Circular RNA circ_001621 promotes osteosarcoma cells proliferation and migration by sponging miR-578 and regulating VEGF expression

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Abstract
Strategies targeted vascular endothelial growth factor (VEGF)-dependent osteosarcoma progression are limited although important progress has been made in illustrating the mechanisms. Here we identified circ_001621 as one of the significantly upregulated circular RNAs (circRNAs) by circRNAs microarrays. We found that patients with high circ_001621 expression had a shorter survival time. Moreover, we found several potential sponge micro RNAs (miRNA) of circ_001621 with Circular RNA Interactome database. Among the candidate sponge, we elucidated the association of circ_001621 and miR-578. In addition, we demonstrated that miR-578 targeted circ_001621 directly. Functionally, we set up the experimental system to investigate the effects of circ_001621/miR-578/VEGF interaction in vitro and in vivo. Results indicated circ_001621-promoted osteosarcoma proliferation and migration via attenuating the inhibition of cyclin-dependent kinase 4 (CDK4) and matrix metalloproteinase 9 (MMP9) by miR-578, respectively. Nude mice experiment was further performed to estimate the promotion of metastasis by circ_001621. The present study evaluated the mechanisms underlying circ_001621 enhanced osteosarcoma progression and provided novel therapeutic targets for advanced osteosarcoma.

Introduction
Osteosarcoma, emerging from mesenchymal cells, is a high-grade primary bone sarcoma and tends to occur in children and young adults. Despite the current regimens combining surgery, chemotherapy, and radiotherapy, the clinical outcome of advanced osteosarcoma is inadequate. Numerous patients suffer from recurrence due to potential or existing distant metastasis. Recently additional strategies have been developed for patients with recurrent osteosarcoma, such as targeted therapies. However, serious side effects and limited response rates make the need for understanding the complex mechanisms involved in osteosarcoma initiation, progression, and metastasis urgently.

As a new subclass of endogenous noncoding RNAs, circRNAs are present in eukaryotic cells and have the capacity of sponging miRNAs, combing with RNA-binding proteins and working as transcription factors. In addition, circRNAs are involved in various cancer types and exhibit multiple effects in regulating carcinogenesis and progression. However, the mechanistic and functional characterization of circRNAs in osteosarcoma is largely unknown.

It has been reported that vascular endothelial growth factor (VEGF) expression associated with the outcome of patients with osteosarcoma. VEGF activates different signaling pathways in angiogenesis, proliferation, migration, and apoptosis. A great number of preclinical experiments and clinical trials targeted VEGF pathways have been performed to exploit novel treatment for refractory cases in past decades, for instance, Sorafenib and Dasatinib. Although great advantages have been made, limited tumor response rates, resistance, toxicity,
and other unforeseen difficulties retard the development of evaluating active agents for osteosarcoma.

In the present study, we aimed to elucidate the correlation of circ_001621 expression and osteosarcoma stages. Furthermore, we attempted to reveal the effects of circ_001621/miR-578/VEGF axis in osteosarcoma progression.

Material and methods

Patients and specimens

Thirty primary osteosarcoma patients who underwent complete resection without pre- or postoperative chemotherapies were recruited at Shengjing Hospital of China Medical University from 2015 to 2018. The study was approved by the Ethics Committee of Shengjing Hospital of China Medical University. All participants provided written informed consent for research purposes and for publication. Thirty patients with an average age of 25.65 years (range from 12 to 41 years). All patients’ slides were reviewed to confirm the diagnosis and to classify the tumor according to Enneking staging system. Eight patients were diagnosed as stage 1, 15 patients were diagnosed as stage 2, and 7 patients were diagnosed as stage 3. Adjacent tissues and tumor tissues were collected simultaneously. All the resected or biopsy specimens were placed immediately into liquid nitrogen and stored at −80 °C.

circRNA microarray analysis

Total RNA from osteosarcoma and adjacent tissues were used for Custom RT2 PCR Arrays (QIAGEN, Hilden, Germany). CircRNA microarray analysis was performed as described10. CircRNAs (fold change > 1.5 and \( P \text{ value} < 0.05 \)) were considered expressed differentially between two groups. Each group was analyzed in triplicate. The data of circRNA microarray profiling have been approved by GEO and the accession code is GSE140256.

