A peptide translated from circPPP1R12A promotes the malignancy of non-small cell lung cancer cells through AKT signaling pathway

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Abstract

Background: Recent literature have indicated that the malignancy of cancer cells is modulated by hsa_circ_0000423 (named circPPP1R12A) through the way of translating protein. Herein, we investigated the role and latent mechanisms of circPPP1R12A in Non-Small Cell Lung Cancer (NSCLC).

Methods: CircPPP1R12A expression was measured by qRT-PCR. The malignancy of NSCLC was determined by CCK-8, TUNEL assay, Wound healing, Transwell and Western blotting assays. The underlying mechanisms of circPPP1R12A were confirmed by Western blotting and qRT-PCR assays.

Results: CircPPP1R12A expression in NSCLC tissues was higher than that of neighboring normal tissues. CircPPP1R12A showed an upregulated expression in NSCLC cells. Upregulation of circPPP1R12A could promote the cell viability of NSCLC cells and reduce the apoptosis of NSCLC cells, while it could not promote cell invasion and migration. The reduction of cell viability and apoptosis was discovered in NSCLC cells with the silencing of circPPP1R12A, but circPPP1R12A knockdown does not inhibit cell invasion and migration. There was something interesting that circPPP1R12A encoding protein circPPP1R12A-73aa was found in NSCLC cells. Mutations in circPPP1R12A-73AA might disrupt the function of circPPP1ra-73AA in A549 and H1299 cells. Next, we found that circPPP1R12A caused the increased growth of NSCLC cells by activating AKT signaling pathway.

Conclusion: In summary, our study proved that NSCLC cell proliferation was promoted by circPPP1R12A-73aa translated from circPPP1R12A through the AKT pathway, which could throw some light on the understanding of the mechanism of NSCLC.

KEYWORDS
AKT pathway, Hsa_circ_0000423, non-small cell lung cancer, proliferation
Non-Small Cell Lung Cancer (NSCLC) is a malignant lung cancer disease with a very high fatality rate because of the lack of effective diagnostic tools and effective treatment methods, especially for patients with advanced cancer. As we all know, air pollution, exposure to chemical carcinogens, ionizing radiation, and chronic lung diseases may contribute to lung cancer. Regardless of extensive exploration, the underlying mechanisms in the physiological and pathological processes of NSCLC remain unclear.

Circular RNAs are covalently closed single-stranded circular transcripts without 5’ caps and 3’ poly(A) tails, which makes circular RNAs resistant to ribonuclease digestion (such as RNase R and exonuclease) and has a longer half-life than linear mRNA. Conserved inter-species evolution is a common feature of most circular RNAs. In general, circular RNAs are expressed at a low level, which means that they may act as splicing noise but have little functional potential. However, various circular RNAs detected by deep sequencing have been experimentally demonstrated to be more abundant than their linear counterparts, sometimes even more than 10 times.

The great majority of circular RNAs consisted of exons are usually located in the cytoplasm, while a small part of circular RNAs made up of introns are located in the nucleus and are usually cell type-specific and tissue-specific expression.

In the traditional view, the necessary elements of eukaryotic translation initiation include the 5 and 3 untranslated regions (UTR). Circular RNAs were once deemed to be non-coding RNAs because of the lack of 5 and 3 ends. Recently, a growing body of evidence has shown that circular RNAs may be involved with multimers, some of which include the initiation codon AUG and appropriately long open reading frames (ORF), which indicates that there is protein coding beyond thought in circular RNAs. Regulatory peptides can actually be encoded by circular RNAs which probably have a hidden proteome. First, artificially constructed engineered circular RNAs can initiate the translation of detectable peptides by recruiting 40s ribosomal subunits in vitro. In 2015, Abe et al. provided compelling evidence that endogenous circular RNAs could act as translation templates. Circular RNAs which contained infinite ORFs could be effectively translated by a rolling cycle amplification mechanism. Finally, protein concatamers were produced by circular RNAs with coding ability. In 2017, hsa_circ_0000615 has been reported by Legnini et al. to be a reverse splicing product of exon 2 of ZNF609, which could encode protein in a splicing-dependent and cap-independent manner during muscle production.

