LncRNA ADAMTS9-AS2 in osteosarcoma inhibits cell proliferation and enhances paclitaxel sensitivity by suppressing microRNA-130a-5p

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Abstract
Introduction: Long noncoding RNA ADAMTS9-AS2 (lncRNA ADAMTS9-AS2) has critical function in tumor growth and drug resistance of various cancers. However, the role and mechanism of lncRNA ADAMTS9-AS2 in osteosarcoma (OS) is still unclear.

Methods: The expression of lncRNA ADAMTS9-AS2 and MicroRNAs-130a-5p (miR-130a-5p) was detected by real-time polymerase chain reaction (RT-qPCR) experiment. In addition, we used the plasmids transfection to construct the lncRNA ADAMTS9-AS2 overexpressed OS cell lines. Subsequently, the cell proliferation ability and the sensitivity to paclitaxel (PTX) in OS cells upon up-regulating lncRNA ADAMTS9-AS2 expression were analyzed via CCK-8 assay, while Western blotting experiment was performed to detect the regulatory mechanism.

Results: We found that lncRNA ADAMTS9-AS2 was down-regulated in OS tissues, and the OS patients with lncRNA ADAMTS9-AS2 downexpression were usually accompanied with a poor prognosis. Subsequently, we discovered that up-regulation of lncRNA ADAMTS9-AS2 inhibited cell proliferation and increased the sensitivity to PTX in OS cells. Interestingly, the Western blot results showed that overexpression of lncRNA ADAMTS9-AS2 could lead to PTEN expression increased, with PI3K and p-AKT expression decreased, indicating that lncRNA ADAMTS9-AS2 could increase the OS cell sensitivity to PTX via regulating PTEN-PI3K/AKT pathway. Furthermore, we identified MicroRNAs-130a-5p (miR-130a-5p) as the downstream target gene of lncRNA ADAMTS9-AS2, which was further confirmed by the luciferase reporter assay. More importantly, our data revealed that miR-130a-5p mimics could partly reverse the influence on cell proliferation and drug sensitivity induced by lncRNA ADAMTS9-AS2 overexpression.

Conclusion: LncRNA ADAMTS9-AS2 exerts its anti-carcinogenesis function by sponging miR-130a-5p, which might be a new therapeutic target for OS treatment.

Keywords
drug sensitivity, lncRNA ADAMTS9-AS2, miR-130a-5p, osteosarcoma, proliferation

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Introduction
Osteosarcoma (OS) is a common malignant tumor in the world, with relatively high incidence and mortality among childhood and adolescents, remaining a big threat to human health.1,2 In recent year, major breakthrough had been made in the comprehensive treatment approaches for OS patients, with targeted therapy and immunization therapy emerging. However, due to the presence of...
constitutive and acquired drug resistances, the OS patients were more likely to have poor prognosis. Consequently, identify a novel bio-marker related to chemotherapy resistance is imperative, which would be a promising way to improve the prognosis of the patients with OS.

The long noncoding RNAs (lncRNAs) are a subcategory of noncoding RNA (ncRNAs). LncRNAs have received increasing attention, as numerous lncRNAs have been confirmed to participate in the progression of malignant tumor. For example, upregulated lncRNA ENST00000470447.1 could suppress cell metastasis ability in oral cancer. More importantly, recent researches show that lncRNAs are actively participated in the regulation of chemotherapy resistance. Therefore, lncRNAs would be a promising bio-marker for the treatment of OS. LncRNA ADAMTS9-AS2 was initially identified in breast cancer with a role of LncRNA. ADAMTS9-AS2 is an antisense transcript of protein coding gene ADAMTS9, which was initially identified in breast cancer and might be involved in reversing tamoxifen resistance. In addition, Yan et al. demonstrated that lncRNA ADAMTS9-AS2 was participated in regulating temozolomide resistance in glioblastoma. However, the mechanism and function of lncRNA ADAMTS9-AS2 in the progression of OS was not fully known.

