the PCR amplifications were performed in reaction volumes of 20 μL containing 10 μL TaqMan 2× Universal PCR Master Mix No AmpErase UNG (Applied Biosystems), 1 μL 20× TaqMan MicroRNA Assay mix (Applied...
Biosystems) and 1.33 μL template cDNA in the same system used for mRNA quantitation. The thermal cycling conditions were, a hot start step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Relative miRNA expression of miR-184 was normalized against the endogenous control, U6, using the comparative delta–delta CT method.

**Luciferase target assay**

For luciferase assay, the 3’ untranslated region (UTR) or coding region of FIH-1 including the binding sites for miR-184 was amplified from HEB cells by using the specific primers. PCR was performed with genome DNA isolated from NCTC1469 cells and the PCR product was then digested with XhoI (NEB). Then, the fragment was inserted into the Xhol-linearized pGL3 (Promega) luciferase reporter vector. To ensure the ligation efficiency, both the PCR products and the Xhol-linearized pGL3 vectors were treated with thermosensitive alkaline phosphatase (NEB) at 80 °C for 20 min. Details of PCR procedures are described as follows: a hot start step at 95°C for 10 min, followed by 40 cycles at 95 °C for 15 s and 55 °C for 45 s, 72 °C for 30 s. To conduct luciferase reporter assay, 5000 cells per well in a 100 μl medium were seeded in 96-well plates. After incubation overnight, the cells were transfected with the modified firefly luciferase vector (500 ng/μl) with lipofectamine TM 2000 Reagent (Invitrogen) according to the manufacturer’s instruction. 48 h after transfection, the firefly and renilla luciferase activities were measured with the Dual-luciferase reporter assay system (Promega). To control the transfection efficiency, Firefly activity was normalized to renilla activity.

**Apoptosis assay**

Cells (50–60% confluent) were transfected with miR-184 inhibitor or negative control (40 μM, 30min) and washed with 1 x PBS twice. Apoptosis was assessed using an Annexin-V FITC –PI Apoptosis Kit (Invitrogen, Carlsbad, CA). This assay employs fluorescein-labeled Annexin-V in concert with propidium iodide (PI) to detect the cells undergoing apoptosis. Briefly, cells were washed with 1xPBS twice and suspended at 2-3 x 10^6 cells/mL in 1 x Annexin-V Binding Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2). Annexin-V FITC and Propidium Iodide Buffer were added to the cells, which were then incubated at room temperature for 15 minutes in the dark. Cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ) within 1 h of staining using the FL1 (FITC) and FL3 (PI) lines.

**Detection of caspase activity**

Cells transtected with HIF-1α-targeted siRNA or random interference siRNA in serum-free culture were collected and digested with trypsin. The cells were centrifuged at 600 x g at 4 °C for 10-15 minutes and washed with PBS once. Then, cell lysate was added in the ration of 2 million cells in 100 μL lysate according to the kit (Beyotime) and cell precipitation was resuspended. The supernatants were lysed on ice for 15 minutes. Then, cell lysates were centrifuged at 4 °C at 16,000-20,000 x g for 10-15 minutes and the supernatant was transferred to a centrifuge tube pre-cooled with ice bath. Next, the detection buffer was added in accordance with the reagent instruction and the sample was added and appropriately mixed. Subsequently, 10 μl 2 mM ice-cold Ac-DEVD-pNA was mixed at 37 °C for 60-120 minutes. When the color significantly changed, absorbance at 405 nm was measured.

**Matrigel invasion assay in vitro**

HEB cells were cultured in serum-free DMEM medium for 24 - 48 h and the cell culture was centrifuged at 12,000 rpm for 15 min. Then, 200 μl supernatant was separated as chemokine and added to the lower chamber of the Boyden chamber. The 8 μm polycarbonate microporous membrane was placed between the upper and lower chambers. Suspension (400 μl) of cells (2 x 10^5) at logarithmic growth phase was added into the upper chamber and cultured at 37°C with 5% CO_2 for 12h. Liquid in the upper chamber was removed and the non-invasion cells on the membrane surface were wiped off with wet cotton swab. After being rinsed with physiological saline and dried, the chamber was fixed with methanol for 30 min and then stained routinely with hematoxylin & eosin. Each group had 3 parallel samples and the number of cell in 5 fields was counted under a 200× microscope. Then, the average cell number was calculated followed by statistical analysis.
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Cell scratch migration test

Cells in logarithmic growth phase were cultured in 6-well plates until confluence. Then, 200 μl sterile pipetting tip was used to scratch the well bottom and PBS was used to wash off cell debris for 3 times. Then, medium containing 10% fresh fetal calf serum was added into each well, followed by incubation at 37 °C in 5% CO₂ for 24 h before being observed under inverted microscope. Scratch width was measured using built-in ruler of the inverted microscope (10 μm). Scratch migration rate = (scratch width at 0 h – scratch width at 12 h) / scratch width at 0 h × 100%.

Statistical Analysis

The data were expressed as the mean ± SEM. The number of independent experiments was represented by “n”. Multiple comparisons were performed using ne-way ANOVA followed by Tukey’s multiple-comparison test, where P<0.05 was considered significant.

