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Brief Communication

Unveiling the prognostic implications of RPLP1 upregulation in osteosarcoma

Xinhui Du[1,2,3*], Hua Wei[4*], Boya Zhang[3], Lon Kai Pang[5], Ruiying Zhao[6], Xu Dong Zhang[7,8], Weitao Yao[3]

[1]School of Basic Medical Sciences, Zhengzhou University, Zhengzhou 450001, Henan, China; [2]Academy of Medical Sciences, Zhengzhou University, Zhengzhou 450001, Henan, China; [3]Department of Bone and Soft Tissue, The Affiliated Cancer Hospital of Zhengzhou University & Henan Cancer Hospital, Zhengzhou 450008, Henan, China; [4]Department of Anesthesiology, Pain and Perioperative Medicine, The First Affiliated Hospital of Zhengzhou University, 1 East Jianshe Road, Zhongyuan District, Zhengzhou 450052, Henan, China; [5]Baylor College of Medicine, Houston, TX 77030, USA; [6]Department of Integrative Biology & Pharmacology, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, TX 77030, USA; [7]Translational Research Institute, Henan Provincial and Zhengzhou City Key Laboratory of Non-coding RNA and Cancer Metabolism, Henan International Join Laboratory of Non-coding RNA and Metabolism in Cancer, Henan Provincial People's Hospital, Zhengzhou University, Zhengzhou 450053, Henan, China; [8]School of Biomedical Sciences and Pharmacy, The University of Newcastle, NSW 2308, Australia. [*]Equal contributors.

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Abstract: Osteosarcoma, a malignant bone tumor characterized by a high rate of metastasis and poor survival, presents a critical need for identifying novel biomarkers associated with metastasis. In this study, we conducted an extensive analysis utilizing transcriptional and clinical data sourced from databases such as GEO, TCGA, CCLE, R2, and Xena. And we discovered that Ribosomal protein LP1 (RPLP1) ranked among the top upregulated genes in relation to osteosarcoma metastasis. Notably, RPLP1 exhibited significant expression in both osteosarcoma cell lines and patient samples. Moreover, multiple osteosarcoma studies revealed a strong correlation between RPLP1 overexpression and worse metastasis-free survival as well as overall survival. Additionally, we observed a consistent association between dysregulation of RPLP1 and reduced overall survival across various tumor types. Knocking down of RPLP1 led to the down-regulation of MYL5 and functional enrichment toward cell cycle and cellular interaction. Based on these findings, we propose that RPLP1 has the potential to serve as a prognostic biomarker, indicating increased metastasis and worse survival outcomes in osteosarcoma. These insights contribute to a better understanding of the disease and may pave the way for future research and therapeutic approaches.

Keywords: RPLP1, osteosarcoma, metastasis, prognosis

Introduction

Osteosarcoma is a highly malignant tumor that primarily affects children and adolescents whose skeletal system is still developing [1]. It mainly arises near large joints such as the knee, hip, or shoulder and can compromise the adjacent joint functions [2, 3]. The standard treatment of osteosarcoma includes sequential neo-adjuvant chemotherapy, wide resection [4, 5], and adjuvant chemotherapy. The introduction of the classic four drugs (Adriamycin, cisplatin, methotrexate, and ifosfamide) has been a significant advancement in the treatment of osteosarcoma, with 5-year overall survival rates for localized osteosarcoma ranging from 60-70% [6, 7]. However, metastasis is relatively common in osteosarcoma, with about 18% of cases diagnosed with metastasis [8]. Distant metastasis is the leading cause of death associated with osteosarcoma, and it presents a significant challenge in the treatment of this disease.

Numerous factors have been proposed to play a role in the development and metastasis of osteosarcoma [9-12]. Alterations within the tumor cells such as genomic changes [13], epigenomic changes [14-16], metabolic reprogramming [17], and deregulation of specific pathways [18, 19] or transcription factors [20, 21] all contribute to osteosarcoma metastasis.

Furthermore, the composition and the functional state changes in the matrix [22] and cells within the tumor microenvironment [23, 24] also promote osteosarcoma metastasis. Several prognostic models have been developed based on these alterations to predict osteosarcoma survival [25, 26]. However, due to the heterogeneity of osteosarcoma and the complexity of these prognostic models, the translational significance of these findings remains limited.

Ribosomal protein LP1 (RPLP1) belongs to the ribosomal protein L12P family and plays a crucial role in the elongation step of protein synthesis [27]. Upregulation of RPLP1 has been reported in various cancer types, including hepatocellular carcinoma [28], breast cancer [29], and cervical cancer [30]. In these cancers, increased RPLP1 expression has been proposed to promote tumor cell tumorigenesis, proliferation, invasion, and migration [31, 32]. The tumor promoting role of RPLP1 has been linked to its effect on tumor cell epithelial-mesenchymal transition [29]. Additionally, deficiency of RPLP1 was reported to result in reactive oxygen species (ROS) accumulation and stress-induced autophagy [31, 32]. However, the specific role of RPLP1 in osteosarcoma has yet to be explored.

To identify key players responsible for osteo sarcoma metastasis, we compared the expression levels of different genes in tumor samples between patients with and without metastasis at diagnosis. Our analysis revealed that RPLP1 was significantly upregulated and correlated with poor survival in osteosarcoma. Moreover, a positive correlation between RPLP1 and MYL5 expression was also revealed in osteosarcoma samples. In summary, we have identified RPLP1 overexpression as a contributing factor in osteosarcoma metastasis, potentially explained by its positive correlation with MYL5 expression.

