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Research article

Genomic nursing science revealed the prolyl 4-hydroxylase subunit alpha 2 as a significant biomarker involved in osteosarcoma

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ABSTRACT

Backgrounds: This study aims to explore the clinical value of P4HA2 (prolyl 4-hydroxylase subunit alpha 2) in Osteosarcoma (OSC), and assess its potential to provide directions and clues for the practice of precision nursing.

Methods: The GSE73166 and GSE16088 datasets were used to explore the P4HA2 expression in OSC. We then used the clinical data of patients obtaining from TARGET database to assess the prognostic value of P4HA2 in OSC. We also evaluated the predictive value of prognostic model based on P4HA2-related genes. Further, GSEA analysis was performed to explore related pathways.

Results: The P4HA2 mRNA expression was higher in OSC than that in normal tissues and other bone cancer samples. Survival analysis found that P4HA2 high expression caused poor overall survival (OS) of patients with OSC and P4HA2 presented a favorable performance for predicting OS. Specifically, P4HA2 high expression statistically influenced the OS of patients with age≥15 years old and those with or without metastasis. Cox regression analysis indicated the independent prognostic value of P4HA2 in OSC, and nomogram analysis revealed its significant contribution to the survival probability of patients. We further established a prognostic model based on P4HA2-related genes, finding that prognostic model had a good prediction ability on OS. These results supported the clinical significance of P4HA2 in OSC. GSEA analysis suggested that P4HA2 was significantly related to the MAPK signaling pathway. In addition, P4HA2-associated natural killer cell-mediated cytotoxicity and T cell receptor signaling pathway were also predicted.

Conclusions: This study revealed that P4HA2 can serve as an important prognostic biomarker for OSC patients, and it may become a promising therapeutic target in OSC treatment.

1. Introduction

Osteosarcoma (OSC) has become the most frequently occurring malignant tumor of bone, with the characteristics of peak frequency in adolescence [1]. The OSC patients generally presented a poor prognosis and high disability rate, which was mainly caused by the tumor metastasis, especially lung metastasis [2]. The 5-year survival rate for patients with lung metastasis was 70%, but which decreased to 30% for patients without lung metastasis [3]. Regarding the OSC treatment, the surgery and combination chemotherapy

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of adjuvant and neoadjuvant have been applied for the osteosarcoma treatment. But the patients always suffered from any adverse reactions in the process of treatment. Despite the multimodal treatments, the 5-year survival rate of OSC patients was still far from satisfactory [4,5]. As such, it was necessary to reveal new biomarker for predicting the prognosis of OSC from the perspective of genetics.

Prolyl-4-hydroxylase subunit 2 (P4HA2), as one of the subtypes of collagen prolyl-4-hydroxylases α isoforms, is required for the collagen biogenesis. Previous studies have revealed that P4HA2 was associated with the epithelial-to-mesenchymal transition promotion, which contributed to the disease progression such as cervical cancer [6] and hepatocellular carcinoma [7]. It has been reported that P4HA2 expression was a predictor of poor outcome in pancreatic cancer [8], breast cancer [9], and cervical cancer [10]. The carcinogenesis of P4HA2 in human cancers have been widely described. Knockdown of P4HA2 significantly inhibited the glioma proliferation, migration, invasion, and suppressed tumor xenograft growth, which was related to the downregulation of collagen expressions and activation of phosphorylated PI3K/AKT [11]. These studies suggested the P4HA2 as a potential therapeutic target for cancer treatment. In addition, P4HA2 was also confirmed as the HIF target [12] and significantly related to the immune infiltrates such as CD8+T cells and PD-L1 in cervical cancer [13]. At present, the potential value of P4HA2 on the patients with OSC has not been revealed.

This study aims to explore the correlation of P4HA2 with the progression of OSC, and evaluate whether it can provide direction and clues for precision nursing practices for this population. We firstly explored the mRNA expression of P4HA2 in OSC and control groups. Then we analyzed the association of P4HA2 expression with the overall survival time of OSC patients. We also assessed the prognostic value of constructed prognostic model based on P4HA2-related genes in OSC. To reveal the underlying mechanism associated with P4HA2 in OSC, we performed the enrichment analysis to detect the possible pathways. In addition, the upstream miRNAs of the P4HA2 that potentially controlled its mRNA expression were predicted. This study revealed a novel prognostic marker in OSC and provided a new perspective on the molecular mechanism of OSC.

2. Methods

2.1. Expression analysis on P4HA2 in OSC

We first obtained the data associated with OSC from the Gene Expression Omnibus (GEO) database. GEO is a public functional genomics data repository supporting MIAME-compliant data submissions. Tools are provided to help users query and download experiments and curated gene expression profiles. According to screening, the GSE16088 and GSE73166 datasets were finally selected. The GSE16088 dataset, containing 14 human OSC tissues and 6 normal tissues (2 kidney tissue, 2 liver tissue, and 2 lymph node tissue), was used to compare the expression difference of P4HA2 between OSC and normal tissues. The GSE73166 including 10 OSC and 8 Ewing sarcoma (EW) samples was used to compare the expression difference of P4HA2 between OSC and other cancer bones. We also conducted the PCR test in vitro to validate the P4HA2 mRNA expression in OSC and normal Mesenchymal Stem Cell (MSC) cells. The procedure for PCR test was presented in the following section.

In addition, the mRNA and protein expressions of P4HA2 across the cell cycle in human osteosarcoma cells (U-2 OS FUCCI) were explored in the Human Protein Atlas (HPA) database. The subcellular location of P4HA2 protein in OSC cells can be found as well. The HPA is a Swedish-based program initiated in 2003 to map all the human proteins in cells, tissues, and organs using an integration of various omics technologies, including antibody-based imaging, mass spectrometry-based proteomics, transcriptomics, and systems biology. All the data in the knowledge resource is open access to allow scientists both in academia and industry to freely access the data for exploration of the human proteome. The HPA program has already contributed to several thousands of publications in the field of human biology and disease and it is selected by the organization ELIXIR (www.elixir-europe.org) as a European core resource due to its fundamental importance for a wider life science community.

