


Prolyl-4-Hydroxylase α Subunit 2 as a Novel Potential Biomarker for Predicting the Prognosis of Epithelial Ovarian Carcinoma

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Objective: Epithelial ovarian cancer (EOC) is one of the leading causes of death worldwide. The aim of this study was to explore the prognostic significance of prolyl-4-hydroxylase α subunit 2 (P4HA2) in patients with EOC.

Patients and Methods: A total of 217 clinical samples (EOC tissues, 167 cases; normal ovarian, 50 cases) were collected and pathologically confirmed using hematoxylin and eosin (H&E) staining. P4HA2 expression in clinical samples was stained by immunohistochemistry (IHC). Relationship between P4HA2 expression and clinicopathological characteristics of EOC patients were analyzed using chi-square test. The differential expression of targets was analyzed in Oncomine database. The prognostic value of P4HA2 was investigated in clinical EOC patients and Kaplan–Meier (KM) Plotter database.

Results: IHC staining showed that P4HA2 was significantly up-regulated in EOC tissues, compared to the normal tissues. Two databases retrieved from Oncomine database further confirmed the up-regulation P4HA2 in EOC. Chi-square test demonstrated that P4HA2 expression was associated with clinical stage ($p=0.036$), tumor grade ($p<0.001$), and residual disease ($p=0.022$). Both in clinical samples and KM Plotter database, high P4HA2 expression was significantly associated with worse progression-free survival (PFS) and overall survival (OS). Cox's proportional hazards regression analysis suggested that high P4HA2 expression were independent risk factors for the survival of EOC patients. Besides, we confirmed the positive correlation between P4HA2 and COL1A1 expression. Moreover, COL1A1 was found to be up-regulated in EOC and also associated with short PFS and OS.

Conclusion: The present study preliminarily proved that P4HA2 expression was associated with clinical outcome in EOC patients. P4HA2 might be a prognostic factor for EOC progression, and has the potential to be a valuable therapeutic target for EOC.

Keywords: P4HA2, prognosis, epithelial ovarian cancer, extracellular matrix, collagen

Introduction

Epithelial ovarian cancer (EOC) is one of the most common gynecological malignancies, and the leading cause of death worldwide.^{1,2} Because there are no symptoms at early stage, the vast majority of EOC patients are already in an advanced stage and have extensive metastasis at the time of diagnosis.^{3,4} Despite significant advancements in the medical technology, diagnosis and treatment for EOC have been achieved over the past two decades, the prognosis of patients has not been significantly improved.⁵ So far, there is no effective treatment for EOC, and the molecular mechanism of its occurrence and development is still not fully understood. Therefore, understanding of

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the pathogenic mechanisms and prognostic factor for EOC progression, and exploring the valuable therapeutic targets for EOC would serve to expand the clinical benefit to a broader patient population.

Proline 4-hydroxylase a subunit 2 (P4HA2) gene is located on chromosome 5q31.1.⁶ As a key enzyme in collagen biosynthesis, P4HA2 plays an important role in maintaining the stability of collagen fiber trimer. Relevant literatures have reported that the high expression level of P4HA2 was associated with the clinical prognosis of many malignant tumors.^{7–11} However, its prognostic value in EOC has not been fully elucidated.

In this study, we found that P4HA2 was markedly up-regulated in EOC and the high expression of P4HA2 predicted poor clinical outcomes. Consistent results were observed in the EOC cohorts downloaded from Oncomine and Kaplan–Meier (KM) Plotter database. Moreover, we preliminarily showed that P4HA2 may have the correlation with collagen biosynthesis. Collectively, our results demonstrated that P4HA2 might be a prognostic factor for EOC progression, and has the potential to be a valuable therapeutic target for EOC.

Patients and Methods

Patients and Tissue Specimens

A total of 217 paraffin-embedded pathological specimens were collected from the Fourth Affiliated Hospital of Hebei Medical University between September 2000 and June 2014. Pathological diagnosis of EOC were confirmed independently by two pathologists from the Department of Pathology in the Fourth Affiliated Hospital of Hebei Medical University. Among them, there were 104 cases of high-grade serous ovarian cancer (HGSOC), 63 other pathological types (clear cell, mucinous, and endometrioid), and 50 normal ovarian tissues from the patients who had no fertility requirement, and underwent hysterectomy plus bilateral adnexal resection due to cervical lesions. All patients with EOC were staged according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. All participants received the whole treatment and were followed up in our hospital. This study was approved by the ethics committee of the Fourth Hospital of Hebei Medical University, and all participants signed informed consent to participate in this study.

