Proteomics-based identification of the role of osteosarcoma amplified-9 in hepatocellular carcinoma recurrence

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Abstract

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies; its recurrence is associated with high mortality and poor recurrence-free survival and is affected by multisystem and multilevel pathological changes. To identify the key proteins associated with tumor recurrence and the underlying mechanisms, proteomic profiling of tumor specimens from early recurrence and nonrecurrence patients was performed in this study. Proteomics was applied to identify differentially expressed proteins during the early recurrence of HCC after surgery. Osteosarcoma amplified-9 (OS-9) was discovered, and the correlation between OS-9 expression and the clinicopathological characteristics of patients was analyzed. Invasion and migration were examined in SMMC-7721 cells with and without OS-9 overexpression. Proteomics was performed once again using SMMC-7721 cells with OS-9 overexpression to further analyze the proteins with altered expression. OS-9 was overexpressed in the early recurrence group, and OS-9 overexpression was associated with high serum alpha-fetoprotein levels and poor recurrence-free survival in 196 patients with HCC. The invasion and migration abilities of OS-9 overexpressed HCC cell line were weakened while treated with HIF-1α or TNF-α inhibitors. Conclusion: Our results suggest that the overexpression of OS-9 is related to HCC recurrence, thereby
INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies; its prevalence has been increasing rapidly, by 2%–3% annually, over the past decade, although the pace has slowed in recent years.[1,2] Moreover, HCC is a leading cause of cancer-related mortality, with a 5-year overall survival rate of 18% for all stages combined.[1] Surgical resection remains the mainstay of curative therapy for HCC at an early stage. The overall survival rate after surgery remains low, due to the high incidence of early recurrence despite the advancement of diagnostic procedures and treatments. Various studies have revealed that almost 70% of patients develop recurrence within 5 years after resection.[3–5] The underlying molecular pathological mechanism of HCC recurrence has not been fully explored and needs to be studied urgently to improve the prognosis after surgery.

Osteosarcoma amplified-9 (OS-9) was first identified by Su et al. and was shown to be amplified and overexpressed in human sarcoma specimens.[6] Similar effects of OS-9 were confirmed in myeloid leukemia in 1998.[7] However, the negative form of OS-9 has been shown in other tumors.[8,9] Additionally, OS-9 interacts with hypoxia-inducible factor 1α (HIF-1α), promotes the oxygen-dependent degradation of HIF-1α, and ultimately reduces the transcriptional activity and expression of HIF-1α, which impairs tumor growth and metastasis.[8–10]

In this study, we investigated the protein OS-9 in the tumor tissues of patients and its relationship with HCC recurrence based on proteomics. Furthermore, the underlying mechanism of OS-9 and HCC recurrence was explored by secondary proteomics.

METHODS

Clinical materials and sample preparation

All clinical samples were collected from patients with HCC who underwent hepatectomy at the First Affiliated Hospital, College of Medicine, Zhejiang University, between 2014 and 2015. Three pairs of samples from patients with early recurrence (<24 months) and non-recurrence (cryopreserved at −80°C) were used for proteomic profiling, and another 196 samples of HCC tissues were formalin-fixed and paraffin-embedded for immunohistochemical (IHC) analysis. All specimens had an individual hospital ID number, and information regarding clinicopathological characteristics, including age, sex, tumor size, lymph node invasion, distant metastasis, alpha-fetoprotein (AFP) level, differentiation degree, and follow-up prognosis, was collected and recorded in detail. This study was performed according to the protocols approved by the Research Medical Ethical Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University.

Proteomic analysis and database search

The protein solution was reduced for 30 min and alkylated for 15 min for digestion. The protein sample was diluted with 100 mM Triethylammonium bicarbonate. Finally, trypsin was added at a 1:50 trypsin-to-protein mass ratio for the first digestion overnight and a 1:100 ratio for the second 4-hours digestion.

