Upregulated lncRNA CASC9 Contributes to Progression of Non-Small Cell Lung Cancer Through Inhibition of miR-335-3p and Activation S100A14 Expression

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Introduction: Non-small cell lung cancer (NSCLC) is a deadly cancer type worldwide and the main sub-type of lung cancer. Cancer susceptibility candidate-9 (CASC9) was reported to be a key player in cancer progression. However, its function and underlying mechanism in NSCLC remain unclear.

Materials and Methods: Expression level of CASC9 in NSCLC tissues and cells was measured with RT-qPCR. Biological roles of CASC9 in NSCLC were analyzed with a series of in vitro experiments. Potential mechanisms of CASC9 in NSCLC were analyzed by predicting and validating the possible targets of CASC9 in NSCLC.

Results: In this study, we found CASC9 expression was upregulated in NSCLC tissues and cell lines. High CASC9 expression was identified as a predictor for poorer overall survival of NSCLC patients. Furthermore, functional assays showed CASC9 knockdown suppressed NSCLC cell proliferation, migration, and invasion, while CASC9 overexpression caused opposite effects. We also found microRNA-335-3p (miR-335-3p) could act as a target of CASC9 in NSCLC and the inhibition effect of CASC9 knockdown on NSCLC progression required the activity of miR-335-3p. In addition, we identified S100 calcium-binding protein A14 (S100A14) acts as a target of miR-335-3p.

Discussion: Taken together, our study suggested CASC9 could promote NSCLC progression via miR-335-3p/S100A14 axis. The CASC9/miR-335-3p/S100A14 regulatory triplets identified in this work might provide new therapeutic strategies for NSCLC treatment.

Keywords: non-small cell lung cancer, CASC9, miR-335-3p, S100A14, ceRNA

Introduction

Non-small cell lung cancer (NSCLC) represents about 85% of all lung cancer cases.¹ NSCLC patients were often diagnosed at late tumor stages, which results in poorer overall survival of cancer patients.²

Long non-coding RNA (lncRNA, above 200 nucleotides in length) is regarded as crucial regulators for cancer expression in recent years.^{3,4} lncRNA cancer susceptibility candidate-9 (CASC9), located at chromosome 8q21.11, was found closely associated with cancer progression. In colorectal cancer, CASC9 expression was found elevated in tumor tissues and correlated with poorer overall survival.⁵ Functionally, CASC9 knockdown inhibits colorectal cancer growth through regulating cleavage and polyadenylation specificity factor subunit 3 and transforming growth factor- β signaling pathway.⁵ Zhang

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et al reported high CASC9 level was a predictor for poorer overall survival of patients with cervical cancer.⁶ Hu and co-authors reported CASC9 could promote ovarian cancer progression in vitro and in vivo through upregulating LIN7A expression.⁷ Moreover, a previous study revealed CASC9 could promote esophageal squamous cell carcinoma metastasis through binding with CREB-binding protein to promote LAMC2 expression.⁸ However, the expression and regulatory mechanism of CASC9 in NSCLC remains to be explored.

S100 calcium-binding protein A14 (S100A14) is a member of S100 protein family and consisted of 104 amino acids.⁹ Meanwhile, the gene that encodes S100A14 protein was mapped to chromosome 1q21.⁹ Several studies have conducted to analyze the roles of S100A14 in cancers and revealed S100A14 functions as an oncogenic role in lung adenocarcinoma and serous ovarian carcinoma.^{10–12} However, the molecules that can regulate S100A14 in NSCLC remain to be explored.

Our study aimed to investigate CASC9 levels in NSCLC tissues and cell lines. Prognostic value of CASC9 on NSCLC patients was analyzed with Kaplan-Meier curve. Effects of CASC9 on NSCLC cell proliferation, migration, and invasion were explored in vitro. Moreover, we further investigated underlying mechanisms by which CASC9 regulates NSCLC progression.

Materials and Methods

Patients

43 NSCLC patients who did not receive anti-cancer treatment were enrolled in this work. Study protocol was approved by the ethics committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Tumor tissues and normal tissues obtained from these patients were snap-frozen in liquid nitrogen and stored at -80°C for further usage. Informed written consent was obtained from all patients. This work was performed in compliance with Declaration of Helsinki. Clinicopathological data including age, gender, tobacco smoking status, alcohol drinking status, tumor stage and tumor size are summarized in Table 1.