Results

Enhanced expression level of miR-184 and HIF-1α in human glioma cells

We examined the expression level of miR-184 in human glioma cells using real-time quantitative RT-PCR. Compared with HEB cells, miR-184 was increased nearly by 64%, 78%, 47%, 87%, and 123% in human glioma cell lines U251, TJ899, A172, TJ861, and TJ905, as normalized to the U6 gene (Fig. 1A). We next examined the protein levels of HIF-1α in these five human glioma cancer cells. Compared with HEB cells, HIF-1α was significantly upregulated in U251, TJ899, A172, TJ861, and TJ905 cells (Fig. 1B).

Inhibition of miR-184 reduced HEB cell line viability

Using a transient transfection method, human glioma cell line HEB was transfected with miR-184 mimics, inhibitor, or negative control for 24, 48, and 72 h. In this study, the mimics were analogs that enhanced miR-184 expression level; inhibitors were analogs that decreased the expression of miR-184. As expected, when miR-184 mimics were transfected into human glioma cell line HEB, cell viability was significantly increased by 25% and 34% at 48 h and 72 h, respectively (Fig. 2A). Conversely, when the miR-184 expression level was
Fig. 2. miR-184 negatively regulates human glioma HEB cell viability. (A) HEB cells were transfected with miR-184 mimics, inhibitor (B), or negative control for 24, 48, and 72 h. Cell viability was determined using the MTT assay. The data represent the means ± SEM of n=6 independent experiments. *P<0.05 versus control.

Fig. 3. Inhibition of miR-184 induced HEB cell apoptosis. Inhibition of miR-184 enhanced cell apoptosis by 1.8-fold versus the negative control in HEB cells (A), as tested using an Annexin V and PI kit. (B) Apoptotic HEB cells increased when transfected with the miR-184 inhibitor, as examined by Hoechst 33342 staining. The white arrow indicates apoptotic cells. As assessed by western blotting, the overexpression of miR-184 elevated the expression level of Bcl-2 and reduced the levels of Bax and Bim (C); the inhibition of miR-184 decreased the level of Bcl2 and increased the levels of Bax and Bim (D). The data represent the means ± SEM of n=3 independent experiments. **P<0.01 versus control.

Inhibited, cell viability was reduced by 15% and 30% at 48 h and 72 h, respectively (Fig. 2B).

Inhibition of miR-184 induced HEB cell apoptosis

We next determined that the inhibition of miR-184 enhanced HEB apoptosis by nearly 1.8-fold versus the negative control (Fig. 3A). Hoechst 33342 staining also showed a higher
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degree of apoptosis in the HEB cells transfected with an miR-184 inhibitor compared with the negative control (Fig. 3B). Furthermore, a western blot analysis of apoptotic protein expression showed that the overexpression of miR-184 restored the expression of Bcl-2 and reduced the levels of Bax and Bim (Fig. 3C). In contrast, the inhibition of miR-184 reduced the levels of Bcl2 and enhanced those of Bax and Bim (Fig. 3D).

miR-184 positively regulated HIF-1α expression by targeting FIH-1

According to TargetScan, FIH-1 is predicted to be a target of miR-184. As shown in Fig. 4A, the nucleotides in the 3’-untranslated region (3’-UTR) of FIH-1 from 41 bp to 47 bp in mammals are highly conserved, and this sequence was predicted to be the target site of miR-184. A luciferase reporter assay was then used to assess whether miR-184 can directly bind to the 3’-untranslated region of FIH-1. The overexpression of miR-184 significantly reduced luciferase activity in HEK 293T cells transfected with the luciferase reporter vector containing the 3’-UTR region (Fig. 4B). To further confirm whether miR-184 could target FIH-1, we used western blotting to detect the expression level of FIH-1. As shown in Fig. 4C, at 48 hours after transfection with miR-184 mimics, the expression level of FIH-1 in HEB cells was decreased by 70%, and HIF-1α was significantly upregulated (Fig. 4C). Conversely, when miR-184 was inhibited in HEB cells, the expression level of FIH-1 was increased, and HIF-1α was decreased (Fig. 4D). These results indicated that FIH-1 is the target gene of miR-
184. We next investigated whether the downregulation of FIH-1 is an integral part of miR-
184-induced HIF-1α overexpression. To this end, an siRNA targeting FIH-1 was selected. 
Transfection of the miR-184 inhibitor alone led to enhanced HIF-1α expression, whereas the 
suppression of FIH-1 by siRNA reduced the protein level of HIF-1α, even in cells that were 
co-transfected with the miR-184 inhibitor (Fig. 4E). Taken together, these results indicate 
that miR-184 positively regulated HIF-1α expression by targeting FIH-1.

