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IDENTIFICATION OF ATXN3 AND UBE2S AS PROGNOSTIC MARKERS FOR OSTEOSARCOMA BY A REGRESSION MODEL FOR INTEGRATING MULTIOMICS

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Abstract – *Objective:* *Osteosarcoma (OS) is a malignant tumor in children, which seriously endangers children's health. However, few studies have been conducted to identify potential targets of OS. The purpose of this study is to identify the protein markers in plasma that are associated with the occurrence, metastasis, and prognosis of OS by combining multinomial analysis and constructing regression models.*

Materials and Methods: *The miRNA expression profile GSE65071 dataset and protein expression (GSE78192) dataset were downloaded from the Gene Expression Omnibus (GEO) database. The differentially expressed proteins (DEPs) and miRNAs (DEMIs) were identified from primary OS vs. normal control, and metastatic OS vs. primary OS, respectively. A miRNA-protein network was constructed based on miRNA-protein pairs. Functional analysis of DEPs involved in miRNA-protein network was utilized to obtain insights into the functions of the network. Subsequently, we screened out the genes differentially expressed similarly in both plasma and osteosarcoma tissues of OS patients. Eventually, genes in miRNA-protein network closely related to survival were identified by the intersection of lasso regression and K-M survival analysis, and Cox regression model was constructed for further analysis.*

Results: *A total of 277 DEMIs and 50 DEPs, 3 DEMIs and 254 DEPs were identified with contact of OS occurs and metastasis. Furthermore, 11 proteins involved in miRNA-protein network were confirmed to exhibit the same dysregulation in both primary OS plasma and tissue and 3 proteins exhibit dysregulation in metastatic patients. Moreover, for patients without metastasis, UBE2S and ATXN3 not only have the same differential expression trend in both plasma and tumor tissues of OS patients, but also are closely related to the survival time of patients.*

Conclusions: *In OS plasma, 11 proteins, including UBE2S, ATXN3 and LARP6 are potential plasma markers for the diagnosis of OS. Totally 3 proteins (UGT3A1, IGF1R and SLC7A1) were the potential plasma markers to predict metastasis of OS. Furthermore, the C-index and the AUC at 4-year of Cox regression model were 0.79 and 0.97, suggesting good reliability and potential of UBE2S and ATXN3 for prognostic prediction and targeted therapy of primary OS.*

KEYWORDS: *Osteosarcoma, Prognosis, ATXN3, UBE2S, Multiomics, Marker.*

LIST OF ABBREVIATIONS: *OS: Osteosarcoma; GEO: Gene Expression Omnibus database; DEPs: differentially expressed proteins. DEMIs: differentially expressed miRNAs; DEGs: differentially expressed genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; STRING: Search Tool for the Retrieval of Interacting Genes database; PPI: Protein-protein interaction; TARGET: Therapeutically Applicable Research To Generate Effective Treatments database; Lasso: least absolute shrinkage and selection operator; COX: proportional hazards model; ROC: receiver-operating characteristic curve.*

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INTRODUCTION

Osteosarcoma (OS), which may originate from mesenchymal stem cells, is the most common primary malignant bone tumors in children and adolescents[1]. OS can occur at any age and is common in adolescents or children under 20 years of age[2]. The application of neoadjuvant chemotherapy in 1970s significantly improved the survival rate of OS patients, five-year survival rate for non-metastatic OS patients increased from less than 20% to 65-70%[3,4]. However, the overall-survival rate did not improve considerably from 1984 to 2004 due to drug resistance and side-effects of chemotherapeutics[5]. Till now, reliable, and noninvasive biomarkers are not available for the detection of the presence or progression of OS, assessment of therapy response, or prediction of upfront prognosis. Therefore, identification of sensitive, specific, and less invasive biomarkers to detect OS at an early stage has become one of the most significant challenges in the management of OS.

Some studies have indicated that potential plasma and tumor tissues microRNAs (miRNA), which are a class of small non-coding RNA molecules negatively modulate protein expression at the post-transcriptional level and associated with tumorigenesis, play important roles in drug resistance, metastasis, recurrence and prognosis in multiple types of tumor[6-9]. The effects of miRNAs on protein expression are diverse. As considerable repressors of gene expression, miRNAs are involved in an abundant range of biological processes such as cell cycle control, apoptosis, and physiological processes by regulating post-transcriptional process of gene expression[10-12]. Recent evidence indicates that miRNAs interact with Argonaute protein family, ribonuclease type III endonuclease and arginine protein in base-pair conformation to form RNA-induced silencing complex (RISC) to degrade or downregulate proteins[13]. Therefore, there are increasingly number of studies are attempting to investigate the mechanism of tumor and the potential of clinical application through the regulation between miRNAs and proteins[14-16]. To identify candidate tumor markers circulating in the blood or cerebrospinal fluid that can improve the diagnosis, patient stratification and early detection of recurrent diseases, Kaid et al[17] screened and analyzed the function and relationship between differential miRNAs and proteins from four tumor cell lines. Furthermore, Erhart et al[18] integrated proteomics/miRNomics to find a reliable immunotherapy target for glioblastoma. Moreover, except that the slope and shape of the protein response curve may change, the inhibition of protein translation by miRNA itself does not affect the level of mRNA[19]. Therefore, integrating plasma proteomics and miRNomics is not only reliable and effective, but also can provide more intuitive diagnosis, stratification, and prognostic evaluation for patients.

