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RESEARCH ARTICLE

Acetylated HOXB9 at lysine 27 is of differential diagnostic value in patients with pancreatic ductal adenocarcinoma

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Abstract Pancreatic ductal adenocarcinoma (PDAC) is the ninth most common human malignancy and the sixth leading cause of cancer-related death in China. AcK27-HOXB9 is a newly identified HOXB9 post-transcriptional modification that can predict the outcome in lung adenocarcinoma and colon cancer well. However, the role of AcK27-HOXB9 in PDAC is unclear. The present study aims to investigate the differential diagnostic role of patients with AcK27-HOXB9 PDAC. Tissue microarrays consisting of 162 pancreatic tumor tissue samples from patients with PDAC and paired normal subjects were used to examine HOXB9 and AcK27-HOXB9 levels and localizations by immunohistochemical analysis and Western blot assay, respectively. HOXB9 was upregulated ($P < 0.0001$), and AcK27-HOXB9 ($P = 0.0023$) was downregulated in patients with PDAC. HOXB9 promoted ($P = 0.0115$), while AcK27-HOXB9 ($P = 0.0279$) inhibited PDAC progression. AcK27-HOXB9 predicted favorable outcome in patients with PDAC ($P = 0.0412$). AcK27-HOXB9 also suppressed PDAC cell migration in a cell migration assay. The results of this study showed that HOXB9 promoted and AcK27-HOXB9 suppressed PDAC progression. The determination of ratio between HOXB9 and AcK27-HOXB9 exhibited potential diagnostic value in patients with PDAC.

Keywords HOXB9; AcK27-HOXB9; PDAC

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a notorious malignancy that ranks as the ninth most common human malignancy and the sixth leading cause of cancer-related death in China [1]. Deaths caused by PDAC are increasing continuously, and PDAC is expected to be the second leading cause of cancer-related death by 2030 [2]. For surgically suitable patients with PDAC, surgery is the current standard treatment, followed by adjuvant gemcitabine-based chemotherapy [3]. Unfortunately, even after standard surgery, the prognosis for patients with PDAC is extremely poor. The median overall survival of patients with PDAC is 17–21 months, and the five-year overall survival is approximately 20% [4]. Considerable efforts have been focused on the pathologic and clinical identification of molecules, which can predict progression and prognosis after PDAC surgery. Thus, finding novel and effective biomarkers is necessary and urgent for the progression and prognosis of patients with PDAC.

HOXB9 is a member of the homeobox-containing (HOX) transcription factor family, which includes 39 genes in humans. HOX transcription factor family genes are classified into four different clusters, namely, HOX A, B, C, and D. All of these genes play important roles in embryonic development [5,6]. Increasing studies have demonstrated that HOX family genes are associated with cancer progression [7,8]. HOXB9 induces angiogenesis, invasion, and lung metastasis in breast cancer [9]. HOXB9 is an important prognostic factor in patients with ovarian cancer [10] and lung cancer [11]. A previous study showed that HOXB9 is upregulated in endometrial cancer, and the researchers predicted a poor prognosis in patients with endometrial cancer [12]. These results strongly suggested

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that HOXB9 is involved in the progression and prognosis of patients with cancer. Wan *et al.* [13] reported that HOXB9 can be acetylated in lysine 27 (AcK27) in 2016. In lung cancer and colon cancer, in contrast to the wild-type HOXB9, AcK27-HOXB9 reduces its capacity to promote cancer cell migration and tumor growth in mice [13,14]. For clinical relevance, increased HOXB9 acetylation in K27 predicts remarkable outcome in patients with lung adenocarcinoma, which is the opposite of the role of HOXB9 [13,14]. Hence, in the present investigation, we wanted to clarify the exact role of HOXB9 and AcK27-HOXB9 in the progression and prognosis of patients with PDAC.

