Combination of hsa_circ_0004674 and lncRNA OIP5-AS1 as a novel clinical biomarker used to predict prognosis in patients with osteosarcoma

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Abstract. Osteosarcoma is a malignant tumor that predominantly occurs in children or adolescents under the age of 20 years old. Metastasis and chemotherapy resistance are two problems in the treatment of osteosarcoma, and the lack of definite biomarkers impairs the course of treatment. In recent years, non-coding RNA, as a biomarker of osteosarcoma, has become an area of research focus. The role of long non-coding RNAs (lncRNAs), such as lncRNA OIP5-AS1, and circular RNAs, such as hsa_circ_0004674, in osteosarcoma have previously been revealed, and the present study investigated their clinical significance. A total of 20 samples were collected from patients with osteosarcoma. The expression levels of lncRNA OIP5-AS1 and hsa_circ_0004674 were analyzed in tumor tissues and patient serum, and their associations with chemotherapy sensitivity, lung metastasis and prognosis were assessed. The results revealed that these two non-coding RNAs were significantly upregulated in the osteosarcoma tissues of patients compared with those in the adjacent tumor tissues. In addition, the expression levels of the two non-coding RNAs were increased in the serum of patients with osteosarcoma compared with those in patients with bone fractures (P<0.01). In patients with lung metastasis or chemotherapy resistance (tumor necrosis rate <90%), the expression levels of the two non-coding RNAs were similarly increased. By plotting the receiver operating characteristic curve, it was revealed that the combination of hsa_circ_0004674 and lncRNA OIP5-AS1 was better than ALP or either non-coding RNA alone in predicting chemotherapy sensitivity and metastasis. Kaplan-Meier survival analysis showed that, in patients with osteosarcoma, higher expression of both non-coding RNAs was associated with worse survival time (log-rank test P=0.006). In conclusion, the combination of hsa_circ_0004674 and lncRNA OIP5-AS1 may be used as a better biomarker than traditional biomarkers, such as ALP, in a clinical setting.

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Introduction

Osteosarcoma is a common high-grade malignant bone tumor in children and adolescents. In recent years, due to the continuous progress of neoadjuvant chemotherapy and other comprehensive treatment strategies, the survival rate of osteosarcoma has increased to 60-70% (1). However, due to the constraints of distant metastasis and chemotherapy resistance, it remains difficult to further improve the survival rate (2). Currently, a large number of studies aim to identify biomarkers to provide evidence for chemotherapy resistance and prognosis.

Long non-coding RNAs (lncRNAs) contain >200 nucleic acid sequences and are not translated into functional proteins (3). lncRNAs have been increasingly explored, and several studies have reported that these RNAs are associated with the migration and invasion of osteosarcoma, including the lncRNAs EPIC1 and NHG1 (4,5). Circular RNA (circRNA) is synthesized by the reverse splicing of an mRNA precursor, resulting in a circular, rather than a linear structure (6). Through the rapid development of high-throughput RNA sequencing technology, more circRNAs related to osteosarcoma have been discovered. Notably circMYO10 has been reported to be associated with chromatin remodeling in osteosarcoma cells, and circTADA2A may be associated with the proliferation and migration of osteosarcoma (7,8). Through high-throughput screening of the whole transcriptome, our previous study revealed that the expression levels of lncRNA OIP5-AS1, hsa_circ_0081001, lncRNA ODRUL and hsa_circ_0004674 were significantly increased in osteosarcoma, and were closely associated with the proliferation, metastasis and drug resistance of the disease (9-12). Upregulation of these RNA indicators may promote tumor proliferation and increase chemotherapy resistance, and may have a tumor-promoting role in osteosarcoma. Some studies

have reported similar conclusions. For example, regulation of the OIP5-AS1/microRNA (miR)-223/CDK14 axis has been shown to have a significant influence on tumorigenesis, which is closely associated with the poor prognosis of patients with osteosarcoma (13). In addition, lncRNA OIP5-AS1 can cause cisplatin resistance in osteosarcoma (14). The present study further explored the clinical significance of these RNAs and their potential for clinical application. The expression levels of these four non-coding RNAs were initially detected in samples. Results of hsa_circ_0081001 and lncRNA ODRUL showed no statistical significance in chemotherapy sensitivity. So the data of hsa_circ_0081001 and lncRNA ODRUL was not referred. The results of lncRNA OIP5-AS1 and hsa_circ_0004674 in pilot study was showed in Figs. 1-3. Therefore, the present study concentrated on the combined use of lncRNA OIP5-AS1 and hsa_circ_0004674 in a clinical setting.

Although previous studies have investigated the expression and mechanism of lncRNA OIP5-AS1 and hsa_circ_0004674 in osteosarcoma cell lines and tissues (9,12), and demonstrated their potential as biomarkers, adequate clinical data have not been obtained. The present study analyzed the serum and tissues of patients with osteosarcoma to explore the association between the two types of non-coding RNAs and the patient data regarding chemotherapy resistance (tumor necrosis rate <90%), distant metastasis and prognosis.