2.2. Prognosis analysis on P4HA2 in patients with OSC

The GSE39055 dataset (N = 36) obtained from the GEO database was firstly used to explore the prognostic value of P4HA2 in OSC. All patients were divided into high and low expression groups according to the cutoff value of P4HA2 determined by the "survminer" R package. Then the Kaplan-Meier analysis was conducted to explore the association of P4HA2 expression with overall survival (OS), and log-rank test was used to compare the survival difference between 2 groups. The influence of P4HA2 expression on the OS was also validated using the data of 85 patients with OSC which was obtained from TARGET database. The main outcome of OS in this study is defined as the time elapsed from study entry to death from any cause [14].

This study further performed a subgroup analysis to explore the association of P4HA2 expression with the OS using TARGET-OSC data. Related subgroups were identified according to sex, age, metastasis status, and specific tumor site. We also conducted the univariate Cox regression analyses to evaluate the association of these variables with OS, and then multivariate Cox regression analysis was conducted to identify the independent factors of OSC. Finally, a nomogram model was established based on the key factors with $P < 0.05$ in multivariate Cox regression analysis. To evaluate the prediction performance of nomogram model on the OS, receiver operating characteristic (ROC) analysis was conducted and *C*-index value was calculated. In addition, the Decision Curve Analysis (DCA) was conducted to assess the clinical net benefit of nomogram model for predicting the OS.

2.3. The expression difference of P4HA2 under different clinical characteristics of OSC patients

Further, we explored the expression difference of P4HA2 among OSC patients based on the different clinical characteristics using the TARGET-OSC dataset. The expression of P4HA2 in different gender, race, metastasis status, metastasis site, and specific tumor region was compared. In addition, we also explored the correlation between P4HA2 and age by Spearman method. The clinical characteristics of patients with OSC are presented in Table .1.

2.4. The mRNA expression validation of P4HA2 in vitro by PCR test

Besides the bioinformatics analysis, this study also verified the mRNA expression difference of P4HA2 in vitro by PCR between human osteosarcoma cells (HOS) and control cells (MSC) which were purchased from the American Type Culture Collection. All cell lines were maintained in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin, and cultured at 37 °C with 5% CO_2.

Then, the total RNA was extracted from cell lines using TRIZOL reagent and then reverse transcribed to cDNA using PrimeScript Reverse Transcriptase following the manufacturer's recommendations. The qPCR was performed with Universal SYBR Green Master Mix using the following PCR conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s, followed by a final elongation step of 10 min at 72 °C. P4HA2 expression was normalized to GAPDH expression calculated using the 2-ΔΔCt methods. The primer sequences were as follows: P4HA2, forward, 5′-GCCAAAGCCCTGATGAGACT-3′, reverse, 5′-GCTCCATC-CACAACACCGTA-3'; GAPDH, forward, 5′-TCATCATCTCTGCCCCCTCT-3′, reverse, 5′-GTGATGGCATGGACTGTGGT-3'.

2.5. The gene set enrichment analysis (GSEA) on P4HA2 in OSC

The significant KEGG pathway associated with P4HA2 in OSC was also analyzed through GSEA. The GSEA analysis can reveal the potential positive and negative pathways associated with P4HA2. It also verified the importance of related pathways originating from the above KEGG analysis on DEGs. The GSEA analysis was conducive to disclosing the possible mechanism involved in OSC.

2.6. The potential therapeutic agent of OSC targeting P4HA2

The potential therapeutic agent for the treatment of OSC were explored through the human disease database MalaCards. To verify whether these agents can target P4HA2, we conducted a molecular docking analysis to explore the binding ability between these agents and P4HA2. The crystal structure of the P4HA2 protein was obtained from the PDB database (https://www.rcsb.org/). The three-dimensional structure of agents was obtained from the ZINC database. AutoDock (v4.2.6) software molecularly docks P4HA2 with agents and the binding activity was analyzed.

2.7. Establishment of a prognostic-related risk score

The importance of P4HA2 on the OS of patients with OSC has been explored. However, more than just one gene participates in the process of disease development. But in most cases, multiple genes interact with each other. Therefore, this study further identified other key biomarkers correlated with P4HA2, and established a prognostic model based on P4HA2-related genes using the TARGET data.

1) First, we assigned all patients into 2 groups according to the median of P4HA2 expression level. The limma R package was used to identify the differential expressed genes (DEGs) between the 2 groups. DEGs with absolute LogFC>1.5 and *P*-value<0.05 were regarded as the key P4HA2-related genes. 2) Because these key DEGs were all significantly related to P4HA2, therefore, collinearity among them needs to be resolved. To preliminarily select crucial prognostic genes from these key DEGs and remove the collinearity,

Table 1
The clinical characteristics of OSC patients.

Characteristics	Group	Counts
Age	Days (Median, Min-Max)	5234 (1299, 11828)
Gender	Male (N)	47
	Female (N)	38
Race	Asian	6
	White	52
	Black	7
Metastasis status	Yes (N)	21
	No (N)	64
Metastasis site	Bone (N)	1
	Lung (N)	5
	Bone + Lung (N)	16
Specific tumor region	Distal (N)	28
	Proximal (N)	23

Note: The clinical data of OSC patients were obtained from the TARGET database.

the least absolute shrinkage and selection operator (LASSO) analysis was then conducted. Through LASSO analysis, the redundant variables can be removed by increasing the penalty factor (λ). In this study, five-fold cross-validation for LASSO analysis was used to determine the optimal λ and core genes. 3) In addition, multivariate Cox regression analysis was conducted to acquire the regression coefficient for each gene and core genes with $P < 0.05$ were selected to establish prognostic prediction model. According to the regression coefficients (β1, β2, β3 …) and gene expression levels (X1, X2, X3 …) of core genes, the risk score of every individual was calculated as following, risk score = β1*X1+β2*X2+ + … βp*Xp [15].

Subsequently, all patients were divided into high- and low-risk groups according to the median risk score. Survival curves were constructed using the Kaplan-Meier methods and log-rank test was conducted to compare the overall survival between the 2 risk groups. ROC analysis was used to evaluate the clinical value of risk score for predicting the 1, 3, 5-year survival and *C*-index value was calculated. For exploring the independent prognostic value of risk score regarding OS, the risk score, gender, race, specific tumor region, metastasis status, and age were enrolled to multivariate Cox regression analysis. The independent factors with $P < 0.05$ were then chosen to construct a nomogram model for predicting the probability of overall survival. The nomogram was generated using the rms package in R software.