Inhibition of HIF-1α by siRNA enhanced the apoptosis of HEB cells

To explore the influence of HIF-1α on HEB cell apoptosis, Hoechst staining, flow 
cytometry, and spectrophotometry were used. Hoechst 33258 fluorescence staining 
showed that the number of apoptotic cells transfected with the HIF-1α-targeting siRNA 
was significantly increased compared to that of the random siRNA group at 48 h of serum 
starvation (Fig. 5A). Consistent with this observation, flow cytometry using Annexin V-PI 
staining in cells treated in the same way demonstrated that the percentage of apoptotic cells 
in the HIF-1α-targeting siRNA group was higher than that in the random siRNA group at 48 h 
(Fig. 5B). Furthermore, the detection of caspase 3 activity using spectrophotometry showed 
that the activity of caspase 3 in the HIF-1α-targeting siRNA group was higher than that in 
the random siRNA group (Fig. 5C). These data indicated that inhibition of HIF-1α by siRNA 
facilitated the apoptosis of HEB cells.

Inhibition of HIF-1α suppressed A172 cell invasion and migration

To test the effect of HIF-1α on A172 cell invasion and migration, a Matrigel invasion 
assay, scratch migration test, and western blotting were performed. The in vitro invasion 
assay showed that the number of transmembrane cells in the HIF-1α-targeting siRNA 
group (34.33 ± 2.68) was lower than that in the random siRNA group (82.13 ± 5.26), with a 
statistically significant difference (Fig. 6A). The results of the scratch migration test showed 
that the migration rate was 15% in the cells transfected with the HIF-1α-targeting siRNA,
which was significantly lower than the migration rate of the random siRNA group (40%), suggesting that reduced HIF-1α protein expression significantly weakened the migration ability of the cells (Fig. 6B). Western blotting showed that MMP-2 and E-cadherin were significantly reduced in the cells transfected with the HIF-1α-targeting siRNA, (Fig. 6C), indicating that HIF-1 interference might induce cell migration. These data demonstrated that the inhibition of HIF-1α suppressed A172 cell invasion and migration.

**Discussion**

miRNAs are non-coding small RNAs that mainly repress mRNA translation in cells. Through their incomplete complementary sequence with a large amount of mRNAs, miRNAs have been proven to have important functions in various oncogenic signaling pathways, such as cell proliferation and migration [22]. miRNAs can be divided into oncogenes and tumor suppressor genes according to their target genes. Glioma is the most common malignant tumor of the central nervous system, accounting for approximately 40% ~ 50% of all intracranial tumors [23]. However, the use of modern microsurgery, radiotherapy, chemotherapy, and other comprehensive treatment measures is still not ideal for the prognosis of glioma patients. Therefore, elucidating the molecular mechanism of glioma occurrence and development, as well as finding key signaling pathways and regulatory factors, has become the focus of brain glioma research [24].

miR-184 was reported to be widely dysregulated in human tumors [25]. In this study, we first explored the expression level of miR-184 in human glioma cells. RT-qPCR revealed that the relative quantity of miR-184 was significantly upregulated in human glioma cells, accompanied by enhanced protein levels of HIF-1α. Furthermore, we also demonstrated that the inhibition of miR-184 inhibited HEB cell growth and enhanced cell apoptosis. More
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The expression of HIF-1α was significantly reduced using a specific siRNA targeting HIF-1α, and this was accompanied by a notable growth inhibition in HEB cells. Flow cytometry and Hoechst staining confirmed that reduced HIF-1α expression enhances cell apoptosis in the absence of serum starvation. HIF-1α can also induce a variety of apoptosis-related proteins, such as the pro-apoptosis proteins BNIP3 and Noxa [28, 29]. IPAS is a negative regulator of HIF-1 and leads to mitochondrial depolarization and caspase 3 activation by binding to Bcl-2 family members Bcl-xL, Bcl-w, and Mcl-1, thereby promoting cell apoptosis [30]. These data confirm the effect of HIF-1 with regard to inhibiting cell apoptosis, which mainly occurs through the mitochondria/caspase pathway.

This study further explored the invasion and migration capability of cells transfected with an HIF-1α-targeting siRNA. Matrigel transmembrane invasion and scratch migration tests showed that reduced HIF-1α protein expression inhibited the invasion and migration ability of HEB cells. Multiple target proteins of HIF-1 are involved in invasion and migration processes, including extracellular matrix degradation target proteins such as urokinase-type plasminogen activator receptor, collagen prolyl hydroxylase, matrix metalloproteinase (e.g., MMP-2), and intercellular adhesion molecules such as E-cadherin. This study also found that the reduced expression of the HIF-1α protein inhibited the expression of MMP-2 and E-cadherin, indicating the important role of HIF-1α in human glioma cell invasion and migration [31].

In conclusion, our data first demonstrate the enhanced expression level of miR-184 in human glioma cells. Furthermore, we also confirmed that miR-184 underexpression inhibits HEB cell viability and induces cell apoptosis. More importantly, FIH-1 is confirmed as the direct target of miR-184 in human glioma cells. By targeting FIH-1, miR-184 positively regulates the expression of HIF-1α, thereby facilitating tumor survival and promoting malignant progression. The pro-survival effect of miR-184 may shed light on the treatment of human glioma cancers.

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Disclosure Statement

The authors have no conflicts of interest to declare.
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