Hematoxylin and Eosin (H&E) Staining

Paraffin-embedded sections were stained with H&E according to the standard protocol. Briefly, paraffin section was dewaxed in xylene, rehydrated in 70–100% graded alcohol solutions, and washed with twice with ddH₂O. Then, section was stained with hematoxylin and eosin in sequence and were observed under light microscope at appropriate magnification.

Immunohistochemical (IHC) Staining

IHC staining of paraffin-embedded tissue sections was processed according to standard protocol. Primary antibodies against P4HA2 (Cat No. 66,604-1-Ig, 1:400 dilution) was purchased from Proteintech (Rosemont, IL, USA).

The results of IHC staining were independently determined by two senior pathologists who were blind to the patients' information. If there was any objection, the third pathologist made the judgment. Each pathological section was observed under Olympus BX-51 microscope (Olympus, Waltham, MA, USA). After positively stained cells count and background evaluation, semi-quantitative P4HA2 expression analysis by IHC was evaluated using a staining score, as Gong et al previously described.¹² The established staining score (range, 0–7) was the sum of staining intensity and proportion of positively stained cells. A final score of 0–4 points indicated no or low expression, and 5–7 points indicated high expression.

Oncomine Analysis

The individual gene expression level of P4HA2 and COL1A1 was analyzed in Oncomine database (<https://www.oncomine.org>). The P4HA2 related data sets were collected according to the following Oncomine screening conditions: (1) Gene name, P4HA2 or COL1A1; (2) Tissue comparison, ovarian cancer vs normal ovarian tissue; (3) data type, mRNA; (4) Significance: $P < 1E-4$; (5) Differential expression level: more than 2-fold change; (6) Threshold: top 10% gene rank. Following screening, two datasets (Hendrix database¹³ and Adib database¹⁴) were included for P4HA2 expression analysis and one TCGA dataset, the Cancer Genome Atlas-Ovarian Serous Cystadenocarcinoma Gene Expression Data set, was included for COL1A1 expression analysis.

KM Plotter Database Analysis

KM plotter integrative data analysis tool (<http://www.kmplot.com>) was used to analyze the prognostic

significance of mRNA in patients with ovarian cancer. To assess prognostic significance of genes, the patient samples were divided into two cohorts (high vs low expression) according to auto select best cutoff. Genes (P4HA2 and COL1A1) were retrieved in the platform database respectively to obtain the KM survival plots, without restriction on the pathological type, clinical stage, and tumor grade. The desired Affymetrix ID (P4HA2, 202733_at; COL1A1, 202311_s_at)¹⁵ in ovarian cancer is valid in KM plotter database, www.kmplot.com.

Statistical Analysis

Statistical analysis was performed with the SPSS 21.0 software package (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism (version 8.0; GraphPad Software Inc., CA, USA). $P < 0.05$ (two-sided) was considered significant. The relationship between the expression level of P4HA2 and the clinicopathological characteristics was analyzed by chi-square test. Survival curves were plotted using the Kaplan–Meier method and compared using the Log rank test. Influence factors for survival were evaluated using univariate and multivariate Cox regression analysis. The correlation between P4HA2 and COL1A1 expression was analyzed using Spearman correlation.

Results

P4HA2 Expression in Patients with EOC

In this study, the EOC tissue (n=167) and normal ovarian tissues (n=50) were pathologically confirmed using H&E staining (Figure 1A). To investigate the role of P4HA2 in EOC, IHC staining was performed, which confirmed the

up-regulation P4HA2 in EOC tissues, compared to the normal tissues (Figure 1B and C). To verify the clinical finding, three dataset was retrieved from Oncomine database (Figure 2). The results showed that the levels of P4HA2 in EOC were higher than that in normal tissues, in all three datasets including Adib dataset ($p=0.002$, Figure 2A), and Hendrix dataset ($p < 0.001$, Figure 2B). Therefore, these above results confirmed the up-regulation of P4HA2 in patients with EOC, indicating the potential role of P4HA2 in EOC progression.