In this report, HOXB9 is upregulated in PDAC compared with the normal pancreatic tissues in 162 clinical patient specimens. In contrast to the wild-type HOXB9, AcK27-HOXB9 is downregulated in cancer tissues. Both HOXB9 and AcK27-HOXB9 are correlated with the pathological grades of PDAC, and their functions are the opposite. AcK27-HOXB9 suppresses PDAC cell migration *in vitro*. The increased AcK27-HOXB9 level is a favorable prognostic factor in patients with PDAC. AcK27-HOXB9 that plays tumor-suppressive role is universal.

Table 1 Clinicopathological characteristics of patients with PDAC (n = 162)

Characteristic	Value	Percentage
Age median (range), year	59 (34–85)	
Sex		
Male	102	62.96%
Female	60	37.04%
TNM category		
T1	6	3.70%
T2	127	78.40%
T3	28	17.28%
Unknown	1	0.62%
N0	80	49.38%
N1	65	40.12%
Unknown	17	10.49%
M0	160	98.77%
M1	2	1.23%
AJCC		
I	66	40.74%
I–II	11	6.79%
II	83	51.23%
IV	2	1.23%
Pathological grade		
I	10	6.17%
I–II	23	14.20%
II	85	52.47%
II–III	26	16.05%
III	12	7.41%
Unknown	6	3.70%
Vascular invasion		
Negative	79	48.77%
Positive	83	51.23%
Lymphatic		
Negative	159	98.15%
Positive	3	1.85%
Survival status		
Alive	24	14.81%
Dead	59	36.42%
Unknown	79	48.77%
Overall survival information		
Yes	66	40.74%
None	96	59.26%

Materials and methods

Patient specimens

Pancreatic cancer and the corresponding paracancerous tissues were collected from the Peking University Third Hospital. Tissue microarrays collected from 162 treatment-naïve patients were obtained from Outdo Biotech Co., Ltd. (Shanghai, China). All study procedures were approved by the Ethics Committee of National Human Genetic Resources Sharing Service Platform (2005DKA21300) and performed in accordance with the *Declaration of Helsinki*. Patient characteristics, including age, sex, TNM category, AJCC stage, invasion sites, and survival status, are shown in Table 1, and the smoking status of the patients was unavailable.

Immunohistochemistry (IHC)

All patient samples were fixed with formalin and embedded with paraffin immediately after collection. Embedded samples were prepared according to a published protocol [20], as follows. Sections were incubated in two washes of xylene for 10 min each. The sections were incubated in two washes of 100% ethanol for 10 min each. Sections were incubated in 95%, 90%, 80%, and 70% ethanol for 5 min each in turn. The sections were washed in dH_2O for 5 min. The slides were brought to a boil in 10 mmol/L sodium citrate buffer with the pH of 6 and then maintained at a sub-boiling temperature for 10 min. The slides were cooled on a bench top for 30 min. The sections were washed in dH_2O three times for 5 min each. The sections were incubated in 3% H_2O_2 for 10 min and then washed in dH_2O two times for 5 min each. Each section was blocked with blocking solution for 1 h at room temperature, and then the blocking solution was removed.

The prepared sections were incubated with HOXB9

(Abcam) or AcK27-HOXB9 (generated by customer request as previous published [13]) antibodies at 4 °C overnight. The antibodies were visualized with the PV9000 2-step plus Poly-HRP Anti-mouse/rabbit IgG Detection System (Zhong Shan Jin Qiao, China) and diaminobenzidine the next day, followed by counter-staining with hematoxylin. All specimens were scored by two independent pathologists, as follows: 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining.

