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Original Contribution

Downregulation of serum miR-194 predicts poor prognosis in osteosarcoma patients

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ABSTRACT

Background: Circulating microRNAs (miRNAs) have promising potential as diagnostic and prognostic biomarkers for osteosarcoma. This study aimed to explore the expression pattern of serum miR-194 and its potential clinical value in patients with osteosarcoma.

Methods: Messenger RNA was isolated from serum sample from 124 osteosarcoma patients, 60 periostitis patients and 60 healthy volunteers. The serum miR-194 level was then examined by quantitative real-time polymerase chain reaction (qRT-PCR). The bioinformatic analysis of the downstream targets of miR-194 was also performed.

Results: The results showed serum miR-194 levels were significantly decreased in osteosarcoma patients compared to those in periostitis patients or healthy controls. Receiver-operating characteristic (ROC) analysis demonstrated that serum miR-194 had a good diagnostic value for identifying osteosarcoma subjects from periostitis patients and normal controls. In addition, serum miR-194 levels were dramatically increased following surgery in osteosarcoma cases. Moreover, low serum miR-194 expression was strongly correlated with positive metastasis and advanced clinical stage, as well as worse survival. Furthermore, serum miR-194 was confirmed to be an independent prognostic biomarker for osteosarcoma. Bioinformatic analysis showed that the downstream targeted genes of miR-194 were closely associated with cancer initiation and development.

Conclusion: In conclusion, our results have demonstrated that serum miR-194 might serve as a novel and promising biomarker for the detection and prognosis of osteosarcoma.

1. Introduction

Osteosarcoma is the most frequent primary bone tumor in children and adolescents, and accounts for approximately 55–60% of malignant bone tumors [1,2]. With the development of combined treatment (neoadjuvant chemotherapy, surgery, and adjuvant chemotherapy), the overall 5-year survival rate has significantly increased to 60–70% over the past decades [3,4]. However, the prognosis remains unfavorable for the patients with metastasis at diagnosis [5,6]. Blood based biomarkers not only contribute to early detection, but also might help monitor therapeutic outcome and disease progression. Therefore, the discovery of novel and effective non-invasive biomarker for osteosarcoma is important and urgently need.

MicroRNAs (miRNAs) are a class of short (approximately 19–25 nucleotides in length), endogenous, single-stranded, noncoding RNAs that regulate target gene expression through transcriptional interference or translational inhibition [7,8]. Accumulating evidence has shown that miRNAs play critical roles in a variety of biological processes, including cell proliferation, differentiation, apoptosis and metastasis [9,10]. MiRNAs have been demonstrated to function as either oncogenes or tumor suppressors [11,12]. In terms of osteosarcoma, many circulating miRNAs such as miR-223 [13], miR-125b [14], miR-300 [15] and miR-95-3p [16], have been previously reported and used as potential markers for cancer diagnosis.

Some previous studies had reported miR-194 played a tumor suppressive role in osteosarcoma. For instance, loss of miR-194 was observed in cancerous tissues and cells, and ectopic miR-194 expression greatly inhibited cell proliferation, migration and stimulated cell apoptosis through inversely regulating CDH2 [17]. Wang et al. found that miR-194 expression was reduced in osteosarcoma tissues and cell lines. Knockdown of miR-194 promoted cell proliferation and invasion by targeting NEAT1, and *vice versa* [18]. Likewise, t Enforced miR-194 expression repressed carcinogenesis *in vitro* and inhibited pulmonary metastasis *in vivo* [19]. However, the potential clinical significance of

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Table 1
Correlation of serum miR-194 expression with different clinicopathological features.

Parameters	Cases	Serum miR-194		*P*
		High	Low	
Age				0.7318
< 50	72	31	41	
≥ 50	52	24	28	
Gender				0.2531
Men	79	32	47	
Women	45	23	22	
Tumor size				0.1307
≤ 8 cm	56	29	27	
> 8 cm	68	26	42	
Histological subtypes				0.5510
Osteoblastic	80	35	45	
Chondroblastic	27	14	13	
Other	17	6	11	
Clinical stage				0.0005***
I/II	64	38	26	
III/IV	60	17	43	
Metastasis				0.0066**
Absent	91	47	44	
Present	33	8	25	
Serum level of ALP				0.7183
Elevated	79	36	43	
Normal	45	19	26	
Serum level of LDH				0.4078
Elevated	67	32	35	
Normal	57	23	34	

Footnote: ALP, alkaline phosphatase; LDH, lactate dehydrogenase
** Indicates $P < 0.01$.
*** Indicates $P < 0.001$.

serum miR-194 for osteosarcoma was unknown. In this study, we aimed to explore the relationship between serum miR-194 levels and the clinical outcomes of osteosarcoma.