The tryptic peptides were dissolved in formic acid and directly loaded onto a homemade reversed-phase analytical column. The peptides were subjected to a nanospray ion source followed by tandem mass spectrometry (MS-MS) in Q Exactive Plus (Thermo Fisher Scientific). Peptides were then selected for MS-MS, and the fragments were detected. A data-dependent procedure that alternated between one MS scan followed by 20 MS-MS scans with a 15.0-s dynamic exclusion was used. The resulting MS data were processed using the MaxQuant search engine (v.1.6.6.0). Tandem mass spectra were searched against the human UniProt database concatenated with a reverse decoy database. The false discovery rate was adjusted to <1%, and the minimum score for modified peptides was set at >40.

IHC staining

IHC staining was performed with formalin-fixed and paraffin-embedded 196 HCC tissues sectioned to a thickness of 4 μm. The slides were placed in an ethylenediaminetetraacetic acid buffer for antigen repair. Forty microliters of diluted primary antibody (1:1000, ab19853; Abcam) was added to the slide and incubated for 30 min at room temperature. The slides were then treated with a secondary antibody for 60 min at room temperature. 3,3’-Diaminobenzidine was used as the chromogenic substrate. Tumor tissues stained for the OS-9 protein were scored using the histochemistry score, which was further used to transform the number of positive cells and their staining intensity in each section into corresponding values to achieve semi-quantitative analysis.
Finally, the median score was regarded as the cutoff and divided into high or low expression.

**Cell culture**

HCC cell lines SMMC7721 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). SMMC7721 cells were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% FBS. All cell lines were cultured at 37°C in a 5% carbon dioxide incubator.

**Protein extraction**

Cell samples were sonicated three times on ice using a high-intensity ultrasonic processor (Scientz), in lysis buffer (8 M urea, 1% protease inhibitor cocktail), after being washed three times with precooled phosphate-buffered saline (PBS). The supernatants were collected via centrifugation for 10 min. The proteins were quantified using the BCATM Protein Assay Kit (Beyotime) and stored at −80°C.

**Western blot analysis**

Equal amounts of protein samples were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore). After blocking with 5% blocking reagent for 1 hour, the membranes were incubated with primary antibodies against OS-9 (1:1000, ab19853; Abcam), HIF-1α (1:1000, ab179483; Abcam), and beta actin (1:5000, ab6276; Abcam) at 4°C overnight. After washing with Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated for 2 hours with horseradish peroxidase-coupled anti-rabbit immunoglobulin G (1:2500, ab6721; Abcam) at room temperature. Densitometry analysis of protein bands was performed using the Millipore ECL Western Blot Analysis Substrate (Merck Millipore). All bands were analyzed using the ImageJ software (Version 2.1.0; National Institutes of Health).

**Cell transfection**

Cell transfection was performed using Lipofectamine 3000 (Invitrogen) following the manufacturer’s instructions. SMMC7721 cells were cultured and uniformly inoculated into 96-well plates at 1 × 10⁴/well. Lentiviruses OS-9 overexpressing lentiviral vectors (Hanbio Biotechnology Co., Ltd.) and controls were purchased. SMMC7721 cells (2 × 10⁴/well) were transiently transfected with lentiviral vectors and collected 24 hours following transfection. The cells with stably depleted expression and overexpression of OS-9 were selected by culturing in a medium containing penicillin and streptomycin. The efficiency of transduction was confirmed with western blotting.

**Tumor invasion and migration assay**

A polycarbonate membrane Transwell (Corning) and Matrigel (BD) were used for the migration assay. Cells were starved for 24 hours before the suspension, lysed, and washed with PBS before being resuspended in serum-free media. In total, 2 × 10⁵ SMMC7721 cells for both OS-9 overexpression and control groups were suspended in 200 μl of serum-free medium and seeded into the upper chamber, while 600 μl of DMEM containing 10% FBS was added to the lower chamber. After incubation for 48 hours at 37°C, the remaining cells on the upper surface were removed. The invading cells were fixed in methanol and stained with 0.5% crystal violet. Cells on the lower surface of the membrane were counted in randomly selected fields under a microscope (Olympus). The experiment was repeated 3 times independently.

