MiR-9-1 Suppresses Cell Proliferation and Promotes Apoptosis by Targeting UHRF1 in Lung Cancer

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Abstract
Lung cancer is listed as the most common reason for cancer-related death all over the world despite diagnostic improvements and the development of chemotherapy and targeted therapies. MicroRNAs control both physiological and pathological processes including development and cancer. A microRNA-9 to 1 (miR-9 to 1) overexpression model in lung cancer cell lines was established and miR-9 to 1 was found to significantly suppress the proliferation rate in lung cancer cell lines, colony formation in vitro, and tumorigenicity in nude mice of A549 cells. Ubiquitin-like containing PHD and RING finger domains 1 (UHRF1) was then identified to direct target of miR-9 to 1. The inhibition of UHRF1 by miR-9 to 1 causes G1 arrest and p15, p16, and p21 were re-expressed in miR-9 to 1 group in mRNA level and protein level. Silence of UHRF1 expression in A549 cells resulted in the similar re-expression of p15, p16, p21 which is similar with miR-9 to 1 infection. Therefore, we concluded that UHRF1 is a new target for miR-9 to 1 to suppress cell proliferation by re-expression of tumor suppressors p15, p16, and p21 mediated by UHRF1.

Keywords
miR-9 to 1, UHRF1, lung cancer, proliferation, apoptosis

Abbreviations
miR-9 to 1, microRNA-9 to 1; UHRF1, ubiquitin-like containing PHD and RING finger domains 1; 3′UTR, 3′untranslated region.

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Introduction

In higher eukaryotes, MicroRNAs (miRNAs) can be used as a posttranscriptional regulator of gene expression.1-3 Recent estimates have found that 20%-30% of all genes are targets of miRNA. Therefore, miRNAs are considered to play a significant role in organisms.4-6 MiRNAs control both physiological and pathological processes such as development and cancer.7-10 MicroRNA-9 to 1 (miR-9-1) is initially identified as an important regulator which plays a pivotal role in nervous system development.11-13 In insects and humans, the mature miR-9 to 1 sequence is found to be widespread.14 In addition, except for *Xenopus laevis*, the mature sequence of miR-9 to 1 in all vertebrates was completely consistent, suggesting that the role of miR-9 to 1 is widely conservative.15 MiR-9 to 1 is expressed almost in the brain and is a mediator in mammalian neurogenesis.16 The function of miR-9 to 1 in nerve system development and differentiation has been extensively identified in several model organisms such as mice and zebrafish.17

An increasing number of literatures suggested that miR-9 to 1 were also involved in tumorigenesis.18-21 In different types of cancer cells, miR-9 to 1 can have different effects on apoptosis and proliferation of cancer cells through targeting different mRNA targets.22-24 miR-9 to 1 is found to over-express in human Hodgkin’s lymphoma cells,25 primary brain tumors,26 cervical cancer,27 and colorectal cancer.28 However, miR-9 to 1 is identified to down-regulate in the ovarian tumor,29 gastric adenocarcinomas,30 breast cancer,31 oral carcinomas,32 Over-expression of miR-9 to 1 suppresses the proliferation in ovarian cancer,33 human medulloblastoma cells,34-36 Therefore, the abnormal expression of miR-9 to 1 in many types of cancer makes it possible to become a disease diagnosis and prognosis marker with important clinical significance.37-40 However, little is known about the pathological function and mechanisms of miR-9 to 1 in lung cancer.