Accumulating studies demonstrated that lncRNAs could serve as a tumor activator or suppressor in different type cancer via sponging miRNA. LncRNA SNHG1, for example, had been reported to act as a tumor promotor in non-small cell lung cancer through miR-145-5p/MTDH axis. According to the results of bio-information, we supposed that microRNAs-130a-5p (miR-130a-5p) might serve as the downstream target of LncRNA ADAMTS9-AS2. Previous studies indicated that miR-130a-5p was closely associated with malignant biological effects of glioma, gastric cancer and so on. However, there is still unclear whether miR-130a-5p is involved in tumor regulation of OS.

In this study, we aimed to identify whether lncRNA ADAMTS9-AS2 functioned as a suppressor in the development of OS. Herein, we first evaluated the expression level of lncRNA ADAMTS9-AS2 in both of OS tissue samples and cell lines. Subsequently, we performed in vitro experiments to estimate the precise role of lncRNA ADAMTS9-AS2 in tumor growth and drug resistance, and confirmed that miR-130a-5p would be the downstream target gene of lncRNA ADAMTS9-AS2 via luciferase experiments. Consequently, we hypothesized that lncRNA ADAMTS9-AS2 could overcome the drug resistance of OS via regulating miR-130a-5p, then further explore its potential mechanism.

Methods

Tissue samples

The OS tissue and adjacent normal tissue samples were obtained from 65 patients who had not been treated with radio- or chemical therapy in our center. And all of the samples were confirmed by veteran pathologists. The medical ethics committee of our center approved this study. This study is a single-center retrospective study, and all patients involved in this research were signed the informed consent voluntarily.

Cell culture

OS cells were obtained from American Type Culture Collection (ATCC, USA). In addition, all of cells were incubated in 10% fetal bovine serum (FBS; Gibco, Gran Island, NY) contained Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, Logan, UT, USA) in a humid atmosphere with 5% CO₂ at 37°C.

Cell transfection

The lncRNA ADAMTS9-AS2 plasmids (called pcDNA-ADAMTS9-AS2) and empty vectors were directly bought from GenePharma Company (Shanghai, China). LncRNA ADAMTS9-AS2 plasmids were transfected into U2-os and MG-63 cells to upregulate the expression of lncRNA ADAMTS9-AS2, while empty vectors were used as negative control (NC). MiR-130a-5p specific mimics and NC (GenePharma) were used for miR-130a-5p upregulation. All the transfection was achieved using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

RNA extraction and quantitative real-time polymerase chain reaction

In brief, total RNA was obtained from cell lines and tissue samples with Trizol reagent (Takara, Dalian, China), then reverse-transcribed into
complementary DNA (cDNA) with the PrimeScript RT Master Mix (TaKaRa). Real-time polymerase chain reaction (RT-qPCR) was performed with the standard SYBR-Green PCR kit (Roche, America), and $2^{-\Delta\Delta C_{t}}$ method was used to analyze the relative expression of target genes.

Primers used in this study were as follows: GAPDH, F: 5′-CAC CCA CTC CTC CAC CTT TG-3′, R: 5′-CCA CCC TGT TGC TGT AG-3′, LncRNA ADAMTS9-AS2, F: 5′-TCT GTT GCC CAT TTC CTA CC-3′, R: 5′-CCC TTC CAT CCT GTC TAC TCT A-3′; MiR-130a-5p, F: 5′-ACA CTC CAG CTG GGT TCA GCT CCT ATA TGA T-3′, R: 5′-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG AAA GGC AT-3′.

**Cell Counting Kit-8 assay**

The transfected cells were planted in 96-well plates. After incubation for 0, 24, 48, 72 and 96 h, Cell Counting Kit-8 (CCK-8, Dojindo) solution was added to each well. A microplate reader (Molecular Devices, Sunnyvale, CA, USA) was applied to detect the absorbance at 450 nm for each well. To date, to assay the drug sensitivity to paclitaxel (PTX), OS cells were also pretreated with different concentration of PTX, then detected the cell viability using CCK-8 assays.

**Luciferase reporter assay**

In order to further elucidate the potential target microRNA of lncRNA ADAMTS9-AS2, publicly available bioinformatic algorithms (StarBase 2.0) was utilized to predict the binding sites between target miRNA and lncRNA ADAMTS9-AS2. The 293-T cells were plated in 24-well plates at the concentration of $5 \times 10^4$ cells/well. Subsequently, cells were transfected with the wild-type lncRNA ADAMTS9-AS2 reporter (lncRNA ADAMTS9-AS2-Wt) or the mutant-type lncRNA ADAMTS9-AS2 reporter (lncRNA ADAMTS9-AS2-Mut). miR-130a-5p mimics or miR-NC were co-transfected into 293-T cells. After 48 h, Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) was used to evaluate the relative luciferase activity.