Material and methods

Cell culture, reagents and cell lines

U2OS, 143B, and MG63 (purchased from the National Collection of Authenticated cell cultures) were cultured at 37°C with 5% CO_2 in DMEM (Sigma, #D6429) containing 10% fetal calf serum (Gibco, #10100147) and 100 UI ml^{-1} penicillin/streptomycin.

RNA extraction and RT-qPCR

Total RNA was extracted from cells using the TRIzol™ Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription and qPCR were conducted with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, k1622) and FastStart Universal SYBR Green Master (Roche, 04913850001). Relative gene expression was calculated using the comparative cycle method (2-ΔΔCt) normalized to the housekeeping gene GAPDH. Primer sequences: RPLP1: F-5'AATGTCCCATCGGGAGCCT3'; R-5'TTTGCTTCTACTTTCTTCTCCTCAG3' and GAPDH: F-5'CTCCTCCTGTTCGACAGTCAGC3'; R-5'CCCAATACGACCAAATCCGTT3'.

Western blot analysis

Cells were harvested and lysed at 4°C in RIPA lysis buffer (Sigma) with protease inhibitor cocktail (Sigma). The protein samples were quantified and subjected to 4-20% Tris-Glycine gels (Thermo Fisher Scientific), and after electrophoresis for 1 hour, they were transferred to nitrocellulose membranes (Bio-Rad). The membranes were probed overnight at 4°C with antibodies specific for RPLP1 and GAPDH after blocking with 5% non-fat dried milk. Then HRP-conjugated secondary antibodies were added and incubated for 1 hour at room temperature and finally detected with Pierce ECL Plus Substrate (Thermo Fisher Scientific). The RPLP1 (pAbs, #21636-1-AP) and GAPDH (mAbs, #5174) antibodies were purchased from Proteintech (Wuhan, China).

IHC staining

Four micrometers thick tumor tissues sections were fixed in 10% formaldehyde, embedded in paraffin, dewaxed in xylene, and rehydrated through a graded series of ethanol. Then, tumor tissue sections were subjected to IHC staining with RPLP antibody (pAbs, #21636-1-AP). The slides were then washed with PBS and treated with diaminobenzidine (DAB) chromogen for 3-5 min that served as the chromogen, and hematoxylin was used for the nuclear counterstain. Afterwards, the sections were dehydrated, cleared, and mounted.

Transcriptional and survival data analysis

The transcriptional and clinical data sets of GSE21257 and GSE133111 were downloaded

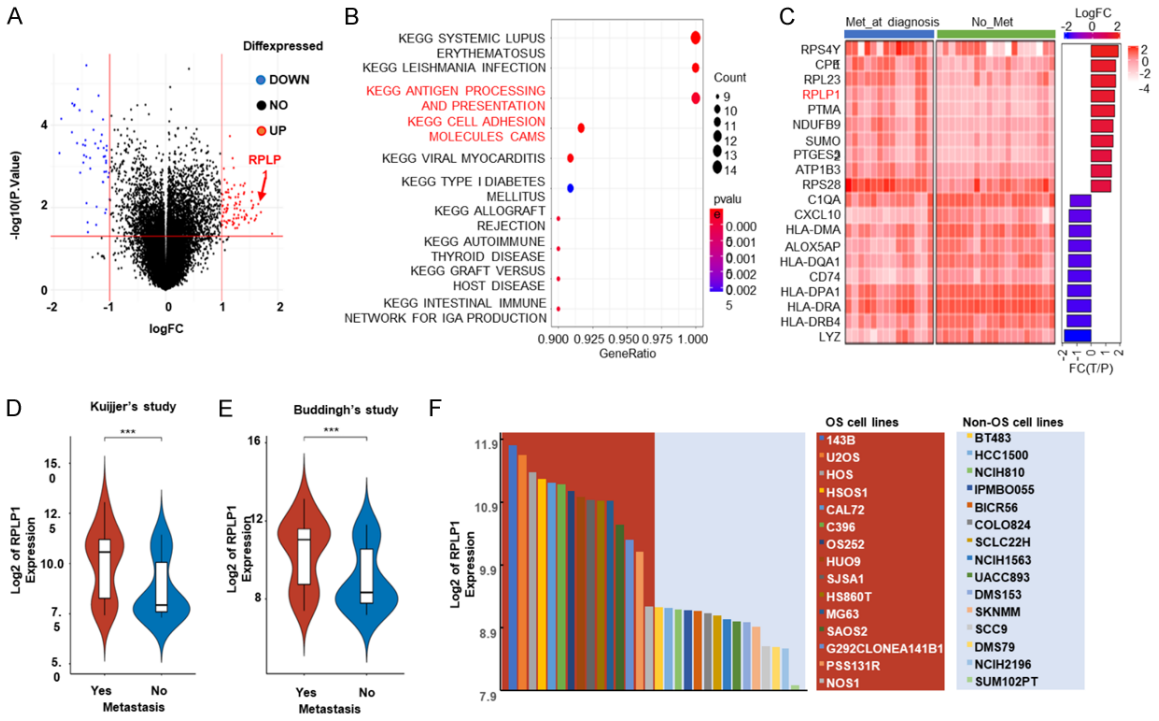

Figure 1. RPLP1 is upregulated in metastatic osteosarcoma. A. The volcano plot of the differentially expressed genes. The upregulated genes were shown in red and the downregulated genes were shown in blue. The RPLP1 was upregulated and shown in red with an arrow. B. The dot plot of the GESA functional enrichment of differentially expressed genes between the samples of osteosarcoma patients diagnosed with metastasis and ones without metastasis. Note that the cell adhesion related pathways were enriched. C. The heatmap of the top 10 differentially expressed genes between the samples in patients with or without metastasis. The RPLP1 was among the top 4 of the upregulated genes. D and E. The violin plots of the RPLP1 expression levels between osteosarcoma patients with or without metastasis. Significant upregulation of the RPLP1 levels in metastatic osteosarcoma patients were consistently shown in both the Kuijjer's and Buddingh's study. ***P < 0.001. F. The bar graph of the RPLP1 expression levels in the osteosarcoma and non-osteosarcoma cell lines. The RPLP1 levels were higher in most of the osteosarcoma cell lines.