However, few studies have been focused on combination analysis of proteomics and miRNomics to find potential protein markers in plasma to investigate the pathophysiological process of OS. In the present study, the miRNAs and proteins differentially expressed in plasma were investigated by using miRNA dataset of GSE65071 and protein microarray dataset of GSE78192 from Gene Expression Omnibus (GEO) database. We integrated plasma proteomics with miRNomics which may help identify reliable and effective markers to provide valuable evaluation for the stratification and prognosis of OS patients. The flowchart of this procedure is presented in Figure 1. After predicting the miRNA-protein interaction pairs, the present study successfully established the miRNA-protein network from primary OS *vs.* normal control, and metastatic OS *vs.* primary OS, respectively. Furthermore, to investigate the differences of gene expression between plasma and tumor tissue, 10 datasets were downloaded from GEO and TARGET database and differentially expressed genes were identified for searching for suitable biomarkers. Gene ontology (GO) functional analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathway enrichment analysis and the interactions between proteins were also performed to construct the miRNA-hubprotein regulatory module to better understand the pathogenesis of OS. Totally, 11 plasma proteins, including UBE2S, ATXN3 and LARP6 are potential plasma markers for the diagnosis and 3 proteins (UGT3A1, IGF1R and SLC7A1) were potential markers to metastasis prediction of OS. Furthermore, the C-index and the AUC at 4-year of Cox module were 0.79 and 0.97 respectively, suggesting a good reliability of UBE2S and ATXN3 for prognosis prediction of primary OS. In this study, we systematically and extensively investigate the plasma miRNA-protein expression profiles in evaluation of OS occurence, metastasis and prognosis.

MATERIALS AND METHODS

Microarray data and preprocessing

The miRNA profile of GSE65071, containing 15 normal plasma, 10 primary OS plasma and 10 metastatic OS, was generated using the platform of GPL19631 (Exiqon human V3 microRNA PCR panel I+II) and the protein profile of GSE78192, containing 3 normal plasma, 3primary OS plasma and 3 metastatic OS plasma, was based on the platform of GPL21502 (Invitrogen ProtoArray v4 human pro-

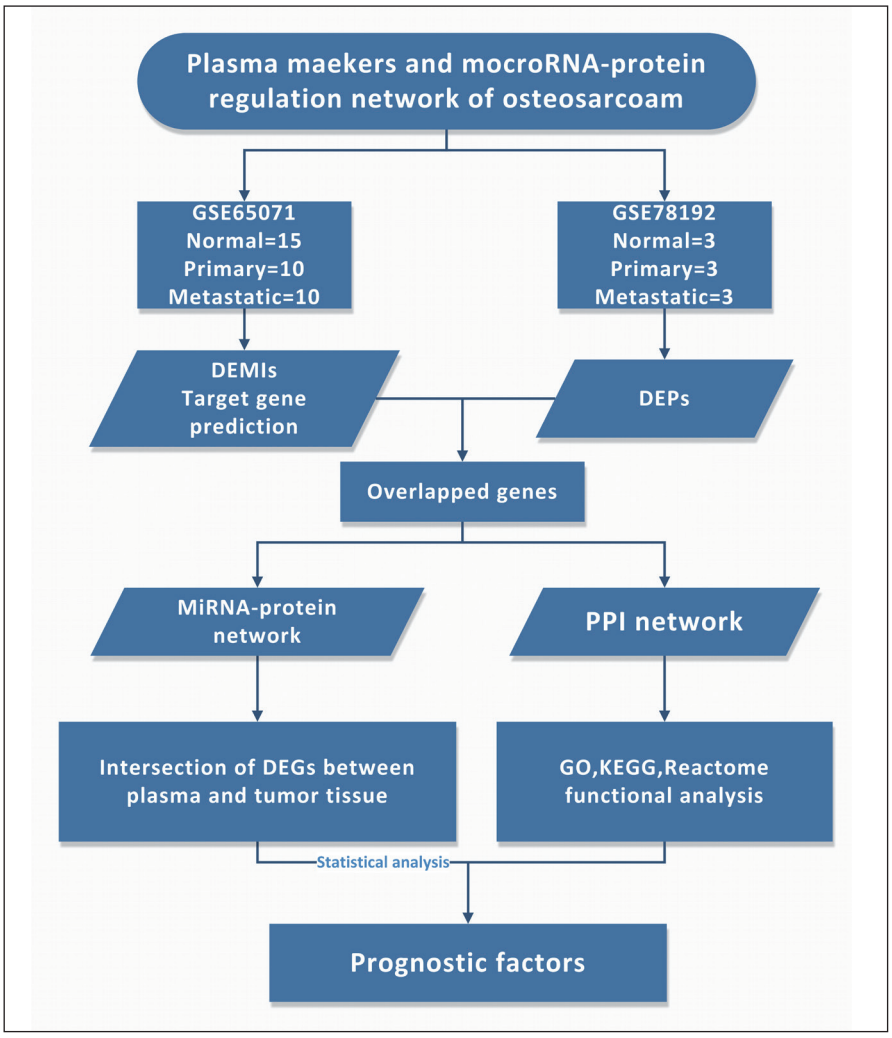

Fig. 1. Overview of workflow of miRNA-protein network analysis. DEMIs, differentially expressed proteins microRNAs; DEPs, differentially expressed proteins. GO, gene ontology; KEGG, kyto encyclopedia of genes and genomes.

tein microarray lot HA20085; Carlsbad, CA, USA). Probe names in protein expression profiles were converted to the gene symbol. In addition, the average of expression was taken when multiple probe names correspond to one gene symbol.