Ubiquitin-like, containing PHD and RING finger domains 1 (UHRF1) is important to maintain the methylation status of DNA.41-43 UHRF1 is a ubiquitin E3 ligase and contains at least 4 domains including a plant homeodomain domain, a ubiquitin-like domain, a RING domain, and a SET and RING associated (SRA) domain.44-47 It was confirmed that UHRF1 could co-locate with DNA methyltransferase protein DNMT1 in the S phase.48 The SRA domain of UHRF1 strongly binds to the physiological substrate for DNMT1.49 UHRF1 recruit DNMT1 to hemimethylated DNA and facilitate DNA methylation.50-52 Further investigations have found that deubiquitylase USP7 and PCNA were also found to form a complex with UHRF1 and influence the E3-ligase activity of UHRF1 and DNA methylation.44,53,54 In normal cells, the expression of hUHRF1 was periodic and highly expressed in the late G1 and G2/M phases.55-57 On the contrary, in cancer cells, the expression of hUHRF1 is always at a high level.58,59 UHRF1 function as an oncogene and were found over-expressed in several cancer types and inversely correlated with survival and prognosis including bladder,60 kidneys,61 breasts,62 cervical,63 ovarian,64 lungs,65 and colorectal cancer.66 The detailed mechanisms may be related to the fact that UHRF1 constitutes a complex that represses tumor suppressor genes expression including MLH1, BRCA1, RB1, and p16INK4A.66-70 UHRF1 mediated tumor suppressor genes such as RASSF1 and CDKN2A inactivation in nonsmall cell lung cancer (NSCLC).71 Knock-down of UHRF1in A549 lung cancer cells results in lower methylation levels of tumor suppressors including CDH13, CYGB, and RASSF1 promoters.72 Moreover, knock-down of UHRF1 was found to inhibit cell proliferation and migration properties.73-75 Down-regulation of ICBP90 and DNMT1 significantly enhanced, p16INK4A and RB1 were observed.76 RNA interference of UHRF1 decreases proliferation and migration, with increased p16INK4A expression in CRC.77 UHRF1 were also regulated by tumor suppressors including TP53 and TP73.78 However, the miRNAs which regulated UHRF1 were little known.

In this study, we constructed miR-9 to 1 over-expressed model and further investigated the biological function and potential target and molecular mechanisms of miR-9 to 1 in NSCLC cell lines.

Materials and Methods

Ethical Approval and Consent to Participate

This study was approved by the Ethics Committee of the School of Medicine, Tongji University (IEC-PAP-15-18). The written informed consents were provided by all patients.

Clinical Tissue Samples

NSCLC and matched noncarcinoma tissue specimens from patients who underwent surgical treatment were obtained between March 2010 to November 2015. Two independent, experienced pathologists confirmed the diagnosis of NSCLC pathologically.

The expression data of miRNAs of 150 paired NSCLC tissues and normal tissues (47 FFPE samples and 103 fresh samples) were procured from GEO datasets GSE36681.

Plasmid Construction and Transfection

The lentivirus vector pLL3.7-miR-9 to 1 was constructed. The sequence was amplified with the primers using primerSTAR®HS (TaKaRa Code DR044A): miR-9 to 1-5′-GCCCTGAGAATCTAAAATCTGGAC-3′, and cloned into Not I/Xho I sites of psi-CHECKTM-2.

psi-CHECKTM-2-UHRF1 to 3′UTR: UHRF1 3′UTR fragment (NM_001048201.1) containing the full-length 3′UTR of UHRF1 was amplified performed with the same polymerase mentioned above by PCR using the primers 5′-AAAACCTCGAGTGGCTTCCCTTCA-3′ and 5′-CCCCGGCGGC GCCTGAGAATCTAAAATCTGGAC-3′ and cloned into the Xho I/Hpa I sites of pLL3.7 vector.

psi-CHECKTM-2-UHRF1 to 3′UTR-mut was constructed by using 4 mutated nucleotides of 3′UTR of UHRF1: forward-mut,
5′-TTCTAATTTAGTTAGTTTGCAGC-3′ and reverse-mut, 5′-AGGCTGCAAACCTAAATTAG-3′.

**Cell Lines**

HEK-293T, NCI-H1299, and A549 cells were cultured in DMEM supplemented with 10% FBS (Gibco BRL). A549 cell and NCI-H1299 cells were infected with pLL3.7 (EV) or pLL3.7-miR-9 to 1 for 24 h, and then the medium was refreshed.

**Dual-Luciferase Reporter Assay**

Dual-luciferase reporter assay was executed in HEK-293T cells. The miR-9 to 1 inhibitor and the control were used to test the inhibitory effect of miR-9 to 1 on UHRF1 3′ UTR.

**Colony Formation Experiment and Tumor Inoculation Assay**

For colony formation assay, lung cancer cells were cultured in the 6 cm diameter culture dish and fixed to stain with 0.5% crystal violet. The colonies numbers in 8 random view fields were counted under an optical microscope.