Cell culture

Human osteoblast line hFOB 1.19 (GNHu14), osteosarcoma cell lines MG63 (TCHu124), and U2OS (SCSP-5030) were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). MNNG/HOS cell line (CRL-1547) was obtained from American Type Culture. Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) (Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) (Gaithersburg, MD, USA). The vendors have claimed that the cells were identified by STR profiling. All cell lines were examined for the presence of Mycoplasma (LookOut® Mycoplasma PCR Detection Kit; Merck & Co., Kenilworth, NJ, USA).

Prediction of miRNA and circRNA targets

Interactions between circRNA and miRNA were predicted with Circular RNA Interactome11. Furthermore, miRDB12,13 was employed to predict the miRNA-binding sites in the three prime untranslated region (3'-UTR) of target genes.

MTT assay

MTT assays were conducted to evaluate the cell viability as previously described14. In brief, cells were seeded at 1 × 10⁴/well in 96-well plates and were plated in 0.1 ml DMEM treated with different factors for 12, 24, 36, and 48 h. At each time point, 10 μl MTT solution (5 mg/mL) was added, followed by incubation for 4 h at 37 °C. Then the medium was replaced by 150 μl dimethyl sulfoxide solution, followed by incubation for another 10 min to solubilize crystals. The optical densities were read at 490 nm using a microplate reader (Life Science, Hercules, CA, USA).

Migration assay

To measure the migratory ability of MG63 and U2OS cells, migration assays were performed using modified Boyden chambers (Merck & Co., Inc., Kenilworth, NJ, USA). A total of 1 × 10⁵ cells in 0.2 mL serum-free DMEM treated with various factors were plated in the upper room of each chamber while the lower room was filled with 0.6 mL DMEM supplemented with 10% FBS. After incubating for 24 h at 37 °C, cells on the upper compartments were removed, whereas the migrated cells in the lower parts were stained, observed, and counted under a high-power microscope.

Western blots

Total proteins were extracted using 100 μl lysis buffer form cells and tissues. Thirty micrograms of lysates resolved with SDS-PAGE gel and were transferred to nitrocellulose membranes through electrobloot. Then membranes were blocked with 5% blocking solution for 1 h, followed by incubation with VEGF (19003-1-AP), cyclin-dependent kinase 4 (CDK4) (11026-1-AP), and matrix metallopeptidase 9 (MMP9) (10375-2-AP) antibodies (Proteintech Group, Inc, Rosemont, IL, USA) overnight at 4 °C. Membranes were washed three times with TBST and incubated with HRP-conjugated secondary antibodies (Merck & Co., Inc., Kenilworth, NJ, USA) for another 1 h. Immunoreactivity was measured using the Western Lighting Ultra (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted by 1 mL TRIZOL (Thermo Fisher Scientific, Waltham, MA, USA) according to the
manufacturer’s protocol. Then 1 mg RNA was reverse transcribed to cDNA in 20 μl system by the RT reaction kit (Promega), was performed using the Mx3000P real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). PCR was carried out as follows: 40 cycles of 94°C for 15 s, 60°C for 10 s, and 72°C for 20 s. All procedures were repeated thrice. Gene expression was normalized to the GAPDH to calculate relative expression

**Hematoxylin and eosin staining**

Hematoxylin and eosin staining has been described previously.

**Immunohistochemical staining**

The paraffin-embedded metastatic foci were deparaffinized, rehydrated, and performed the heat-induced antigen retrieval (Tris buffer, pH 8.0). The slides were incubated in 1:20 35% H2O2 in distilled water for 20 min at room temperature, followed by incubation with the mouse monoclonal anti-VEGF antibody (1:500; Abcam, Cambridge, MA, USA). The VEGF expression was detected by the DAB kit (MAIXIN Bio, Fuzhou, China). The slides were counterstained with hematoxylin. Stained slides, negative and positive controls were analyzed under a bright-field microscope (BX43; OLYMPUS, Tokyo, Japan).