Differential expression analysis

The miRNAs in the GSE65071 dataset were annotated using the REF_ID, PROBE_ID, PANEL, WELL, miRNA_ID, POT_ID, and SEQUENCE by the GPL19631. The proteins in the GSE78192 dataset were annotated by the GPL21502 and BioDBnet (https://biodbnet-abcc.ncifcrf. gov/db/db2db. php). Only miRNAs and proteins with|log2 fold change (FC)|> 1. 0 and *p*-value< 0. 05 were regarded as DEMIs and DEPs. DEMIs and DEPs were identified using LIMMA (version: 3. 42. 2), an R package that processes the normalized data and analyzes gene differential expression.

miRNA-protein network construction and analysis

Target genes of DEMIs were predicted and analyzed through the miRNET (www.mirnet.ca), a web tool based on eleven different miRNA databases[20]. To demonstrate how the miRNAs regulate proteins in plasma of OS, the DEPs, targeted by DEMIS, were selected to construct the miRNA-protein network, and the miRNA-protein subnetwork was generated using the Molecular Complex Detection (MCODE) plug-in Cytoscape.

Functional analysis of genes involved in miRNA-protein network

Gene Ontology (GO), which includes the cellular component (CC), molecular function (MF), and biological process (BP)[21], Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathway en-

richment analysis[22] were used to identify primary function and pathways of DEPs involved in miRNA-protein network using the ClueGO plug-in Cytoscape.

Protein-protein interaction (PPI) network construction and analysis

In order to identify the interaction network of genes involved in miRNA-protein network of OS, Search Tool for the Retrieval of Interacting Genes database (STRING) was utilized to construct the PPI network[23]. PPI networks of differentially expressed genes were visualized using Cytoscape software 3.7.1 (http://cytoscape.org/). In the networks, nodes represent proteins and edges represent interactions between proteins. Subnetwork was identified by MCODE.

DEGs in tumor tissue

To further investigate the difference of DEGs between plasma and tumor tissue. The DEGs between primary OS and normal bone were selected from GSE14359 by LIMMA package. For metastatic OS, DEGs in GSE85537 were used to compare with DEGs in plasma to obtain the genes of overlapping part. Taking $p<0.05$ and $|\log FC| > 1$ as the threshold. Subsequently, we verified whether DEGs with the same expression trend in plasma and tumor tissue had a significant effect on the overall survival rate and disease-free survival of OS patients in GSE39055 and Therapeutically Applicable Research To Generate Effective Treatments (TARGET) database. The Kaplan-Meier one-way survival, least absolute shrinkage and selection operator (lasso), proportional hazards model (COX) and time-dependent receiver-operating characteristic (ROC) curve were performed by R package survival (version 3.1-11), survminer (version 0.4.6), glmnet (version 4.0) and survivalROC (version 1.0.3). A p-value < 0.05 was considered as statistically significant.

RESULTS

Differential expression analysis

Finally, 277 DEMIs, 50 DEPs were detected in OS *vs.* normal, 211 DEMIs, 26 DEPs up-regulated and 66 DEMIs, 23 DEPs down-regulated (Figure 2). Compared with primary OS, a total of 3 DEMIs, 254 DEPs were determined between metastatic OS. The top miRNAs and proteins are listed in ***Table S1-S4***.

miRNA-protein network construction and analysis

We use miNET website to select DEPs that involved in DEMI target genes and visualize them on Cytoscape software. Circles are DEPs and the triangles are DEMIs. Light color means logFC>1, and dark color means logFC<-1. The thicker the edge will be if the combined_score is higher ***(Figure S1)***. In total, 36 DEPs are targeted by multiple DEMIs (Table 1). One module was identified, including PASK, TTK, has-mir-192-5p and has-mir-215-5p. In metastatic OS *vs.* primary OS, 15 proteins were targeted by multiple DEMIs (Table 2).

Functional analysis and pathway analysis of genes in miRNA-mRNA network

A total of 36 DEPs were involved in the miRNA-protein network. To further analyze the functional characteristics of the 36 DEPs in the network, we used the ClueGO plug-in Cytoscapeto to perform GO, Reactome and KEGG pathway analysis. Overall, 39 functional enrichment terms from GO were observed to be divided into 2 parts, including 35 biological processes (BPs) and 4 molecular functions (MFs) ***(Figure S2)***. Top 5 BPs included "protein autophosphorylation", "long-term synaptic potentiation", "cell death in response to oxidative stress", "intrinsic apoptotic signaling pathway in response to oxidative stress", and "negative regulation of cellular response to insulin stimulus". MFs included "tau protein binding", "protein phosphorylated amino acid binding", "phosphotyrosine residue binding" and "protein kinase A binding". In our analysis, 34 KEGG pathways and 32 Reactome pathways were also observed to be significantly enriched ***(Figure S3)***. Major pathways associated with osteosarcoma tumor included "Foxo signaling pathway", "Relaxin signaling pathway" and "AGE-RAGE signaling pathway in diabetic complications".