from the GEO database. We obtained the expression datasets for RPLP1 from the TCGA database for cancer samples and from the CCLE database for cell lines, respectively. The expression of RPLP1 and clinical information of three osteosarcoma studies (Aqelan PMID: 22350417, Kuijjer PMID: 22454324, and Buddingh PMID: 21372215) were downloaded from the R2 website. Survival analysis and Kaplan-Meier plots of kidney renal clear cell carcinoma, kidney renal papillary cell carcinoma, and liver hepatocellular carcinoma based on the RPLP1 expression level were performed and downloaded from the KMplot website. The transcriptional and survival data of Target-OS study were downloaded from the Xena database respectively.

Bioinformatic analysis

The bioinformatic analysis and graphic plot were performed with R v4.2.1 (https://www.R-project.org/). Packages used included the ggpubr, ggplot2, survminer, tidyverse, ggsci, patchwork, limma, clusterProfiler, pheatmap, and ggrepel.

Results

The RPLP1 is upregulated in metastatic osteosarcoma

In order to explore the potential mechanisms underlying osteosarcoma metastasis, we employed bioinformatic tools to analyze the transcriptional changes in osteosarcoma patients with or without metastasis using dataset GSE-21257 from the R2 database. Our analysis revealed a total of 137 significantly differentially expressed genes, as summarized in Supplementary Table 1. The volcano plot was generated to visualize these gene expression changes (**Figure 1A**). Furthermore, the Gene Ontology (GO) functional enrichment analysis

showed that the differentially expressed genes were enriched in biological processes and molecular functions related cellular interaction (Supplementary Figure 1). Then, the Gene Set Enrichment Analysis (GSEA) highlighted pathways associated with cellular adhesion and antigen processing (**Figure 1B**). Both sets of functional analysis results collectively pointed to cellular interaction pathways whose alterations play fundamental roles in tumor cell metastasis. The top 10 up- or down-regulated genes were extracted and plotted in the heatmap (**Figure 1C**). Three of the top 4 upregulated genes were found to be associated with ribosomal function. Among these, RPLP1 emerged as a key molecule of interest for further investigations.

To validate the upregulation of RPLP1 in metastatic osteosarcoma, we conducted a series of bioinformatic analyses using publicly available sequencing data. Our analyses consistently revealed a significant upregulation of RPLP1 in metastatic osteosarcoma patient samples compared to localized patient samples. This finding was observed in both Kuijjer's study (**Figure 1D**) and Buddingh's study (**Figure 1E**). We further examined RPLP1 expression levels from CCLE database and found its levels were notably higher in most of the osteosarcoma cell lines compared to other cancer cell lines (**Figure 1F**).

Overall, our findings establish a correlation between RPLP1 and osteosarcoma metastasis, providing validation for its upregulation in metastatic osteosarcoma samples.

The expression of RPLP1 is significantly elevated in osteosarcoma and associated with poorer survival outcomes

To further validate the observed dysregulation of RPLP1 expression, we assessed the RPLP1 expression levels in osteosarcoma cell lines and tissue samples obtained from our center. In comparison to the immortalized osteoblast cell line hFOB, there are markedly higher expression of both RPLP1 mRNA and protein levels in all three osteosarcoma lines (U2OS, MG36 and 143B) (**Figure 2A** and Supplementary Figure 2). Similarly, we observed upregulated RPLP1 protein levels in tumor samples from PDX models than their corresponding normal tissues (**Figure 2B**). Additionally, when examining osteosarcoma patient samples along with their adjacent non-tumor tissue samples, we consistently detected significant upregulation of RPLP1 protein levels in tumors while the normal counterpart showed no such increase (**Figure 2C**). To complement these findings, we performed IHC staining of RPLP1 on the osteosarcoma samples. Strong positive staining for RPLP1 was observed in all four tumor samples in comparison to their paired adjacent non-tumor tissues (**Figure 2D** and Supplementary Figure 3).

To broaden the scope of our study, we expanded our analysis to include public datasets, specifically examining RPLP1 levels in tumor and normal control samples from Aqeilan's study. The osteosarcoma tumor samples showed significant upregulation of RPLP1 compared to the normal control samples, as visualized through a violin graph (**Figure 2E**). To investigate the prognostic implications of RPLP1 in osteosarcoma, we conducted survival analysis using the expression levels of RPLP1 in patients from Kuijjer's study. Patients with high levels of RPLP1 expressed exhibited significantly worse overall survival and metastasis free survival (**Figure 2F** and **2G**). Similarly, a trend towards decreased overall survival probability was observed in the TARGET osteosarcoma dataset (Supplementary Figure 4A), although the difference did not reach statistical significance ($P = 0.09$).

We further evaluate the prognostic significance of RPLP1 across different cancer types with bioinformatic analyses. Our results revealed that highly expressed RPLP1 was associated with worse overall survival in renal clear cell carcinoma (Supplementary Figure 4B), renal papillary cell carcinoma (Supplementary Figure 4C), and hepatocellular carcinoma (Supplementary Figure 4D). In summary, our comprehensive analyses confirmed the upregulation of RPLP1 in osteosarcoma using different models and demonstrated its prognostic significance in this malignancy. Elevated RPLP1 expression not only associated with poorer overall survival in osteosarcoma, but also in various cancer types.