**Immunofluorescence analysis**

The expression of VEGF and nuclei was detected by immunofluorescence staining with specific antibodies. MG63 cells expressing control, circ_001621 or miR-578 and circ_001621 and miR-578 simultaneously grew to 60–80% confluence on coverslips in six-well plates. The cells were fixed, permeabilized, and blocked following the previously described protocol. The cells were incubated with primary antibody, monoclonal anti-VEGF antibody (1:100) at 4°C overnight. The cells were washed with 1× PBS three times, incubated with DAPI (4’,6-diamidino-2-phenylindole) and a FITC-conjugated goat anti-mouse IgG secondary antibody (1:100) at room temperature for 45 min. The VEGF was visualized and photographed using the Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany). Five fields of view were taken pictures. The primary antibody, the secondary antibody, and DAPI were purchased from Abcam (Cambridge, MA, USA).

**Statistical analysis**

According to each methods’ standard practice, the present data were gain and analyzed. No statistical method was used to predetermine sample size. The data from all experiments were presented as means plus standard deviation (s.d.). No samples were excluded from the analysis. The association between circ_001621 and miR-578 expression was analyzed by Spearman’s correlation coefficient. The differences were evaluated by one-way analysis of variance with LSD test, and \( P < 0.01 \) indicated by ‘**’ or ‘###’ respectively were statistically significant. Statistical analysis was conducted using GraphPad version 7.0 (San Diego, CA, USA).
expression negatively correlated with miR-578 expression in osteosarcoma. The inverse association was in line with the inhibition of microRNA-578 expression by circ_001621 (Fig. 1h).

These results indicated that circ_001621 was involved in the promotion of osteosarcoma malignancy and inhibited miR-578 expression.

Table 1 Differentially expressed circRNAs in osteosarcoma.

| circRNA      | Fold change | Trend |
|--------------|-------------|-------|
| Circ_0000006 | 54.12       | Up    |
| Circ_0046264 | 40.00       | Up    |
| Circ_0096041 | 13.27       | Up    |
| Circ_0078767 | 11.09       | Up    |
| Circ_0094088 | 9.15        | Up    |
| Circ_0049271 | 5.14        | Up    |
| Circ_010220  | 27.31       | Down  |
| Circ_0000253 | 15.13       | Down  |
| Circ_0020378 | 5.13        | Down  |
| Circ_0000979 | 2.59        | Down  |

CircRNAs (fold change > 1.5 and P value < 0.05) were considered expressed distinctively compared with adjacent groups. Each group was analyzed in triplicate.

**Results**

**circ_001621 expression predicts shorter overall survival of osteosarcoma and correlates with miR-578 negatively**

Ten circRNAs were found to express differentially in 30 frozen samples of osteosarcoma compared with paired adjacent tissues, among which the difference in circ_001621 (circ_0000006) was the most significant (Table 1).

The expression of circ_001621 in osteosarcoma increased compared with that in adjacent tissues (Fig. 1a). Furthermore, circ_001621 expression in osteosarcoma cells MG63, U2OS, and HOS was higher than that in osteoblast cell hFOB 1.19 (Fig. 1b). Importantly, the high expression of circ_001621 was associated with poor survival (Fig. 1c) and advanced stages of osteosarcoma (Table 2). No significant correlation was identified between circ_001621 expression and the other features, such as gender, age, or family history.