Protein-protein interaction (PPI) network construction and analysis of primary OS

PPI network of 36 DEPs involved in miRNA-protein network was constructed by STRING web. MCODE identified one significant module in the PPI network ***(Figure S4)***. The module consisted of 6 target genes, including GSK3A, PRKCZ, PRKCA, IKBKB, PDPK1, IRS1, and GSK3A was identified as hub-gene in the module.

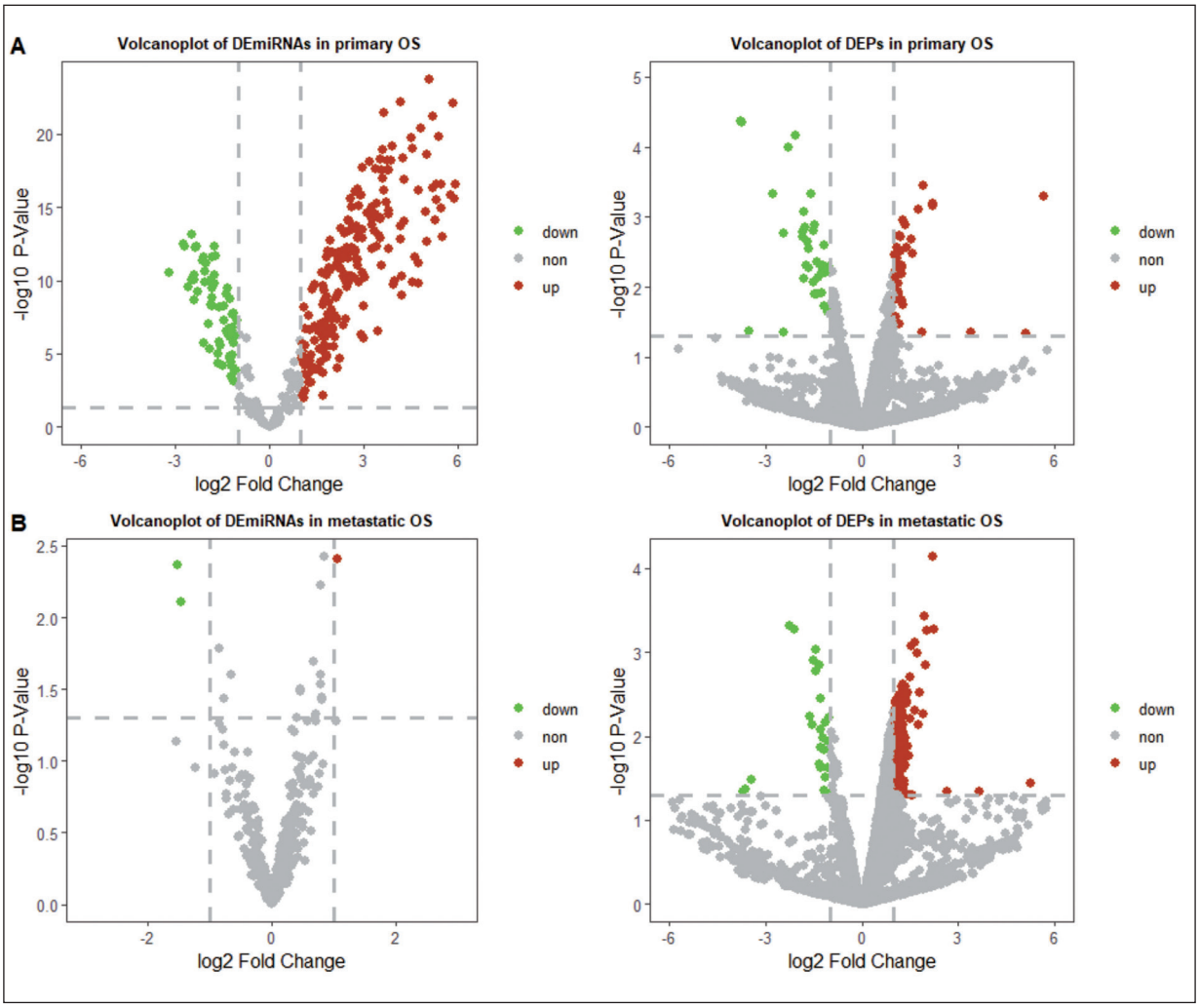

Fig. 2. Volcano plot of DEMIs and DEPs. *A*, Volcano plots of DEMIs and DEPs in primary OS *vs.* normal. *B*, Volcano plots of DEMIs and DEPs in metastatic OS *vs.* primary OS. Red dots represent upregulated and green dots represent downregulated DEMIs and DEPs.

DEGs in tumor tissue and survival analysis

Genes with the same expression trend in both plasma and tumor tissue are considered as overlapped genes. A total of 11 overlapped genes in primary OS and 3 overlapped genes in metastatic OS (Table 3).

TABLE 1. Totally 36 DEPs were involved in Primary OS vs. normal miRNA-protein network.