Materials and methods

Patients and specimens. Tumor specimens were collected from patients with primary osteosarcoma who were treated at Shanghai Tenth People's Hospital (Shanghai, China) between June 2014 and December 2019. The age of patients was between 5 and 32 years old, with an average age of 15.25 years. The study received ethical approval (approval no. SHSY-IEC-4.1/21-300/01) from the Institutional Review Board of Shanghai Tenth People's Hospital Affiliated to Tongji University. All patients/guardians participating in the present study provided written informed consent. For children, the study was explained in simple language and written informed consent was obtained from their guardian. Enrolled patients were diagnosed with osteosarcoma, and pathologists determined that the pathological subtype was conventional osteosarcoma. The conventional osteosarcoma is the most frequent variant, which arises from the medullary bone (15). All patients received two cycles of preoperative chemotherapy and at least four cycles of postoperative chemotherapy. The dominating chemotherapy protocol was IOR/OS-4 (high-dose methotrexate, 8-12 g/m^2/day; cisplatin, 100 mg/m^2/day; Adriamycin, 30 mg/m^2/day; and high-dose ifosfamide, 2-3 g/m^2/day) (16). In addition, the inclusion criteria also included patients that had not received any treatment before and who cooperated with regular follow-up every month. Exclusion criteria were as follows: i) The patient had an infectious disease; ii) patients with difficulties in venous blood collection; iii) the patient was >3 years old or >60 years old; and iv) patients with other serious physical or mental diseases. The total number of conventional osteosarcoma cases was 28. Patients were excluded who were lost to follow-up (n=3) or who died from serious post-operative complications (n=1). Considering that incomplete surgical resection also affects the prognosis, patients with local tumor recurrence were also excluded (n=4). Finally, 20 patients (Table I) were included in the present study. Postoperative follow-up was performed every 2-3 months. Tumors and adjacent (normal) tissue were collected and placed in liquid nitrogen for immediate storage at -196˚C. Chemotherapy response (tumor necrosis) was graded based on the amount of tumor necrosis in the resected specimen. Tumor specimens with a necrosis rate of <90% were defined as chemotherapy resistant (17) and the results of the necrosis rate were evaluated by pathologists. The surgical margin in the bone tissue was 3 cm outside the tumor, and 5 cm in soft tissue. Adjacent tissues were taken from outside the surgical margin. Pathological results indicated that all surgical margins were negative. The preoperative serum samples refer to the 5 ml venous blood samples taken before chemotherapy. For comparison, serum samples were collected from 8 patients with bone fractures who had not yet undergone surgery (Table II). These patients had no ailment other than bone fractures and were range from 13 to 31 years old. Their average age was 21.125 years old. After the blood samples were collected, they were centrifuged at 194 x g for 5 min at 25˚C. The serum (supernatant fraction) was then collected into a 1.5-ml Eppendorf (EP) tube and stored at -20˚C.

RNA extraction. TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) was added to mung tissues with a volume of ~3 ml, which were ground using a grinding rod for full lysis. Subsequently, 200 μl chloroform was added to the lysate, thoroughly vortexed and centrifuged at 14,548 x g for 10 min at 4˚C. After centrifugation, the solution was divided into three layers, and the upper layer was aspirated into a new 1.5-ml EP tube. Subsequently, 500 μl isopropanol was added to the aspirated supernatant, thoroughly vortexed and centrifuged at 14,548 x g for 20 min at 4˚C. Afterward, the supernatant was removed to retain the precipitate. Prechilled ethanol (80%) was added to wash the precipitate, and the precipitate was centrifuged at 14,548 x g for 2 min at 4˚C. After removing the supernatant, the mixture was placed at room temperature until the precipitate became transparent. Next, 30 μl RNase-free water was added to dissolve the precipitate. The previous step was repeated. A whole blood total RNA extraction kit (Aidlab Biotechnologies, Ltd.) was used for serum RNA extraction, and the extraction method was performed according to the manufacturer's instructions.

Reverse transcription-quantitative PCR. A PrimeScript™ RT Master Mix RT kit (Takara Biotechnology Co., Ltd.) was used to generate cDNA from RNA according to the manufacturer's protocols. Specify a sample volume of 20 μl and edit the protocol parameters to match those shown below: i) Pre-denaturation at 95˚C for 30 sec; ii) cycling stage, 95˚C for 5 sec, then 60˚C for 10 sec, repeat this step 39 times; iii) melt curve stage. The expression levels of hsa_circ_0004674 and lncRNA OIP5-AS1 were detected using a TB green kit (Takara Biotechnology Co., Ltd.). The primer sequences are presented in Table III, and GAPDH was used as the internal reference. The $2^{-\Delta\Delta Cq}$ method was used for quantification (18); ΔCq is the difference in Cq values between the gene of interest and the endogenous control.

Table I. Patient information.

Patient no.	Sex	Age, years	Tumor location	Progression-free survival, months	Lung metastasis	Chemotherapy resistance	Expression of hsa_circ_0004674	Expression of lncRNA OIP5-AS1
1	Male	12	Proximal fibula	26	Yes	No	Low	High
2	Male	14	Proximal tibia	4	Yes	No	High	High
3	Female	8	Proximal tibia	12	Yes	Yes	High	High
4	Male	18	Distal femur	2	Yes	No	High	High
5	Male	20	Scapula	11	Yes	Yes	High	High
6	Female	5	Distal femur	12	Yes	Yes	High	High
7	Male	11	Proximal tibia	28	Yes	No	High	Low
8	Male	14	Proximal tibia	18	Yes	No	High	Low
9	Male	17	Distal femur	48	Yes	Yes	High	High
10	Female	6	Distal femur	4	Yes	No	High	Low
11	Female	28	Distal femur	13	Yes	Yes	High	High
12	Male	14	Distal femur	14	Yes	Yes	Low	High
13	Male	18	Proximal tibia	36	No	No	Low	Low
14	Male	17	Distal femur	9	Yes	Yes	Low	Low
15	Male	16	Proximal tibia	60	No	No	Low	Low
16	Female	15	Distal femur	30	No	Yes	Low	Low
17	Male	13	Distal tibia	22	No	No	Low	Low
18	Male	32	Distal femur	30	No	No	Low	Low
19	Female	17	Proximal humerus	32	Yes	Yes	Low	Low
20	Female	10	Proximal fibula	26	No	No	Low	Low

Low refers to patients with a lower expression compared with that in the serum of control individuals. High refers to patients with a higher expression compared with that in the serum of control individuals. All patients were diagnosed with conventional osteosarcoma.