According to previous reports, hsa_circ_0000423 (named circPPP1R12A) encodes a new type of protein that can increase the malignancy of colon cancer through the Hippo-Yap signaling pathway. However, it is not clearly known about the functions of circPPP1R12A in NSCLC. Hence, we need to attain the objective of confirming whether circPPP1R12A regulates NSCLC cell pathogenesis and metastasis in the study.

2 | MATERIALS AND METHODS

2.1 | Human tumor and normal tissues

Twenty pairs of patient specimens including NSCLC tissues and adjacent normal tissues were provided from the Ningbo First Hospital. The human sample collection was obtained with informed consent. All experimental protocols were approved by the Medical Ethics Committee of Ningbo First Hospital.

2.2 | Cell culture

Human normal lung epithelial cells (BEAS-2B), A549 and H1299 were gained from the American Type Culture Collection (ATCC). Cells were grown in Dulbecco’s modified eagle medium (DMEM) containing 10% fetal bovine serum (Gibco), penicillin (100 U/ml) (Gibco) and streptomycin (100 mg/ml) (Gibco) at 37°C under a humid atmosphere containing 5% carbon dioxide.

2.3 | Real-time PCR

Trizol reagent (Beyotime) was used to isolate total RNA from tissues or cells. cDNA was synthesized by the PrimeScript RT Reagent Kit (Takara). The quantitative real-time Polymerase Chain Reaction (qRT-PCR) was performed on the ABI 7500 Fast Real-Time PCR System (Life Tech). The 2^ΔΔCt method was used to calculate gene expression and normalized to GAPDH. The sequences of primers were listed as follows: circPPP1R12A (Forward, 5’-ACAGCAGCAGGCTAGAAGAAAG-3’; Reverse: 5’-TGTCCTAAGCAGGAAAACACA-3’) and (Forward, 5’-GCCTCTCTGTCCTCTCCTGTC-30; Reverse, 50- ATCCGTTGACTCC GACCTTCAC-3’).

2.4 | Cell proliferation

Cell growth of A549 and H1299 cells was determined by Cell Counting Kit-8 (CCK8) assays (Beyotime) according to a previous study.

2.5 | Wound healing assay

100% confluent A549 and H1299 cells were scratched by the pipette tips. The open area was captured and assessed by using Image J software (National Institutes of Health, USA) after 24 h later.

2.6 | Cell invasion assay

Transwell experiment was performed in a Boyden chamber (Corning), and Matrigel was used to coat the upper surface of the chamber.
There were approximately $2 \times 10^5$ cells in the serum-free medium added into the upper chamber. After 24 h, cells fixed with 4% formaldehyde and were stained with 0.1% crystal violet (Beyotime) for 15 min. Then, a cotton swab was used to remove the cells on the upper compartment. Finally, images were photographed and the relative numbers of invasion cells were calculated by Image J (National Institutes of Health).

2.7 TUNEL assay

Cells cultured on the coverslip were washed by Phosphate Buffered Saline (PBS) and then fixed by 4% paraformaldehyde for 30 min. 0.3% Triton X-100 was used to penetrate cytomembrane. Terminal Deoxynucleotidyl Transferase mediated dUTP Nick-End Labeling (TUNEL) reagent was used to assist the vector to enter the cell.20 The data were represented by mean±SD. The Student’s test was used to evaluate the significance of difference. Related research adopts Pearson correlation analysis. *$p<0.05$, **$p<0.01$, ***$p<0.001$, versus the control were considered to be statistically significant. Each experiment was repeated three times. Information of primary antibody was listed in Table 1.

2.10 Statistics

The data were represented by mean±SD. The Student’s test was used to evaluate the significance of difference. Related research adopts Pearson correlation analysis. *$p<0.05$, **$p<0.01$, ***$p<0.001$, versus the control were considered to be statistically significant. Each experiment was repeated three times.