2. Materials and methods

2.1. Patients

This study was approved by the Ethics Committee of our hospital. All participants provided their written informed consent before serum sample collection, and all specimens were handled and made anonymous according to the ethical and legal standards. We enrolled 124 patients (79 men, 45 women) diagnosed with high-grade osteosarcomas by core needle biopsy. Of all subjects, 80 patients were with osteoblastic osteosarcomas, 27 with chondroblastic osteosarcomas, 15 with fibroblastic osteosarcoma, 2 with telangiectatic osteosarcoma. Tumor grade was classified following the Tumor Node Metastasis (TNM) Classification of the Union for International Cancer Control (UICC). Moreover, 60 periostitis patients and 60 healthy subjects were enrolled as controls. The clinical information of all osteosarcoma patients was summarized in Table 1.

2.2. Serum sampling and RNA extraction

Up to 4 ml blood sample was withdrawn from all subjects and centrifuged at 12000 rpm at 4 °C for 10 min, then the serum supernatant was transferred into EDTA tube and stored at −80 °C. The miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) was performed to isolate total RNA from sera following the manufacturer's protocol. Total RNA concentration was assessed by measuring absorbance at A260/280 with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

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

The reverse transcription reaction was performed using the One Step Prime Script miRNA cDNA Synthesis Kit (Takara, Dalian, China). The resulting cDNAs were subjected to qRT-PCR using specific primers and SYBR Green PCR master mix (Applied Biosystems, CA, USA) in the StepOnePlus Real Time PCR (Applied Biosystems, CA, USA). qRT-PCR was repeated in triplicate for each sample. The cel-miR-39 was used as an internal control for normalization of data, and the serum miR-194 level was calculated and determined using the $2^{-\Delta\Delta Ct}$ method.

2.4. Bioinformatic analysis

For the bioinformatic analysis of the downstream targeted genes of miR-194. Gene Ontology and KEGG pathway enrichment analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource (https://david-d.ncifcrf.gov/). STRING online database (http://string-db.org) and Cytoscape software (Version 3.7.0, Institute of Systems Biology, Seattle, WA, USA) were used to identify key downstream targeted genes of miR-194 associated with tumorigenesis. Cytoscape MCODE plug-in was used for searching clustered subnetworks.

2.5. Statistical analysis

All statistical analyses were carried out using MedCalc 16.4.3 (MedCalc, Ostend, Belgium) and SPSS v21.0 software (SPSS Inc., Chicago, IL, USA). The Kruskal-Wallis test was used to analyze the difference of serum miR-194 expression between groups. The association between the serum miR-194 expression and clinicopathological variables was evaluated with Chi-square test. Receiver operating characteristic (ROC) curves and the area under the curve (AUC) were used to estimate the diagnostic value of serum miR-194. The Kaplan-Meier method plus log-rank test were used to calculate the survival curves. Multivariate Cox's proportional hazard regression test was used to assess the effect of serum miR-194 expression and clinical parameters on patient survival. Overall survival (OS) was defined as the time from diagnosis of osteosarcoma to any cause of death. Disease-free survival (DFS) was defined as the time from diagnosis of osteosarcoma to the date of the first sign of relapse. P value < 0.05 was regarded as statistically significant.

3. Results

3.1. Downregulation of serum miR-194 in patients with osteosarcoma and its diagnostic value

The serum miR-194 levels were detected in serum samples collected from all subjects by qRT-PCR. The serum miR-194 expression was dramatically lower in patients with osteosarcoma than that in healthy volunteers ($P = 0.008$) and periostitis patients ($P = 0.021$, Fig. 1A). However, no significant difference in serum miR-194 expression levels was found among different histological subtypes of osteosarcoma ($P > 0.05$, Fig. 1B).

ROC curve was then built to evaluate the diagnostic accuracy of serum miR-194. Fig. 2A showed that the AUC value of serum miR-194 for identifying osteosarcoma patients from healthy controls was 0.855, and the specificity and sensitivity were 84.2% and 79.1%, respectively. Similarly, Fig. 2B revealed that serum miR-194 had a specificity of 72.8% and a sensitivity of 79.1% to distinguish the osteosarcoma subjects from periostitis patients, with an AUC of 0.810.