Western blot assay

Western blot assay was performed according to a published protocol [15], as follows. The tissue was placed in round-bottom microcentrifuge tubes, for 0–5 mg piece of tissue. Approximately 300 μL of cold lysis buffer was added to the tube, then homogenized with an electric homogenizer, and centrifuged for 20 min at 12 000 rpm at 4 °C in a microcentrifuge. The supernatant was aspirated and placed in a fresh tube kept on ice. The protein concentration was determined for each tissue lysate. The amount to protein to load was determined, and an equal volume of 2 × Laemmli sample buffer was added. Then, each lysate was boiled at 100 °C for 5 min. Equal amounts of protein were loaded into the wells of the SDS-PAGE gel, along with the molecular weight marker. The gel was run for 2–3 h at 100 V. Then, the protein from the gel was transferred to the PVDF membrane. The membrane was blocked for 1 h at room temperature by using a blocking buffer. The membrane was incubated with appropriate dilutions of primary antibody in the blocking buffer at 4 °C. The membrane was washed in three washes of TBST for 5 min each. Then, the membrane was incubated using the recommended dilution of the conjugated secondary antibody in blocking buffer at room temperature for 1 h. The membrane was washed in three washes of TBST for 5 min each. An image was acquired using image scanning methods for colorimetric detection.

Antibodies for the following proteins were used: α-actin (Sigma-Aldrich), HOXB9 (Abcam), and AcK27-HOXB9 (generated by customer request as previously published).

Cell culture

The human pancreatic adenocarcinoma cell line ASPC-1 was purchased from ATCC (Rockefeller, MD, USA). ASPC-1 cell was cultured in Dulbecco's modified eagle medium, supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37 °C with 5% CO_2. When the cells reached 80%–90% confluency, they were passed by dissociation with 0.25% trypsin-EDTA solution (Gibco) for 1–2 min. For transient transfection, 70%–80% confluent cells were transfected with indicated plasmids by using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen).

Cell migration

Migration assays were performed using the Boyden chamber technique. Cell suspension containing 1×10^5 cells/mL in 100 μL of serum-free medium was added into the upper surface of the wells. The lower wells contained 600 μL of the same medium with 20% FBS. After 8 h of migration at 37 °C, the migrated cells were fixed with 4% formaldehyde and stained by 0.5% crystal violet through the inserts. The cells were subsequently counted under the Olympus microscope in five random fields of the counting chamber.

Statistical analysis

The correlation between HOXB9 and AcK27-HOXB9 expression levels and patients' clinic pathological characteristics were analyzed by Chi-squared test. Kaplan–Meier analysis was applied to analyze the overall survival of 162 cases of patients with PDAC grouped by the expression levels of HOXB9 or AcK27-HOXB9. Student's *t*-test was applied for a single comparison of two groups. Data were presented as mean ± SEM. All statistical analyses were performed in GraphPad Prism 6.01. Results were considered statistically significant at $P < 0.05$.

Results

HOXB9 was upregulated, while AcK27-HOXB9 was downregulated in patients with PDAC

HOXB9 is involved in various cancers, such as breast cancer, lung cancer, and ovarian cancer. In 162 PDAC tumor specimens and paired normal pancreatic tissues, IHC analysis revealed that HOXB9 was expressed both in the cytoplasm and nuclei of pancreatic cells but mainly in nuclei (Fig. 1A, as indicated by the arrows). Compared with normal pancreatic tissues, HOXB9 was upregulated in tumor tissues (Fig. 1A). Subsequently, semiquantitative analysis confirmed that HOXB9 increased in PDAC compared with that of the control tissues ($P < 0.0001$, Fig. 1B). Simultaneously, we examined the level of AcK27-HOXB9 in the same cohort, and the results showed that AcK27-HOXB9 was mainly expressed in cytoplasm but not in the nuclei of pancreatic cells (Fig. 1C, as indicated by the arrows). In contrast to the HOXB9 level, AcK27-HOXB9 was downregulated in PDAC compared with the normal pancreatic tissues (Fig. 1C). Semiquantitative analysis also showed a significant AcK27-HOXB9 downregulation in PDAC ($P = 0.0023$, Fig. 1D). Western blot assay also showed that HOXB9