3 RESULTS

3.1 CircPPP1R12A was involved in NSCLC cell proliferation

CircPPP1R12A, which is first identified in colon cancer and plays a notable role in colon cancer, upregulates colon cancer malignancy through the Hippo-YAP signaling pathway.19 However, its role in NSCLC requires explanation. Therefore, the purpose of our study is to investigate the role of circPPP1R12A in NSCLC. Compared to the normal tissues nearby, circPPP1R12A expression was upregulated in NSCLC tumor tissues (Figure 1A). CircPPP1R12A expression was increased in A549 and H1299 cells compared to BEAS-2B cells (Figure 1B). Subsequently, we constructed circPPP1R12A overexpressing vector (Lv-circPPP1R12A) in A549 and H1299 cells to overexpress circPPP1R12A. CircPPP1R12A expression in A549 and H1299 cells were upregulated after Lv-circPPP1R12A transfection (Figure 1C). Circular RNAs play a crucial part in the malignant phenotype of cancer cells, such as proliferation, invasion and migration.21-24 For example, hsa_circ_0020095 enhances colon cancer proliferation, invasion and migration.24 Hsa_circ_0022160 accelerates esophageal squamous cell proliferation, migration and invasion.23 Interestingly, upregulation of circPPP1R12A significantly promoted the cell viability of A549 and H1299 (Figure 1D).

| Reagent or Resource | Source   | Identifier |
|---------------------|----------|------------|
| Anti-β-Actin        | Beyotime | AA128      |
| Anti-AKT            | Proteintech | 10176-2-AP |
| Anti-p-AKT          | Proteintech | 66444-1-lg |
| Anti-MAPK           | CST      | 8690       |
| Anti-p-MAPK         | CST      | 4370       |
| Anti-YAP            | CST      | 14074      |
| Anti-TAZ            | CST      | 83669      |
| Anti-STAT           | CST      | 9177       |
| Anti-p-STAT         | CST      | 14994      |
| Anti-p-NF-κB        | CST      | 3033       |
| Anti-NF-κB          | CST      | 8242       |

2.8 Plasmids, small interference RNAs and transfection

CircPPP1R12A overexpressing vector (Lv-circPPP1R12A), HA-circPPP1R12A overexpressing vector (Lv-HA-circPPP1R12A), HA-circPPP1R12A-mut overexpressing vector (Lv-HA-circPPP1R12A-Mut), HA-circPPP1R12A-73aa overexpressing vector (Lv-HA-circPPP1R12A-73aa) and the negative control plasmid (pLVX-ires-GFP) were designed according to a previous study19 and synthesized by Genomeditech, Shanghai, China. CircPPP1R12A expression was instantly knockdown by human circPPP1R12A-specific small interference RNAs (si-circPPP1R12A), which were generated by Genomeditech, Shanghai, China. Lipofectamine 3000 transfection reagent was used to assist the vector to enter the cell.20 After 48 h, real-time PCR was used to detect the transfection efficiency of each group of cells.

The data were represented by mean±SD. The Student’s test was used to evaluate the significance of difference. Related research adopts Pearson correlation analysis. *$p<0.05$, **$p<0.01$, ***$p<0.001$, versus the control were considered to be statistically significant. Each experiment was repeated three times.

2.9 Western blotting

Cells were lysed in RIPA lysis buffer (Beyotime) supplemented with PMSF (Beyotime), and total proteins were harvested. Protein was separated by 8%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane. Next, block all membranes with 5% skimmed milk (Biotech) and then use the primary antibody for detection. Subsequently, all membranes were cultured in enzyme-labeled anti-rabbit/mouse IgG (Biotech Biotechnology). Finally, the enhanced chemiluminescence (ECL) system (Millipore) was used to observe all protein bands. The protein abundance was normalized by GAPDH, and the band density was evaluated by ImageJ software. Each experiment was repeated three times. Information of primary antibody was listed in Table 1.
However, the apoptosis, invasion and migration of A549 and H1299 cells was not changed after upregulation of circPPP1R12A (Figure 1E–G). These results manifested that circPPP1R12A might promote A549 and H1299 cell proliferation but not invasion and migration.

### 3.2 Silencing of circPPP1R12A reduced NSCLC cell proliferation

Small interference RNAs to circPPP1R12A (si-circPPP1R12A) was established to silence circPPP1R12A expression for the sake of...
further identifying whether circPPP1R12A regulated NSCLC cell proliferation. In A549 and H1299 cells, circPPP1R12A expression was reduced distinctly by si-circPPP1R12A (Figure 2A). Based upon the previous part of the experiment (Figure 1), A549 and H1299 cell viability were confirmed first, and the results verified that A549 and H1299 cell viability would be inhibited by si-circPPP1R12A (Figure 2B). TUNEL assay results proved that si-circPPP1R12A would increase the apoptosis of A549 and H1299 cells (Figure 2C). Crucially, circPPP1R12A silencing could not decrease A549 and H1299 cell invasion and migration (Figure 2D,E).