3.2. Serum miR-194 levels were upregulated in osteosarcoma patients following surgery

As illustrated in Fig. 3A, serum miR-194 levels in post-operative

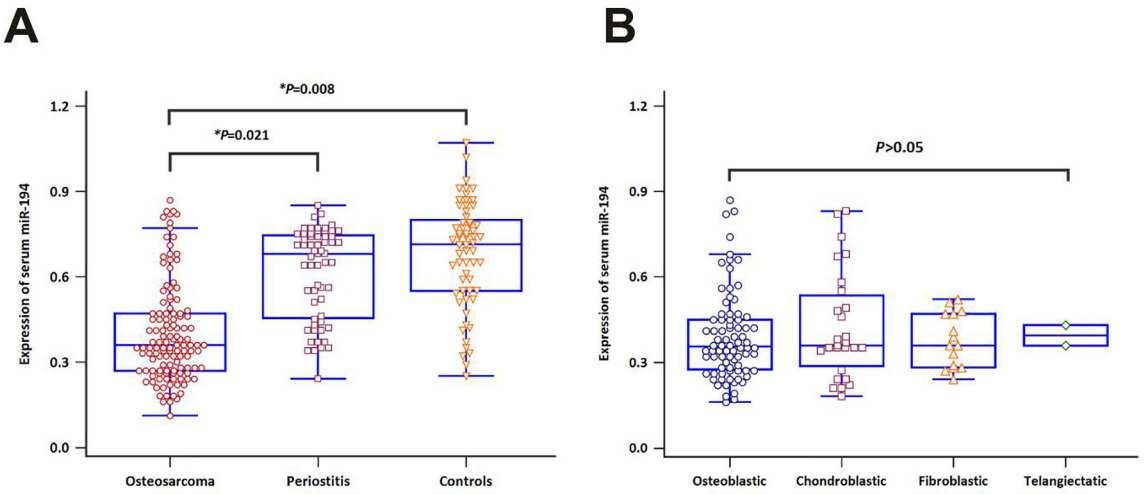

Fig. 1. The serum miR-194 levels in osteosarcoma subjects were significantly lower than those in periostitis patients and healthy controls (A). There was no significant difference in serum miR-194 expression levels between osteoblastic osteosarcomas and chondroblastic osteosarcomas (B).

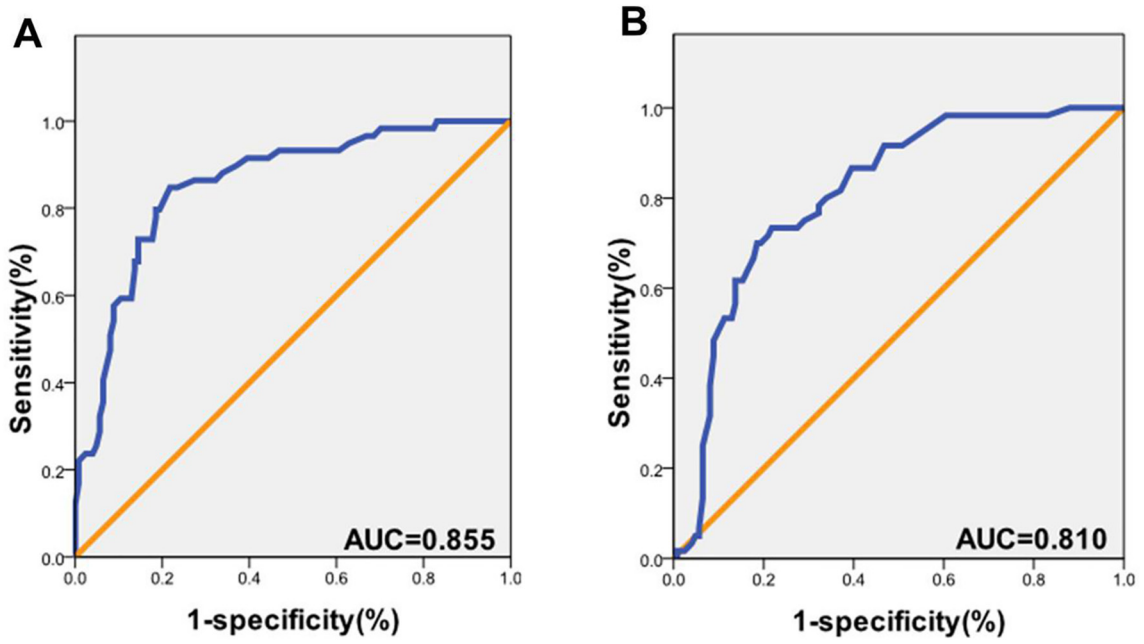

Fig. 2. ROC curve of using serum miR-194 expression to distinguish osteosarcoma subjects from normal controls (A). ROC curve of using serum miR-194 expression to distinguish osteosarcoma subjects from periostitis patients (B).