**RNA-immunoprecipitation**

The Magna RIPTM RNA kit (Millipore, Bedford, MA, USA) was used for RNA-immunoprecipitation (RIP) assay. In brief, the treated cells were lysed with RIPA solution supplement with protease inhibitor and RNA enzyme inhibitor. Cell extraction was incubated with IgG and MS2 antibodies. Protein samples were digested, and immuno-precipitated RNA was harvested. The expression level of purified RNA was detected by RT-qPCR.

**Western blot analysis**

The RIPA lysis buffer (Beyotime Biotechnology, China) with protease inhibitor (Roche, China) was applied for the extraction of total proteins. After that, equal amounts of proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred into polyvinylidene difluoride (PVDF) membrane. Blocking was performed with 5% BSA, and then the membrane was incubated with primary antibodies and secondary antibodies. The blot signals were visualized by chemiluminescent detection system.

**Statistical analysis**

Data were shown as the mean ± standard deviation (SD). The SPSS 20.0 software (Chicago, IL, USA) was used for statistical analyses. Differences among groups were measured by the student’s t-test and the one-way analysis of variance. $P<0.05$ was considered statistically significant. All the experiments were conducted three times.

**Results**

**Down-regulation of lncRNA ADAMTS9-AS2 was related to poor prognosis in OS**

First of all, to determine the regulatory role of lncRNA ADAMTS9-AS2 in OS, RT-qPCR was performed. Our data displayed that lower-expression of lncRNA ADAMTS9-AS2 accounted for 74% (37/50) of OS specimens (Figure 1(a)). Compared with that in the adjacent non-tumor tissues, the expression of lncRNA ADAMTS9-AS2 was significantly down-regulated in OS tissues (Figure 1(b)). Besides, cumulative survival results exhibited that patients with lower-expression of lncRNA ADAMTS9-AS2 might have shorter survival time (Figure 1(c)). Furthermore, OS patients with low expression level of lncRNA ADAMTS9-AS2 were more likely to have poor histological differentiation (Figure 1(d)), advanced
enneking (Figure 1(e)) and distant metastasis (Figure 1(f)).

In order to detect the relationship between the clinicopathological characteristics and the expression level of lncRNA ADAMTS9-AS2 in OS, we divided the tissue samples into two groups based on the median of the relative lncRNA ADAMTS9-AS2 expression. In this study, we discovered that the expression of lncRNA ADAMTS9-AS2 was closely associated with larger tumor size ($P = 0.037$), poor histological differentiation ($P = 0.030$), distant metastasis ($P = 0.017$), and the advanced enneking stage ($P = 0.036$; Table 1). Taken together, these results consistently indicated that lncRNA ADAMTS9-AS2 might serve as a biomarker of poor prognosis.

**Overexpression of lncRNA ADAMTS9-AS2 suppresses cell proliferation and increases sensitivity to PTX in OS**

Since lncRNA ADAMTS9-AS2 was proven to be down-regulated in OS and capable of predicting poor prognosis, we subsequently speculated that lncRNA ADAMTS9-AS2 could act as a suppressor in OS progression. To further investigate its potential biological function, the expression of lncRNA ADAMTS9-AS2 in OS cells was determined by RT-qPCR assay. Then, the results showed that the expression of lncRNA ADAMTS9-AS2 in OS cell lines was all down-regulated relative to the normal Hfo1b.19 cell line, with U2-OS and MG-63 cell lines showing the lowest expression (Figure 2(a)). The lncRNA ADAMTS9-AS2 overexpressed U2-OS and MG-63 cell lines via plasmids transfection. Subsequently, our data proved that compared with the blank group (OS cells without treatment), the expression of lncRNA ADAMTS9-AS2 was not changed in the NC group (OE-vector), but the OE-lncRNA ADAMTS9-AS2 group with dramatical overexpression levels of lncRNA ADAMTS9-AS2, implying that the lncRNA ADAMTS9-AS2 overexpressed cell lines had been successfully constructed (Figure 2(b) and (c)).