Zheng et al. reported that circPPP1R12A promoted colon cancer cell proliferation, invasion and migration. Different from theirs, our results indicated that circPPP1R12A could only enhance NSCLC cell viability but not the ability of

![Figure 2](image-url)

**FIGURE 2** CircPPP1R12A knockdown reduced the proliferation of A549 and H1299 cells. (A) RT-PCR was used to detect the expression of circPPP1R12A in A549 and H1299 cells transfected with si-control or si-circPPP1R12A. (B) CCK-8 assay was used to confirm A549 and H1299 cell viability after si-control or si-circPPP1R12A transfection. (C) The apoptosis of A549 and H1299 cells was determined by TUNEL assay. (D) Transwell assay was used to confirm A549 and H1299 cell invasion after si-control or si-circPPP1R12A transfection. (E) Wound healing assay was used to determine the migration of A549 and H1299 cells after si-control or si-circPPP1R12A transfection.
invasion and migration, which might be related to the different metabolism of each type of cancer.

### 3.3 | CircPPP1R12A promoted NSCLC proliferation through encoding small peptides

It has been reported by Zheng et al.\(^{19}\) that circPPP1R12A encoded a novel small peptide to promote the malignancy of colon cancer. CircPPP1R12A was annotated originally in circular RNA Db database. 73-aa peptide was encoded by a short 216-nt small ORF of circPPP1R12A (named circPPP1R12A-73aa in this study). The cyclization of circPPP1R12A produced the tandem initiation codon AUG, which bound to the overlapping genetic code to begin translation. The sequence of circPPP1R12A-73aa was shown in Figure 3A. In order to study how the circPPP1R12A-73aa in A549 and H1299 cell acted on, we constructed a few HA-labeled vectors for circPPP1R12A (Figure 3B,C). CircPPP1R12A expression in A549 and H1299 cells was overexpressed after transfection with Lv-HA-circPPP1R12A or Lv-HA-circPPP1R12A-mut, but not in A549 and H1299 cells after transfection with Lv-HA-control or Lv-HA-circPPP1R12A-73aa (Figure 3D). In addition, the level of HA-labeled-circPPP1R12A-73aa peptide in A549 and H1299 cells was confirmed by Western bolt after transfection with Lv-HA-control, Lv-HA-circPPP1R12A, Lv-HA-circPPP1R12A-mut or Lv-HA-circPPP1R12A-73aa respectively, which showed circPPP1R12A could encode a small peptide (Figure 3F-H). These came to a result that circPPP1R12A, annotated as a circular RNA, actually encoded an unknown peptide. A549 and H1299 cell lines with stable transfection of the above four vectors were established so that we could determine whether circPPP1R12A promoted the cell viability of A549 and H1299 cells via circPPP1R12A-73aa.

CCK-8 results gave evidence that Lv-HA-circPPP1R12A and Lv-HA-circPPP1R12A-73aa enhanced the viability of A549 and H1299 cells. We could see an increase in cell viability in A549 and H1299 cells. Nevertheless, the viability of A549 and H1299 cells did not achieve an enhancement by Lv-HA-circPPP1R12A-mut (Figure 3E). Even more interesting was that the four vectors had no effect on the invasion of A549 and H1299 cells (Figure 3I). In summary, circPPP1R12A, annotated as a circular RNA, actually encoded an unidentified peptide to promote the proliferation of NSCLC cells.