Cell Lines

NSCLC cells (A549, H1299) and lung epithelial cell line (BEAS-2B) were obtained from American Type Culture Collection (ATCC). These cells were incubated in DMEM contains 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a 37°C humidified incubator with 5% CO_2 .

Table 1 Correlations of CASC9 Expression and Clinicopathological Features of NSCLC Patients

Variable	No.	CASC9 Expression		χ^2	P value
		High	Low		
Sex					
Male	24	15	9	0.604	0.437
Female	19	14	5		
Age (years)					
≥ 50	22	12	10	0.660	0.417
< 50	21	14	7		
Tumor size (cm)					
≥ 5	28	21	7	5.121	0.024
< 5	15	6	9		
Smoking status					
Non-smoker	20	11	9	0.010	0.920
Smokers	23	13	10		
Drinking status					
Non-drinker	17	10	7	0.103	0.748
Drinker	26	14	12		
Tumor stage					
I–II	18	7	11	4.714	0.030
III	25	18	7		

Cell Transfection

pcDNA3.1 contains sequence of CASC9 or S100A14 expression vector (pCASC9 or pS100A14) was constructed by GenScript (Nanjing, Jiangsu, China). miR-335-3p mimic (5'-UUUUUCAUUAUUGCUCCUGACC-3') and corresponding negative control (miR-NC, 5'-GUCUCAUCGUCCUUAUUCUUAU-3') were purchased from GenePharma (Shanghai, China). Small interfering RNA targeting CASC9 (si-CASC9, 5'-CAGCAAAGCAAUGGAAGCAU GUAUU-3') and siR-NC (5'-UUCUCCGAACGUGUCAC GUTT-3') were also bought from GenePharma. Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc.) after incubation these cells to about 80% confluence. Cells were collected for following analyses after 48 h transfection.

RNA Extraction and Quantitative Real-Time PCR (RT-qPCR)

RNA was isolated from tissues and cells with Trizol reagent (Invitrogen). After concentration quantification using NanoDrop-1000 (Thermo Fisher Scientific, Inc.), RNA was reverse transcribed into complementary DNA

using Reverse Transcription kit (Takara, Dalian, Liaoning, China). RT-qPCR was performed at ABI 7500 system (Applied Biosystems, Foster City, CA, USA) using SYBR Green (Takara) with primers: CASC9: F: 5'-TTG GTCAGCCACATTCATGGT-3', R: 5'-AGTGCCAATGA CTCTCCAGC-3'; S100A14: F: 5'-GTCGGTCAGCCAA CGCAGAG-3', R: 5'-CAGGCCACAGTTGCTCGG-3'; MMP-2: F: 5'-TGATTCTGGTCGCTCAGATG-3', R: 5'-C TTGTTTCCAGGAAGGTGA-3'; MMP-9: F: 5'-AGG ACCATGGGGATCCTTAC-3', R: 5'-AACACAAGGCTG CCCATTAC-3'; E-Cadherin: F: 5'-TAACCGATCAG AATGAC-3', R: 5'-TTTGTGACGGGAGCTCAGGAT-3'; N-Cadherin: F: 5'-CAACTTGCCAGAAAACCTCCAGG-3', R: 5'-ATGAAACCGGGCTATCTGCTC-3'; GAPDH: F: 5'-GGGAGCCAAAAGGGTCATCA-3', R: 5'-TGATG GCATGGACTGTGGTC-3'; miR-335-3p: F: 5'-UUUUUC AUUAUUGCUCUGACC-3', R: 5'-CCAGTCTCAGGG TCCGAGGTATTC-3'; U6 snRNA: F: 5'-CTCGCTTCG GCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'. Relative expression level was calculated using 2- $\Delta\Delta C_t$ method.