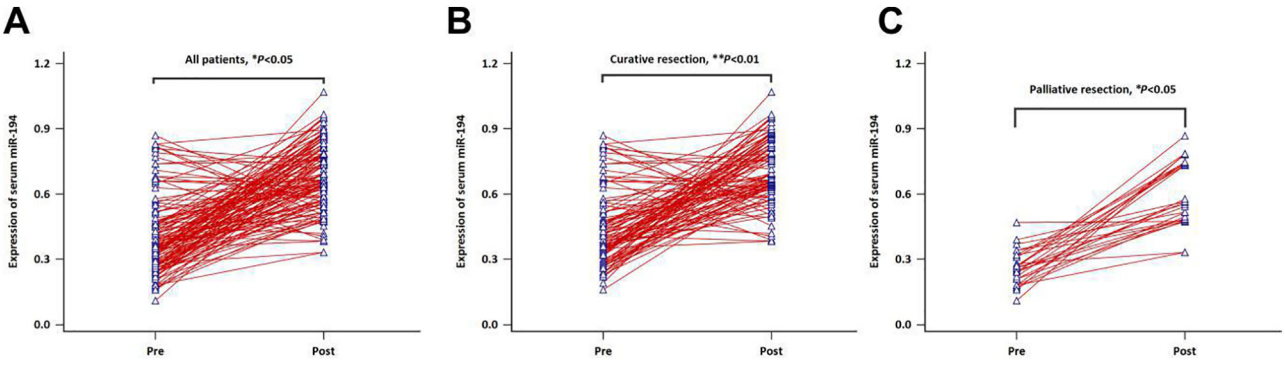

Fig. 3. Comparison of miR-194 levels in all 124 paired blood samples before and after surgery (A). Comparison of miR-194 levels in 98 paired blood samples (curative resection) before and after surgery (B). Comparison of miR-194 levels in 26 paired blood samples (palliative resection) before and after surgery (C).

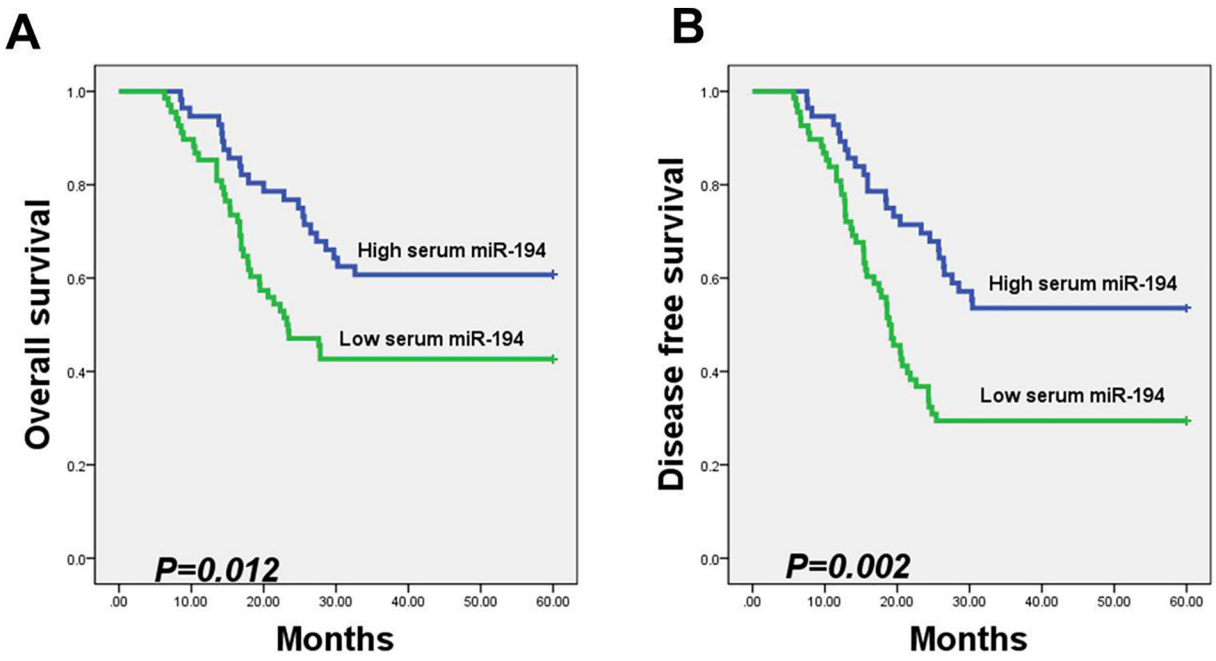

Fig. 4. Osteosarcoma patients with lower serum miR-194 level suffered a significantly shorter OS and DFS than those with higher serum miR-194 level.