2.8. The upstream miRNAs prediction of P4HA2

Due to the negative regulation of miRNA on mRNA in a sequence-specific manner at the posttranscriptional level, hence, we further explored the potential upstream miRNAs of P4HA2 that possibly controlled its mRNA expression. The DIANA, miRDB, miRWalk, and Target Scan databases were used to predict the consistent upstream miRNAs. The potential pathway associated with the upstream miRNAs was explored through the DIANA database. DIANA provides hundreds of thousands of high quality manually curated experimentally validated miRNA: gene interactions, enhanced with detailed meta-data. The miRDB is an online database for miRNA target prediction and functional annotations. All the targets in miRDB were predicted by a bioinformatics tool, MirTarget, which was developed by analyzing thousands of miRNA-target interactions from high-throughput sequencing experiments. The miRWalk is a freely accessible, regularly updated comprehensive archive supplying the largest available collection of predicted and experimentally verified miRNA-target interactions. Target Scan predicts biological targets of miRNAs by searching for the presence of conserved 8mer, 7mer, and 6mer sites that match the seed region of each miRNA.

2.9. Statistical analysis

The data in this study were analyzed with SPSS 23.0. Statistical differences between groups were evaluated using the *t*-test or analysis of variance (ANOVA). Survival analysis was conducted by Kaplan-Meier method and survival difference between groups was compared with log-rank test. Prognostic performance was analyzed with ROC analysis, Cox regression, and nomogram analysis. Correlation between 2 variables was detected with the Spearman method. $P < 0.05$ was regarded as the statistical significance.

3. Results

3.1. The expression of P4HA2 in OSC

We first explored the mRNA expression level of P4HA2 in OSC. It was found that the expression of P4HA2 mRNA was higher in OSC tissues compared with that in normal tissues (GSE16088) (Fig. 1A). The expression of P4HA2 was also higher in the OSC group than that in other bone cancer (GSE73166) (Fig. 1B). We then validated the mRNA expression of P4HA2 in HOS and MSC cells using PCR (Fig. 1C).

We also evaluated the mRNA expression of P4HA2 in the whole cell cycle of OSC cells (U2-OS FUCCI). As observed in Fig. 2A, the mRNA expression of P4HA2 was increasing with the time of the cell cycle, however, the difference showed no statistical significance during the whole cell cycle. No significant difference in P4HA2 protein expression was observed during the whole cell cycle (Fig. 2B). In addition, we explored the subcellular location of P4HA2 protein and the result indicated that P4HA2 was mainly localized to the endoplasmic reticulum and vesicles in U2-OS cells (Fig. 2C).

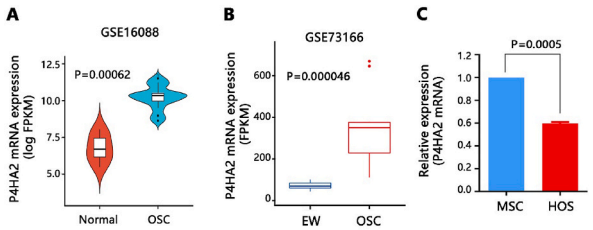

Fig. 1. The mRNA expression analysis. (A) The mRNA expression of P4HA2 in human osteosarcoma tissues and normal tissues was validated using GSE16088 dataset (from GEO database). Normal (B) The mRNA expression of P4HA2 in OSC and other bone cancer samples in GSE73166 dataset (from GEO database). (C) The mRNA expression of P4HA2 in OSC cell (HOS) and control cell (MSC) was validated by PCR.

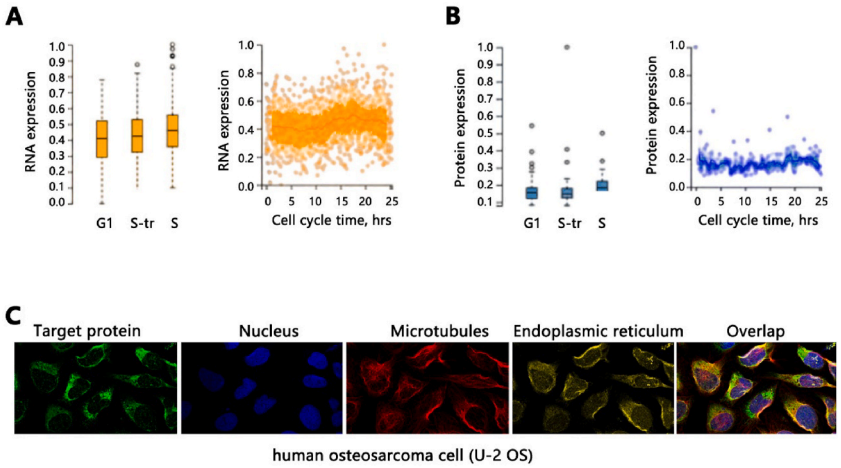

Fig. 2. The expression and subcellular location of P4HA2 in OSC cell (U2-OS). (A) The mRNA expression of P4HA2 across cell cycle. (B) Protein expression of P4HA2 across cell cycle. (C) The location of P4HA2 protein in OSC cell.

3.2. The prognostic value of P4HA2 in OSC

This study further explored the prognostic impact of P4HA2 expression on patients with OSC using the GSE39055 dataset (N = 36), finding that P4HA2 high expression was related to a shorter OS (Fig. 3A). We also validated the association of P4HA2 expression with OS of patients with OSC using the data from TARGET database (N = 85), and similar unfavorable effect of P4HA2 on OS was found (Fig. 3B).

In addition, we performed a subgroup analysis on the survival of patients based on patients' age, gender, metastasis status, and specific tumor region. The clinical characteristics of patients with OSC was shown in Table .1. The results showed that the expression of P4HA2 did not influence the OS of female, male patients (Fig. 4A), and those with age<15 years old (Fig. 4B). However, high expression of P4HA2 was significantly unfavorable to the prognosis of patients with age≥15 years old. In addition, the prognostic influence of P4HA2 was not related to different specific tumor regions (Fig. 5A) and metastasis status (Fig. 5B).