Western Blot

Tissues and cells were collected and lysed with RIPA buffer to extract protein samples. BCA kit (Beyotime) was used to quantify protein concentration. Then, protein samples were isolated at SDS-PAGE, transferred to PVDF membrane, and treated with anti-S100A14 (ab251779, Abcam, Cambridge, MA, USA) and anti-GAPDH (ab181602, Abcam) at 4°C for overnight. Then, secondary antibody (ab6721) was added and incubated for 4 h. Band signals were visualized using BeyoECL kit and analyzed with Image J software.

Immunohistochemistry (IHC) Analysis of S100A14 in NSCLC Tissues

Protein levels of S100A14 in NSCLC tissues and normal tissues analyzed with the IHC method were analyzed at Human Protein Atlas.

Cell Proliferation Assay

Cell Counting Kit-8 (CCK-8, Beyotime, Haimen, Jiangsu, China) assay was used to measure cell viability. Cells were seeded into 96-well plate at the density of 5×10^3 cells/well and incubated for 0, 24, 48, and 72 hrs. At these time points, CCK-8 reagent was added to each well and

incubated for another 4 hrs. Microplate reader was used to measure optical density at 450 nm.

Wound Healing Assay

Cell surface was scratched with pipette tip after incubation of these cells in serum-free medium to 90% confluence. PBS was used to remove cell debris, and then observed under a microscope at 0 hrs and 24 hrs after wound creation.

Transwell Invasion Assay

DMEM was used to resuspend collected cells and then filled into upper chamber of Matrigel-coated insert (BD Bioscience, San Jose, CA, USA), while DMEM contains 10% FBS was filled into lower chamber. 48 hrs later, non-invasive cells were removed, while invasive cells were fixed by methanol, stained with crystal violet, and counted under microscope.

Bioinformatic Analysis

miRNA targets of CASC9 were predicted at DIANA LncBase v_2, and we found miR-335-3p was a putative target. Subsequently, TargetScan was used to predict targets of miR-335-3p and revealed S100A14 was a potential target.

Luciferase Activity Reporter Assay

Wild-type (wt) sequence of CASC9 or S100A14 contains a binding region for miR-335-3p was inserted into psi-CHECK-2 (Promega, Madison, WI, USA) to generate wt-CASC9 or wt-S100A14. Mutant luciferase vectors (mt-CASC9 or mt-S100A14) were constructed using site-direct mutagenesis kit (Takara) to lose miR-335-3p binding sites. Cells were co-transfected with luciferase vectors and miRNAs using Lipofectamine 2000. 48 hrs later, dual-luciferase activity system (Promega) was utilized to measure relative luciferase activity using *Renilla* luciferase as internal control.

RNA Immunoprecipitation (RIP) Assay

RIP assay was performed using Magna RIP Kit (EMD Millipore, Billerica, MA, USA) to analyze the co-enrichment of CASC9, miR-335-3p, and S100A14. Cells were lysed using RIP buffer to obtain cell pellets. Then, anti-Ago2 antibody-conjugated magnetic beads were used to incubate with these pellets. RNA sample was isolated from these pellets after treatment with Proteinase K and RNase-free DNase. qRT-PCR was performed to detect CASC9, miR-335-3p, and S100A14 expression.

Statistical Analysis

Results obtained from three independent experiments were analyzed with GraphPad Prism (GraphPad Prism Software Inc, San Diego, CA, USA) and then expressed as mean $\pm$ SD. Student's *t*-test and one-way analysis of variance with Tukey post-hoc test were used for difference analyses. Kaplan-Meier curve with Log-rank test was used to analyze the effect of CASC9 on overall survival of NSCLC patients. Pearson's coefficient was used to analyze correlations of miR-335-3p with CASC9 or S100A14. Chi-square test was used to analyze the correlation of CASC9 expression and clinicopathological parameters. P value of less than 0.05 was regarded as statistically significant.

Results

CASC9 Expression Level Was Elevated in NSCLC

Firstly, we found CASC9 was highly expressed in NSCLC tissues compared with normal tissues (Figure 1A). Using the median expression level of CASC9 as cut-off value, these patients were classified into high or low groups. Kaplan-Meier curve showed high CASC9 expression was a predictor for poorer overall survival of cancer patients (Figure 1B). Then, we analyzed the correlations of CASC9 expression and clinicopathological parameters. It was found the high CASC9 expression was significantly correlated with tumor size and tumor stage, while it did not have correlations with other collected clinicopathological parameters (Table 1). In addition, we showed CASC9 expression level was elevated in NSCLC cells compared with normal cell (Figure 1C).