In the migration experiment, all of the steps were the same as those in the invasion experiment, except that Matrigel was not used in the upper chamber.

**Reverse-transcription polymerase chain reaction for messenger RNA analysis**

Total RNA was extracted from SMMC-7721 cells overexpressing OS-9 and control cells using 1 ml of TRizol reagent (Invitrogen). Quantitative real-time polymerase chain reaction (PCR) was carried out using ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems) and DNA Master SYBR Green Kit (Takara Bio, Japan) according to the manufacturer’s instructions. The specific primer pairs used for the target genes are listed in Table 1. The PCR reaction conditions were as follows: 40 cycles of denaturation at 95°C for 30 s, annealing at 95°C for 10 s, and extension at 60°C for 30 s. All reactions were performed in triplicate. The expression values were calculated using the 2^−ΔΔCt method, and all results were normalized to the messenger RNA (mRNA) expression of glyceraldehyde 3-phosphate dehydrogenase, which was used as an internal control.

**Bioinformatic methods**

Proteins were classified by Gene Ontology (GO) annotation into the following three categories:
biological process, cellular compartment, and molecular function. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to identify enriched pathways. These pathways were classified into hierarchical categories according to the KEGG website. For each category of proteins, the InterPro database and a two-tailed Fisher’s exact test were used to test the enrichment of the differentially expressed proteins against all of the identified proteins.

For hierarchical clustering based on differentially expressed protein functional classifications, we first collated all categories obtained after enrichment, along with their p values, and then filtered for the categories that were enriched in at least one of the clusters. This filtered p value matrix was transformed by the function $x = -\log_{10}(p)$ (p value). Finally, the x values were z-transformed for each functional category. These z scores were then clustered using a one-way hierarchical clustering in Genesis. Cluster membership was visualized with a heat map using the “heatmap.2” function in the R package “networkD3.”

Inhibition of signaling proteins interplay with OS-9

Inhibitors including HIF-1α and tumor necrosis factor alpha (TNF-α) were obtained (VH-298, MedChemExpress; HY-100947, Methylthiouracil; HY-B0513, MedChemExpress). OS-9 overexpression or control SMMC-7721 were treated with the two inhibitors at 20 μM or 20 μM, respectively, and then invasion and migration assay were administrated.

Statistical analysis

The SPSS software (version 23.0; SPSS) was used for data analysis. Measurement data are presented as mean ± SD, and a t-test was used for intergroup comparisons. A χ² test was used for intergroup comparisons of count data; Fisher’s exact test was used when the sample size was less than 5. The postoperative recurrence-free and overall survival rates were expressed using Kaplan-Meier curves, and the log-rank χ² test was used for comparisons between high and low expression groups. Multivariate Cox regression analysis was used to analyze the recurrence-free survival rate. All statistical tests were bilateral, and statistical significance was set at $p < 0.05$.

RESULTS

Proteomics was applied to discover differentially expressed proteins during the early recurrence of HCC after surgery

Among all the 3723 proteins, 17 were up-regulated in the HCC tissues from patients with early recurrence (n = 3), while 18 were up-regulated in the tissues of matched patients with nonrecurrence ($p < 0.05$) (Figure 1). All 35 proteins with expression changes greater than 1.5-fold were analyzed (Table 2). The change in OS-9 expression exceeded 300-fold and was largest among all proteins.
OS-9 expression was correlated with some clinicopathological features in patients with HCC

The patients were equally divided into a low OS-9 expression group (n = 98) and a high OS-9 expression group (n = 98) based on the median expression levels of OS-9 as the cutoff value in IHC analysis. Representative IHC results are shown in Figure 2A–D. The correlations between OS-9 expression and the clinicopathological characteristics of patients with HCC were analyzed using the chi-square test (Table 3). The serum AFP levels of patients were higher in the high OS-9 expression group than in the low OS-9 expression group (p < 0.05). The high OS-9 expression group showed a higher rate of HCC recurrence than the low OS-9 expression group (p < 0.001) (Figure 2E,F). However, OS-9 expression did not significantly differ by age, sex, hepatitis B virus infection, tumor size, histological differentiation, vascular invasion, or the number of tumors.