Possible correlation of RPLP1 with MYL5 in osteosarcoma

In our quest to explore the biological function of RPLP1 in osteosarcoma progression, we analyzed differentially expressed genes following

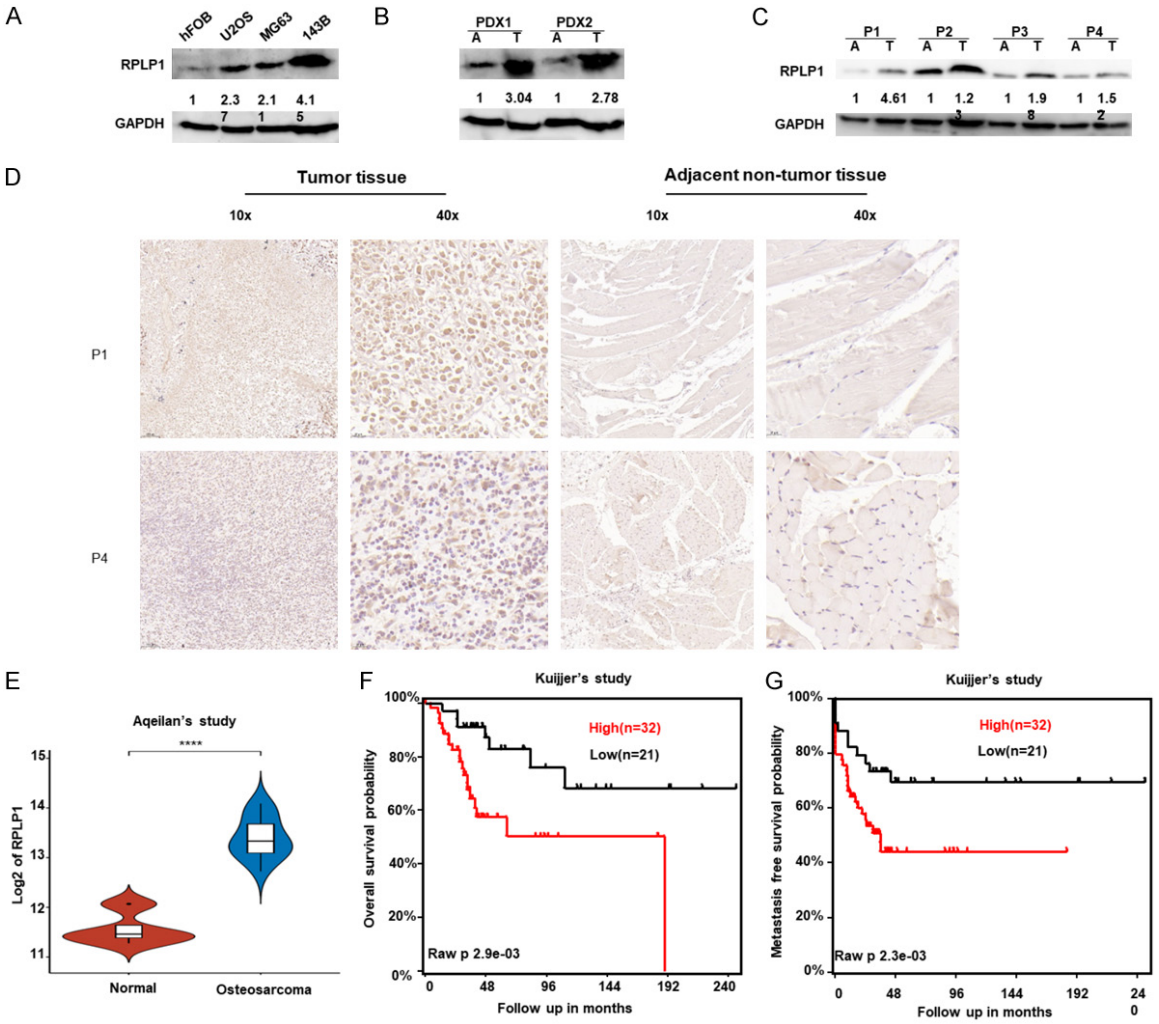

Figure 2. The RPLP1 is highly expressed in osteosarcoma and correlated with worse survival. (A) The protein levels of RPLP1 were upregulated in osteosarcoma cell lines (A), PDX model tissues (B) and patient tumor tissue (C). (C) Four pairs of osteosarcoma patient tumor tissue and adjacent non-tumor tissue were used to detect the RPLP1 protein levels with Western blot. All the tumor tissues showed higher levels of RPLP1 comparing with the paired adjacent non-tumor tissues. (D) The IHC staining of the RPLP1 in the paired osteosarcoma and adjacent non-tumor tissue (P1 and P4). The tumor tissues were strongly positive for RPLP1 while the adjacent non-tumor tissues were weak positive or negative for RPLP1. (E) The violin plot of the relative RPLP1 levels of osteosarcoma in the Aqeilan's study. Bioinformatic analysis showed that the expression of RPLP1 was upregulated in osteosarcoma samples comparing with the normal control samples. ****P < 0.0001. (F and G) The survival analysis of the Kuijjer's study showed significant differences in the overall survival and metastasis free survival between osteosarcoma patients with high and low expression of RPLP1. Osteosarcoma with higher RPLP1 levels presented with significantly worse survival.