### 3.4 | CircPPP1R12A-73aa enhanced NSCLC cell proliferation via activating AKT signaling pathway

Due to the limitations of funding and experimental conditions, we have adopted Western blotting to find the signal pathway regulated by circPPP1R12A-73aa. CircPPP1R12A was reported by Zheng et al.\(^{19}\) to encode a novel small peptide to promote the malignancy of colon cancer through Hippo-YAP signaling. Accordingly, the first and the foremost was to detect the YAP and TAZ expression. However, there was not changed by circPPP1R12A or circPPP1R12A-73aa, in A549 cells transfected above four vectors. Then, we found that p-NF-κB, p-MAPK and p-STAT in A549 cells remained unchanged. Interestingly, we found that p-AKT was increased by Lv-HA-circPPP1R12A and Lv-HA-circPPP1R12A-73aa (Figure 4A-G). After that, we affirmed by AKT inhibitor Perifosine (5 μM)\(^{25}\) the role of p-AKT played in A549 and H1299 cells transfected with Lv-HA-circPPP1R12A-73aa or Lv-HA-control. As shown in Figure 4B,C, the effect of circPPP1R12A-73aa on A549 and H1299 cells was suppressed by perifosine. Perifosine resulted in a decrease of viability induced by circPPP1R12A in A549 and H1299 cells (Figure 4H,I). These results showed a phenomenon that circPPP1R12A-73aa strengthened NSCLC cell proliferation via activating AKT signaling pathway.

### 4 | DISCUSSION

Although NSCLC is a clinical disease with high morbidity and mortality, its clinical research has never stopped moving forward. The analysis of its specific mechanism has not been clearly clarified, which becomes a huge impediment to the prevention and treatment of NSCLC.\(^{26}\)

Circular RNAs have been been proved to regulate the progression of many cancers. To name only a few, Jie et al.\(^{27}\) have reported that hsa-circ_0023028 contributed to the progression of laryngeal squamous cell carcinoma. Du et al.\(^{28}\) have also reported that hsa_circ_0095868 upregulated by M6A promotes the tumorigenesis of hepatocellular carcinoma. Our study focused on the role of circPPP1R12A in NSCLC. CircPPP1R12A accelerated the proliferation, invasion and migration of colon cancer as Zheng et al.\(^{19}\) reported. In our study, the expression of circPPP1R12A was increased in tumor tissues. These results, which exerted a tremendous fascination on us, were confirmed that our conjecture was correct. We found that compared with BEAS-2B cells (human normal lung epithelial cells), the expression of CircPPP1R12A was upregulated in A549 and H1299 cells. The increased expression of circPPP1R12A in A549 and H1299 cells might have a bearing on the tumor pathogenesis of NSCLC cell.

Consequently, we constructed Lv-circPPP1R12A and si-circPPP1R12A to study the role of circPPP1R12A in NSCLC cells. CircPPP1R12A in A549 and H1299 cells was overexpressed, and its upregulation could promote the viability of A549 and H1299 cells. Afterwards, the apoptosis of A549 and H1299 cells was not changed by circPPP1R12A. The viability of A549 and H1299 cells was inhibited by the silencing of circPPP1R12A. It is reported that circular RNAs could promote the viability of various cancer cells. Hsa_circ_0071452 promotes the immunosuppression and anti-PD-1 of lung adenocarcinomas and squamous cell carcinomas through the miR-181a-5p/CARM1 axis.\(^{30}\) Hsa_circ_0006089 has been reported by Chen et al.\(^{31}\) to improve the viability of gastric cancer cells through modulating miR-770-5p/CDK6 axis. Previous study and our results demonstrated that circular RNAs regulated cancer cell proliferation through a different signaling pathway.
Second, we conducted researches to ferret out the role of circPPP1R12A in A549 and H1299 cell invasion and migration. To our surprising, upregulation or knockdown of circPPP1R12A have no impact on A549 and H1299 cell invasion and migration. It is generally known that the proliferation, migration and invasion of tumor cells will be usually influenced by circular RNAs.