### Table 2

Thirty-five unique proteins (>1.5-fold change and p value < 0.05) were identified between early recurrence and nonrecurrence patients

| Accession | Protein name                                | Ratio | Regulated type | p value |
|-----------|---------------------------------------------|-------|----------------|---------|
| 63252870  | Protein OS-9 isoform 4 precursor            | 344.984 | Up             | 0.002   |
| 20149496  | EF-hand domain-containing protein D1 isoform 1 | 1.989   | Up             | 0.008   |
| 28976154  | Trafficking protein particle complex subunit 6B isoform 2 | 1.923   | Up             | 0.014   |
| 208879470 | Eukaryotic translation elongation factor 1 epsilon-1 isoform 2 | 1.742   | Up             | 0.017   |
| 530405632 | E3 ubiquitin-protein ligase ARIH1 isoform X1 | 1.732   | Up             | 0.046   |
| 295986608 | Immunoglobulin lambda-like polypeptide 5 isoform 1 | 1.721   | Up             | 0.028   |
| 5174411   | CD5 antigen-like precursor                  | 1.679   | Up             | 0.016   |
| 399788586 | Negative elongation factor C/D              | 1.664   | Up             | 0.001   |
| 530391089 | Tenasin isoform X4                          | 1.654   | Up             | 0.012   |
| 530411127 | Bleomycin hydrolase isoform X1              | 1.627   | Up             | 0.021   |
| 88900491  | Neutral alpha-glucosidase AB isoform 3 precursor | 1.625   | Up             | 0.041   |
| 5032057   | Protein S100-A11                            | 1.581   | Up             | 0.045   |
| 530398611 | Chromodomain-helicase-DNA-binding protein 4 isoform X1 | 1.575   | Up             | 0.009   |
| 4504349   | Hemoglobin subunit beta                     | 1.574   | Up             | 0.028   |
| 530373812 | Cohesin subunit SA-1 isoform X1             | 1.532   | Up             | 0.007   |
| 530427497 | Unconventional myosin-If isoform X2         | 1.517   | Up             | 0.034   |
| 258679498 | Carbonic anhydrase 1                        | 1.505   | Up             | 0.036   |
| 530366108 | Conserved oligomeric Golgi complex subunit 2 isoform X1 | -1.504  | Down           | 0.019   |
| 8051636   | Exportin-T                                  | -1.512  | Down           | 0.013   |
| 355930315 | Protein scribble homolog isoform b          | -1.535  | Down           | 0.008   |
| 300244569 | Serine/arginine-rich splicing factor 11 isoform 2 | -1.602  | Down           | 0.005   |
| 171460928 | 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase | -1.604  | Down           | 0.01    |
| 4504505   | Peroxisomal multifunctional enzyme type 2 isoform 2 | -1.621  | Down           | 0.033   |
| 4557481   | Canalicular multispecific organic anion transporter 1 | -1.657  | Down           | 0.005   |
| 24586679  | Histone H2B type 1-A                        | -1.674  | Down           | 0.047   |
| 530395424 | Solute carrier family 22 member 18 isoform X2 | -1.802  | Down           | 0.011   |
| 530425831 | Ral GTPase-activating protein subunit alpha-2 isoform X2 | -1.804  | Down           | 0.047   |
| 11068137  | Hydroxyacid oxidase 1                       | -1.885  | Down           | 0.04    |
| 548923675 | 2-hydroxyacyl-CoA lyase 1 isoform d         | -2.003  | Down           | 0.017   |
| 530362126 | Fatty-acid amide hydrolase 1 isoform X1      | -2.007  | Down           | 0.023   |
| 530386343 | Transferrin receptor protein 2 isoform X1    | -2.153  | Down           | 0.022   |