RPLP1 knockdown in a tumor model, and identified 403 genes that exhibited significant differences (Supplementary Table 2). The volcano plot was generated to visualize these differentially expressed genes (DEGs) (**Figure 3A**). The GO functional enrichment analysis demonstrated a significant enrichment of deferentially expressed genes in biological processes related to the cell cycle (Supplementary Figure 5). Additionally, GSEA indicated a significant enrichment in the cell adhesion pathway (**Figure 3B**). To identify the specific molecule responsible for the alterations in the cell adhesion pathway following RPLP1 knockdown, we examined the overlapping genes between the DEGs and the KEGG focal adhesion pathway gene list. This analysis revealed a total of 10 overlapping genes, which were depicted in a Venn diagram (**Figure 3C**) and in a heatmap (**Figure 3D**). Among these 10 overlapping genes, MYL5 was

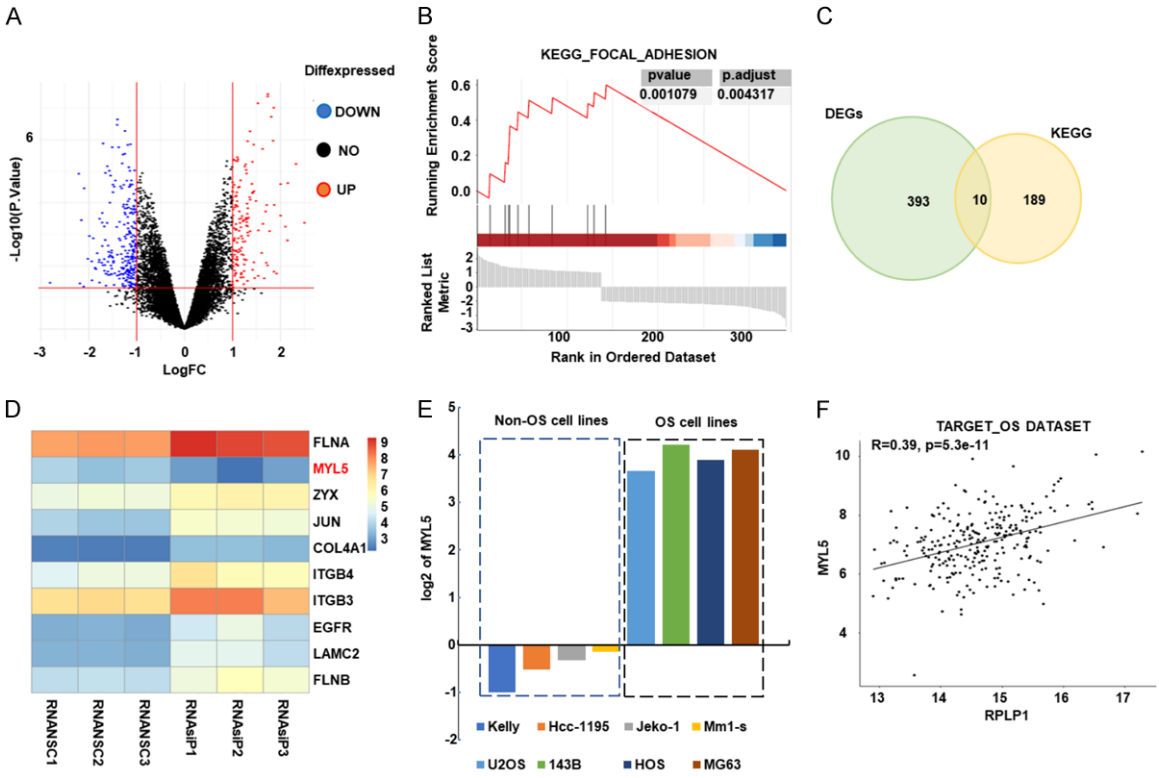

Figure 3. Possible correlation of RPLP1 with MYL5 in osteosarcoma. (A) The volcano plot of differentially expressed genes after RPLP1 knockdown. The upregulated genes were labeled in red while the downregulated genes were labeled in blue. (B) The GSEA functional enrichment analysis showed that differentially expressed genes were significantly enriched in the KEGG_FOCAL_ADHESION pathway. (C) The Venn diagram shows the overlap between the differentially expressed genes (A) and the list of genes in the KEGG_FOCAL_ADHESION pathway (B). (D) The heatmap of the 10 overlapping genes. MYL5 (shown in red color) was the only downregulated genes after RPLP1 knockdown. (E) The bar graph of the relative expression of MYL5 in different cancer cell lines. The MYL5 expression levels were higher in osteosarcoma cell lines (U2OS, 143B, HOS, and MG63) compared with non-osteosarcoma cell lines (Kelly, Hcc-1195, Jeko-1, and Mml-s). (F) Correlation of RPLP1 with MYL5 in osteosarcoma. Significant positive correlation between RPLP1 and MYL5 were detected in osteosarcoma samples from the TARGET osteosarcoma dataset.

the only downregulated gene following RPLP1 gene knockdown.

Subsequently, we tried to assess the potential correlation between MYL5 and RPLP1 in osteosarcoma. We examined the expression levels of MYL5 in different tumor cell lines. Consistently, higher expression levels of MYL5 were observed in osteosarcoma cell lines compared to other tumor cell lines (**Figure 3E**). In addition, correlation analysis performed using osteosarcoma dataset revealed a positive correlation between the expression levels of RPLP1 with MYL5 in osteosarcoma tissue samples (**Figure 3F**).

To summarize, our findings suggest that MYL5 expression might be regulated by RPLP1 in tumors, and in osteosarcoma specifically, MYL5 exhibits a positive correlation with RPLP1.