Table II. Patient information of the control group.

Patients	Sex	Age, years	Site of fracture
1	Male	23	Femur
2	Female	29	Femur
3	Female	18	Humerus
4	Male	13	Ulna and radius
5	Male	23	Clavicle
6	Male	17	Radius
7	Female	15	Tibia
8	Male	31	Tibia and fibula

Table III. PCR primer sequences.

Gene	Accession number	Oligo sequence, 5'-3'
hsa_circ_0004674	NM_001391981	F: GTTGACCAAGC AAGCTTCCAG R: GGTACTTGCAG GTTTTACTGGG
lncRNA OIP5-AS1	NR_026757	F: TGCGAAGATGG CGGAGTAAG R: TAGTTCCTCTC CTCTGGCCG
GAPDH	NM_001289745	F: TCTCTGCTCCT CCTGTTCGA R: GCGCCCAATA CGACCAAATC

F, forward; R, reverse; lncRNA, long non-coding RNA.

Statistical analysis. Statistical analyses were performed and diagrams were generated using SPSS 22.0 statistical software package (IBM Corporation). Paired and unpaired Student's t-tests were performed to assess associations between expression and clinicopathological parameters. The detection of expression levels of serum alkaline phosphatase (ALP) is a necessary test for patients when they are admitted to hospital, and ALP data were obtained from the hospital laboratory reports. The alkaline phosphatase test is performed on fully automated analyzers which are based on the principle of photometry. It was noted that most of the tumor cells in the tissue samples were necrotized after preoperative chemotherapy; therefore, preoperative serum instead of surgically obtained tumor specimens were used when judging chemotherapy sensitivity. Receiver operating characteristic (ROC) curves of hsa_circ_0004674, lncRNA OIP5-AS1 and ALP were plotted to discuss their differences. Progression-free survival (PFS) was calculated using the Kaplan-Meier survival analysis and was evaluated by the log-rank test and Breslow test. P<0.05 was considered to indicate a statistically significant difference.

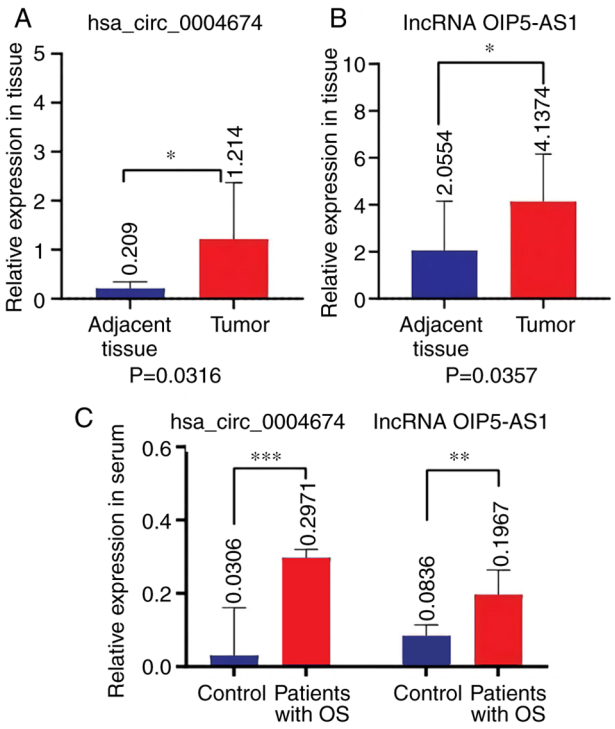

Figure 1. Expression levels of non-coding RNAs in OS. The expression levels of (A) hsa_circ_0004674 and (B) lncRNA OIP5-AS1 in adjacent tissues and tumor tissues of patients with OS (n=20, adjacent tissues and tumor tissues are paired); *P<0.05 (paired Student's t-test). (C) Expression levels of hsa_circ_0004674 and lncRNA OIP5-AS1 in the serum of healthy individuals (n=8) and patients with OS (n=20); **P<0.01, ***P<0.001 (unpaired Student's t-test). lncRNA, long non-coding RNA; OS, osteosarcoma.

Results

Expression levels of hsa_circ_0004674 and lncRNA OIP5-AS1 are significantly increased in the tumor tissues and serum of patients with osteosarcoma. The tumor and adjacent tissues of each patient (n=20) were obtained during surgery. The analysis of expression showed that the levels of hsa_circ_0004674 and lncRNA OIP5-AS1 were significantly higher in tumor tissues than those in adjacent tissues (P=0.0316 and 0.0357; Fig. 1A and B). These results indicated that these two non-coding RNAs were highly expressed in tumor tissues from patients with osteosarcoma. Serum samples from patients with bone fractures and preoperative serum samples from patients with osteosarcoma were then assessed. The results revealed that the expression levels of hsa_circ_0004674 and lncRNA OIP5-AS1 were significantly elevated in the serum of patients with osteosarcoma (Fig. 1C).

Expression levels of hsa_circ_0004674 and lncRNA OIP5-AS1 are significantly increased in the tumor tissues and serum of patients with osteosarcoma and lung metastasis. The expression levels of two non-coding RNAs in the tumor tissues and serum of patients with osteosarcoma and lung metastasis were compared. The results revealed that the expression levels of hsa_circ_0004674 and lncRNA OIP5-AS1 were increased in the tumor tissues of patients with lung metastasis compared with the levels in patients without metastasis (P=0.036 and 0.007; Fig. 2A and B). The expression levels of