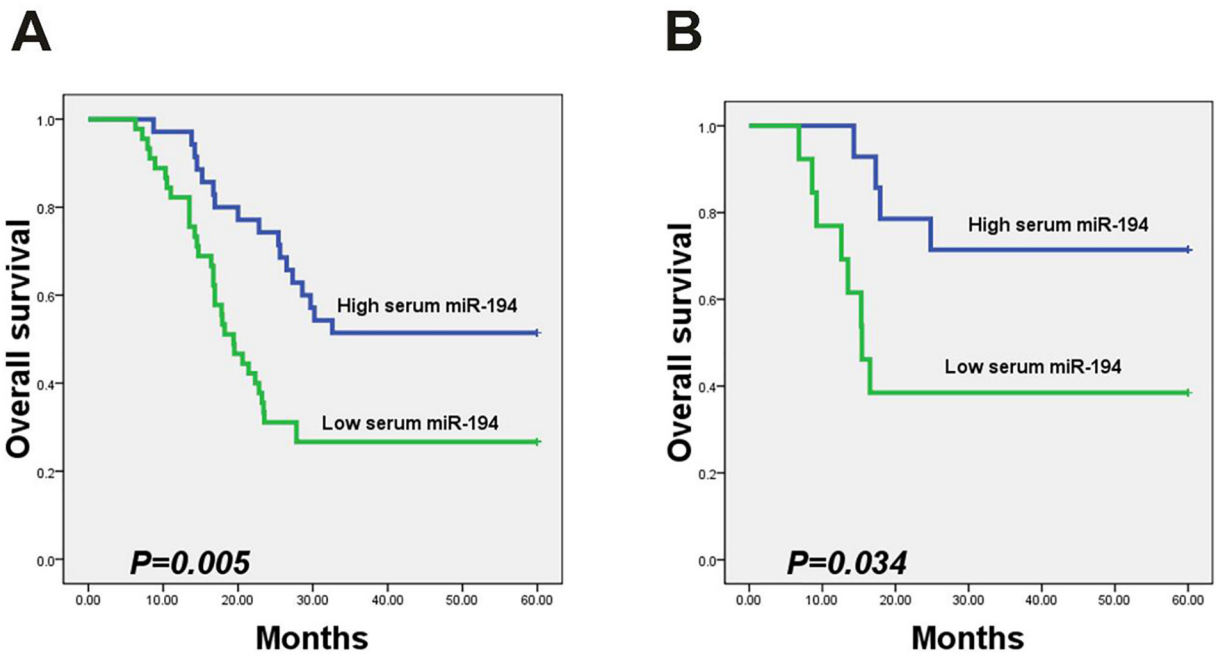

Fig. 5. Osteoblastic osteosarcoma patients with lower serum miR-194 level suffered a significantly shorter OS than those with higher serum miR-194 level (A). Chondroblastic osteosarcoma patients with lower serum miR-194 level suffered a significantly shorter OS than those with higher serum miR-194 level (B).

samples were greatly elevated compared with the matched pre-operative samples ($P < 0.05$). Among all osteosarcoma subjects, 98 cases underwent potentially curative surgeries and 26 cases underwent palliative resections. We found serum miR-194 levels in patients with curative surgeries were sharply elevated ($P < 0.01$, Fig. 3B). Also, a significant increase in miR-194 expression was observed in patients with palliative resections ($P < 0.05$, Fig. 3C).

3.3. Association between serum miR-194 expression and clinicopathological features

Table 1 showed the associations of serum miR-194 expression with various clinical parameters. The median fold-change value was used to divide all patients into high serum miR-194 expression group (n = 55) and low miR-194 expression group (n = 69). Low serum miR-194 expression was highly correlated with positive metastasis ($P = 0.0066$) and advanced clinical stage ($P = 0.0005$). However, no significant difference was found between serum miR-194 and other clinical features including age, gender, tumor size, histological subtypes, serum levels of alkaline phosphatase and lactate dehydrogenase (all $P > 0.05$).

Table 2
Multivariate analysis of prognostic factors associated with OS/DFS.

Parameters	Multivariate analysis	
	HR (95% CI)	*P* value
Overall survival		
Serum miR-194	3.65 (1.58–5.89)	0.014
Metastasis	3.23 (1.42–5.14)	0.018
Clinical stage	4.17 (1.86–6.50)	0.007
Disease free survival		
Serum miR-194	4.39 (1.90–6.91)	0.005
Metastasis	3.86 (1.71–6.23)	0.011
Clinical stage	4.92 (2.26–7.82)	< 0.001