According to the prediction of Circular RNA Interactome, miR-578 was one of the sponges of circ_001621 (Fig. 1d). The previous study has shown that miR-578 was low expression in BRCA-related breast cancer, and involved in the regulation of focal adhesion kinase, VEGF, and hypoxia-inducible factor-1 (HIF-1) related signaling cascades19. However, the effects of miR-578 in osteosarcoma progression remain largely unknown. Compared with adjacent tissues, miR-578 expression was downregulated in osteosarcoma tissues (Fig. 1e). These results were in accordance with those from cell lines, in which miR-578 expression in MG63 and U2OS reduced apparently compared with that in hFOB 1.19 (Fig. 1f).

To investigate the relationship between circ_001621 and miR-578 expression, correlation analysis was performed. Result in Fig. 1g shows that circ_001621 expression negatively correlated with miR-578 expression in osteosarcoma.

**miR-578 inhibits the proliferation and migration of osteosarcoma cells by targeting VEGF**

Previous studies have revealed that multiple miRNAs hampered VEGF expression, resulting in suppression of osteosarcoma cells proliferation and migration20,21. miR-578 mimic impeded cell proliferation (Fig. 3a) and migration significantly (Fig. 3b, c). In contrast, miR-578 inhibitor boosted cell proliferation (Fig. 3a) and migration significantly (Fig. 3b, c). One of the explanations for the results is miR-578 retarded VEGF-mediated singling pathways. For example, a previous study provided evidence that VEGF contributed to regulation of cell proliferation by targeting the expression of CDK422 and promoted cell migration by elevating MMP9 expression23. Results in Fig. 3d, e shows that CDK4 and MMP9 expression increased significantly in MG63 cells expressing miR-578 while those declined in cells expressing miR-578 inhibitor. Identical results were seen in U2OS cells (Fig. 3f, g).

**circ_001621 promotes the proliferation and migration of osteosarcoma cells**

MTT assay and transwell assay were used to access the proliferation and migration of osteosarcoma cells, separately. Figure 4a shows that cells expressing circ_001621 proliferated significantly compared with those expressing...
control. The number of migrated cells increased apparently in the circ_001621 group compared with that in control (Fig. 4b, c). Western blot and qRT-PCR were performed to examine the expression of VEGF, CDK4, and MMP9. Results showed that circ_001621 upregulated VEGF, CDK4, and MMP9 expression remarkably in MG63 and U2OS cells (Fig. 4d–g). The observations suggested that the circ_001621 augmented proliferation and migration through the promotion of VEGF, CDK4, and MMP9.

**circ_001621 promotes the proliferation and migration of osteosarcoma cells via abolishing the inhibition of VEGF by miR-578**

It is known that circRNAs/miRNAs/mRNAs interactome was involved in the initiation and progression of
osteosarcoma. To analyze the roles of circ_001621 and miR-578 in VEGF-mediated proliferation and migration, cells were transfected with control, circ_001621, or the combination of circ_001621 and miR-578. As shown in Fig. 5a, cells in group circ_001621 proliferated significantly. However, the proliferation of cells in the combination group changed little compared with that of cells in control group. In addition, migration of MG63 cells expressing circ_001621 increased obviously while that of cells expressing circ_001621 and miR-578 changed hardly compared with that of cells in control group (Fig. 5b). Similar results were observed in U2OS cells (Supplementary Fig. A) To further investigate the mechanisms underlying the hindrance of promoted proliferation and migration, western blot and qRT-PCR was performed. Results showed that the co-transfection of circ_001621 and miR-578 attenuated the enhanced VEGF, CDK4, and MMP9 expression significantly (Fig. 5c, d). Likewise, VEGF, CDK4, and MMP9 enhanced in U2OS expressing circ_001621 while the enhancement was impaired by expressing circ_001621 and miR-578 (Supplementary Fig. b, c). In line with the results of western blots and the qRT-PCR assay, the immunofluorescence assay showed the circ_001621 promoted VEGF expression in MG63 cells (Fig. 5h). Alternatively, VEGF expression reduced in cells expressing circ_001621 and miR-578. These results indicated that circ_001621 promoted VEGF-mediated proliferation and migration via targeting miR-578.