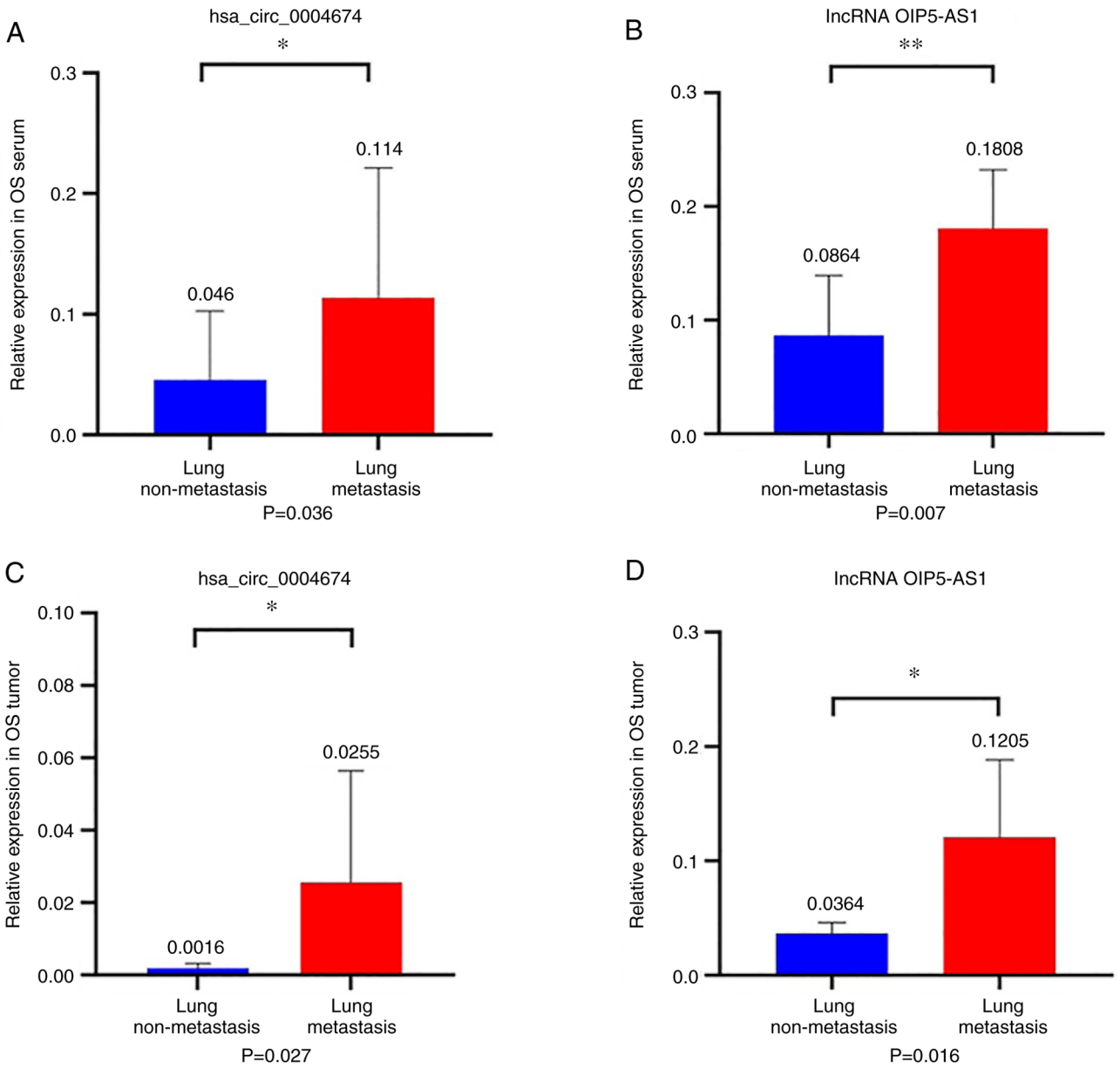

Figure 2. Expression levels of non-coding RNAs in patients with OS, with (n=14) or without (n=6) lung metastasis. The expression levels of (A) hsa_circ_0004674 and (B) lncRNA OIP5-AS1 in the serum of patients with OS, with and without lung metastasis. The expression levels of (C) hsa_circ_0004674 and (D) lncRNA OIP5-AS1 in the tumor tissues of patients with OS, with and without lung metastasis. *P<0.05 and **P<0.01 (unpaired Student's t-test). lncRNA, long non-coding RNA; OS, osteosarcoma.

hsa_circ_0004674 and lncRNA OIP5-AS1 were also significantly increased in the serum of patients with lung metastasis compared with the levels in patients without metastasis (P=0.027 and 0.016; Fig. 2C and D). These findings indicated that the non-coding RNAs hsa_circ_0004674 and lncRNA OIP5-AS1 were significantly associated with lung metastasis in patients with osteosarcoma.

hsa_circ_0004674 and lncRNA OIP5-AS1 expression is associated with the sensitivity of patients with osteosarcoma to chemotherapy. The serum samples used for these experiments were collected from the patients with osteosarcoma before treatment. The results revealed that there was a significant increase in the expression of the two non-coding RNAs in the serum from chemoresistant patients, and suggested that the expression levels of these two non-coding RNAs could provide evidence for determining whether patients were sensitive to chemotherapy ((P=0.038 and 0.048; Fig. 3A and B).