Symbol	*Regulation*
TG, SELENOI, PRKD2, PDPK1, GRK7, LARP6, HOMER2, PRKCZ, UBE2S, IRS1, STK25, SMAD2, CSNK1G2, STK24, SMAD3, MAPK1, SNAP25, GSK3A	up
THOP1, STK16, ZAP70, PRKCA, CLK3, IKBKB, ATF2, ATXN3, LUC7L, COPZ1, TTK, TOM1, DYRK3, PASK, MAPK14, CENPB, PRKACA, RTKN	down

Then, the lasso regression analysis, Kaplan-Meier one-way survival, COX proportional hazards model and time-dependent ROC methods were used for analysis. Reliably, both K-M one-way survival and lasso regression found that two genes (ATXN3 and UBE2S) were significantly correlated with overall-survival (Figure 3), which has low correction (correlation=0.272 and variance inflation factor (VIF)=1.032824) in Figure 4B. Subsequently, multivariate Cox regression module was applied to predict the prognosis, and the sample characteristics of

TABLE 2. In total, 15 DEPs were involved in Metastatic OS vs. Primary OS miRNA-protein network.

Symbol	*Regulation*
ZNF616, UGT3A1, TNIP1, SLC7A1, RPS6KA3, PLEKHA3, NDST1, MAP3K8, IGF1R	up
DRAM1, DDIT4, COX20, CDKN1B, HOMER2	down

TABLE 3. Overlapped genes between plasma and tumor tissue. Totally 11 overlapped DEPs were obtained in primary OS and 3 DEPs in metastatic OS.

Prim vs. Normal	*Met vs. Prim*
PRKCA, DYRK3, HOMER2, TOM1, IRS1, PRKCZ, IKBKB, CENPB, UBE2S, LARP6, ATXN3	UGT3A1, IGF1R, SLC7A1

the multivariate Cox regression module were verified by testing cohort based on 109 patients without gene-expression data from TARGET database (Figure 4A). To accelerate the clinical application, the expression values of UBE2S and ATXN3 were weighted by the multivariate Cox regression coefficient were transformed into a risk score as follows: The risk score = 48*exp (0.5907*expression value of UBE2S) + (-1.0533*expression value of ATXN3). Multivariate Cox analysis showed that the predictive power of ATXN3 in the module was mutually independent (Figure 4C). In order to evaluate the prediction efficiency of the Cox model, Concordance index (c-index), time-dependent ROC curve (AUC) and K-M one-way survival analysis were applied in 63 patients with complete expression information from TARGET database. Notably, the AUCs of the time-dependent ROC curves of the COX module were 0.97 at 4-year overall-survival, and the *p*-value of the log-rank test at 4-year was lower than 0.0001 (Figure 4D-E). To investigate the link among risk score, overall-survival and biomarkers, we ranked patients in ascending order of risk score and displayed patient clinical information and expression values on the same abscissa (Figure 5). Consistent with multivariate Cox regression analysis, high-risk group of modules corresponded to poor prognosis and ATXN3 demonstrated greater consistency with patient outcomes.

DISCUSSION

The expression level of proteins can be regulated by miRNAs in a variety of ways, resulting in the differential expression of proteins that may not be consistent with the trend of mRNA[24]. Previous studies have confirmed that it is feasible and useful of a combination of proteomics and miRNomics approach for the investigation of tumor factors in medulloblastoma and glioma[17,18]. Integrating proteomics/miRNomics has been reported as a reliable method for exploring mechanisms of local microenvironmental changes[25]. The discovery of the pathogenesis and development of OS will provide important clues in the diagnosis, treatment and prediction of prognosis. In the present study, in order to find plasma markers closely related to OS occurrence, metastasis and prognosis, we downloaded two types of microarray datasets: protein expression profile and miRNA expression profile from the GEO database and integrated plasma proteomics/miRNomics. Compare to normal group, a total of 277 DEMIs and 50 DEPs were identified in osteosarcoma plasma, containing 211 up-regulated, 66 down-regulated miRNAs and 27 up-regulated, 23 down-regulated proteins. In the PPI network