circ_001621 promotes the metastasis of osteosarcoma in vivo

To investigate the effect of circ_001621 on osteosarcoma metastasis in vivo, cells posttransfected with control, circ_001621 alone or combination of circ_001621 and miR-578 were injected to nude mice through tail vein. Three weeks later, lungs and livers were collected. Figure 6a shows the HE staining of lung metastasis and the number of metastatic foci in each lung per group. The number of lung metastatic foci increased (13 ± 1.8) in circ_001621 group while that decreased (4.8 ± 1.5) in the combination group compared with control (8 ± 1.5). Meanwhile, the represent HE staining of liver metastasis and the number of metastatic foci in each liver per group were shown in Fig. 6b. circ_001621 enhanced liver metastasis (11.7 ± 3) while the combination suppressed liver metastasis (5 ± 1.4) compared with control (7.2 ± 2). In addition, the VEGF expression in lung metastasis was confirmed by immunohistochemistry (Fig. 6c). Over-expression of circ_001621 elevated VEGF expression while the co-expression of circ_001621 and miR-578 deregulated VEGF expression. In addition, circ_001621 promoted expression of VEGF, CDK4, and MMP9 evidently while co-transfection of circ_001621 and miR-578 blocked the promotion significantly (Fig. 6d, e).

Discussion

In recent years, an increasing number of studies have revealed the diverse roles of circRNAs in multiple biological processes, especially in the initiation, progression, and metastasis of cancers. For example, circTADA2A sponged miR-203a directly and upregulated CREB expression, leading to osteosarcoma progression and metastasis. circFAT1 impeded the suppression of Yes-associated protein 1 by miR-375 and promoted osteosarcoma tumorigenesis. circNASP sponged miR-1253 and provoked forkhead box F1, enhancing proliferation and invasion of osteosarcoma. Previous studies suggest

| Variables       | Description | No. of patient | circ_001621 expression | χ²   | P value |
|-----------------|-------------|---------------|------------------------|------|---------|
| Gender          | Male        | 19            | Low: 8, High: 11       | 0.706| 0.401   |
|                 | Female      | 11            | Low: 2, High: 9        |      |         |
| Age(years)      | <25         | 13            | Low: 5, High: 8        | 0.271| 0.602   |
|                 | ≥25         | 17            | Low: 5, High: 12       |      |         |
| Family history  | Yes         | 4             | Low: 1, High: 3        | 0.144| 0.704   |
|                 | No          | 26            | Low: 9, High: 17       |      |         |
| TNM grade       | I           | 8             | Low: 7, High: 1        | 15.434| 0.0004* |
|                 | II          | 15            | Low: 1, High: 14       |      |         |
|                 | III         | 7             | Low: 2, High: 5        |      |         |

*P < 0.05.

T primary tumor, N regional lymph nodes, M metastasis.
that the functions of circRNAs remain largely unknown. In the present study, we focused on the role and underlying mechanisms of circ_001621 in osteosarcoma progression.

The expression of circ_001621 has been determined to be upregulated in osteosarcoma tissues and cells. Meanwhile, circ_001621 expression correspond with advanced stages of osteosarcoma. More importantly, highly expressed circ_001621 correlated with shorter overall survival. Although other evidence related to circ_001621 distribution was unavailable, the present data indicated that circ_001621 may contribute to osteosarcoma progression.

miRNAs play important roles in circRNAs/miRNAs/mRNAs networks, in which miRNAs serve as circRNAs sponge and target mRNAs. In our study, miR-578 was downregulated in osteosarcoma tissues and cells. Furthermore, miR-578 expression related to circ_001621 negatively and declined in cells expressing circ_001621, suggesting that circ_001621 sponged miR-578. Apart from our study, a previous study has revealed that miR-578 was involved in induction of apoptosis. Downregulation of miR-578 elevated expression of Max dimerization protein 1 and the Sin3A associated protein 18, causing pancreatic cancer cells apoptosis. miR-578 also associates with focal adhesion, VEGF and HIF-1 in