Combination of hsa_circ_0004674 and lncRNA OIP5-AS1 is better than ALP in predicting chemotherapy sensitivity and metastasis. The expression levels of hsa_circ_0004674, lncRNA OIP5-AS1 and ALP were analyzed from the serum. The associations between the expression levels of these markers and metastasis or chemotherapy sensitivity were assessed, and receiver operating characteristic (ROC) curves were plotted. The two non-coding RNAs were more sensitive than ALP in predicting chemosensitivity. Compared with a single index, the combination of hsa_circ_0004674 and lncRNA OIP5-AS1 had better specificity in predicting chemosensitivity

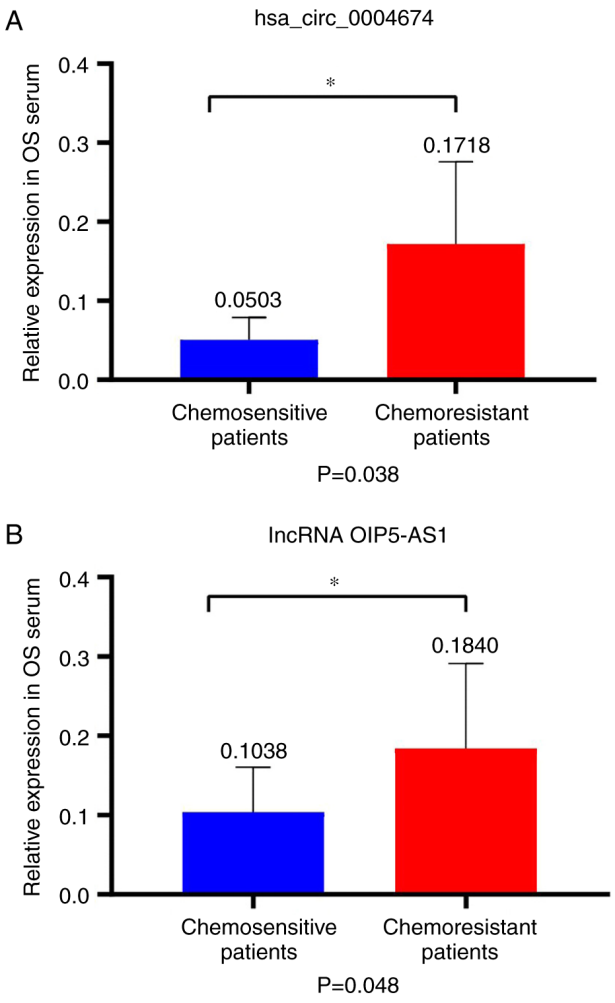

Figure 3. Association between the expression levels of non-coding RNAs in OS and sensitivity to chemotherapy. (A) Expression levels of hsa_circ_0004674 in the serum of patients. (B) Expression levels of lncRNA OIP5-AS1 in the serum of patients. Chemosensitivity (n=11) indicated that the tumor necrosis rate was ≥90%, and chemoresistance (n=9) indicated that the tumor necrosis rate was <90%. *P<0.05 (unpaired Student's t-test). lncRNA, long non-coding RNA; OS, osteosarcoma.

(Fig. 4A; Table IV). In terms of predicting metastasis, the area under the curve (AUC) value of hsa_circ_0004674 was lower than that of ALP, which may indicate that ALP is better in reflecting tumor metastasis. However, AUC values close to 0.9 also indicated a good specificity of hsa_circ_0004674 in predicting metastasis. The AUC values of hsa_circ_0004674, lncRNA OIP5-AS1 and ALP were similar, and all of them had good predictive function in metastasis. However, the combination of hsa_circ_0004674 and lncRNA OIP5-AS1 had the best predictive value (Fig. 4B; Table V).

Combination of hsa_circ_0004674 and lncRNA OIP5-AS1 as clinical biomarkers to predict the prognosis of patients. Based on the aforementioned results, it was hypothesized that the high expression of the two non-coding RNAs may indicate a poor prognosis for patients. The mean values of RNA expression in serum from patients with fractures (control) were considered to be normal compared with those from patients with tumors. Subsequently, the association between the expression levels of lncRNA OIP5-AS1 and patient prognosis was assessed

Table IV. AUC values for chemotherapy sensitivity.

Variable	AUC
ALP	0.519
hsa_circ_0004674	0.574
lncRNA OIP5-AS1	0.685
Combination	0.722

ALP, alkaline phosphatase; AUC, area under the curve; lncRNA, long non-coding RNA.

Table V. AUC values for metastasis.

Variable	AUC
ALP	0.867
hsa_circ_0004674	0.858
lncRNA OIP5-AS1	0.883
Combination	0.983

ALP, alkaline phosphatase; AUC, area under the curve; lncRNA, long non-coding RNA.

(Fig. 5A). Patients with lower expression than mean expression in control individual were assigned to the low expression group, whereas the remaining patients (higher expression than mean expression in control individual) were assigned to the high expression group. The PFS of all patients was assessed and survival curves were generated. The PFS time of all patients ranged between 1 and 60 months, with a mean average of 27.95 months. Excluding deaths from other causes and loss to follow-up, 20 patients were successfully assessed, 13 of whom developed postoperative tumor recurrence or metastasis. A total of 5 patients died during the assessment period, and the remaining patients survived until the end of the study. As shown in Fig. 5A, patients with low serum lncRNA OIP5-AS1 expression had a better prognosis than those with high serum expression, and this difference was significant according to the log-rank test (P=0.009) and the Breslow test (P=0.02). The mean PFS time in the low expression and high expression groups was 39.667 and 15.778 months, respectively.

The same method was used to generate the survival curve according to the expression levels of hsa_circ_0004674 (Fig. 5B). A total of 10 out of the 20 patients were assigned to the high expression group, as their expression levels were greater than those in the control group, and there were 10 patients in the low expression group. The mean PFS time was 40.014 months in the low expression group and 17.200 months in the high expression group, and the difference was significant according to the log-rank test (P=0.015) and the Breslow test (P=0.012).