We also performed Cox regression analysis to detect the prognostic factors of OSC. We enrolled the patient's age, gender, race, metastasis status, specific tumor region, and P4HA2 expression level into the Cox regression. The univariate Cox analysis indicated that P4HA2 expression, age, and metastasis status were significant influencing factors of patient's OS (Table .2). Multivariate analysis further showed that P4HA2 expression and age were independent prognostic factors. We then enrolled the 2 independent factors (P4HA2 expression and age) into the nomogram analysis to reveal their importance in the survival probability. The analysis (Fig. 6A) indicated that P4HA2 expression presented a larger contribution to the survival probability of patients compared with age, and P4HA2 expression contributed 100 scores. ROC analysis indicated that P4HA2 and nomogram model had similar AUC (Delong test P = 0.661) and *C*-index values (Fig. 6B). DCA analysis also showed the similar clinical net benefit between P4HA2 and nomogram model (Fig. 6C). These results all suggested the importance of P4HA2 in the overall survival of patients and it may be an important biomarker in OSC.

3.3. The expression difference of P4HA2 under different clinical characteristics

We then explored the expression difference of P4HA2 under different clinical characteristics using the TARGET-OSC dataset. The analysis (Fig. 7A-7B) indicated that the patient's gender, age, race, metastasis status, metastasis site, and specific tumor region did not influence the expression of P4HA2.

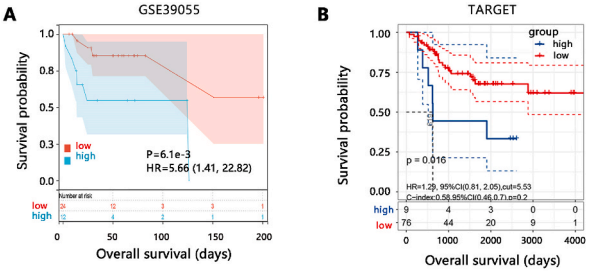

Fig. 3. The influence of P4HA2 on the overall survival of patients with OSC using (A) GSE39055 dataset (N = 36) and (B) TARGET-OSC dataset (N = 85), respectively. The GSE39055 dataset was obtained from GEO database.

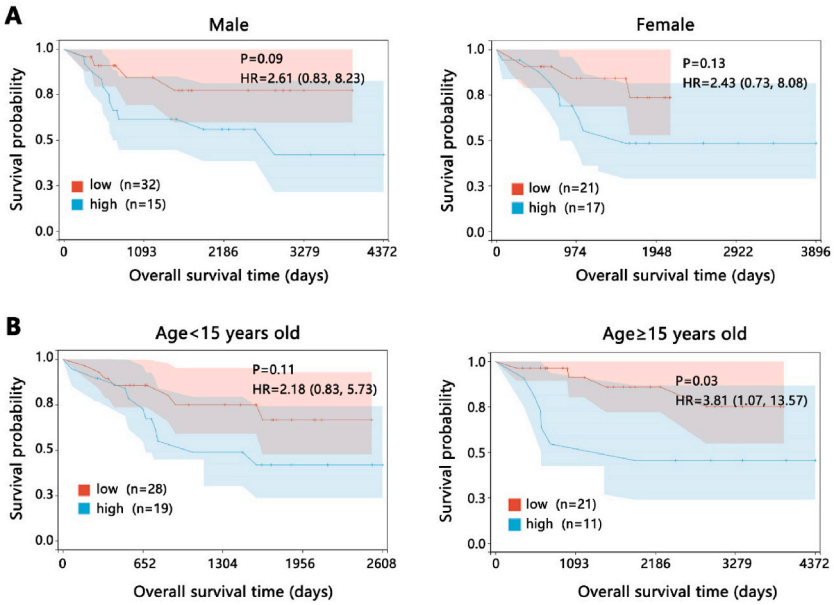

Fig. 4. Prognostic analysis of P4HA2 in patients with osteosarcoma grouped by (A) gender and (B) age. The survival data of osteosarcoma patients were obtained from TARGET database.

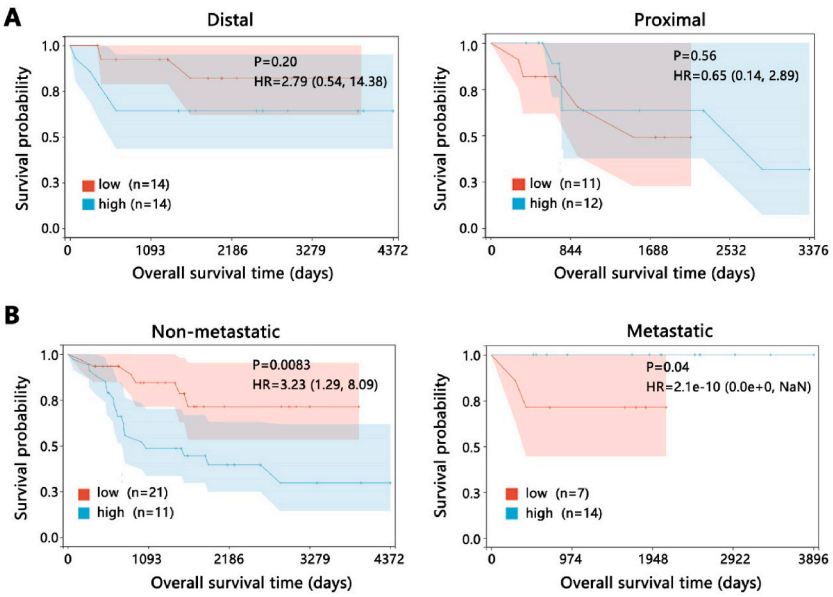

Fig. 5. Prognostic analysis of P4HA2 in patients with osteosarcoma grouped by (A) specific tumor region and (B) metastasis status. The survival data of osteosarcoma patients were obtained from TARGET database.

3.4. The pathway analysis on P4HA2 in OSC

Due to the important clinical role of P4HA2 in OSC, we further explored the significant pathways associated with P4HA2 involved in OSC progression through GSEA analysis (Fig. 8A-8C). A total of 84 positive and 18 negative pathways associated with P4HA2 were observed. The GSEA analysis indicated that the MAPK pathway was positively related to P4HA2 expression, indicating that P4HA2 high expression might activate the MAPK signaling pathway, thus affecting the prognosis of patients. Natural killer (NK) cell-mediated cytotoxicity and T cell receptor signaling pathway were also predicted.