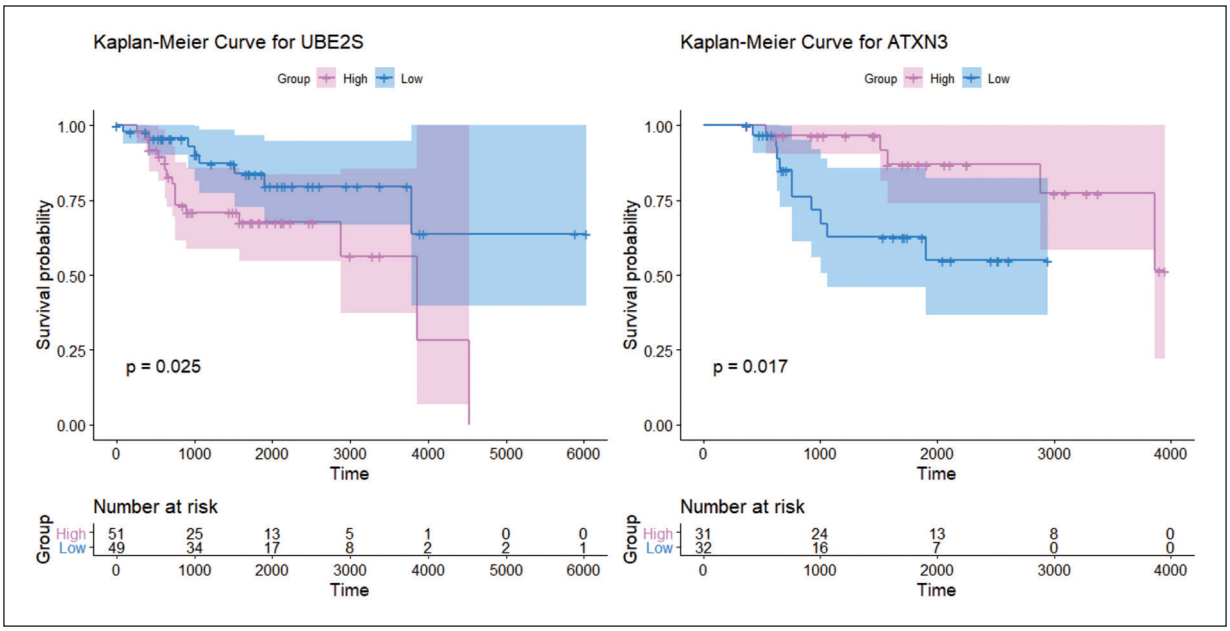

Fig. 3. K-M survival analysis of 11 proteins overlapped in plasma and tumor tissue. Low expression of UBE2S is related to poor prognosis and p=0.025. High expression of ATXN3 is significantly associated with better overall survival and p=0. 017.