CASC9 Overexpression Promotes NSCLC Cell Proliferation, Migration, and Invasion

To explore the roles of CASC9 in NSCLC, gain-of-function experiments were performed at first. The results showed pCASC9 transfection significantly increased CASC9 level in NSCLC cells (Figure 2A). CCK-8 assay showed CASC9 overexpression promotes NSCLC cell proliferation (Figure 2B). Additionally, wound-healing assay and transwell invasion assay showed CASC9 overexpression promoted NSCLC cell migration and invasion abilities compared with control groups (Figure 2C and D). Analysis of the related markers including MMP-2, MMP-9, E-Cadherin, and N-Cadherin in these groups showed that MMP-2, MMP-9, N-Cadherin expressions were increased, while E-Cadherin expression was decreased by pCASC9 (Figure 2E).

CASC9 Knockdown Inhibits NSCLC Cell Proliferation, Migration, and Invasion

To further investigate the functions of CASC9, we knock-down its expression in NSCLC cell using si-CASC9 (Figure 3A). CCK-8 assay demonstrated CASC9 knock-down inhibits NSCLC cell growth (Figure 3B). Moreover, the introduction of si-CASC9 hinders NSCLC cell migration and invasion abilities (Figure 3C and D). After the knockdown of CASC9, MMP-2, MMP-9, N-Cadherin expressions were decreased, while E-Cadherin expression was increased in NSCLC cells (Figure 3E).

Figure 1 CASC9 was elevated expression in NSCLC.

Notes: (A) RT-qPCR results showed CASC9 was upregulated in NSCLC tissues compared with normal tissues. (B) Kaplan-Meier curve showed that high CASC9 expression was a predictor for poor overall survival of cancer patients. (C) RT-qPCR results showed CASC9 was upregulated in NSCLC cell lines compared with normal cell line. *** $P < 0.001$.

Abbreviations: RT-qPCR, quantitative real-time PCR; NSCLC, non-small cell lung cancer; CASC9, cancer susceptibility candidate-9.

Figure 2 CASC9 overexpression promotes NSCLC cell proliferation, migration, and invasion.

Notes: (A) RT-qPCR results showed CASC9 was upregulated in NSCLC cell lines after pCASC9 transfection. (B) Cell counting kit-8 assay showed that CASC9 overexpression accelerates NSCLC cell proliferation. (C) Wound-healing assay showed CASC9 overexpression increased cell migration ability. (D) Transwell assay showed pCASC9 transfection enhanced invasion ability. (E) Expression of MMP-2, MMP-9, N-Cadherin, and E-Cadherin in NSCLC cells after pCASC9 transfection. ***P < 0.001; **P < 0.01.

Abbreviations: RT-qPCR, quantitative real-time PCR; NSCLC, non-small cell lung cancer; CASC9, cancer susceptibility candidate-9.

CASC9 Directly Regulate miR-335-3p Expression

Then, we analyzed mechanisms of CASC9 in NSCLC, and we showed miR-335-3p was a putative target for CASC9 (Figure 4A). RT-qPCR showed transfection of miR-335-3p mimic decreases CASC9 levels in NSCLC cells (Figure 4B). Dual-luciferase activity reporter assay

validated interactions between CASC9 and miR-335-3p (Figure 4C). Moreover, we showed miR-335-3p expression was decreased in NSCLC tumor tissues and cell lines compared with normal counterparts (Figure 4D and E). Importantly, we showed CASC9 and miR-335-3p were inversely correlated in NSCLC tissues (Figure 4F).

Figure 3 CASC9 knockdown inhibits NSCLC cell proliferation, migration, and invasion.

Notes: (A) RT-qPCR results showed CASC9 was downregulated in NSCLC cell lines after si-CASC9 transfection. (B) Cell counting kit-8 assay showed that CASC9 downregulation inhibits NSCLC cell proliferation. (C) Wound-healing assay showed CASC9 downregulation decreased cell migration ability. (D) Transwell assay showed si-CASC9 transfection decreased invasion ability. (E) Expression of MMP-2, MMP-9, N-Cadherin, and E-Cadherin in NSCLC cells after si-CASC9 transfection. *** $P < 0.001$; ** $P < 0.01$.