Table 2
Univariate and multivariate analysis of overall survival in patients with osteosarcoma.

	Univariate		Multivariate	
	HR (95% CI)	P	HR (95% CI)	P
P4HA2 expression	1.287 (1.023–2.047)	0.013	1.751 (1.026–2.990)	0.040
Age	0.881 (0.804–0.966)	<0.001	0.841 (0.742–0.955)	0.007
Gender	1.008 (0.470–2.161)	0.984	0.838 (0.349–2.011)	0.692
Race	1.119 (0.462–2.721)	0.803	1.013 (0.385–2.667)	0.979
Metastasis status	0.200 (0.047–0.848)	0.029	0.313 (0.071–1.376)	0.124
Metastasis site	0.489 (0.046–5.183)	0.552	0.707 (0.024–20.727)	0.841
Specific tumor region	1.767 (0.632–4.942)	0.278	5.935 (0.530–66.406)	0.148

Note: The clinical data of OSC patients were obtained from the TARGET database.

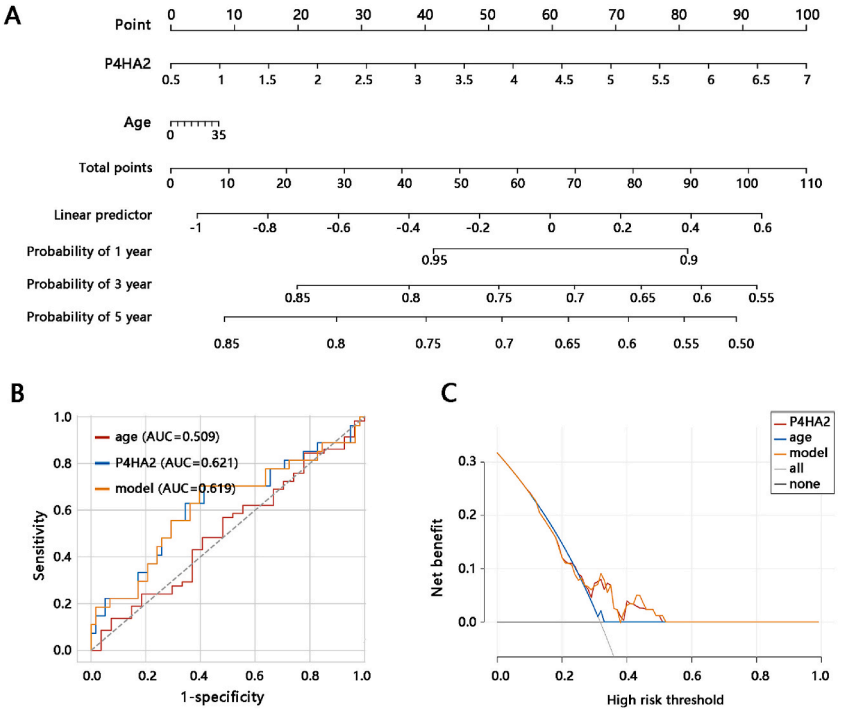

Fig. 6. Clinical value evaluation of nomogram model. (A) Nomogram model was used to predict the 1-, 3-, and 5-year overall survival in TARGET-osteosarcoma dataset. The independent prognostic factors including P4HA2 and age were enrolled into the Nomogram analysis. (B) ROC analysis was conducted to assess the prediction performance on OS status. Delong test: P (model vs age) = 0.086, Delong test: P (model vs P4HA2) = 0.661. C index for P4HA2 = 0.621, C index for age = 0.491, C index for model (P4HA2+age) = 0.618. (C) DCA analysis was conducted to assess the clinical net benefit for predicting the survival status.

3.5. The potential therapeutic agent of OSC targeting P4HA2

Above results have demonstrated that P4HA2 was a potential target of OSC. Therefore, exploring the targeting agent for the treatment of OSC had importance clinical value. Through the human disease database, the main 5 therapeutic agents were found for the treatment of OSC, including Zoledronic acid, Lithium carbonate, Acetylsalicylic acid, Heparin, bovine, and Reviparin. These agents have been approved to enter the phase 4 clinical trial. We further conducted the molecular docking analysis to reveal the potential binding ability between P4HA2 and these agents, finding that only Lithium carbonate successfully bound to the P4HA2 (Fig. 9A-9B). This result suggested that Lithium carbonate may be a useful therapeutic agent for OSC by targeting P4HA2.

3.6. The prognostic value of P4HA2-related prognostic model

The above results have revealed the importance of P4HA2 expression level on the OS of OSC patients, we further explored other important biomarkers significantly correlated with P4HA2. All the TARGET-OSC patients were divided into 2 groups according to the median value of P4HA2 expression, then we identified the differential expressed genes (DEGs) between the 2 groups. A total of 1001 DEGs were detected and 116 DGEs with absolute Log2(FC) > 1.5 were enrolled into the further LASSO analysis. The LASSO analysis

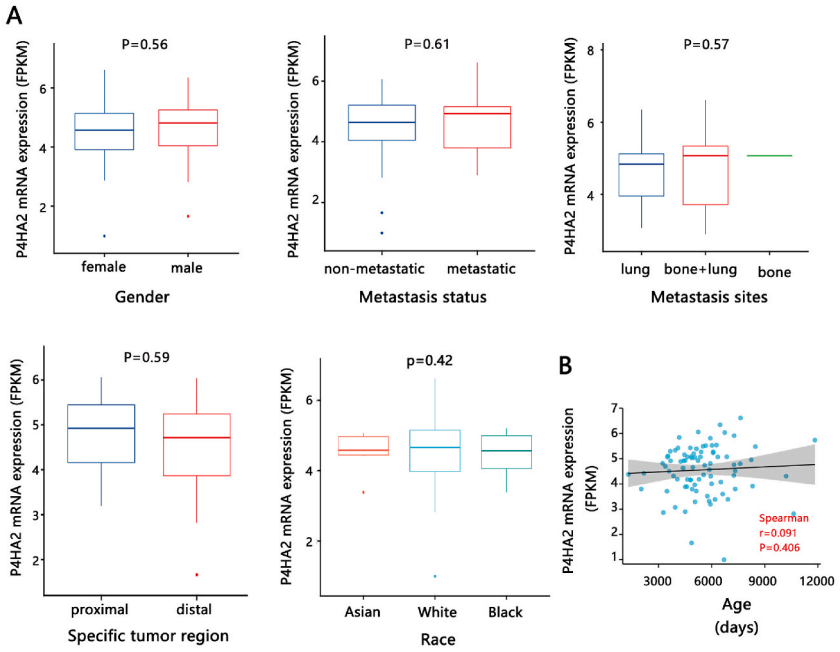

Fig. 7. The expression difference of P4HA2 under different clinical characteristics. The clinical data of OSC patients was obtained from TARGET database. (A) The P4HA2 expression difference between different groups. (B) The correlation between age and P4HA2 expression level by Spearman method.