Discussion

The mechanism studies of osteosarcoma metastasis pose significant challenges due to the rarity and complexity of the disease [33]. The genomic landscape of osteosarcoma is characterized by high instability and heterogeneity [34]. While somatic or germline mutations in genes like *TP53* and *RB1* have been identified to have tumor-promoting roles, drawing definitive conclusions is difficult due to the low recurrence of these mutations in tumor samples [35, 36]. In fact, multiple whole-genome sequencing or whole-exome sequencing studies conducted on osteosarcoma samples have indicated that copy number alterations and

structural rearrangements, rather than somatic mutations, are the predominant genomic changes in osteosarcoma [37]. Therefore, studying transcriptional changes may hold greater promise in unraveling the mechanisms underlying osteosarcoma metastasis.

In this study, we employed bioinformatic tools to analyze the differentially expressed genes associated with osteosarcoma metastasis. Among the genes identified, RPLP1, a ribosomal protein, ranked highly as an upregulated gene. Ribosomal proteins (RPs) are RNA-binding proteins that play a crucial role in ribosome biosynthesis and protein translation [38]. Beyond their conventional functions, growing evidence suggests that RPs also possess extra-ribosomal functions, contributing to the regulation of cell proliferation, migration, and invasion [39]. Previous studies have proposed that RPLP1 is upregulated and promotes tumor progression and metastasis in various cancer types. However, its precise role in osteosarcoma remains unexplored.

Through our investigation using osteosarcoma cell lines and tissue samples from our center, we observed the upregulation of RPLP1 in osteosarcoma. Our findings were further validated by analyzing public datasets through bioinformatic analyses, which consistently demonstrated the upregulation of RPLP1. In various studies, overexpression of RPLP1 in osteosarcoma samples was associated with reduced overall survival and metastasis-free survival. Consistent with the proposed tumor-promoting role of RPLP1 in osteosarcoma, we also observed a correlation between high levels of RPLP1 expression and poorer survival outcomes in different malignancies.

Myosins are ATP-dependent actin-based molecular motors critical for diverse cellular processes like intracellular trafficking, cell motility, and cell invasion [40]. Myosin light chain 5 (MYL5) has been reported to be upregulated in multiple malignant tumors such as glioblastoma [41], colon cancer [42], cervical carcinoma [43], and breast cancer [44] and has been implicated in tumor metastasis. Mechanically, MYL5 has been shown to positively regulate HIF-1alpha and promotes cancer cell metastasis [43].

We investigated the transcriptional changes occurring after RPLP1 knockdown in tumor cells, and the analysis revealed significant enrichment of DEGs in pathways associated with cellular interactions. Notably, the well-established metastasis-promoting protein MYL5 [43] showed significant downregulation. Furthermore, a positive correlation was observed between the expression levels of RPLP1 and MYL5 in osteosarcoma samples, suggesting a potential synergistic role in osteosarcoma.

For the first time, this study presents the novel discovery of the upregulation of ribosomal protein RPLP1 in osteosarcoma, along with its prognostic significance. We consistently observed recurrent overexpression of RPLP1 in most of the osteosarcoma cell lines and tumor samples across multiple datasets. The prognostic value of RPLP1 was further confirmed in various datasets. It is important to note that our findings are primarily based on bioinformatic analysis of different datasets on osteosarcoma. Therefore, a detailed exploration of the molecular mechanisms underlying the role of RPLP1 in osteosarcoma was not included in this study, which represents a limitation and deserves further investigation.

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Disclosure of conflict of interest

None.

Address correspondence to: Weitao Yao, Department of Bone and Soft Tissue, The Affiliated Cancer Hospital of Zhengzhou University & Henan Cancer Hospital, Zhengzhou 450008, Henan, China. E-mail: zlyyyaoweitao1402@zzu.edu.cn; Xu Dong Zhang, Translational Research Institute, Henan Provincial and Zhengzhou City Key Laboratory of Non-coding RNA and Cancer Metabolism, Henan International

Join Laboratory of Non-coding RNA and Metabolism in Cancer, Henan Provincial People's Hospital, Zhengzhou University, Zhengzhou 450053, Henan, China. E-mail: xu.zhang@newcastle.edu.au