| 4503987   | Gamma-glutamyl hydrolase precursor          | -2.19   | Down           | 0.003   |
| 530402736 | Conserved oligomeric Golgi complex subunit 3 isoform X6 | -2.343  | Down           | 0.025   |
| 55770895  | Protein LAP2 isoform 7                      | -2.71   | Down           | 0.027   |
| 116875826 | HD domain-containing protein 2              | -246.486 | Down           | 0.008   |
FIGURE 1  Differential proteins expression in early recurrence group (n = 3) and non–early recurrence group (n = 3). (A) Volcano plot of the differentially expressed proteins. Gray dots represent genes that are not differentially expressed in the early recurrence group and non–recurrence group; red dots and blue dots represent genes that are up-regulated and down-regulated significantly in early recurrence group. (B) Heat map of the differentially expressed proteins. Red rectangles mean that genes are up-regulated in these samples, and blue ones mean down-regulated. (C) Kyoto Encyclopedia of Genes and Genomes pathway enrichment for differentially expressed proteins. The sizes of the circle represent the number of genes enriched in pathways, and the color of the circle means significance. (D) Protein–protein interaction network of differentially expressed proteins with osteosarcoma amplified-9 (OS-9); the combined score of interactions are more than 0.4. Abbreviations: ABCC2, ATP binding cassette subfamily C member 2; AD1, acireductone dioxygenase 1; AMFR, autocrine motility factor receptor; CA1, carbonic anhydrase 1; CD5L, CD5 molecule like; CHD4, chromodomain helicase DNA binding protein 4; DERL2, derlin 2; DERL3, derlin 3; DREL1, derlin 1; EEF1E1, eukaryotic translation elongation factor 1 epsilon 1; EFHD1, EF-hand domain family member D1; ERBIN, erb2 interacting protein; ERLEC1, endoplasmic reticulum lectin 1; FAF2, Fas associated factor family member D1; ERBB, erb2 interacting protein; HBB, hemoglobin subunit beta; HAO1, hydroxyacid oxidase 1; HDDC2, HD domain containing 2; HIST1H2BA, histone cluster 1, H2ba; HSD17B4, hydroxysteroid 17-beta dehydrogenase 4; IGLL5, immunoglobulin lambda like polypeptide 5; NELFCD, negative elongation factor complex member C/D; OS-9, osteosarcoma amplified-9; P4HB, prolyl 4-hydroxylase subunit beta; S100A11, S100 calcium binding protein A11; SCRIB, scribble planar cell polarity protein; SEL1L, SEL1L adaptor subunit of ERAD E3 ubiquitin ligase; SRSF11, serine and arginine rich splicing factor 11; SYVN1, synoviolin 1; TFR2, transferrin receptor 2; TNC, tenascin C; TRAPPc6B, trafficking protein particle complex subunit 6B; VCP, valosin containing protein; XPOT, exportin for tRNA;
Correlations between OS-9 expression and HCC recurrence. (A,B) Representative immunohistochemistry (IHC) staining on surgical specimens of high OS-9 expression. (C,D) Representative IHC staining on surgical specimens of low OS-9 expression. (E) Scatter plot of the expression of OS-9 using histochemistry score of tumor tissues in both recurrence and nonrecurrence groups. Patients in the early recurrence group showed higher expression of OS-9 in the tumor tissue significantly ($p < 0.01$). (F) Recurrence-free survival of patients with HCC stratified by OS-9 expression, the high OS-9 expression in the tumor tissue group expressed significantly poor recurrence-free survival ($p < 0.001$).
OS-9 predicted poor overall survival in patients with HCC