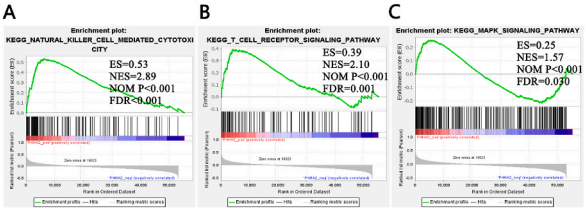

Fig. 8. Enriched KEGG pathways positively correlated with P4HA2 expression in OSC. GSEA was performed using the hallmark gene sets from MSigDB. (A) Natural killer (NK) cell-mediated cytotoxicity; (B) T cell receptor signaling pathway. (C) MAPK signaling pathway.

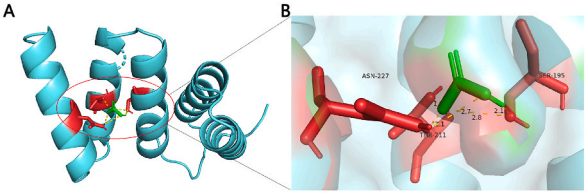

Fig. 9. The binding analysis between Lithium carbonate and P4HA2 by molecular docking analysis. (A) Overall view. (B) Local enlarged view.

finally generated 4 key markers (AP000851.2, KIF25, LINC01549, RHBDL2) that significantly correlated with the patient's overall survival by setting the $\lambda = 0.12$ (Fig. 10A-10C).

We further conducted Cox regression analysis on the 4 key markers. According to their regression coefficients and gene expression levels, the risk score of every patient was calculated using the above formula:

$$\text{Risk score} = 0.010*\text{AP000851.2} + 0.095*\text{KIF25} + 0.010*\text{LINC01549} + 0.017*\text{RHBDL2}.$$

Then all patients were divided into low and high risk-score groups according to the median risk score. Kaplan-Meier analysis showed that patients in the high-risk group had lower overall survival time (Fig. 11A). Time-independent ROC analysis showed that the 4-genes risk score had a favorable prediction performance for 1, 3, and 5-year survival of patients with OSC (Fig. 11B). The *C*-index value of risk score was 0.730.

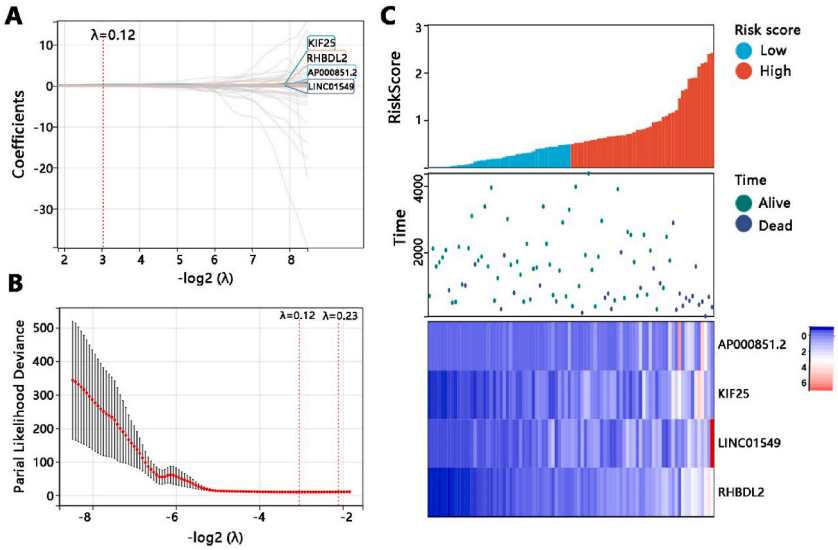

Fig. 10. Construction of LASSO regression model. (A) LASSO regression model was developed from 116 P4HA2-related genes and 4 key genes were determined. (B) Calculating the tuning parameter (λ) based on the partial likelihood deviance with Fivefold cross-validation. The optimal log λ value is indicated by the vertical red line in the plot. (C) Survival and expression details. Each dot represents a patient, and the heatmap represents the 4 genes expression levels. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