Although each single indicator was shown to have prognostic potential, a new strategy of combining two RNAs for prognosis was adopted in the present study. Since the AUC value of the combination was the largest (Fig. 4), this new strategy should be able to reflect the prognosis more accurately. Highly expressed lncRNA OIP5-AS1 and hsa_ circ_ 0004674

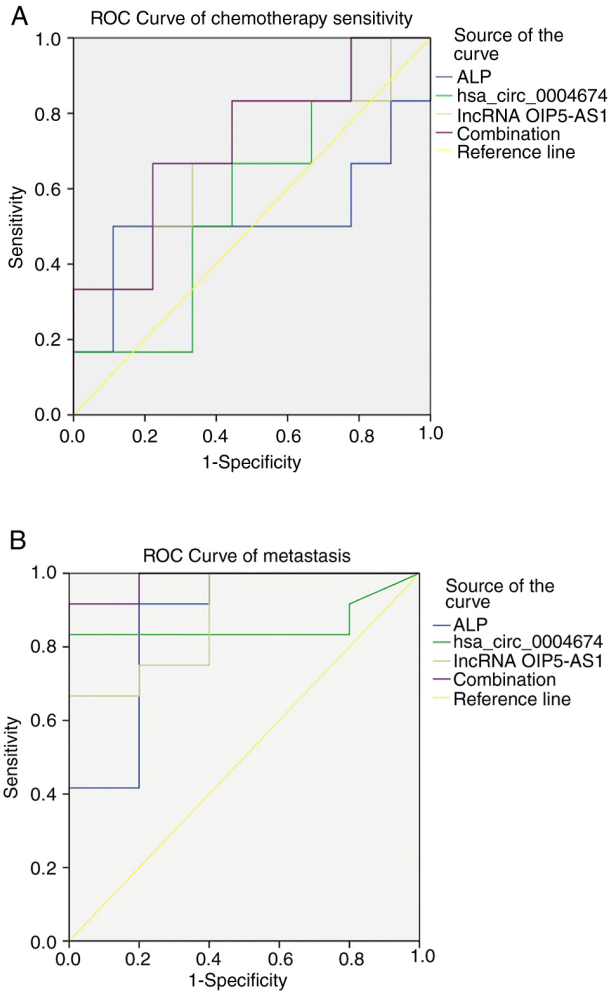

Figure 4. ROC curve of chemosensitivity and metastasis. (A) ROC curve analysis of hsa_circ_0004674, lncRNA OIP5-AS1, ALP, and the combination of hsa_circ_0004674 and lncRNA OIP5-AS1 for sensitivity to chemotherapy. A tumor necrosis rate ≥90% was considered a good response to chemotherapy. The combination of two non-coding RNAs had the best predictive value, while ALP had little value in this respect. (B) ROC curve analysis of hsa_circ_0004674, lncRNA OIP5-AS1, ALP, and the combination of hsa_circ_0004674 and lncRNA OIP5-AS1 for metastasis. All potential markers had great value in predicting metastasis. The AUC of the combination of the two non-coding RNAs was much larger than that of the others. AUC, area under the curve; lncRNA, long non-coding RNA; ROC, receiver operating characteristic.

are considered to be risk factors. Therefore, only when the two indicators are both lower compared with the mean expression in control individuals can the patient be included in the low expression group. If any one of the two indicators is higher compared with the mean expression of the control group, the patient will be assigned to the high expression group Under this new strategy, there were 12 patients in the high expression group and 8 patients in the low expression group. A survival curve was then plotted according to PFS (Fig. 5C). The mean PFS time was 45.46 months in the low expression group and 17.67 months in the high expression group. The results showed that by comparing the expression levels of both lncRNA OIP5-AS1 and hsa_ circ_ 0004674 between patients and the healthy subjects, the survival time could be more effectively predicted compared with analyzing a single indicator (log-rank test P=0.006; Breslow test P=0.012).

The results of the three survival curves indicated that combination of the two RNAs had the smallest P-value, thus suggesting that the combination of hsa_circ_0004674 and lncRNA OIP5-AS1 was a better tool than either of them alone in predicting patient prognosis and survival.

Discussion

Currently, surgical resection of primary lesions combined with neoadjuvant chemotherapy is the main treatment for osteosarcoma, and neoadjuvant chemotherapy has greatly improved the survival time of patients in recent years. However, once metastasis occurs, the 5-year survival rate of patients is <20% (19). Chemotherapy serves an important role in preventing metastasis (20), and patients who are resistant to chemotherapy are more inclined to have a poor prognosis due to metastasis. To date, there are no reliable biomarkers to reflect the postoperative chemotherapy or distant metastasis of osteosarcoma. Effective biomarkers can assist doctors in optimizing patients' therapeutic schedules. In addition, biomarkers serve an important role in the development of new drugs. Researchers can therefore select patients to participate in clinical trials according to the results of the biomarkers, which in turn could accelerate the research and development process (21).

Non-coding RNAs have remained a popular topic in the research of osteosarcoma biomarkers in recent years. The most studied non-coding RNAs include lncRNAs, circRNAs and microRNAs. lncRNAs and circRNAs are closely associated with the metastasis and drug resistance of osteosarcoma (22). Multiple studies have reported correlations *in vitro* (23,24). There have also been some reports on the expression levels of single non-coding RNAs in patients with osteosarcoma. However, due to the individual differences of patients with osteosarcoma and the complexity of the disease, it is likely that a single non-coding RNA would not be effective in predicting prognosis.