Tumor inoculation assay was performed in BALB/c nude mice at the age of 6 to 8 weeks. The tumor growth was investigated every 3 days for 4 weeks and the volume was recorded by measuring the length (L) and width (W) with calipers and was calculated with the formula: volume = 1/2(L × W^2).

**Total RNA Extraction and Quantitative PCR (qPCR)**

Total RNA was extracted from lung cancer cells using Trizol reagent and qPCR was performed with RT Kit in compliance

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**Figure 1.** The expression and clinical significance of miR-9 to 1 in NSCLC. (A) miR-9 to 1 expression level in 47 paired FFPC tumor samples and normal lung tissues from NSCLC patients. Raw data was from GSE36681. (B) qRT-PCR was used to quantify miR-9 to 1 expression level in 103 paired frozen tumor samples and normal lung tissues from NSCLC patients. (C) Fold change of miR-9 to 1 expression level in 56 paired frozen tumor samples and 8 normal lung tissues from NSCLC patients. (D) qRT-PCR was used to quantify miR-9 to 1 expression level in 56 frozen tumor samples and 8 normal lung tissues from NSCLC patients. (E, F) Kaplan–Meier survival analysis was used to evaluate the miR-9 to 1 prognostic value for OS (E) and PFS (F) of 56 NSCLC patients.

Abbreviations: NSCLC, nonsmall cell lung cancer; miR-9 to 1, microRNA-9 to 1; FFPC, formalin-fixed paraffin-embedded; qRT-PCR, quantitative real-time polymerase chain reaction; OS, overall survival; PFS, progression-free survival.
with the recommendations. Specific stem-loop RT primers were used for qPCR as the following: 5'-GGCCGGCTTTTGGTATCTAGC-3' and 5'-GTGCAAGGTCGAGGTTCTG-3' for mature miR-9 to 1, 5'-CTCGTACGGCAAGCAACACA-3' and 5'-AAACCTCAGATTTTGGT-3' for RNU6; 5'-TGTCAAGGTTGGAAGAAT-3' and 5'-GCCAGGTCTACATCGTC-3' for UHRF1. RNU6 was used as the internal control.

**Western Blot (WB) and Immunohistochemistry**

Total protein was extracted using a lysis buffer. The concentration was examined using standard procedures for WB. The blots were incubated with antibodies against UHRF1 (PA5-27969, Abcam; 1:2000), p16 (MA5-14260, 1:500), p14 (MA5-14260, 1:1000), p15 (MA1-12294, 1:1000), and GAPDH (ab9485, Abcam; 1:2000).

Paraffin-embedded tissues were acquired from the sacrificed nude mice and fixed in PBS with 4% formaldehyde for 24 h. Samples were dehydrated and then embedded in paraffin before being sectioned at 8 μM and used for H&E staining.

**Proliferation and Interference Experiment**

Cell counting kit-8 reagent was used for cellular proliferation assay. The data of optical density (OD) value at 450 nm were recorded through a microplate reader.

**Statistical Analysis**

Levels of gene expression were recorded as mean ± standard deviation (SD). The independent t-test and the χ² test were used to calculate the difference between 2 groups or the difference among different groups, respectively. Univariate survival analysis of overall survival (OS) or progression-free survival (PFS) of NSCLC patients was analyzed using the Kaplan-Meier method assay. All statistical analyses were carried out using the SPSS 21.0 software (SPSS Inc.).

**Results**

### The Level of miR-9 to 1 Expression in miRNA Microarray Profiling Data

The present study first performed an in silico analysis using GEO database data. Analysis using GSE36681 data showed that the miR-9 to 1 level was both significantly reduced in FFPE (n = 47; FC = 0.56, P = .0026; Figure 1A) and fresh (n = 103; FC = 0.53, P = .02; Figure 1B and C) samples of lung cancer compared with that in normal controls.

### Validation of the Reduced miR-9 to 1 Level in NSCLC Tissue Samples

The levels of miR-9 to 1 expression were also found to be lower in all NSCLC tumor biopsies (n = 56) compared with those in normal lung tissues (n = 8) and the differences were showed to be statistically significant (FC = 0.38, P = .02; Figure 1D).