Abbreviations: RT-qPCR, quantitative real-time PCR; NSCLC, non-small cell lung cancer; CASC9, cancer susceptibility candidate-9; si-CASC9, small interfering RNA targeting cancer susceptibility candidate-9; siR-NC, negative control small interfering RNA.

S100A14 Was the Direct Target of miR-335-3p

Afterward, we explored targets of miR-335-3p using TargetScan. We found S100A14 ranks top among all the predicted targets and therefore was selected for analyses (Figure 5A). RT-qPCR indicated miR-335-3p

overexpression decreased S100A14 levels in NSCLC cells (Figure 5B). Western blot indicated S100A14 protein levels were also suppressed by miR-335-3p mimic (Figure 5C). To further confirm the connection of miR-335-3p and S100A14, dual-luciferase activity reporter assay was performed. We showed miR-335-3p mimic

Figure 4 CASC9 acts as competing endogenous RNA for miR-335-5p.

Notes: (A) Binding module between CASC9 and miR-335-3p. (B) CASC9 expression in NSCLC cells with synthetic miRNAs transfection. (C) Relative luciferase activity in NSCLC cells with synthetic miRNAs and luciferase activity vectors transfection. RT-qPCR results showed miR-335-3p was downregulated in NSCLC (D) tissues and (E) cell lines. (F) Correlation of CASC9 and miR-335-3p in NSCLC tissues. ***P < 0.001; **P < 0.01.

Abbreviations: RT-qPCR, quantitative real-time PCR; NSCLC, non-small cell lung cancer; CASC9, cancer susceptibility candidate-9; miR-335-3p, microRNA-335-3p; miR-NC, negative control microRNA; wt, wild-type; mt, mutant.

inhibited luciferase activity in cells transfected with wt-S100A14 (Figure 5D). RIP assay further indicated that CASC9, miR-335-3p, and S100A14 were co-enriched by anti-Ago2 (Figure 5E). The analysis of S100A14 expression in NSCLC tissues and cells using qRT-PCR indicated S100A14 expression was elevated in tumor tissues and cell lines compared with normal groups (Figure 5F and G). Western blot showed S100A14 protein levels were also increased in both NSCLC tissues and cells investigated (Figure 5H and I). We further analyzed the protein level of S100A14 in tumor tissues using IHC assay and confirmed S100A14 was increased expression in NSCLC tissues (Figure 5J). Moreover, we found a significant inversely relationship between miR-335-3p and S100A14 in tumor tissues (Figure 5K).

CASC9 Promotes NSCLC Progression via S100A14

At length, we investigated whether S100A14 was responsible for the roles of CASC9 in NSCLC through co-transfecting of si-CASC9 and pS100A14. We showed S100A14 level could be elevated by pS100A14 and inhibited by si-CASC9 (Figure 6A). S100A14 overexpression promoted NSCLC cell proliferation, migration, and invasion (Figure 6B–D). We also showed restoration of S100A14 partially reversed the effects of si-CASC9 on NSCLC progression (Figure 6B–D).

Discussion

lncRNAs have been revealed to play crucial roles in contributing NSCLC progression, however only few of them have been functionally characterized.⁴ For instance,

Figure 5 S100A14 was the direct target for miR-335-5p.

Notes: (A) Binding module between S100A14 and miR-335-3p. (B) S100A14 expression in NSCLC cells with synthetic miRNAs transfection. (C) S100A14 protein expression in NSCLC cells with synthetic miRNAs transfection. (D) Relative luciferase activity in NSCLC cells with synthetic miRNAs and luciferase activity vectors transfection. (E) RIP assay revealed CASC9, miR-335-3p, and S100A14 were co-enriched in anti-Ago2 pellets. RT-qPCR results showed S100A14 was increased in NSCLC (F) tissues and (G) cell lines. Western blot to analyze S100A14 protein expression in NSCLC (H) tissues and (I) cell lines. (J) IHC assay to detect S100A14 expression in NSCLC tissues and normal tissues. (K) Correlation of S100A14 and miR-335-3p in NSCLC tissues. ***P < 0.001; **P < 0.01.