3.4. Correlation between serum miR-194 expression and prognosis of osteosarcoma

Kaplan-Meier survival analysis demonstrated that patients with high serum miR-194 expression had significantly longer OS and DFS than those with low serum miR-194 expression ($P = 0.012$, Fig. 4A; $P = 0.002$, Fig. 4B). Then, we found the OS rates of osteoblastic osteosarcoma or chondroblastic osteosarcoma patients with high serum miR-194 expression were significantly higher than those with low expression ($P = 0.005$, Fig. 5A; $P = 0.034$, Fig. 5B). In addition, multivariate Cox regression analysis identified serum miR-194 (OS: HR = 3.65, 95% CI = 1.58–5.89, $P = 0.014$; DFS: HR = 4.39, 95% CI = 1.90–6.91, $P = 0.005$), metastasis (OS: HR = 3.23, 95% CI = 1.42–5.14, $P = 0.018$; DFS: HR = 3.86, 95% CI = 1.71–6.23, $P = 0.011$) and clinical stage (OS: HR = 4.17, 95% CI = 1.86–6.50, $P = 0.007$; DFS: HR = 4.92, 95% CI = 2.26–7.82, $P < 0.001$) as independent prognostic factors in osteosarcoma patients (Table 2).

3.5. Bioinformatic analysis of the downstream targets in miR-194

The downstream targeted genes of miR-194 were obtained from TargetScan7.1. The GO analysis showed that GO: 0000122~negative regulation of transcription from RNA polymerase II promoter, GO: 0045893~positive regulation of transcription, DNA-templated, GO: 0045944~positive regulation of transcription from RNA polymerase II promoter, GO: 0006366~transcription from RNA polymerase II promoter and GO: 0006351~transcription, DNA-templated were top biological processes (Fig. 6A). GO: 0005654~nucleoplasm, GO: 0005634~nucleus, GO: 0005829~cytosol, GO: 0005737~cytoplasm and GO: 0013020 ~membrane were the top enriched cellular components (Fig. 6B). GO:0005515~protein binding, GO:0003700~transcription factor activity, sequence-specific DNA binding, GO:0044212~transcription regulatory region DNA binding, GO:0000978~RNA polymerase II core promoter proximal region sequence-specific DNA binding and GO:0003677~DNA binding were the top enriched molecular functions (Fig. 6C). The KEGG pathways analysis showed that ubiquitin mediated proteolysis, TGF-beta signaling pathway, pathways in cancer, Wnt signaling pathway and signaling pathways regulating pluripotency of stem cells were the top enriched pathways (Fig. 6D). Based on the STRING online database and Cytoscape software, protein-protein interaction (PPI) network complex was constructed. Many central node genes such as HIF-1a, YAP1, AKT2 which have been demonstrated to play an oncogenic role in tumorigenesis of osteosarcoma were identified (Fig. 7A–D).