### The Association Between miR-9 to 1 Expression and Clinical Characteristics

Next, miR-9 to 1 expression was further analyzed in NSCLC samples based on clinical characteristics (including age, sex, lymph node metastasis, TNM stage, tumor differentiation, smoking history, vascular invasion, invasion of lung membrane, and tumor diameter. Univariate analysis showed that the expression of miR-9-1 was significantly associated with tumor diameter [P = .009], lymph node metastasis [P = .006], and TNM stage [P = .012] in NSCLC patients (Table 1). However, there is no significant association between miR-9-1 expression and age, sex, invasion of the lung membrane, tumor differentiation, vascular invasion, or smoking history (P > .05; Table 1).

### Association of miR-9-1 Levels and Clinicopathological Data with Survival of NSCLC Patients

We next studied the correlation of miR-9-1 levels and clinicopathological data with the survival of patients with NSCLC.

**Table 1. The Association Between miR-9 to 1 Expression and Clinical Characteristics.

| Factor                        | Variable | N  | miR-9 to 1 expression (mean ± SD) | P-value |
|-------------------------------|----------|----|----------------------------------|---------|
| Age                           | ≥ 60     | 27 | 0.613 ± 0.029                    | .254    |
|                               | < 60     | 29 | 0.788 ± 0.026                    |         |
| Gender                        | Male     | 26 | 0.652 ± 0.035                    | .238    |
|                               | Female   | 30 | 0.759 ± 0.068                    |         |
| Smoking history               | Ever     | 18 | 0.812 ± 0.085                    | .065    |
|                               | Never    | 38 | 0.698 ± 0.025                    |         |
| Lymph node metastasis         | Positive | 25 | 0.538 ± 0.021                    | .006*   |
|                               | Negative | 31 | 0.833 ± 0.045                    |         |
| Tumor differentiation         | Poorly   | 16 | 0.621 ± 0.033                    | .079    |
|                               | Moderately | 11 | 0.756 ± 0.036                   |         |
|                               | Well     | 29 | 0.812 ± 0.024                    |         |
| TNM stage                     | III-IV   | 18 | 0.561 ± 0.067                    | .012*   |
|                               | I-II     | 38 | 0.804 ± 0.055                    |         |
| Invasion of lung membrane     | Positive | 29 | 0.651 ± 0.014                    | .068    |
|                               | Negative | 22 | 0.762 ± 0.036                    |         |
|                               | Unknown  | 5  | 0.801 ± 0.082                    |         |
| Vascular invasion             | Positive | 25 | 0.621 ± 0.035                    | .068    |
|                               | Negative | 29 | 0.712 ± 0.051                    |         |
|                               | Unknown  | 2  | 0.798 ± 0.082                    |         |
| Diameter                      | ≥ 5 cm   | 26 | 0.522 ± 0.031                    | .009*   |
|                               | < 5 cm   | 30 | 0.813 ± 0.032                    |         |

*P < .05.
The results revealed that a reduced miR-9-1 level was associated with a shorter OS ($P = .0005$; Figure 1E) and DFS ($P = .008$; Figure 1F) of patients with NSCLC.

**miR-9 to 1 Suppression of Lung Cancer Cell Proliferation In Vitro**

To investigate the function of miR-9 to 1 in A549 and NCI-H1299 cells, a CCK-8 assay was performed in stable miR-9 to 1-infected A549 and NCI-H1299 cells. The results showed that the proliferation rate in miR-9 to 1 group was significantly reduced to 49.65% and 55.6% when compared to the EV group in A549 ($P < .01$) and NCI-H1299 ($P < .05$) cells, respectively (Figure 2A and B). Moreover, overexpression of miR-9 to 1 significantly increased the cellular apoptosis of A549 and NCI-H1299 cells compared with the control (Figure 2C and D).

To further evaluate the effect of miR-9 to 1 on cell proliferation, we conducted colony-forming experiments on the A549 cell line. The results showed that the colony-forming ability of the miR-9 to 1 group was significantly inhibited. The colony number of more than 50 cells produced by a single cell in the miR-9 to 1 group was significantly lower when compared to that in the control group (46.37%; $P < .01$) (Figure 2E and F).