After that, we collected the OE-lncRNA ADAMTS9-AS2 cells for CCK-8 assay and detected that upregulation of lncRNA ADAMTS9-AS2 significantly inhibited proliferation of U2-OS and MG-63 cells (Figure 2(d) and (e)). Furthermore, to detect the influence of lncRNA ADAMTS9-AS2 on drug sensitivity, we also detected the cell viabilities of OE-lncRNA ADAMTS9-AS2 OS cells in the
presence of PTX, with concentration ranged from 0–40 μg/mL. Compared to OE-vector group, OS cells showed increased sensitivity to PTX in OE-lncRNA ADAMTS9-AS2 group, as the concentration of IC50 was lower (Figure 2(f) and (g)). These findings consistently elucidated the role of lncRNA ADAMTS9-AS2 in OS progression.

LncRNA ADAMTS9-AS2 could inhibit the PETN/PI3K/AKT signaling pathway

We subsequently detected the expression of PETN/PI3K/AKT signaling pathway, which was highly related to cell proliferation and drug resistance. As shown in Figure 3, the expression of PTEN protein was dramatically increased, while PI3K and p-AKT proteins were reduced, after the overexpression of lncRNA ADAMTS9-AS2.

MicroRNAs-130a-5p (miR-130a-5p) expression showed negative relationship with lncRNA ADAMTS9-AS2 in OS patients

To identify the way through which lncRNA ADAMTS9-AS2 developed its carcinogenic function, we further predicted that miR-103a-5p might be the potential target of lncRNA ADAMTS9-AS2 via StarBase website. In addition, our data indicated that miR-103a-5p was overexpressed in 76% (38/50) of OS tissues (Figure 4(a)). Similarly, compared with the adjacent normal tissues, the miR-103a-5p expression in OS tumors was remarkably up-regulated (Figure 4(b), \( P < 0.0001 \)). Moreover, The correlation analysis showed that the lncRNA ADAMTS9-AS2 expression was negatively correlated with the miR-103a-5p (Figure 4(c), \( r = -0.6123, P < 0.001 \)).

### Table 1. Correlation of lncRNA ADAMTS-9-AS2 expression with clinicopathological factors in osteosarcoma.

| Clinicopathological features | Number of cases | lncRNA ADAMTS-9-AS2 expression | \( P^* \) |
|-----------------------------|-----------------|-------------------------------|----------|
|                             |                 | Low (n=25)                    | High (n=25) |
| Age (years)                 |                 |                               |           |
| <18                         | 28              | 13                            | 15        |
| ≥18                         | 23              | 12                            | 10        |
| Gender                      |                 |                               |           |
| Male                        | 31              | 17                            | 14        |
| Female                      | 19              | 8                             | 11        |
| Tumor size (cm)             |                 |                               |           |
| >5                          | 17              | 12                            | 5         |
| ≤5                          | 33              | 13                            | 20        |
| Anatomic site               |                 |                               |           |
| Humerus/scapula/rib         | 15              | 8                             | 7         |
| Femur/fibula/tibia          | 35              | 17                            | 18        |
| Histological differentiation|                 |                               |           |
| Well                        | 20              | 6                             | 14        |
| Moderate                    | 17              | 9                             | 8         |
| Poor                        | 13              | 10                            | 3         |
| Enneking stage              |                 |                               |           |
| I                           | 16              | 4                             | 12        |
| II                          | 23              | 13                            | 10        |
| III                         | 11              | 8                             | 3         |
| Distant metastasis          |                 |                               |           |
| Yes                         | 11              | 9                             | 2         |
| No                          | 39              | 16                            | 23        |
| LDH (U/L)                   |                 |                               |           |
| <500                        | 30              | 16                            | 14        |
| ≥500                        | 20              | 9                             | 11        |
| ALP (U/L)                   |                 |                               |           |
| <500                        | 34              | 14                            | 20        |
| ≥500                        | 16              | 11                            | 5         |

*\( P < 0.05 \) indicated statistically significant.
MicroRNAs-130a-5p is the downstream target of lncRNA ADAMTS9-AS2 in OS