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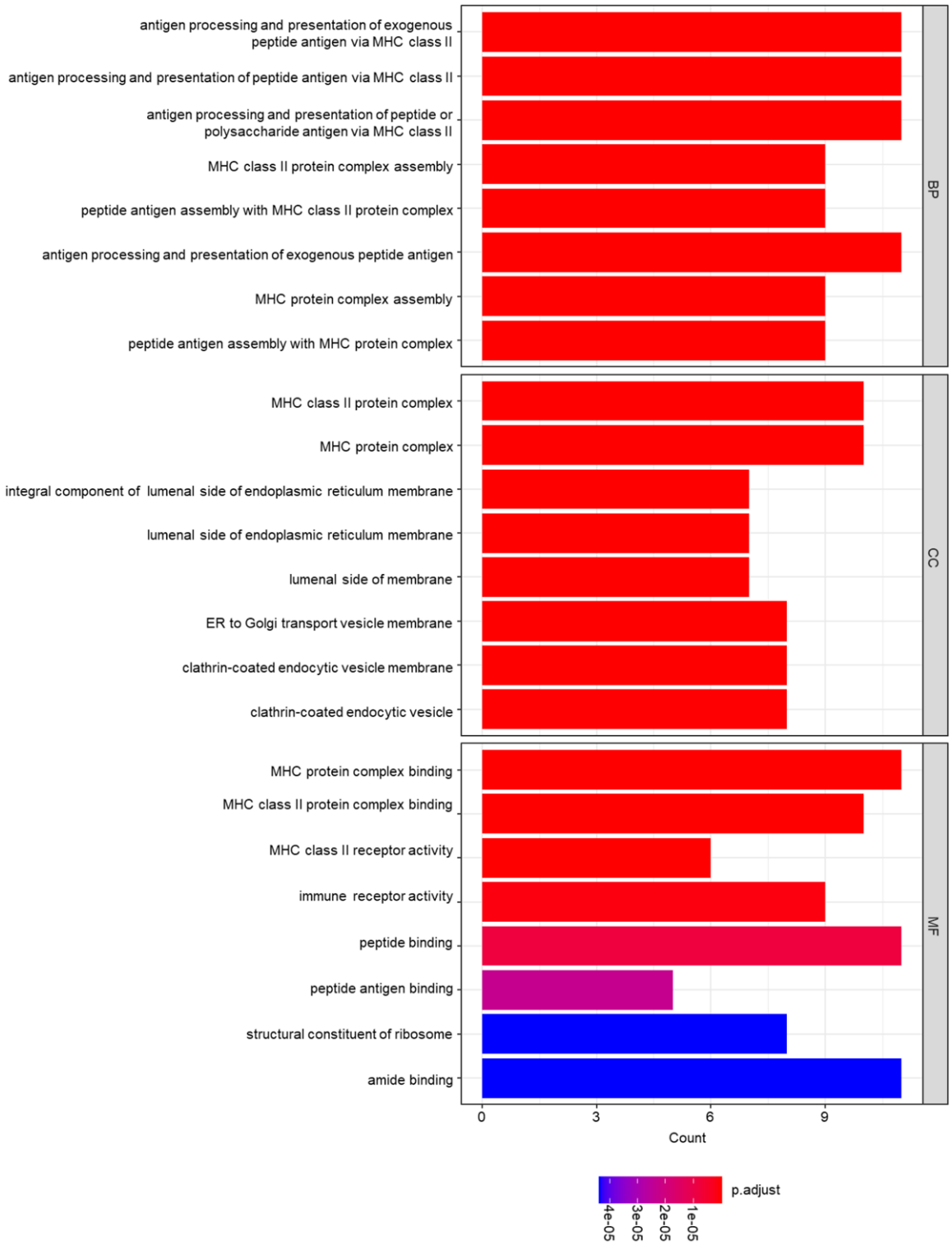
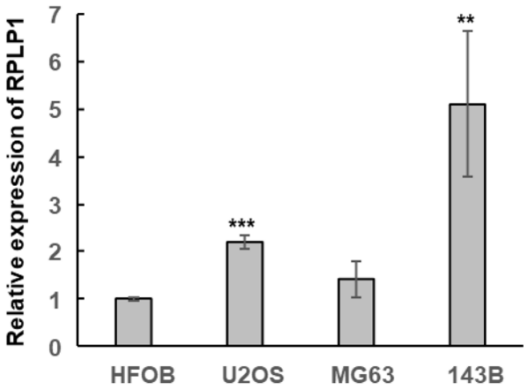
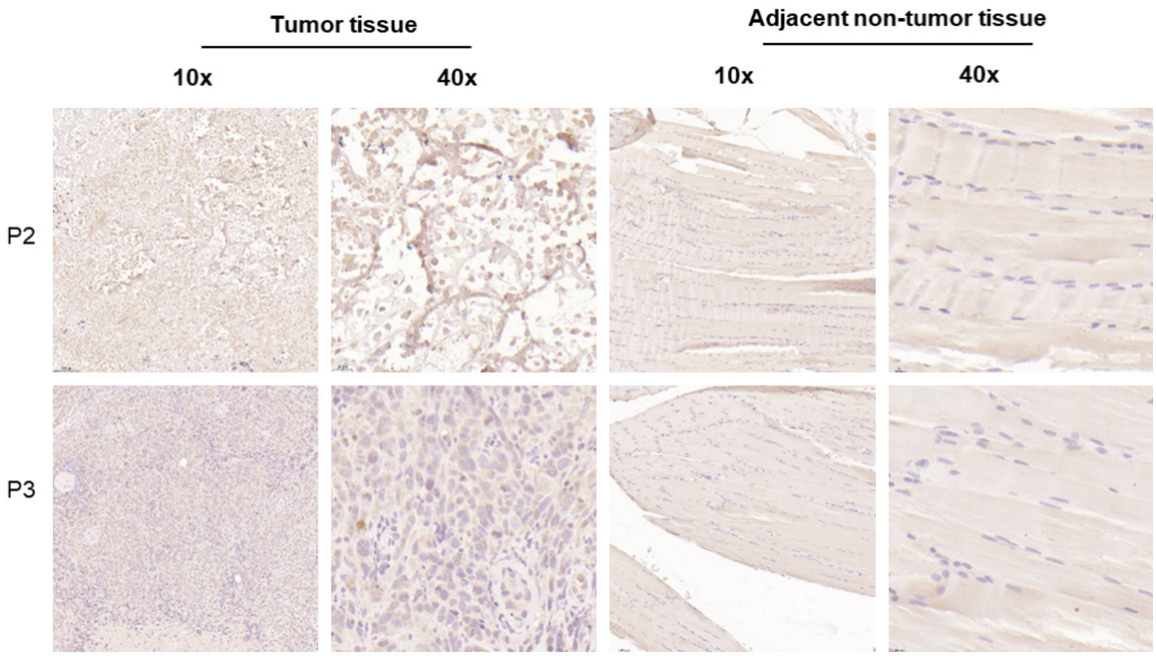