Relationship Between P4HA2 and Clinicopathologic Characteristics of Patients with EOC

To investigate the association of P4HA2 with EOC progression, we analyzed the relationship between P4HA2 expression and clinicopathological characteristics of the patients with EOC. As shown in Table 1, the P4HA2 expression had no significant association with the age ($p=0.956$) and clinical histology ($p=0.203$) of EOC patients. Notably, the high expression of P4HA2 was significantly associated with clinical stage ($p=0.036$), grade ($p < 0.001$), and residual disease ($p=0.022$).

P4HA2 Expression is Associated with Poor Prognosis in Patients with EOC

Subsequently, to explore the influence of P4HA2 expression on patients' survival, KM survival analysis was performed in clinical samples and KM plotter database, respectively. As shown in Figure 3A and B, the high

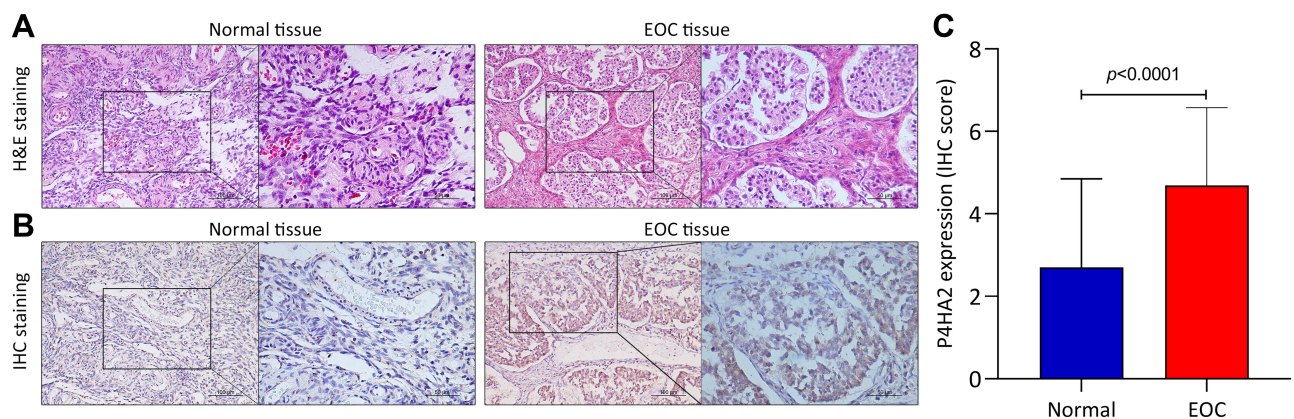


Figure 1 The up-regulation of P4HA2 in clinical samples of patients with EOC. **(A)** The normal ovarian tissues and EOC tissue were pathologically confirmed using H&E staining. Representative images were shown at 200 \times magnification (scale bar, 100 μ m) and 400 \times magnification (scale bar, 50 μ m). **(B)** The expression of P4HA2 in normal ovarian tissues and EOC tissue were detected using IHC staining. Representative images were shown at 200 \times magnification (scale bar, 100 μ m) and 400 \times magnification (scale bar, 50 μ m). **(C)** Semi-quantitative P4HA2 expression in normal ovarian tissues and EOC tissue by staining score.

Abbreviation: EOC, epithelial ovarian cancer.