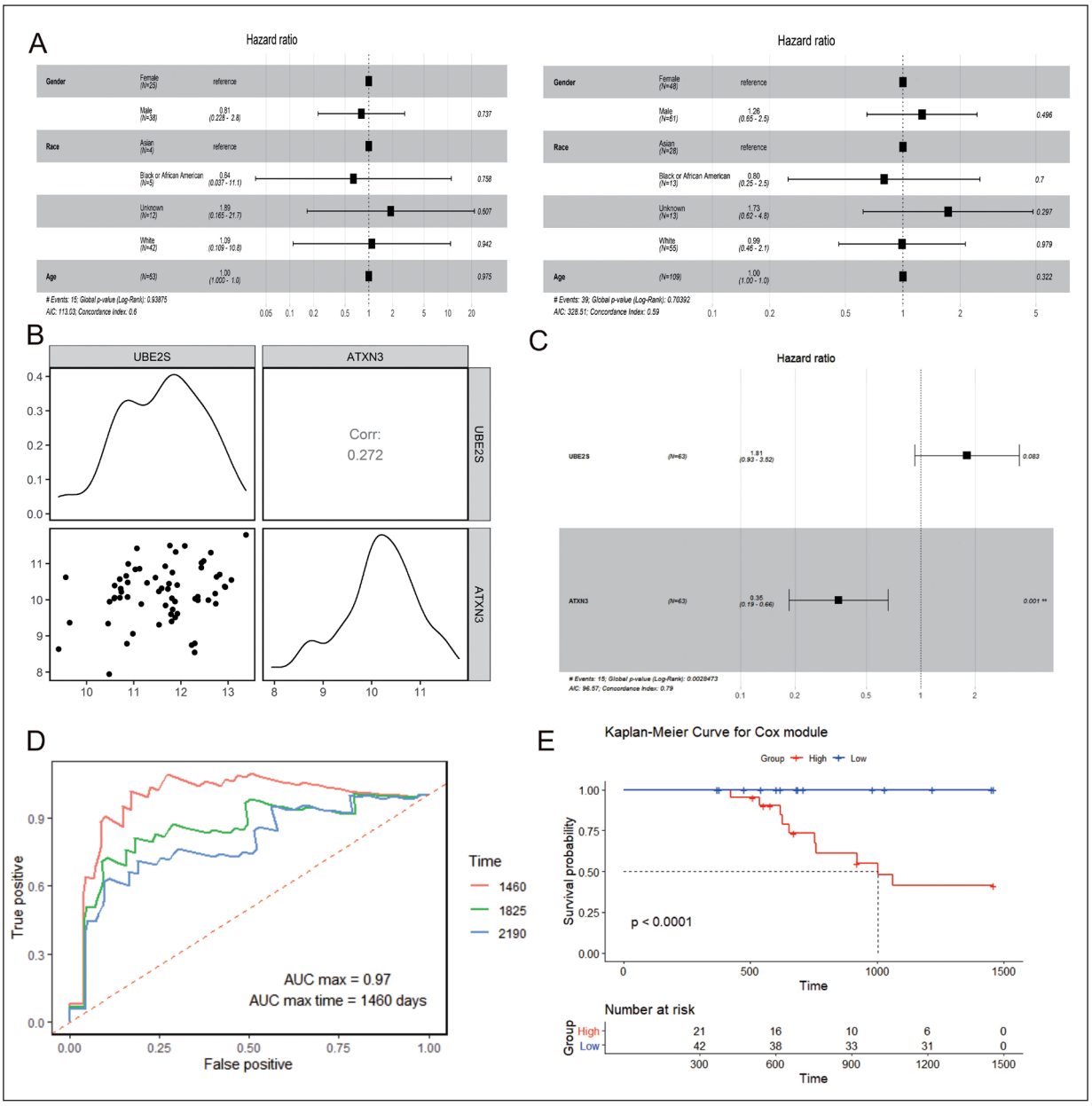

Fig. 4. Sample characteristics and development of the multivariate Cox regression in the TARGET discovery and testing datasets. *A,* Sample characteristics of the multivariate Cox regression. The left is multivariate Cox regression module in discovery dataset and the right is characteristics of multivariate Cox regression module in testing dataset. *B,* Correlation coefficient of ATXN3 and UBE2S (cor=0.272). *C,* Development of DEGs from K-M survival analysis, forest plot of multivariate Cox regression models in primary OS. *D,* ROC curve of multivariate Cox regression module. The AUC at 4-year is 0.97. *E,* Four-year survival analysis of OS between high- and low-risk groups stratified by the module in the discovery dataset.

PRKCA and MAPK1 were seen as core genes by MCODE in Cytoscape. MAPK1 (mitogen-activated protein kinase 1), involving in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development, was found to be significantly targeted by multiple DEMIs. Our results are consistent with previous reports that overexpression of MAPK1 is related to OS[26,27]. We constructed the miRNA-protein network to search for proteins that are most plausible plasma biomarkers related to the occurrence and metastasis of OS. Finally, by integrating plasma proteomics/miRNomics, 36 proteins, including MAPK1, SMAD2, DYRK3 and PASK, not only show significant differences in protein expression, but also are the target genes of multiple DEMIs, which strongly proves their association with OS. Totally 15 proteins, including IGF1R, SLC7A1 and TNIP1, are recognized as hub proteins that show significant correlation with OS metastasis.

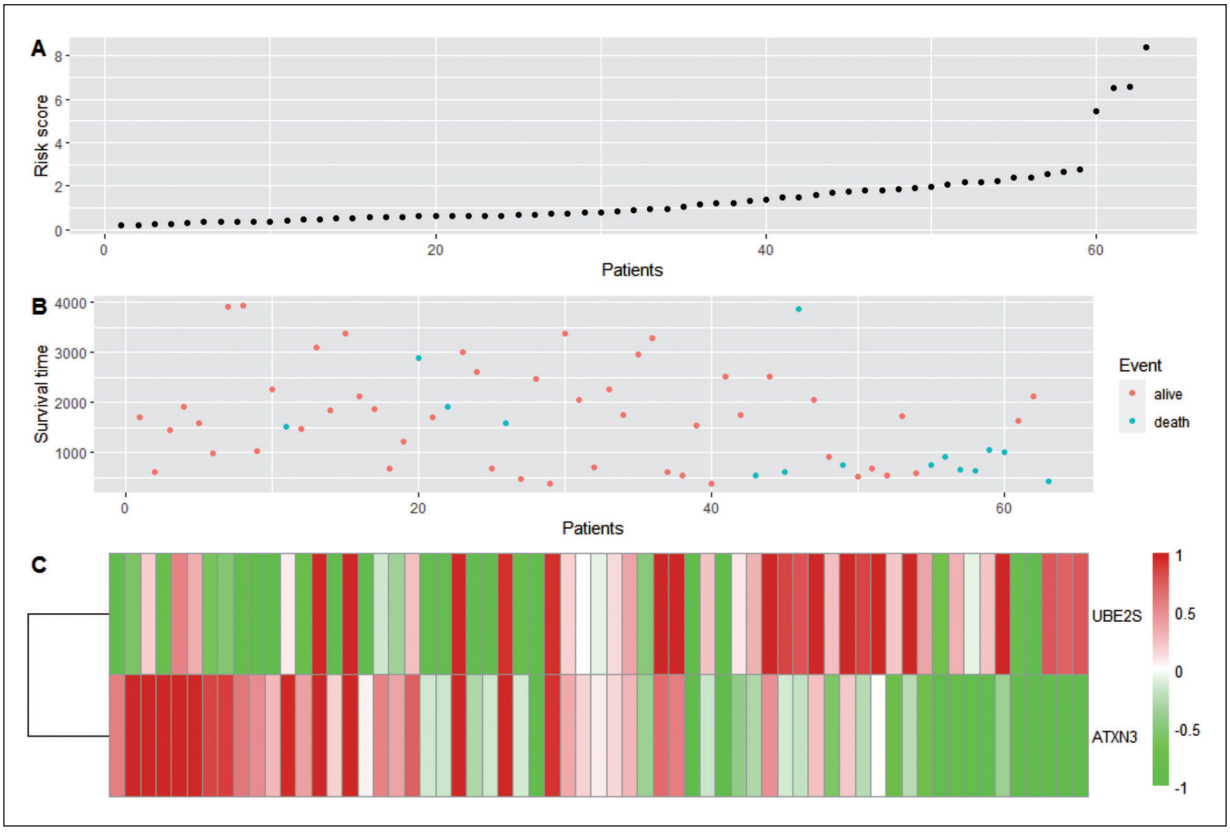

Fig. 5. Two-gene prognostic signature biomarker characteristics in the discovery dataset cohort. *A,* Risk scores distribution in discovery cohort. *B,* Association of clinical characteristics with risk scores with red dots represent alive and blue dots represent death. *C,* Heatmap of the UBE2S and ATXN3 differentially expressed between high- and low-risk, with red indicating higher expression and green indicating lower expression.