Abbreviations: RT-qPCR, quantitative real-time PCR; NSCLC, non-small cell lung cancer; S100A14, S100 calcium binding protein A14; miR-335-3p, microRNA-335-5p; miR-NC, negative control microRNA; wt, wild-type; mt, mutant.

Li et al showed LINC00968 was elevated expression in NSCLC.¹³ Xia et al showed lncRNA PLAC2 was decreased expression in NSCLC tissues and correlated with poorer overall survival of cancer patients.¹⁴ Also, they reported force PLAC2 expression could inhibit NSCLC cell migration and invasion via targeting miR-21.¹⁴ Another work showed knockdown of cancer susceptibility 15 could inhibit NSCLC progression via regulating epithelial-mesenchymal transition associated molecules.¹⁵

In our work, we showed CASC9 level was elevated in both tumor tissues and cell lines. Also, we showed high CASC9 expression was an indicator for poorer overall survival of NSCLC patients, suggesting CASC9 might be a prognostic biomarker. Through a series of in vitro experiments, we showed CASC9 could promote NSCLC cell proliferation, migration, and invasion. By analyzing the epithelial-mesenchymal transition (EMT) markers and two matrix metalloproteinases (MMPs), we showed MMP-2, MMP-9, N-Cadherin expression can be increased, while

Figure 6 CASC9 promotes NSCLC progression through S100A14.

Notes: (A) RT-qPCR to detect S100A14 expression after si-CASC9+pcDNA3.1, si-CASC9+pS100A14, siR-NC+pcDNA3.1, or siR-NC+pS100A14 transfection. (B) Cell counting kit-8 assay showed that S100A14 overexpression accelerates NSCLC cell proliferation. (C) Wound-healing assay showed S100A14 overexpression increased cell migration ability. (D) Transwell assay showed pS100A14 transfection enhanced invasion ability. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Abbreviations: RT-qPCR, quantitative real-time PCR; NSCLC, non-small cell lung cancer; CASC9, cancer susceptibility candidate-9; S100A14, S100 calcium-binding protein A14; si-CASC9, small interfering RNA targeting cancer susceptibility candidate-9; siR-NC, negative control small interfering RNA.

E-Cadherin was decreased by CASC9 overexpression. These results suggested CASC9 might function as an oncogenic lncRNA in NSCLC.

lncRNA-miRNA-mRNA regulatory triplets proposed by Salmena have been well recognized.^{2,16} For instance, CASC9 functions as competing endogenous RNA to regulate TWIST2 expression through sponging miR-215 in breast cancer.⁶ According to bioinformatic analysis results and luciferase activity reporter assay, we identified miR-335-3p as a target of CASC9. Through RT-qPCR analysis, we showed miR-335-3p mimic transfection could decrease CASC9 expression level in NSCLC cells. miR-335-3p has

reported decreased expression in childhood acute lymphoblastic leukemia, and correlated with poor overall survival of cancer patients.¹⁷ Also, miR-335-3p was revealed can be regulated by lncRNAs EAT1 and MALAT1 to affect cancer progression and drug resistant.¹⁷ Moreover, miR-335-3p was reported could be regulated by LINC00969 to affect intervertebral disk degeneration.¹⁸ We further investigated the targets of miR-335-3p using TargetScan. We revealed miR-335-3p may directly target S100A14, an oncogene in lung adenocarcinoma.^{10,11} Additionally, we showed S100A14 was increased expression in NSCLC tissues and cell lines using RT-qPCR analysis, Western

blot, and IHC assay. RIP assay further confirmed the connections of CASC9, miR-335-3p, and S100A14. Functionally experiments showed CASC9 could promote NSCLC malignant behaviors via regulating S100A14.

Conclusion

In conclusion, our work showed CASC9 functions as an oncogenic lncRNA in NSCLC through regulating miR-335-3p/S100A14 axis. Our work may provide novel therapeutic targets for NSCLC treatment.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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