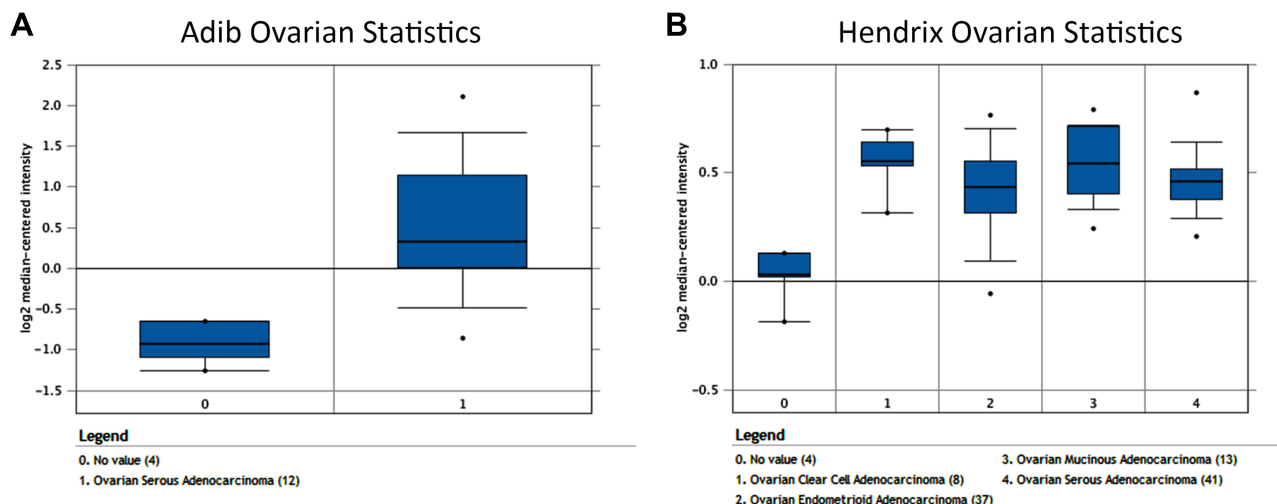


Figure 2 The up-regulation of P4HA2 in EOC patients from Oncomine database. (A) The expression of P4HA2 mRNA in Adib ovarian dataset. (B) The expression of P4HA2 mRNA in Hendrix ovarian dataset.
Abbreviation: EOC, epithelial ovarian cancer.

P4HA2 expression is significantly associated with worse PFS ($p=0.0093$, Figure 3A) and OS ($p=0.0374$, Figure 3B) in clinical samples. Through the KM plotter integrative data analysis, we further verified that high P4HA2 levels predicted PFS ($p<0.001$, Figure 3C) and OS ($p<0.001$, Figure 3D) in EOC, which consistent with the findings from clinical samples. Furthermore, to verify the influence of P4HA2 expression on patients’ survival, Cox’s

proportional hazards regression model was performed by adjusting clinical parameters (Table 2). Both the univariate and multivariate analysis showed that high clinical stage (III–IV), large residual disease (>1 cm), and high P4HA2 expression were independent risk factors for the survival of patients with EOC. Moreover, as HGSOc is the most common type of ovarian cancer, we compared the expression of P4HA2 in HGSOc with other pathological types.

Table 1 Association Between P4HA2 Expression and Clinicopathologic Characteristics of EOC Patients

Characteristics	Cases	P4HA2 Expression		
		Low Expression	High Expression	P-value
Age (years)				
<50	78	47	31	0.956
≥50	89	54	35	
Clinical stage				
I–II	59	42	17	0.036
III–IV	108	59	49	
Grade				
G1	41	34	7	<0.001
G2–G3	126	67	59	
Clinical histology				
Serous	104	59	45	0.203
Others	63	42	21	
Residual disease (cm)				
≤1	66	47	19	0.022
>1	101	54	47	