As was suggested by pathway enrichment analysis, based on KEGG and Reactome database, genes involved in miRNA-protein network demonstrated that "Foxo signaling pathway", "Relaxin signaling pathway" and "AGE-RAGE signaling pathway in diabetic complications" probably played an important role in OS occurrence. GO functional analysis demonstrated abnormal functions related to Serine/threonine phosphorylation probably affect the development of osteosarcoma. Serine/threonine phosphorylation is involved in dozens of human cancers, including colon cancer[28]. Previous researches are consistent with our results, serine/threonine kinase and its receptor related proteins have also turned out to be involved in the development of OS[28-31]. In order to verify our results, we searched the relevant literature from Pubmed. Between primary OS and normal, the differential expression of MAPK1[26,27], SMAD2[32,33], IRS1[34], SMAD3[35], MAPK14[36], ATF2[37], PDPK1[38], TTK[39], IKBKB[40] and CENPB[41] have been confirmed to be related to OS by previous studies. In addition, overexpression of IGF1R[42], DDIT4[43], CDC42[44] and CDKN1B[45] have also been shown to play important roles in the metastasis of OS . In another group of metastatic OS and primary OS, 3 DEMIs and 254 DEPs are confirmed, containing 1 up-regulated, 2 down-regulated miRNAs and 239 up-regulated, 15 down-regulated proteins. In GO analysis, the DEPs were also substantially enriched in the Serine/threonine phosphorylation, suggesting an important role of these pathways in osteosarcoma. Infection and tumor suppressor gene disorder related pathways were identified as the most significant in KEGG analysis.

In addition, we analyzed the DEGs, involved in miRNA-protein network and expressing the same trend in both tumor tissue and plasma, by lasso regression analysis, Kaplan-Meier one-way survival, COX and time-dependent ROC methods. The multivariate Cox module based on ATXN3 and UBE2S, which was screened by both lasso regression analysis and Kaplan-Meier one-way survival were associated with prognosis and the log-rank of Cox model was also significant (p=0.003). Notably, the AUCs of the time-dependent ROC curves of the module was 0.97 at 4-year overall-survival, and the p. value of the log-rank test at 4-year was lower than 0.0001, showing the high reliability for prognostic evaluation. Ataxin-3 (ATXN3), a protein in the Josephin family of deubiquitinases, is involved in neurological dysfunction-re-

lated diseases such as Machado-Joseph disease[46]. Upregulation of ATXN3 in lung, breast, testicular cancer and cancer stem cell is often associated with poor prognosis[47-50] while behaving as a protective factor in colon cancer[51]. However, the biological function of ATXN3 in osteosarcoma remains unclear to date. In our result, ATXN3 played an important role in the "FOXO-mediated transcription" and "FOXO-mediated transcription of oxidative stress, metabolic and neuronal genes", which involved in the process of numerous tumorigenesis. The loss of FOXO proteins in osteoclast progenitors does increase proliferation, osteoclast formation, and bone resorption that may result in proliferation of osteosarcoma cells[52]. In addition, ATXN3 activates the manganese superoxide dismutase (SOD2) gene by interacting with the forkhead box O (FOXO) transcription factor FOXO4, supporting that ATXN3 plays an important role in regulating the antioxidant stress response via SOD2[53]. Similar to our results, high expression of ubiquitin-conjugating enzyme E2S (UBE2S), a family of E2 protein in the ubaiquitination process, can promote multiple cancers progression and symbolizes a poor prognosis[54-56]. Lys11-linked chains are efficient proteasomal degradation signals, assembled by UBE2S specifically and cleavage by ATXN3[57]. High expression of UBE2S is probably due to the inhibition of ATXN3 by other dysregulation. In all, these results are supposed to explain the disorder of microenvironment in plasma of osteosarcoma patients and provide targets for future diagnosis, treatment, and prognosis prediction. Further investigation for the factors we identified might help improve osteosarcoma diagnosis, treatment, and prognosis assessment in the future.

CONCLUSIONS

Our study identified several reliable plasma markers for prediction of diagnosis and prognosis in primary and metastatic OS patients through bio-informatics analysis. Finally, 11 and 3 proteins, selected from plasma miRNA-protein network and overlap of tumor tissue and plasma, were potential plasma markers for diagnosis and metastatic prediction of OS. Furthermore, C-index and the AUC at 4 year of Cox module were 0. 79 and 0. 97, suggesting independent effect of UBE2S and ATXN3 in primary OS. The finding may potentially enable more precise and personalized cancer treatment in the future.

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Author contributions:
QQ conceived and designed the studies. JL performed the data mining. JL drafted the
manuscript and QQ revised the manuscript and contributed to knowledge.

Data availability:
The original data of the study are from the public databases of GEO and TARGET, and all data can be downloaded and used free of charge. A variety of data analysis methods is freely available on the R platform.

Ethical statement:
Not applicable.

Conflict of Interest:
The study was conducted without any potential conflict of interest.

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