![Fig. 2 miR-578 targets VEGF in osteosarcoma.](image-url)

- **A** Prediction of putative-binding sites between miR-578 and VEGF by miRDB.
- **B** Negative correlation between miR-578 expression and VEGF.
- **C** Luciferase reporter assay indicated that VEGF DEL abrogated the inhibition of VEGF promoter activity. **P < 0.01, vs control.**
- **D-g** VEGF expression in cells expressing control, miR-578 or miR-578 inhibitor was detected by western blot and real-time PCR, respectively. Results represented the mean ± s.d. of three independent experiments. **P < 0.01, vs control.**

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Fig. 3 miR-578 inhibits the growth and migration of osteosarcoma cells by targeting VEGF. a The proliferation of cells expressing control, miR-578 or miR-578 inhibitor for 24 h was determined by MTT assay. b, c Migration of cells expressing control, miR-578 or miR-578 inhibitor was detected by the transwell assay. d–g The indicated proteins in cells expressing control, miR-578 or miR-578 inhibitor were detected by western blot and real-time PCR. Results represented the mean ± s.d. of independently repeated experiments. **P < 0.01, vs control. miR-578 in, miR-578 inhibitor.
BRCA-related breast cancer angiogenesis. Various functions of miR-578 may be due to tissue-specific expression profiles of miRNAs and proteins in different cancers. Another possible explanation is circ_001621 regulates signaling cascades temporally and spatially in cancer.

It is well accepted that angiogenesis is fundamental to tumor growth, invasion, and metastasis. As a pivotal tumor-derived angiogenic factor, VEGF plays multiple roles in carcinogenesis, progression, and recurrence of osteosarcoma, such as promotion of angiogenesis, inflammation, and vascular permeability, comprising...
fundamental signaling pathways\textsuperscript{29–31}. An extensive amount of research promotes understanding of VEGF pathways, leading to development of several VEGF targeted compounds. For instance, Bevacizumab\textsuperscript{32} combined with chemotherapy, Apatinib\textsuperscript{33}, Sirolimus combined with chemotherapy\textsuperscript{34} were used to treat recurrent/advanced osteosarcoma. Up to now there are several obstacles retard development of novel targeted therapies. Drug sensitivity, resistance, variety of subtypes of tyrosine kinase receptors expression and activation of alternative signaling pathways in osteosarcoma can defeat VEGF inhibition. The present findings that circ_001621 promoted osteosarcoma progression via VEGF-CDK4-MMP9 axis extended knowledge of VEGF-related pathways. To explore therapeutic potential of circ_001621, further investigation on circ_001621 expression in different subtypes of osteosarcoma and stage-specific osteosarcoma is necessary. Moreover, difference between

\begin{itemize}
\item \textbf{Fig. 5} circ_001621 promotes the cells proliferation and migration via abolishing the inhibition of VEGF by miR-578. 
\item a Proliferation in cells expressing control, circ_001621 or combination was detected by the MTT assay. 
\item b Migration of MG63 cells expressing control, circ_001621 or combination was detected by the transwell assay. 
\item The indicated genes expression in MG63 cells were detected by western blot (c) and real-time PCR (d), separately. Results represented the mean ± s.d. of three independent experiments. **P < 0.01, vs control, ##P < 0.01, vs circ_001621.
\item e Immunofluorescent stain of VEGF in the indicated MG63 cells. Green, VEGF; Blue, DAPI.
\end{itemize}
tissue and serum expression of circ_001621 during osteosarcoma cannot be ignored. In addition, it is worth estimating association of circulating VEGF and circ_001621 expression because circulating VEGF expression correlated with the development of lung metastasis.

In conclusion, this study identified circ_001621 as an oncogene in osteosarcoma progression, at least partly, by activating VEGF-dependent proliferation and migration via sponging miR-578. These results provide new insight into the role of circ_001621 and miR-578 and the regulation of VEGF signaling cascades in osteosarcoma.

Conflict of interest
The authors declare that they have no conflict of interest.
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