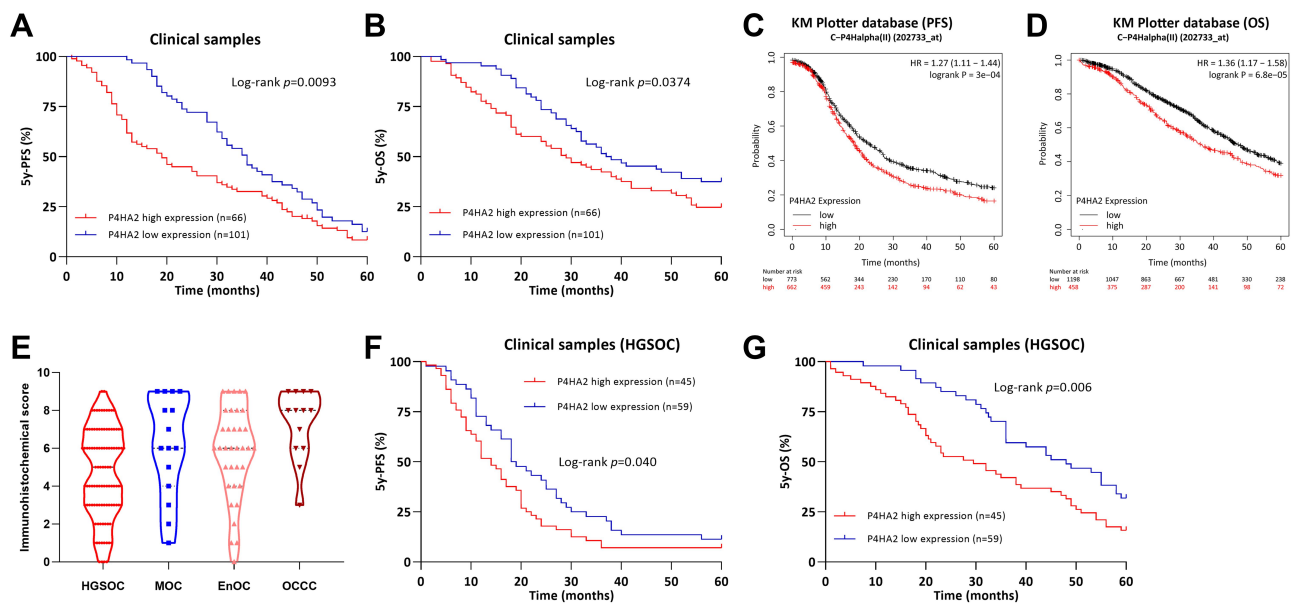


Figure 3 P4HA2 expression is associated with poor prognosis in patients with EOC. (A and B) Kaplan–Meier curves of progression-free survival (A) and overall survival (B) in EOC patients categorized by P4HA2 expression; (C and D) the Kaplan–Meier plotter platform (<http://www.kmplot.com>) resulted that patients with the high P4HA2 expression was significantly associated with short progression-free survival (C) and poor overall survival (D) in EOC; (E) The P4HA2 expression in different pathological types. (F and G) Kaplan–Meier curves of progression-free survival (F) and overall survival (G) in HGSOC patients categorized by P4HA2 expression. **Abbreviations:** EOC, epithelial ovarian cancer; HGSOC, high-grade serous ovarian cancer; MOC, mucinous ovarian cancer; EnOC, endometrioid ovarian cancer; OCCC, ovarian clear cell carcinoma.

As shown in Figure 3E, there was no significant difference in the expression of P4HA2 among different pathological types of EOC. In the patients with HGSOC, the analysis of clinical samples showed that the high P4HA2 expression is also significantly associated with worse PFS ($p=0.040$, Figure 3F) and OS ($p=0.006$, Figure 3G). Overall, these results suggested that P4HA2 expression is associated with poor prognosis in patients with EOC.

COL1A1 is often found to be associated with metastatic HGSOC, which is the most common type of ovarian cancer, thus we investigated the relationship of P4HA2 with COL1A1. As expected, COL1A1 was also up-regulated in EOC from Oncomine database (Figure 4A). More importantly, P4HA2 expression was found to be positively correlated with COL1A1 expression ($R=0.43$,

$p<0.001$, Figure 4B). Similarly, KM plotter integrative data analysis indicated that high COL1A1 expression is significantly associated with worse PFS ($p=0.0093$, Figure 4C) and OS ($p=0.0374$, Figure 4D). These results provided additional evidence for the role of P4HA2 in EOC.

Discussion

EOC is a significant cause of cancer related morbidity and mortality in women.¹⁶ Due to the special anatomic location of the ovary, the disease is usually lack of clinical symptoms in the early stage, leading to the poor outcome for patients in advanced stage.¹⁷ There are many mechanisms are responsible for the development and progression of EOC, including cell proliferation,¹⁸ apoptosis inhibition,¹⁹ tumor stroma formation,²⁰ and peripheral