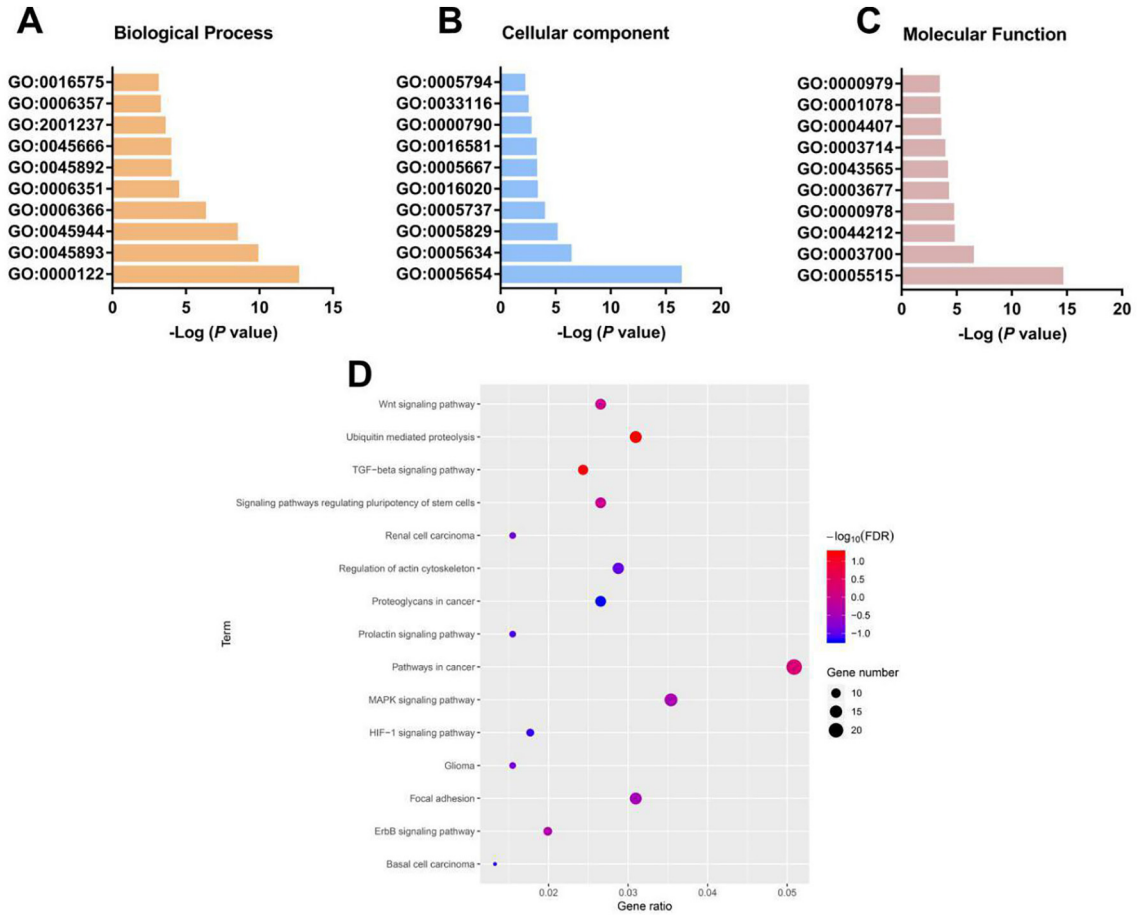

Fig. 6. GO and KEGG analysis of the downstream targeted genes of miR-194.

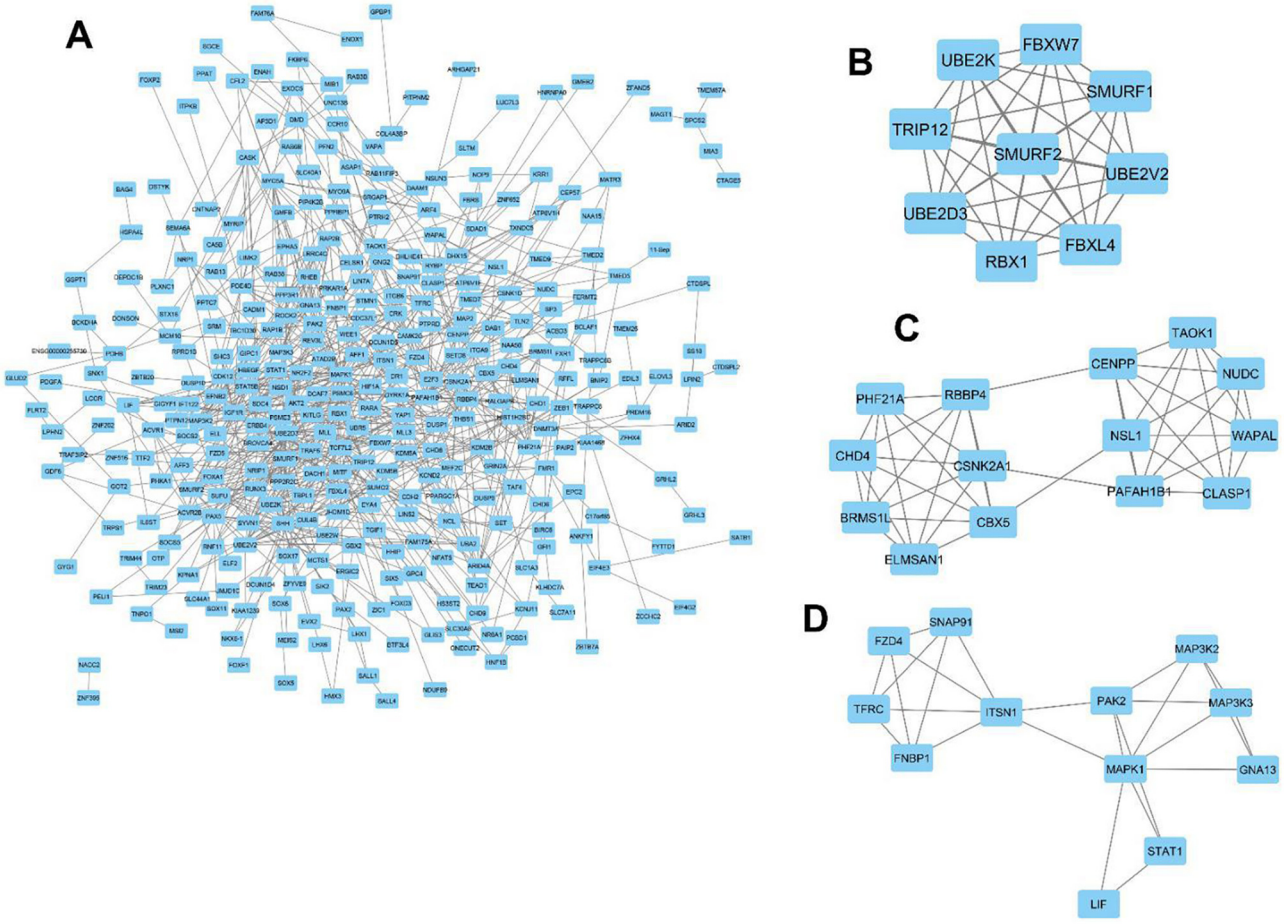

Fig. 7. PPI analysis of the downstream targeted genes of miR-194.