Univariate and multivariate Cox analyses were performed to evaluate the prognostic factors for HCC recurrence Table 4). The univariate analysis revealed that patient age >60 years, serum AFP levels > 20 μg/l, tumor size > 10 cm, poor/medium-poor pathological differentiation, microvascular invasion, and high OS-9 expression were significant risk factors that could increase the recurrence rate in patients with HCC. The multivariate analysis showed that patient age > 60 years, tumor size > 10 cm, poor/medium-poor pathological differentiation, vascular invasion, and high OS-9 expression were significantly associated with tumor recurrence.
FIGURE 3  OS-9 promoted SMMC-7721 cell migration, and invasion. (A,B) OS-9 overexpression efficiency verification after lentiviruses transfection. (C) Effects of OS-9 overexpression group on cell migration capacity by Transwell assays in SMMC-7721 cells. (D) Effects of control group on cell migration capacity. (E) Effects of OS-9 overexpression group on cell invasion capacity. (F) Effects of control group on cell invasion capacity. (G) Results from Transwell assay revealed an increased migratory and invasion capacity of OS-9 overexpression SMMC-7721 cell lines (p < 0.01). Abbreviations: NC, control; OE, overexpressed
OS-9 promoted SMMC-7721 cell migration and invasion

*In vitro* assays were performed to explore the potential function of OS-9 in HCC cells. The overexpression of OS-9 using lentiviruses was verified to be feasible in SMMC-7721 cells (Figure 3A,B). The transwell assay revealed the increased migratory (Figure 3C,D; \( p < 0.01 \)) and invasive (Figure 3E,F; \( p < 0.01 \)) capacity of SMMC-7721 cells with OS-9 overexpression.
Potential interacting proteins directly targeted by OS-9 in SMMC-7721 cells

To determine the molecular mechanisms by which OS-9 regulates HCC recurrence, MS was used. Potential interacting proteins were analyzed between SMMC-7721 cells with stable OS-9 overexpression and those with the vector control. The expression of 268 proteins was up-regulated, and that of 140 proteins was down-regulated, in the overexpression group compared with that in the vector control group (fold change ≥ 1.5; p < 0.05). Several significant proteins, including mixed lineage kinase domain-like (MLKL), tumor necrosis factor alpha-induced protein 3 (TNFAIP3), JunB proto-oncogene (JUNB), caspase-7 (CASP7), enolase1 (ENO1), enolase2 (ENO2), enolase3 (ENO3), and lactate dehydrogenase A (LDHA), were differentially expressed (Figure 4).

Functional classification of differentially expressed proteins by bioinformatic analysis

Based on the data shown previously, a systematic bioinformatic analysis (protein function annotation) was carried out to determine GO terms and KEGG pathways in which the differentially expressed proteins were significantly enriched. GO, KEGG, and protein domain analyses indicated the involvement of differentially expressed proteins and signaling pathways in regulation of the HIF-1 and TNF signaling pathways (Figure 4).

mRNA expression in the HIF-1 and TNF signaling pathways

OS-9 overexpression significantly promoted the expression of mRNAs involved in the HIF-1 and TNF signaling pathways in SMMC-7721 cells. The mRNA expression levels of MLKL, TNFAIP3, JUNB, CASP7, TNF-α, and tumor necrosis factor receptor–associated factor 6 (TRAF6) increased more than 2-fold in SMMC-7721 cells with OS-9 overexpression, which represented activation of the TNF signaling pathway. Moreover, the expression of ENO1, ENO2, ENO3, LDHA, and aldolase, fructose-bisphosphatase A (ALDOA) was elevated by more than 3-fold in this group, indicating activation of the HIF-1 signaling pathway (Figure 5).