As lncRNAs have been acknowledged to regulate expression of certain miRNAs by sponging them at specific binding sites. The binding sites between lncRNA ADAMTS9-AS2 and the miR-130a-5p were also illustrated (Figure 5(a)). More importantly, the results of luciferase reporter assay confirmed that miR-130a-5p was predicted to be the potential downstream target of lncRNA ADAMTS9-AS2 (Figure 5(b)). In addition, RIP assay showed a significantly higher abundance of microRNA-130a-5p in the ADAMTS9-AS2 group, while no evident change was observed after the binding sites in ADAMTS9-AS2 were mutated (Figure 5(c)). To further explore the association between lncRNA ADAMTS9-AS2 and miR-130a-5p, a luciferase reporter assay was performed. The results showed that the luciferase activity increased significantly when the binding sites were mutated, indicating that miR-130a-5p was the downstream target of lncRNA ADAMTS9-AS2.

Figure 2. Overexpression of lncRNA ADAMTS9-AS2 suppresses cell growth and increases sensitivity to PTX in OS cells: (a) the expression level of lncRNA ADAMTS9-AS2 in OS cell lines (143B, Saos-2, MG-63, HOS, os-732 and U2-OS cells) and normal Hfob1.19 cell line, detected by RT-qPCR assay, ((b) and (c)) the upregulation efficiency of lncRNA ADAMTS9-AS2 by specific-plasmids interference in OS cell lines, ((d) and (e)) the effect of lncRNA ADAMTS9-AS2 on proliferation of OS cells in vitro using CCK-8 assay, and ((f) and (g)) the effect of lncRNA ADAMTS9-AS2 on PTX sensitivity of OS cells using CCK-8 assay. All experiments were repeated at least three times.

***P<0.001.

Figure 3. LncRNA ADAMTS9-AS2 could regulate the PTEN/PI3K/AKT signaling pathway: the expression of drug resistance–related PTEN, PI3K, AKT, and p-AKT proteins via Western blotting.
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and miR-130a-5p, we increase the expression of miR-130a-5p in OE-lncRNA ADAMTS9-AS2 MG-63 cells, using miR-130a-5p mimics (Figure 5(d)). From the results of CCK-8 assay, miR-130a-5p could partly rescue the influence on cell proliferation and drug sensitivity induced by lncRNA ADAMTS9-AS2 overexpression (Figure 5(e) and (f)). Taken together, these findings suggest that miR-130a-5p might be a downstream gene of lncRNA ADAMTS9-AS2 in OS.

Discussion

OS, a common type of malignant cancer, is one of the deadly diseases for mankind. To date, PTX is a widely used chemotherapy drug in treatment of various cancers, including OS. However, the presence of primary or acquired PTX resistance contributed more to the high death rate of OS. In recent year, the biological technologies and researches had made a significant progress, but the underlying mechanisms related to PTX resistance was still unclear.

Identifying the differentiate expressed genes would be a promising way to clarify the precise molecular mechanism of PTX resistance. Emerging studies confirmed that IncRNAs have critical function in the chemotherapy resistance of various cancer, including gastric cancer, esophageal cancer, and hepatocellular cancer.
Recently, numerous studies identified lncRNA ADAMTS9-AS2 as a tumor suppressor gene, including gastric cancer\(^\text{25}\) and colorectal cancer.\(^\text{26}\) For example, Pan et al.\(^\text{26}\) revealed that the ADAMTS9-AS2/miR-32/PHLPP2 regulatory axis might act as an important therapeutic target for the treatment of colorectal cancer. Similarly, in this study, the results of RT-qPCR assay indicated that the expression level of lncRNA ADAMTS9-AS2 was lower than that in the adjacent normal tissues. After statistical analysis, we further discovered that the OS patients with ADAMTS9-AS2 down-regulation were more likely to have larger tumor size, poor histological differentiation, the advanced Enneking stage, distant metastasis, and shorter survival time. Taken together, lncRNA ADAMTS9-AS2 might function as a tumor suppressor in OS progression.