**miR-9 to 1 Suppression of Lung Cancer Cell Proliferation In Vivo**

In order to further verify the results of in vitro cell experiment, we injected $7 \times 10^6$ EV cells or miR-9 to 1 overexpression cells into
the skin of the right front leg and the lower part of the left leg, respectively. Tumor mass was observed in all (8 of 8) mice (half male and half female) in the EV group within 7−10 days after inoculation (3 representative mice in Figure 3A and B). All mice were killed at 5 weeks after inoculation, and the tumor weight was weighed. Four weeks after inoculation, the average tumor weight in miR-9 to 1 group was found to be significantly lower than that in the control group (Figure 3A and B). Tumor size was measured every twice a week when a tumor was obvious and our results showed that tumor volumes in miR-9 to 1 group achieved 55.36% and 36.49% of the control (both P<.05) at 4 and 5 weeks after inoculation, respectively (Figure 3B and C), which suggested that miR-9 to 1 suppress cell proliferation of NSCLC cells both in vitro and in vivo.

**miR-9 to 1 Target UHRF1 in Lung Cancer Cells**

To investigate the mechanisms of miR-9 to 1 inhibition effect on proliferation and tumorigenicity of A549 cells, we then searched for potential target genes by using the online prediction algorithm of Targetscan, Targetscan 5.1, Pictar, Mirbase, microiRNA, and RNA22, and found that UHRF1 is found as a putative target of miR-9 to 1.
To establish that miR-9 to 1 targets and regulates the expression of UHRF1, we constructed 3′UTR of UHRF1 downstream of renilla luciferase reporter gene in psi-CHECK2 vector, and then constructed 4 nucleotide mutations in the possible binding region of miR-9 to 1 seed sequence (hereinafter referred to as UHRF1-3′UTR MUT) (Figure 4A and B). Our results indicated that the relative luciferase activity in the reporter that UHRF1 contained wild-type 3′UTR was significantly decreased after miR-9 to 1 overexpression when compared to the EV group ($P < .01$) (Figure 4C). However, the relative luciferase activity of UHRF1 to 3′UTR-mut was recovered to the same level when compared to the control psi-CHECK2 group (Figure 4C).

UHRF1 expression decrease after miR-9 to 1 overexpression was reversed when adding miR-9 to 1 inhibitor with a final concentration of 50 nM ($P < .05$) (Figure 4D). The mRNA level of A549 and NCI-H1299 cells were measured by qPCR and the UHRF1 level was reduced to 46.48% and 49.52% at A549 and NCI-H1299 groups, respectively, as compared with the EV control group (both $P < .05$) (Figure 4E). The protein level of UHRF1 at miR-9 to 1 group was also measured by WB, and the protein reduced to about 1/3 and 1/2 at miR-9 to 1 group as compared with EV group at A549 and NCI-H1299 cell, respectively (Figure 4F). Transfection with UHRF1 coding sequence (CDS) at A549 cell overcome the suppression induced by miR-9 to 1 since the construct contained no 3′UTR.

When we used miR-9 to 1 mimics to transfection of A549 cells, the protein of UHRF1 was reduced in a dose-dependent manner (Figure 4G).

Figure 4. UHRF1 is a direct target of miR-9 to 1. (A) mRNAs microarray analysis to identify key differentially expressed genes after overexpression of miR-9 to 1 in A549 cells. (B) Structure and cloning site of the psi-CHECK-2 vector. (C) Mutations of 4 nucleotides were successfully performed by DNA sequencing. (D) Dual-luciferase assays were performed. Each sample was 6 well repeat. (E) Transient transfection of miR-9 to 1 mimics or its negative control (NC) at indicated concentration in A549 cells and protein were harvested 48 h after transfection. (F) Quantitative PCR results showed the UHRF1 mRNA was reduced at miR-9 to 1 stable transfection of A549 and NCI-H1299 cells. (G) UHRF1 protein was decreased in miR-9 to 1 group at A549 and NCI-H1299 cells. (H) Rescue experiments indicate that UHRF1 rescued the inhibition state in the A549 cell line by re-introducing of 3′UTR free UHRF1 coding sequence carried by the pBabe vector. The UHRF1 was detected by its antibody. GAPDH served as a loading control.