Interplay between HIF-1α or TNFα signaling pathway and OS-9

OS-9 overexpression significantly induced up-regulation of HIF-1 expression in SMMC-7721 cells by western blot results (Figure 6A,B). Transwell assay were also adopted in OS-9 overexpression and control SMMC-7721 cell line with or without HIF-1α or TNF-α inhibitors. The results showed that invasion and migration of OS-9 overexpressed cell line (Figure 6C,D) were weakened using HIF-1α inhibitor (Figure 6E,F). There are also significant changes for migration and invasion of OS-9 overexpressed HCC cell line using TNF-α inhibitor (Figure 6G,H).

DISCUSSION

Increasing evidence has demonstrated that proteins play important roles in the recurrence and metastasis of HCC, which is accompanied by changes in the expression patterns of various proteins. In this study, proteomics was performed using tumor specimens from early recurrence and nonrecurrence patients; the change in OS-9 expression exceeded 300-fold and was largest among all of the proteins investigated. Based on these results, more clinical samples were collected, and IHC analysis was performed to identify the critical
FIGURE 5  (A–K) The relative messenger RNA (mRNA) expression level of lineage kinase domain-like MLKL (A), tumor necrosis factor alpha–induced protein 3 (TNFAIP3) (B), JunB proto-oncogene (JUNB) (C), and tumor necrosis factor receptor–associated factor 5 (TRAF5) (D), TNF-α (E) and caspase-7 (CASP7) (F) could be observed to increase more than 2-fold ($p < 0.01$). The relative expression of enolase1 (ENO1) (G), enolase2 (ENO2) (H), enolase3 (ENO3) (I), aldolase, fructose-bisphosphate A (ALDOA) (J), and lactate dehydrogenase A (LDHA) (K) were also elevated more than 2-fold ($p < 0.01$).
ROLE OF OSTEOSARCOMA AMPLIFIED-9 IN HCC RECURRENCE

Progress has been made in treatment strategies for HCC; however, the recurrence-free survival of patients with HCC has not improved. Novel biomarkers are urgently needed to improve the prognosis of these patients. The function of OS-9, an endoplasmic reticulum lectin, in tumor regulation is rarely analyzed, and contradictory conclusions have been reached. Overexpression of OS-9 has been observed in osteosarcomas, and OS-9 was reported to be co-amplified with CDK4 in three of five sarcoma tissues. OS-9 acts as a part of the endoplasmic reticulum-associated degradation (ERAD) machinery and aids in the transfer of misfolded proteins. Sun et al. discovered that the long non-coding RNA ENST00000480739 negatively regulates HIF-1α expression by up-regulating OS-9 expression in pancreatic ductal adenocarcinoma, and that the down-regulation of HIF-1α expression through OS-9 indicates underlying tumor metastasis. However, no study has indicated a correlation between OS-9 and HCC.

Numerous unfolded/misfolded proteins are degraded by ERAD, which is regarded as an important ER-folded.
os-9 in regulating cellular activity and molecular function. To determine GO terms and KEGG pathways in which the differentially expressed proteins are significantly enriched, we carried out the following three enrichment analyses: GO classification, KEGG pathway, and protein domain. GO functional enrichment analysis of differentially expressed genes showed that the upregulated genes in HCC cells were primarily involved in monosaccharide catabolic/biosynthetic and peptidase regulator activity–related processes, and the downregulated genes were primarily involved in the biological processes of calcium ion binding or protein complex binding. KEGG analysis showed that the expression of OS-9 regulated the development of HCC. The overexpression of OS-9 was related to up-regulation of the HIF-1 and TNF signaling pathways. However, the regulation of proteins related to microRNAs (miRNAs) in cancer cells and the Wnt signaling pathway was also observed in the raw data of our proteomics results.