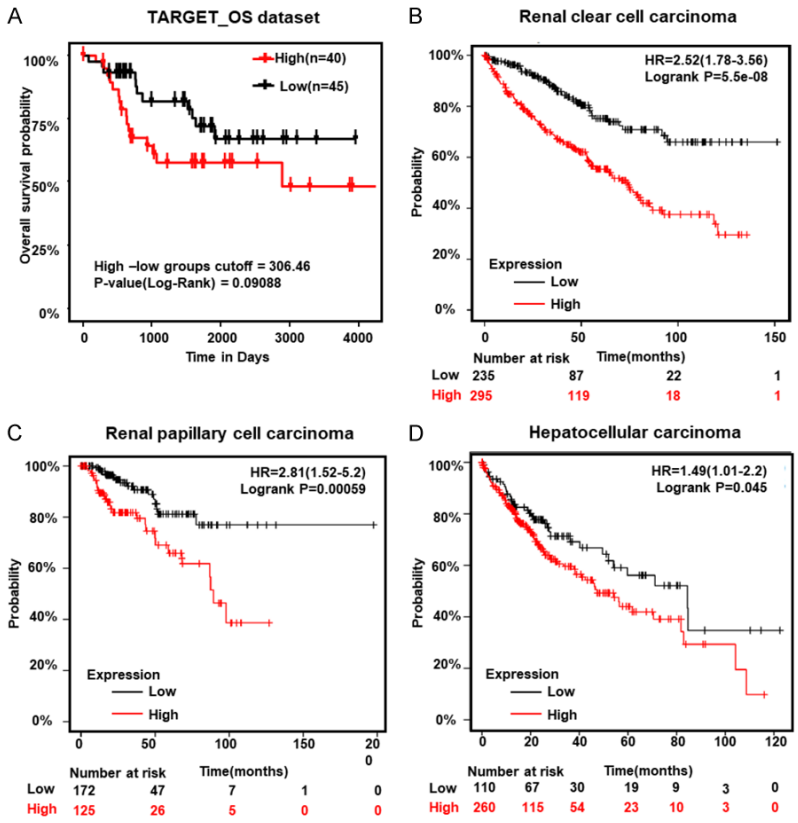
Supplementary Figure 1. The GO enrichment analysis of differentially expressed genes between samples of osteosarcoma metastatic patients and ones without metastasis. The differentially expressed genes were enriched in cellular contact related biological process such as antigen processing and MHC protein complex assembly.



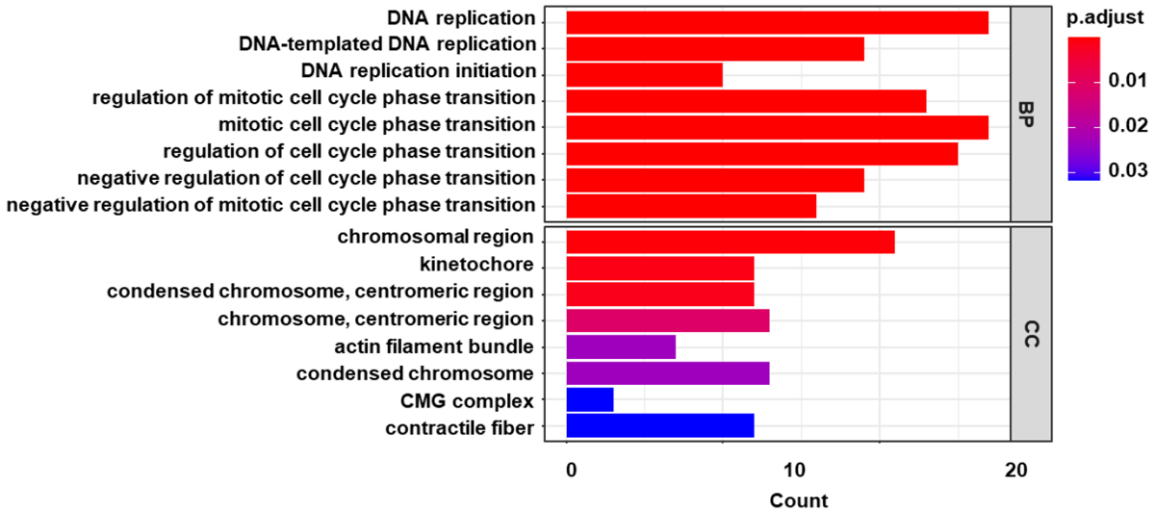
Supplementary Figure 2. The relative expression levels of RPLP1 in osteosarcoma cell lines assessed with qRT-PCR. The RPLP1 is significantly upregulated in U2OS and 143B cell lines comparing with hFOB cells. Each experiment was conducted with 3 biological repeats. **P < 0.01, ***P < 0.001.



Supplementary Figure 3. The IHC staining of the RPLP1 in the paired osteosarcoma and adjacent non-tumor tissue (P2 and P3). The tumor tissues were strongly positive for RPLP1 while the adjacent non-tumor tissues were weak positive or negative for RPLP1.



Supplementary Figure 4. Survival analysis of RPLP1 in different tumor types. A. The survival analysis of the TARGET_OS dataset. Osteosarcoma with high RPLP1 levels showed worse overall survival though the differences were not statistically significant (P = 0.09). B-D. The survival analysis of renal clear cell carcinoma, renal papillary cell carcinoma and hepatocellular carcinoma based on RPLP1 expression levels. Significantly worse survivals were observed in RPLP1 high group in all the three cancer types.



Supplementary Figure 5. The GO functional enrichment analysis showed differentially expressed genes were enriched in cell cycle related pathways.