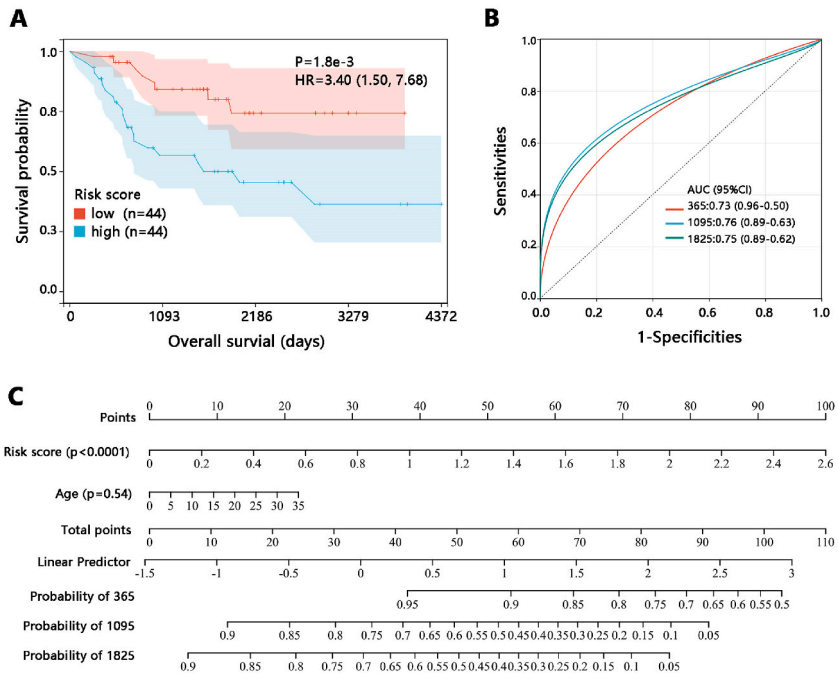

Fig. 11. Clinical value assessment on the prognostic model based on P4HA2-related genes. (A) Kaplan–Meier analysis of the overall survival in the high- and low-risk groups of the TARGET-osteosarcoma cohort. (B) Time-dependent ROC analysis of the risk score for the overall survival and survival status in the TARGET-osteosarcoma cohort. (C) Nomogram of prognostic model developed to predict the 1-, 3-, and 5-year overall survival in the TARGET-osteosarcoma cohort.

We then performed a Cox regression analysis to investigate whether the risk score was an independent predictor of overall survival in patients with OSC. The Cox analysis including risk score, age, gender, race, metastasis status, and specific tumor region, indicated that the risk score was still an independent prognostic factor (Table .3). Further, the independent risk factors including 4-genes risk score and age were used to develop a nomogram model for predicting the OSC prognosis (Fig. 11C). The *C*-index of the nomogram model was 0.745, which was higher than that of single 4-genes risk score.

Table 3
Multivariate analysis of overall survival in patients with osteosarcoma.

Variables	B	Wald	P	HR (95% CI)
Risk score	2.116	12.547	0.000	8.296 (2.573–26.749)
Gender	0.351	0.216	0.642	1.420 (0.323–6.234)
Race	0.848	1.227	0.268	2.334 (0.521–10.462)
Specific tumor region	−1.167	2.720	0.099	0.311 (0.078–1.246)
Metastasis status	0.180	0.037	0.848	1.197 (0.191–7.518)
Age	0.120	4.372	0.037	1.127 (1.008–1.261)

Note: The clinical data of OSC patients were obtained from the TARGET database.

3.7. The upstream miRNAs analysis of P4HA2

Due to the significance of abnormal P4HA2 mRNA expression on OS in OSC, we explored the potential upstream markers involved in the abnormal expression of P4HA2 mRNA. MicroRNAs are highly conserved and abundant small non-coding RNAs inhibiting gene expression by inducing target mRNA degradation and/or the inhibition of translation. Hence, exploring the upstream miRNAs of P4HA2 that potentially regulated its expression was extremely important. We initially predicted 38, 57, 19809, and 1124 miRNAs in DIANA, miRDB, miRWalk, and Target Scan databases, respectively. A total of 11 consistent miRNAs among 4 databases were screened out, namely hsa-let-7b-5p, hsa-miR-30a-5p, hsa-let-7a-5p, hsa-let-7i-5p, hsa-let-7f-5p, hsa-let-7d-5p, hsa-miR-520d-5p, hsa-miR-9-5p, hsa-miR-98-5p, hsa-let-7e-5p, and hsa-let-7c-5p.

In addition, the DIANA tool was used to explore the significant KEGG pathways associated with 11 upstream miRNAs of P4HA2. KEGG pathway analysis (Table .4) indicated that these miRNAs were mainly involved in the TGF-beta signaling pathway, Adherens junction, and MAPK signaling pathway. According to the above pathway analysis, the common MAPK pathway seemed to be the most important term. The significance of the MAPK signaling pathway associated with P4HA2 in OSC needed to be determined further. However, we found no correlation between upstream miRNAs and immune-related pathways.

4. Discussion

Precision nursing is an emerging method, which takes the personal genome, environment, and life style into consideration, to prevent and treat the disease. The objective of precision nursing is to reveal the risk biomarkers of disease through analyzing the genomic profiles, and provide the appropriate treatment for the special populations at the right time. According to these important biomarkers, nursing personnel can apply genetic and genomic knowledge to provide risk assessment, risk management, treatment selection, and treatment decision-making for individuals, families, communities, and populations throughout the entire lifecycle [16]. By analyzing genes and genomic profiles, healthcare personnel could perform deep phenotyping to provide accurate nursing practices for appropriate patients at the right time.

In this study, we revealed a significant biomarker associated with OSC progression and confirmed its significant clinical role. At present, emerging studies have documented that P4HA2 was a prognostic biomarker candidate for several human cancers. In hepatocellular carcinoma (HCC), P4HA2 was determined as a partial EMT-related gene and its overexpression was significantly associated with poor prognosis of HCC patients [7]. P4HA2 was also markedly up-regulated in cervical cancer and up-regulation of P4HA2 correlated with the shorter overall survival and relapse-free survival (RFS), and knockdown of P4HA2 suppressed the EMT process [6]. In this study, we confirmed that high expression of P4HA2 independently predicted poor prognosis of patients with OSC. We also established a prognostic model based on P4HA2 using the LASSO Cox regression, a broadly selected machine learning algorithm used to minimize the risk of overfitting. The prognostic model presented a good predictive value in OSC. These researches revealed the importance of P4HA2 in OSC and implied its potential as a promising therapeutic target in human cancers.

It has been reported that P4HA2 was involved in the synthesis and degradation of collagen in the tumor microenvironment. A previous study showed that low expression of P4HA2 correlated with poor survival in patients with pancreatic cancer, and indicated the involvement of collagen deposition in the restraint of tumor [8]. Another study revealed the high expression and negative prognosis influence of P4HA2 in breast cancer, and silencing P4HA2 expression inhibited the tumor growth and metastasis, accompanied by reduced deposition of collagen I and IV [17]. These researches indicated the significance of P4HA2 in the EMT process and collagen deposition. However, the potential function of P4HA2 in collagen deposition of OSC was not elucidated.

This study further explored the potential pathway associated with P4HA2 in OSC. The enrichment analysis revealed the

Table 4
The KEGG pathway analysis on 11 microRNAs.