The transcriptions of HIF-1 have been shown to activate a wide repertoire of genes that promote tumor growth and metastasis and is associated with poor clinical outcomes. HIF-1 can regulate the expression of various target genes and promote hypoxia, which activates the transcription of genes responsible for angiogenesis, glucose metabolism, proliferation, invasion, and metastasis in HCC.16–18 Moreover, HIF-1 is a particularly crucial protein for shifting the metabolic program from oxidative phosphorylation to glycolysis, and its expression was up-regulated in SMMS-7721 cells with OS-9 overexpression in our study.16 The potential role of HIF-1α in the barrier-modulation function of OS-9 was determined by Sun et al.; however, HIF-1α levels were not affected by OS-9 overexpression or direct physical interaction in this study.19 Similarly, we observed activation of the HIF-1 signaling pathway rather than overexpression of the HIF-1 protein. The expression of ENO1, ENO2, ENO3, ALDOA, and LDHA was more than 2-fold higher in the OS-9 overexpression group.

TNF-α has been revealed to be a vital cytokine for tumor progression, as well as apoptosis, inflammation, and immunity, and is produced and released by activated macrophages.20 Zhao et al. revealed that TNF-α inhibits the expression of miR-497 and promotes the self-renewal and metastasis phenotypes of HCC cells through the nuclear factor kappa B (NF-kB)/miR-497/SALL4 axis, which is associated with poor prognosis in patients with HCC.21 The knockdown of TNF-α could inhibit the activation and proliferation of hepatic progenitor cells through the tumor necrosis factor receptor 2 (TNFR2)/signal transducer and activator of transcription 3 signaling pathway to inhibit hepatocellular carcinogenesis.22 Two distinct surface receptors were discovered: TNFR1 and TNFR2. An extensive cross-talk among the apoptosis, NF-κB, and JNK signaling pathways generated from TNFR1, which initiates most of the biological activities of TNF-α, has been confirmed.23 Ligand
binding to TNFR2 could lead to activation of the NF-κB, p38 mitogen-activated protein kinase, extracellular signal–regulated kinase, and phosphatidylinositol 3-kinase pathways, which are related to cell proliferation, migration, and survival and the modulation of regulatory T-cell function.\textsuperscript{23,24} In our study, TNFAIP3, JUNB, CASP7, TNF-α, and TRAF5 expression was more than 2-fold higher in the OS-9 overexpression group; this indicates that the expression of these proteins serves as a biomarker in human HCC tissues. Furthermore, elevated TNFAIP3 levels can lead to oncogenic properties.\textsuperscript{25,26} To further examine the interplay between HIF1-α or TNF and OS-9, inhibitors of these two pathways were adopted. The Transwell array results revealed that the overexpression of OS-9 could enhance the migration and invasion capacity of the HCC cell line, blocking the two proteins prevented OS-9 from inducing migration and invasion in HCC where OS-9 is overexpressed.

In conclusion, the results of our study suggest the effect of OS-9 on the tumorigenesis, development, and adverse prognosis of HCC. A higher OS-9 expression is related to higher AFP levels and leads to a lower recurrence-free survival rate. The overexpression of OS-9 enhances the invasion and migration of SMMS-7721 cells. According to MS, GO, and KEGG analyses, the HIF-1 and TNF signaling pathways are activated in tumor cells with OS-9 overexpression. The expression of various key proteins in these pathways is significantly changed. This study provides a potential protein biomarker that could help improve the prognosis and recurrence-free survival of patients with HCC after surgery.

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CONFLICT OF INTEREST
Nothing to report.

AUTHOR CONTRIBUTIONS
Data management, statistical analysis, and manuscript draft: Xuyong Wei and Mengfan Yang. Cohort identification, experimental implementation, and data management: Binhua Pan, Xiaobing Zhang, Hanchao Lin, Wangyao Li, Wenzhi Shu, Kun Wang, Abdul Rehman Khan, Xuanyu Zhang, and Beini Cen. Project administration: Xiao Xu performed. All authors searched the literature, designed the study, interpreted the findings, and revised the manuscript.

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