The present study analyzed two non-coding RNAs that were significantly upregulated in the serum and tumor tissues of patients with osteosarcoma. Before treatment, the expression levels of hsa_circ_0004674 and lncRNA OIP5-AS1 in the serum may provide strong evidence regarding the sensitivity of patients with osteosarcoma to chemotherapy. If the expression levels of hsa_circ_0004674 or lncRNA OIP5-AS1 in the serum are high, the risk of metastasis and a poor response to chemotherapy may also be increased. In addition, the expression levels of these two RNAs may be useful when estimating the prognosis of patients after surgery. In addition, through the expression of hsa_circ_0004674 and lncRNA OIP5-AS1, the possibility of metastasis in patients can be predicted. The present results agreed with those of previous studies. lncRNA OIP5-AS1 plays a key role in doxorubicin resistance by sponging miR-200b-3p to upregulate the expression of FN1 (9). In addition, it has been suggested that hsa_circ_0004674 may mediate chemotherapy resistance by regulating the circRNA/miR-490-3p/ABCC2 or circRNA/miR-1254/EGFR axes (12). The present study indicated that the possibility of metastasis or cellular response to chemotherapy may be predicted by monitoring the expression levels of both RNAs; however, some problems need to be solved before clinical

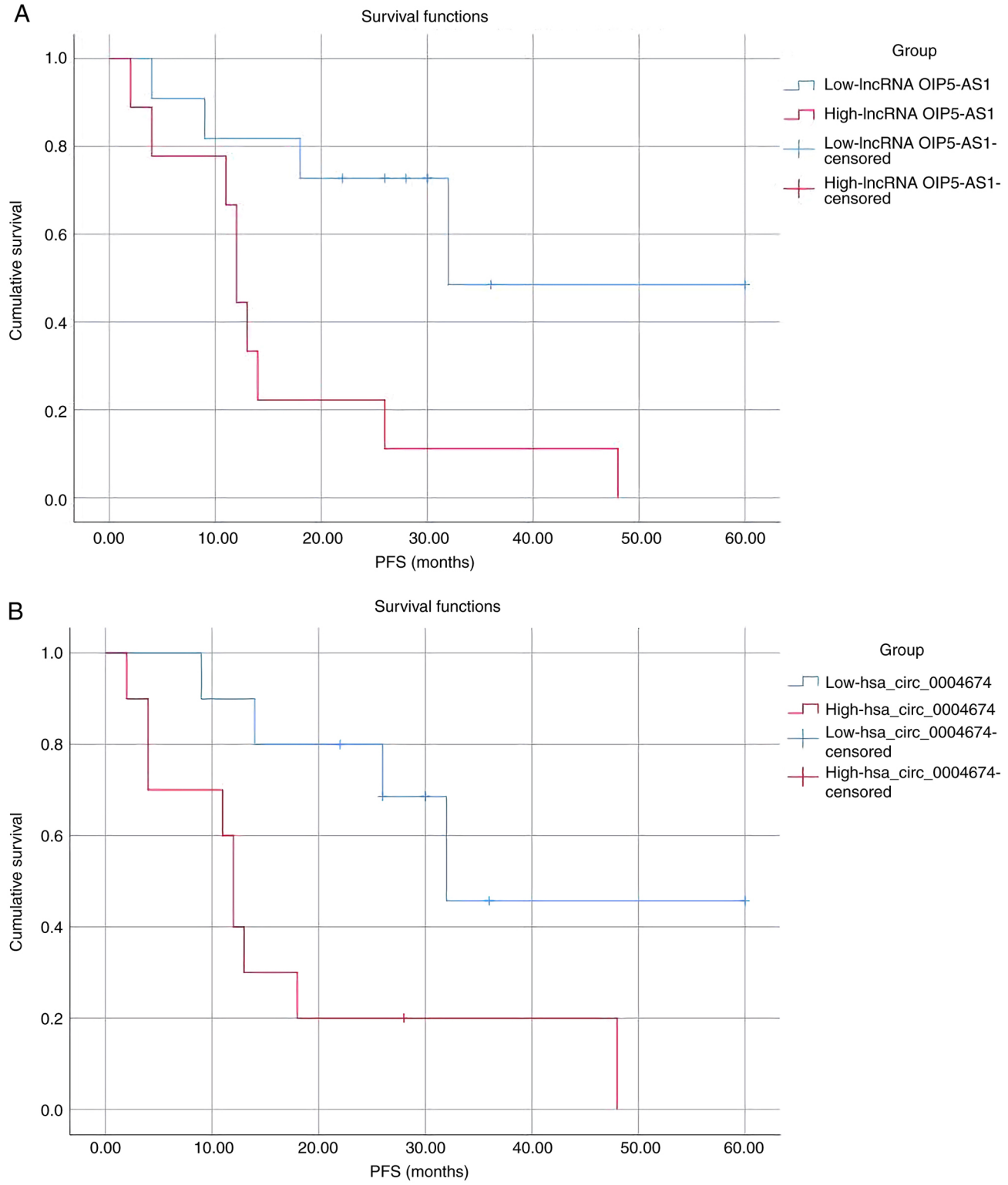

Figure 5. Continued.

application. First, an interval value should be set for healthy individuals, which will require a large control sample that covers all population characteristics. Clinically, patient conditions may change, for example, from chemotherapy sensitivity to chemotherapy resistance. Therefore, it must be determined as to whether the change in the expression levels of the two RNAs is consistent with the changes in clinical characteristics, as well as the specific statistical significance. Finally, the benefit of a combined biomarker must be determined in patients. We plan to address these concerns in future research. However, the current results suggested that these two RNAs are feasible and potential biomarkers. Compared with traditional single biomarkers, the combination of multiple serum RNA indicators has higher sensitivity and specificity for predicting disease progression (25). Elevated ALP has been observed in most patients with osteosarcoma. A number of