4. Discussion

Our study has demonstrated that abnormally decreased serum miR-194 level was found in osteosarcoma patients compared with periostitis patients or healthy controls. ROC analysis demonstrated that serum miR-194 expression could well screen osteosarcoma patients from periostitis patients, as well as healthy controls. In addition, serum miR-194 levels were greatly upregulated in patients after receiving surgical treatment. Moreover, low serum miR-194 was closely associated with unfavorable clinicopathological parameters and clinical outcome. Furthermore, bioinformatic analysis revealed that many downstream targeted genes of miR-194 were correlated with tumorigenesis. Therefore, serum miR-194 might serve as a promising biomarker for the diagnosis and prognosis of osteosarcoma.

Besides osteosarcoma, miR-194 was also found to act as a tumor suppressor in various cancer types. For instance, low expression of miR-194 was found in laryngeal squamous cell carcinoma tissues and cell lines, and its upregulation suppressed tumorigenesis through inversely regulating Wee1 [20]. Moreover, *in vitro* and *in vivo* evidence revealed that the miR-194 could restrain cancer cell proliferation, invasion and promote cell apoptosis *via* silencing KDM5B expression in esophageal squamous cell carcinoma [21]. In non-small cell lung cancer, miR-194 expression was lower in cancer tissues and reduced miR-194 was positively linked to shorter survival. Ectopic miR-194 expression significantly inhibited the tumorigenic properties of cancer cells both *in vitro* and *in vivo* by degrading FOXA1 [22] or CUL4B [23]. In hepatocellular carcinoma, a reduction in miR-194 levels were found in cancer tissues and its downregulation strongly associated with aggressive clinical variables. MiR-194 overexpression or MAP4K4 inhibition significantly decreased cancer cell proliferation and induced cell apoptosis *in vitro* [24]. Also, Zhai and colleagues reported low miR-194 expression occurred more frequently in late stage, type I patients with endometrial cancer, and deregulated miR-194 was correlated with poorer survival of patients [25]. In clear cell renal cell carcinoma (ccRCC), miR-194 was remarkably downregulated in ccRCC tissues compared to non-neoplastic tissue adjacent to osteosarcoma, and there was significant association between low miR-194 expression and poorer clinical variables, as well as worse prognosis [26].

In contrast, miR-194 was found to display increased expression in prostate cancer and ovarian carcinoma. Das et al. showed the association between elevated miR-194 expression and tumor aggressiveness and metastasis, and knockdown of miR-194 significantly decreased cancer cell viability and growth *in vitro* and *in vivo* by regulating SOCS2 [27]. Also, miR-194 expression was increased in tumor tissues of ovarian cancer patients, and overexpression of miR-194 promoted tumorigenesis through directly silencing PTPN12 expression [28].

Interestingly, the role of miR-194 in carcinogenesis of some tumor types was controversial and conflicting results had been reported. In gastric cancer, reduced miR-194 expression was observed in tissues and cell lines. *In vitro* and *in vivo* experiments showed that miR-194 upregulation had an inhibitory effect on tumor aggressiveness by inhibiting KDM5B expression [29]. Conversely, serum miR-194 levels were highly expressed in mice with diffuse-type gastric cancer, suggesting miR-194 worked as an oncogene [30]. Similarly, Le and colleagues revealed miR-194 overexpression not only dramatically suppressed cell migration/invasion, but also inhibited tumor growth in xenograft [31]. Another study showed that higher miR-194 expression was associated with poorly differentiation, and miR-194 upregulation greatly stimulated the

oncogenic activities of cancer cells [32]. Thus, the role of miR-194 in carcinogenesis needs further exploration.

5. Conclusions

Taken together, our study has demonstrated that serum miR-194 is downregulated in patients with osteosarcoma. Low serum miR-194 expression is associated with poor prognosis, indicating that serum miR-194 might be one of the potential biomarkers for predicting prognosis of osteosarcoma.

Ethic approval

This study was approved by the Ethics Committee of the Affiliated Stomatological Hospital of Nanchang University.

Inform consent

All participants provided their written informed consent before serum sample collection, and all specimens were handled and made anonymous according to the ethical and legal standards.

Declaration of competing interest

All authors declare that they have no conflict of interest.

Acknowledgement

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