Abbreviations: miR-9 to 1, microRNA-9 to 1; PCR, polymerase chain reaction; UHRF1, ubiquitin-like containing PHD and RING finger domains 1; 3′UTR, 3′untranslated region.
**MiR-9 to 1 Induces the Expression of Tumor Suppressor Genes Including p15, p16, and p21**

UHRF1 is involved in some tumor suppressor genes inactivation by hypermethylation of its promoter region. And inhibition of UHRF1 is also related to the re-expression of tumor suppressors. We next studied whether UHRF1 down-regulation by miR-9 to 1 can re-active the expression of tumor suppressors. Among tumor suppressor genes, we search for some cell cycle inhibitors and found that p15, p16, and p21 are strikingly re-expressed in miR-9 to 1 group in mRNA level (Figure 5A to D) and protein level (Figure 5E). These results are similar to the interference of UHRF1 by specific stem-loop RNA mediated by the pLKO vector (Figure 5F).

**Expression and Their Correlation of miR-9 to 1 and UHRF1 in NSCLC**

Considering the close association of miR-9 to 1 with UHRF1 in lung cancer cell lines, we next studied the expression and correlation of miR-9 to 1 and UHRF1 in NSCLCs FFPE samples.
from the human tissue specimen. The results indicated that the expression of UHRF1 was significantly higher in NSCLC samples (n = 56) compared with normal lung tissues (n = 36) (FC = 9.65, p < .001; Figure 6A). Moreover, we observed a negative correlation between miR-9 to 1 and UHRF1 expression in NSCLC samples (n = 56) (Figure 6B).

**Clinical Significance of UHRF1 and miR-9 to 1 in NSCLC**

We then evaluated the prognostic value of UHRF1 and miR-9 to 1 expression in patients with NSCLC. The OS of NSCLC patients with a low expression of UHRF1 was prolonged when compared to NSCLC patients with high expression of UHRF1 (P = .037; Figure 6C).

We next explored the clinical significance of UHRF1 expression together with miR-9 to 1. Our results indicated that NSCLC patients with UHRF1 high and miR-9 to 1 low levels show significantly decreased OS (P < .001) (Figure 6D), suggesting that UHRF1 and miR-9 to 1 might have potential prognostic value and could be useful as tumor biomarkers for the diagnosis of NSCLC patients.

**Discussion**

MiRNAs are noncoding RNA molecules, which regulate gene expression through translational repression or deregulating of messenger RNAs.79-82 Accumulating evidence suggests miRNAs can play various roles in lung cancers.83-85 In the
In this study, we search for the miR-9 to 1 direct target by informatics through a public prediction website and identified that UHRF1 is the putative target of miR-9 to 1. UHRF1 to MiR-9 to 1-2, and miR-9 to 1-3.86 MiR-9 to 1 is the well-studied miRNA in vitro, and tumorigenesis in vivo, which suggests that miR-9 to 1 function as a tumor suppressor in the lung cancer cell. MiR-9 to 1 has 3 loci in the genome, including miR-9 to 1, miR-9 to 1-2, and miR-9 to 1-3.86 MiR-9 to 1 is the well-studied miRNA in vitro, and tumorigenesis in vivo, which suggests that miR-9 to 1 suppresses lung cancer cell proliferation in vitro and in vivo. MiR-9 to 1 suppresses cell proliferation and induces apoptosis through directly targeting UHRF1 to re-activation of tumor suppressor genes p15, p16, and p21.

In summary, in this study, we identified that miR-9 to 1 suppressed lung cancer cell proliferation in vitro and in vivo. MiR-9 to 1 suppresses cell proliferation and induces apoptosis through directly targeting UHRF1 to re-activation of tumor suppressor genes p15, p16, and p21.

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Author Contribution

CYJ, YSM, and DF designed the study. All authors performed the experimental analyses and interpreted the data. CYJ, YSM, and DF wrote the manuscript. CYJ, WX, JBL, GXJ, and FS contributed equally to this work. All authors contributed to the final version of the manuscript and approved the final manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval and Consent to Participate

This study was approved by the Ethics Committee of Shanghai Tenth People’s Hospital, Tongji University School of Medicine (IEC-PAP-15-18). All patients provided their written informed consent. The authors are accountable for all aspects of the work (if applied, including full data access, the integrity of the data, and the accuracy of the data analysis) in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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**Supplemental Material**

Supplemental material for this article is available online.

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