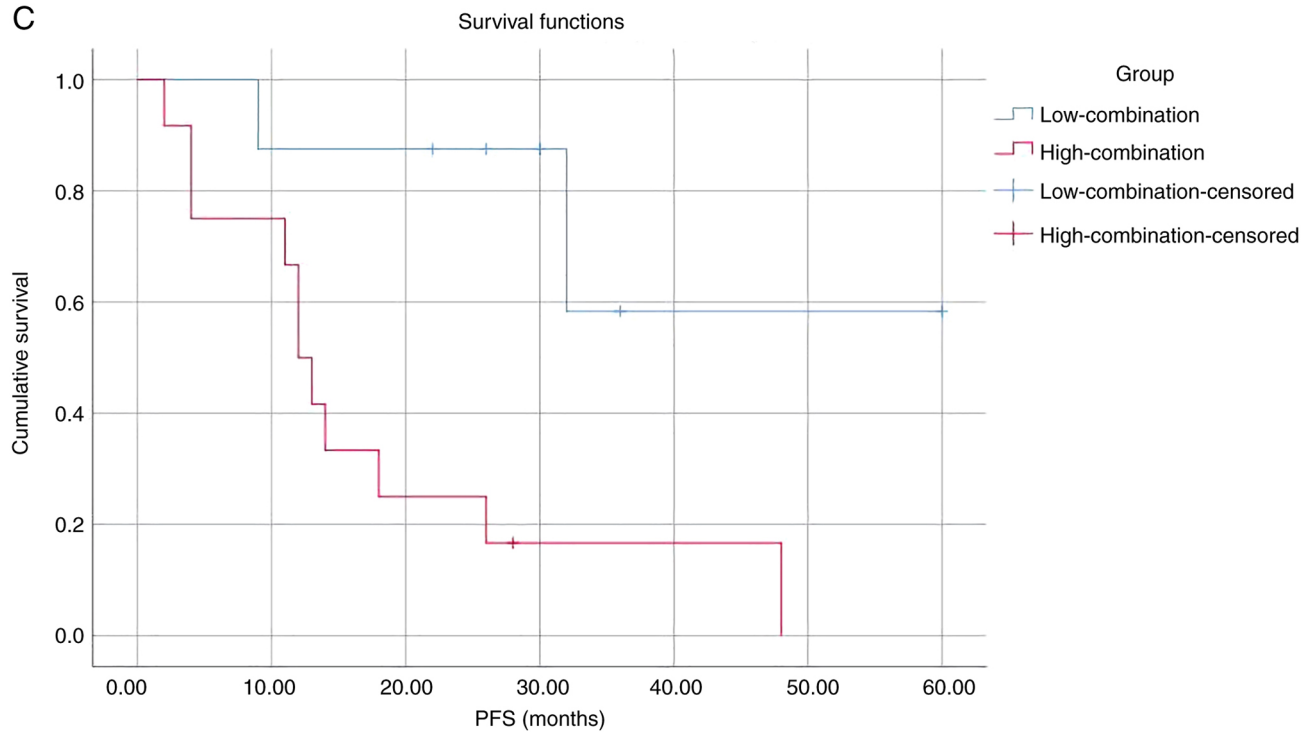

Figure 5. Survival curve of patients with osteosarcoma. (A) Association between the expression levels of lncRNA OIP5-AS1 in the serum of patients and PFS. Low indicates that the expression of lncRNA OIP5-AS1 was lower than that in the control group. The remaining subjects were assigned to the high expression group [high (n=9) and low (n=11)]. (B) Association between hsa_circ_0004674 expression levels in the serum of patients and PFS. Low indicates that the expression of hsa_circ_0004674 was lower than that in the control group. The opposite was true for the high expression group [high (n=10) and low (n=10)]. (C) Association between the expression levels of the two non-coding RNAs in the serum of patients and PFS. Low indicates that the expression levels of both non-coding RNAs were lower than those in the control group. The remaining subjects were assigned to the high expression group [high (n=12) and low (n=8)]. lncRNA, long non-coding RNA; PFS, progression-free survival.

studies (26,27) have explored the relationship between ALP and clinical prognosis, and the association between ALP levels and survival outcomes has been shown to be significant. The increase in ALP is associated with shorter survival times, increased incidence of lung metastasis and a poor chemotherapeutic response (26). The present study suggested that the combination of hsa_circ_0004674 and lncRNA OIP5-AS1 was a better biomarker than ALP with regard to predicting patient prognosis and survival.

Notably, the present study has some limitations. First, the sample size of the present study was small, and samples from the same individual at different treatment stages were lacking. This limited the further exploration of the association between changes in expression levels and disease progression. In the future, we aim to explore the association between serum changes at different time periods post-surgery and clinical progression. A follow-up study of this cohort is still ongoing. Second, the insufficient follow-up time in some patients did not fully demonstrate the potential of the combined use of these RNAs to predict long-term survival outcomes. There remain some unresolved problems, and it could not be ruled out whether there were other factors, such as inflammation and chemotherapy drugs, that may have led to false-positive results (28). As the subject of this study was conventional osteosarcoma, the results cannot be used to assess non-conventional osteosarcoma at present. The molecular mechanism would be the focus of follow-up research to try to solve this problem step by step and screen out biomarkers of osteosarcoma.

In conclusion, in the present study, two non-coding RNA markers were identified, and their clinical value in predicting the chemosensitivity and distant metastasis of conventional osteosarcoma was elucidated. The AUC values suggested that the combined use of hsa_circ_0004674 and lncRNA OIP5-AS1 was more valuable than the use of the conventional ALP marker or any one of the non-coding RNA markers alone. Through the comprehensive use of two non-coding RNA markers, the survival time of patients could be effectively estimated in the clinic. The present study provides a basis for further exploration of the role of these two non-coding RNAs in the diagnosis and treatment of osteosarcoma.

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Availability of data and materials

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

Authors' contributions

All the authors made a significant contribution to this manuscript and have read and approved the final manuscript. CZ and YZ conceptualized the study. ST and KZ designed the methodology. ST and YZ wrote the original draft preparation, and reviewed and edited. JH, ST and YZ acquired and analysed the data. CZ and JH supervised the study. CZ and ST confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The study received ethical approval (approval no. SHSY-IEC-4.1/21-300/01) from the Institutional Review Board of Shanghai Tenth People's Hospital Affiliated to Tongji University (Shanghai, China). All patients/guardians participating in the present study provided written informed consent. For children, the study was explained in simple language and written informed consent was obtained from their guardian.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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