**FIGURE 3** CircPPP1R12A promoted the proliferation of A549 and H1299 cells by encoding a small peptide. (A) The pattern diagram of 1138-nt circPPP1R12A and a 73-aa (~10 kDa) protein (circPPP1R12A-73aa) was shown. (B and C) A CMV-induced expression vector contained the HA-labeled circPPP1R12A sequence (Lv-HA-circPPP1R12A). Lv-HA-circPPP1R12A with start codon mutant (ATG/ACG) (Lv-HA-circPPP1R12A-Mut). A CMV-induced expression vector contained the HA-labeled circPPP1R12A-73aa sequence (Lv-HA-circPPP1R12A-73aa). The pattern diagram of these vectors was shown. (D) CircPPP1R12A expression was confirmed in A549 and H1299 cells after Lv-HA-control, Lv-HA-circPPP1R12A, Lv-HA-circPPP1R12A-Mut and Lv-HA-circPPP1R12A-73aa transfection. (E) CCK-8 assay was used to detect the cell viability of A549 and H1299 cells after Lv-HA-control, Lv-HA-circPPP1R12A, Lv-HA-circPPP1R12A-Mut and Lv-HA-circPPP1R12A-73aa transfection. (F) Western blotting was used to detect circPPP1R12A-73aa peptide after Lv-HA-control, Lv-HA-circPPP1R12A, Lv-HA-circPPP1R12A-Mut and Lv-HA-circPPP1R12A-73aa transfection. (G and H) The quantitative analysis of HA protein expression in A549 and H1299 cells. (I) Transwell assay was used to determine A549 and H1299 cell invasion after Lv-HA-control, Lv-HA-circPPP1R12A, Lv-HA-circPPP1R12A-Mut and Lv-HA-circPPP1R12A-73aa transfection.
instance, hsa_circ_0026416 promotes the malignant phenotypes of colorectal cancer via the miR-485-3p/PDL signaling pathway.\textsuperscript{32} Hsa_circRNA_102034 ameliorates breast cancer cell malignant phenotypes through the miR-106a-5p/STAT3 axis.\textsuperscript{33} However, our results demonstrated clearly that circPPP1R12A only affected the proliferation of NSCLC, but not migration and invasion. Recent studies generalize a conclusion that the proliferation, metastasis and invasion of cancer cells could be probably interfered with by diverse factors. For instance, silencing of lncGBCDR do not affect the invasion and migration of gallbladder cancer cells.\textsuperscript{34} We can see an enhancement in the proliferation of triple-negative breast cancer and inhibition of their invasion and migration causing by FBXO22.\textsuperscript{35}
These studies suggest that proliferation, invasion, and migration of cancer cells changes are not consistent.

In fact, circRNAs can be divided into noncoding circRNAs and coding circRNAs. In 2015, Abe et al. provided strong evidence that endogenous circular RNAs could be used as a translation template. In the decellularized E. coli translation system, circular RNAs with infinite ORF were efficiently translated by rolling circle amplification (RCA). These results testified that it is possible to translate circular RNAs without poly (A) tail or cap structure into protein. Since the discovery, a mounting number of evidences manifest that circular RNAs can encode regulatory proteins/peptides. These functional proteins/peptides have an impact on tumor occurrence, invasion and metastasis. CircPPP1R12A encodes a novel small peptide to promote the malignant phenotypes of colon cancer as well as encoding small peptides in A549 and H1299 cells come to light. After ATG in ORF is mutated to ACG, the translation of circPPP1R12A can be inhibited. It was interesting that the mutation of ORF could inhibit the function of circPPP1R12A in A549 and H1299 cells. Regulation of the Hippo-YAP signaling pathway had been successfully realized through a small peptide encoded by circPPP1R12A.

In consequence, we carried out detection of the protein expression of YAP and TAZ in A549 and H1299 cells transfected with Lv-HA-circPPP1R12A-73aa. What we found was that YAP/TAZ, NF-kB, MAPK and STAT pathway did not changed in NSCLC cells, whereas we found that p-AKT was increased by circPPP1R12A-73aa in A549 cells. AKT acts a pivotal part in NSCLC cell pathogenesis and metastasis. AKT signaling pathway reduces the chemosensitivity of NSCLC to cisplatin. Hsa_circ_0007580 promotes NSCLC cell growth, invasion and proliferation via AKT pathway. Furthermore, AKT inhibitor could inhibit the role of circPPP1R12A-73aa in A549 and H1299 cells. These results were a validation of the conjecture that circPPP1R12A-73aa enhanced the proliferation of NSCLC cells through the AKT pathway.

5 | CONCLUSION

To summarize, our study claimed that circPPP1R12A encoded a small peptide to promote the proliferation of NSCLC cell via AKT pathway. Our research also contributed novel insights and ideas to the clinical prediction and treatment of NSCLC, which might become the key to breakthrough progress in the future.

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Weijun Zhao, Yibo Xue and Zixuan Chen carried out the experiments and collected data. Weijun Zhao conceived, designed the study, Yandan Zhang and Yonggang Zhu, draft the manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

All authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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