To further conform the biological function of lncRNA ADAMTS9-AS2 in OS development, we constructed the lncRNA ADAMTS9-AS2-overexpressed OS cells via plasmids transfection. The results of CCK-8 assay indicated that overexpression of lncRNA ADAMTS9-AS2 could markedly inhibit cell proliferation ability of OS. Moreover, we discovered that up-regulation of lncRNA ADAMTS9-AS2 significantly increased cellular susceptibility to PTX in both Saos-2 cells and MG-63 cells. Taken together, these results consistently exhibited that lncRNA ADAMTS9-AS2 was closely associated with the tumor growth and drug resistance of OS, but its potential regulated mechanisms remained unclear.

Phosphatase and tensin homolog (PTEN) is closely related to tumor proliferation inhibition and apoptosis induction, which is a negative regulator of PI3K/AKT signaling pathway.\(^\text{27,28}\) More importantly, increasing studies identified that the PI3K/AKT signaling pathway could activate the NF-κB signaling pathway, which was involved in up-regulating the expression level of drug resistance related P-gp protein.\(^\text{29}\) For instance, miR-19b-3p had been proved to inhibit the PI3K/AKT signaling pathway contributed to the reversal of drug resistance.
saracatinib-resistance in breast cancer. Therefore, in this study, the Western blotting assay was applied to determine whether PTEN-P38K/Akt pathway was involved in lncRNA ADAMTS9-AS2-induced PTX resistance of OS cells. And our data revealed that overexpression of lncRNA ADAMTS9-AS2 could lead to PTEN up-regulated, while P38K and p-AKT proteins were significantly down-regulated, indicating that lncRNA ADAMTS9-AS2 might inhibit cell proliferation and enhance PTX sensitivity via suppressing PTEN-P38K/AKT pathway.

Moreover, to further research the downstream of lncRNA ADAMTS9-AS2 on regulating OS cells, we screened in the StarBase and identified miR-130a-5p as the downstream gene of lncRNA ADAMTS9-AS2. In fact, miR-130a-5p was initially deciphered with a role in promoting cell proliferation, migration, and invasion in various cancers, such as glioma and gastric cancer. Interestingly, Sun et al. discovers that miR-130a-5p could exert anti-tumor effects via binding to SOX4, while SOX4 is identified as an important regulatory factor of P38K/AKT signaling pathway. In addition, another study demonstrated that miR-130a exerted neuroprotective effects against ischemic stroke through activating PTEN/P38K/AKT pathway. Therefore, we hypothesized that lncRNA ADAMTS9-AS2 would increase the sensitivity to PTX in OS cells via regulating miR-130a-5p/P38K/AKT axis. In this study, we found that miR-130a-5p was up-regulated in OS tissues, and its expression level was negatively related to lncRNA ADAMTS9-AS2. In addition, we successfully validated miR-130a-5p as lncRNA ADAMTS9-AS2 target downstream gene via luciferase reporter assay. Furthermore, we discovered that supplement of miR-130a-5p mimics could partly rescue the influence on cell proliferation and drug sensitivity induced by lncRNA ADAMTS9-AS2 overexpression.

However, there are still some limitations in this research. First, the potential downstream of lncRNA ADAMTS9-AS2/miR-130a-5p axis was not fully clarified. Second, the in vivo experiments need further exploration to confirm the biological role of lncRNA ADAMTS9-AS2 in OS progression. Third, in this study, we only elucidated the association between the expression level of lncRNA ADAMTS9-AS2 and the clinicopathological features of OS patients, but without knowledge of sensitivity and specificity for lncRNA ADAMTS9-AS2 in prognosis prediction of OS patients. In total, these data consistently indicated that lncRNA ADAMTS9-AS2 could function as a critical regulator in overcoming PTX resistance of OS cells via regulating miR-130a-5p/P38K/AKT axis.

**Conclusion**

To sum up, this study confirmed that lncRNA ADAMTS9-AS2 sponged miR-130a-5p, then elicited its impact on cell proliferation and drug sensitivity in OS via regulating PTEN-P38K/Akt pathway, which presents a new clue to help figure out the potential cure for patients with OS in the future.

**Declaration of conflicting interests**

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

**Ethical approval**

Ethical approval for this study was obtained from the institutional ethical review board of Gansu Provincial People’s Hospital, Gansu Province, China.

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**Informed consent**

Written informed consent was obtained from all subjects before the study.

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