Table 2 Cox Regression Analysis for Overall Survival in Patients with EOC

Characteristics	Univariate Analysis			Multivariate Analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (<50 vs ≥50 years)	1.387	0.912–2.110	0.126	1.485	0.948–2.324	0.084
Clinical stage (III–IV vs I–II)	1.514	1.236–1.855	<0.001	1.29	1.017–1.636	0.036
Grade (G2–G3 vs G1)	0.925	0.720–1.190	0.546	0.88	0.673–1.151	0.352
Clinical histology (Serous vs others)	0.976	0.767–1.242	0.842	0.891	0.661–1.201	0.450
Residual disease (>1 vs ≤1 cm)	1.858	1.392–2.478	<0.001	1.704	1.268–2.289	<0.001
P4HA2 expression (High vs low)	4.495	2.258–8.948	<0.001	3.35	1.619–6.934	0.001

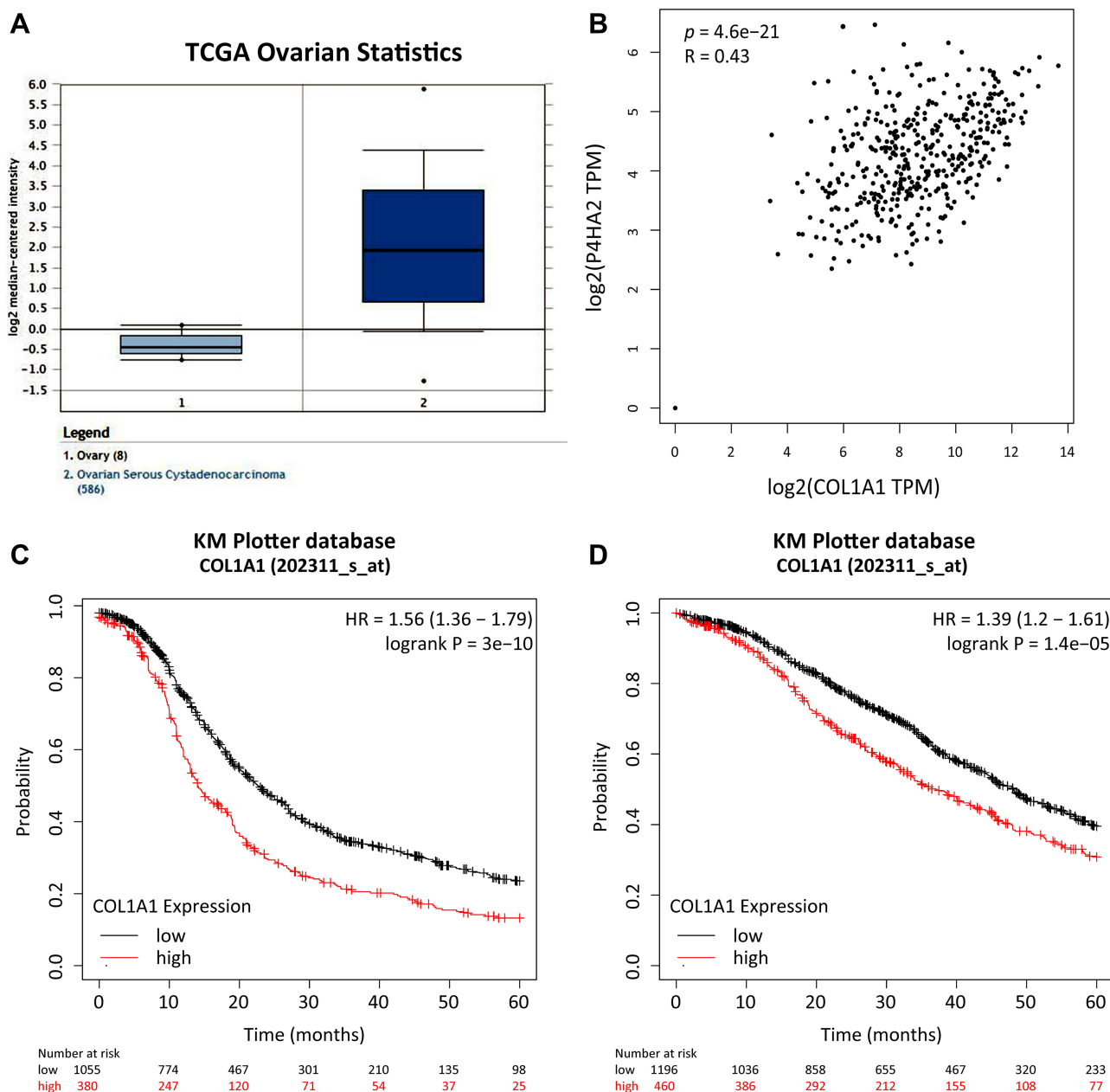


Figure 4 The relationship between P4HA2 with COL1A1. **(A)** The expression of COL1A1 mRNA in TCGA ovarian dataset; **(B)** the correlation analysis between P4HA2 and COL1A1 expression was analyzed using Spearman correlation in the GEPIA database; **(C and D)** the Kaplan–Meier plotter platform (<http://www.kmplot.com>) resulted that patients with the high COL1A1 expression was significantly associated with short progression-free survival **(C)** and poor overall survival **(D)** in EOC.

Abbreviation: EOC, epithelial ovarian cancer.

blood vessels.²¹ Although these aspects are regulated by various factors, there are still lack of effective biomarkers and targets to guide clinical treatment. Therefore, it is very important to identify novel prognostic biomarkers and therapeutic targets in EOC.

In this study, the association between the P4HA2 expression and the clinical outcomes of EOC patients was investigated. The results showed that the expression levels of P4HA2 were statistically up-regulated in

EOC tissue compared to normal ovarian tissues. Meanwhile, univariate and multivariate analysis demonstrated that patients with high expression levels of P4HA2 had a worse prognosis, indicating that P4HA2 might be act as an independent prognostic factor in EOC progression. This finding was consistent with the previous studies, including melanoma⁷ cervical cancer,⁸ liver cancer,⁹ lung cancer,¹⁰ and breast cancer.¹¹