Pathway ID	Pathway name	*P*-value
hsa04350	TGF-beta signaling pathway	6.54e-06
hsa05205	Proteoglycans in cancer	1.93e-05
hsa04520	Adherens junction	4.67e-05
hsa04014	Ras signaling pathway	1.88e-03
hsa04010	MAPK signaling pathway	2.12e-03

involvement of the MAPK signaling pathway. The survival analysis indicated that P4HA2 high expression caused a poor prognosis in patients with osteosarcoma, which might be related to the activation of the MAPK signaling pathway. No study reported the function of P4HA2 through the MAPK signaling pathway in OSC. However, the role of MAPK in osteosarcoma has been widely investigated. Previous studies have suggested that the MAPK pathway was significantly associated with apoptosis [18,19], phosphorylation [20], and the angiogenesis process [21] in osteosarcoma. Whether the P4HA2 can affect the apoptosis, phosphorylation of MAPK pathway, and angiogenesis process in osteosarcoma by MAPK pathway, needed further investigations. Additionally, several studies reported the important role of the MAPK pathway in Ewing sarcoma. The activation of the MAPK signaling pathway was associated with the cytotoxicity enhancement [22] of the anti-tumor drug, cell death [23,24] induction, and migratory ability reduction of Ewing's sarcoma cell [25].

Moreover, natural killer (NK) cell-mediated cytotoxicity positively associated with P4HA2 was also predicted. P4HA2 has been identified as a hypoxia-immune-related prognostic biomarker [13]. It can be activated by HIF-1 to induce extracellular matrix remodeling under hypoxic conditions and promote cancer metastasis [17]. Our results may suggest the metastasis risk of patients with P4HA2 high expression. Especially, the immune escape is one of the main reasons for cancer metastasis. In our pathway analysis, P4HA2 high expression was positively related to the NK cell-mediated cytotoxicity. However, the NK cells are efficient in controlling the spreading of cancer metastasis [26]. We speculated that P4HA2 high expression caused the potential metastasis risk of cancer cells, which might be sensed by NK cells and further stimulated NK cells to respond to control the immune escape. In addition, a previous study has found that Ewing's sarcoma cell was exquisitely sensitive to expanded NK cells [27], and the irradiation significantly enhanced the NK cell killing on the tumor [28]. Therefore, high expression of P4HA2 may promote the sensitivity of OSC cells to the NK cells, and enhance the anti-tumor activity of NK cells. The immune-related pathway associated with P4HA2 in OSC needs to be further validated.

Due to the significant role of P4HA2 in OSC, we explored the regulatory factors that potentially regulated the mRNA expression of P4HA2. MicroRNAs can bind to the 3′ untranslated region (3′-UTR) of mRNA targets to control gene transcription and translation [29]. The microRNAs are highly conserved and abundant small non-coding RNAs that negatively regulate gene expression in a sequence-specific manner [30]. In this study, 5 miRNAs that may regulate the mRNA expression of P4HA2 were predicted and presented prognostic potential (data not shown), and KEGG analysis indicated that they were also enriched in the MAPK signaling pathway. Presently, few studies reported the regulation between these miRNAs and the MAPK pathway. Dai et al. found that hsa-let-7i-5p can be downregulated by the downregulation of APE1 in OSC cells (HOS), and hsa-let-7i-5p might participate in the MAPK signaling pathway and the development of OSC by interaction with APE1 [31]. Xue et al. found that higher expression of hsa-let-7d-5p was independently associated with a poor prognosis of esophageal adenocarcinoma, and the gene functional enrichment analysis revealed that it participated in the MAPK signaling pathway [32]. Ying et al. showed that has-miR-9-5p could bind to the BRAF mRNA 3′UTR and inhibit the transcription and translation of BRAF, thereby suppressing the proliferation, migration, and invasion of choroidal melanoma cell lines [33]. While BRAF is a serine/threonine kinase involved in the MAPK pathway. It followed that has-miR-9-5p can interact with BRAF kinase in the MAPK pathway and then influence the cancer progression. Yi et al. found that overexpression of has-miR-9-5p suppressed the MAPK pathway by inhibiting CXCR4, thereby reducing the high glucose-induced injury [34]. The hsa-miR-30a-5p has been proved to significantly correlate with inflammation. Fu et al. showed that overexpression of hsa-miR-30a-5p significantly suppressed inflammatory responses, which was related to the inhibition of the MAPK signaling pathway [35]. Choi et al. indicated that hsa-miR-30a-5p could exert greater anti-inflammatory effects by inhibiting MAPK signaling pathway and then reduce neuronal apoptosis [36]. However, no research revealed the regulatory relationship between the MAPK pathway and hsa-miR-520d-5p. Previous studies have revealed the vital role of these miRNAs in the development of disease, and the detailed regulation in OSC needed further exploration.

Above all, the present study screened a significant biomarker (P4HA2) associated with OSC progression. We also revealed the importance of the MAPK signaling pathway involved in OSC. The potential mechanisms such as apoptosis, angiogenesis, and cytotoxicity associated with P4HA2 and MAPK pathway needed further investigation. In addition, the prognostic value of P4HA2 in patients with OSC should be validated in a clinical cohort with a larger sample size. Some potential information might not be excavated until now, and the future studies needs to mine more potential information in clinical data to provide more clues and basis for precise nursing. In practice, we can combine the general data, clinical symptoms, biochemistry test, molecular biomarker, and psychological states, to develop individualized nursing, diagnosis, and treatment programs.

5. Conclusions

This study identified the P4HA2 as an independent prognostic biomarker associated with OSC. We found that P4HA2 was abnormally expressed in OSC and its high expression independently predicted poor prognosis of patients. These results suggested the clinical importance of P4HA2 in the OSC progression. By performing the enrichment analysis, we found that P4HA2 was significantly enriched in the MAPK signaling pathway. In addition, we detected 11 upstream miRNAs that potentially controlled the P4HA2 mRNA expression. Our results revealed a novel biomarker for the OSC treatment and provided a new perspective on the molecular mechanism of OSC.

Data availability

Data will be made available on request.

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Ethical approval and consent to participate

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Hua-ping Chen: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Xiao Han:** Writing – original draft, Methodology, Investigation. **Hui-ping Sun:** Writing – original draft, Validation, Data curation. **Tao Xie:** Writing – original draft, Formal analysis. **Xiao-liang Fan:** Writing – review & editing, Writing – original draft, Supervision, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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