Many previous studies have shown that extracellular matrix (ECM) is important for the occurrence and development of malignancies.^{22,23} Moreover, collagen is an important part of ECM and forms the scaffold of tumor micro-environment,⁹ thus, it plays a critical role in tumor infiltration and metastasis.²⁴ Collagen biosynthesis and deposition are a multi-step process, which is regulated by a variety of post-transcriptional modifying enzymes, especially the collagen pro4-hydroxylase.²⁵ P4HA2 as the member of the collagen pro4-hydroxylase family could increase collagen deposition.¹¹ Many previous studies have shown that high expression of P4HA2 is associated with poor prognosis of malignancies. P4HA2 promoted proliferation, and particularly invasiveness in melanoma.⁷ Epithelial–mesenchymal transition (EMT) was involved in cervical cancer progression regulated by P4HA2.⁸ Feng et al have demonstrated that HBx-elevated P4HA2 enhanced the collagen deposition in the liver in vivo and in vitro, leading to liver fibrosis and liver cancer progression.⁹ A similar finding was found in lung cancer.¹⁰ Moreover, increased mRNA levels of P4HA2 correlated with poor clinical outcome in breast cancer patients.¹¹ In this study, we confirmed that P4HA2 was highly expressed in EOC and associated with poor prognosis. Moreover, given that HGSOC is the most common type of ovarian cancer, the expression of P4HA2 in HGSOC comparing with other pathological types were analyzed. However, no expression difference was found among different pathological types, indicating that the P4HA2 may be a common biomarker for different pathological types of EOC. Certainly, this speculation still needed to be verified in the further investigation.

COL1A1 was often found to be associated with metastatic HGSOC, which is the most common type of ovarian cancer, thus we investigated the relationship of P4HA2 with COL1A1. As expected, COL1A1 was found to be up-regulated in EOC and also associated with poor progression in EOC. More importantly, P4HA2 expression was found to be positively correlated with COL1A1 expression. These results provided additional evidence for the role of P4HA2 in EOC.

In conclusion, the present study preliminarily proved that P4HA2 expression was associated with clinical outcome in EOC patients. P4HA2 might be a prognostic factor for EOC progression, and has the potential to be a valuable therapeutic target for EOC.

Ethical Approval

This study was approved by the Medical Ethics Committee of the Fourth Hospital of Hebei Medical University. All the procedures were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

All authors declare that they have no conflict of interest.

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