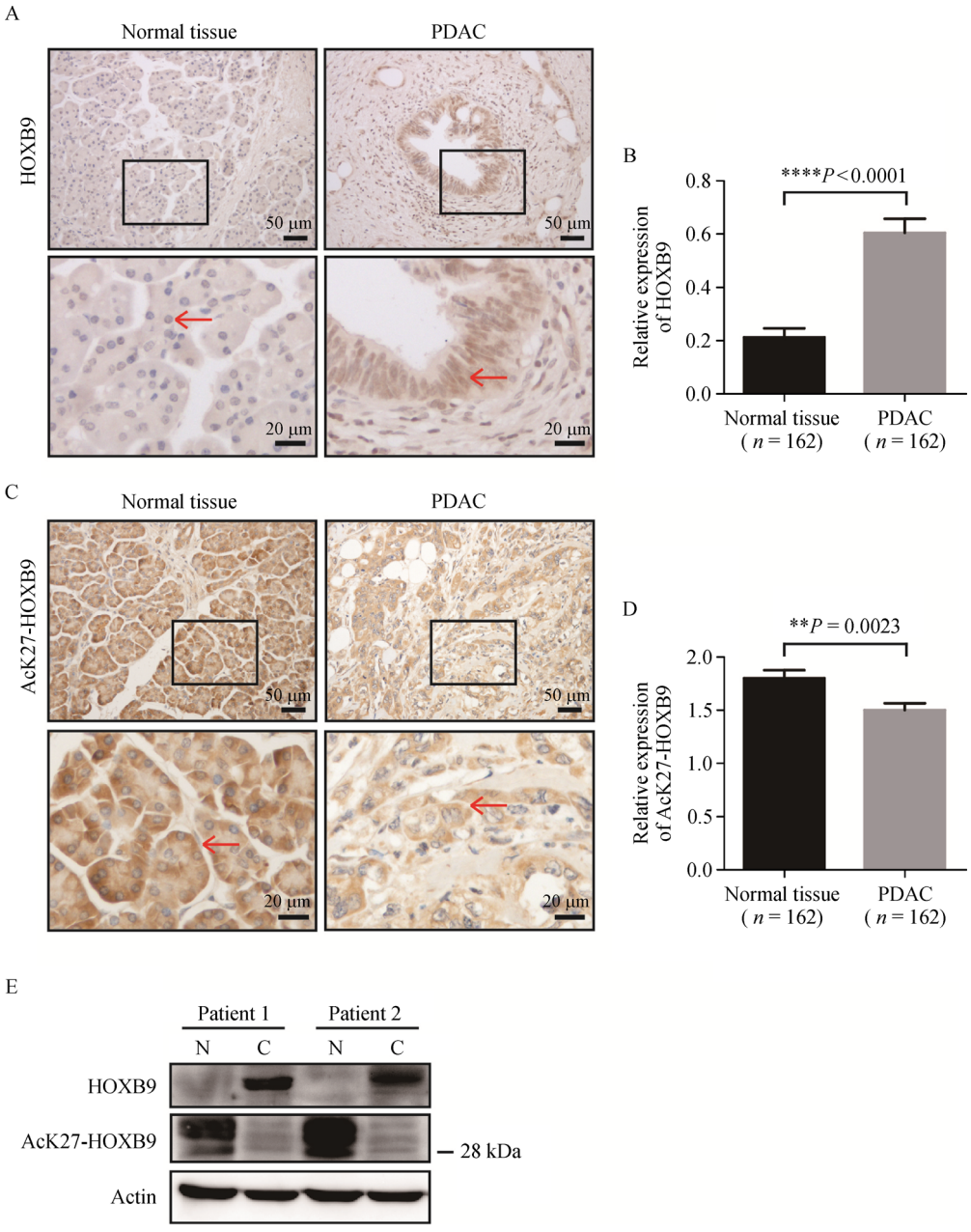

Fig. 1 Expression levels of HOXB9 and AcK27-HOXB9 in the tissues of patients with PDAC and paired normal subjects. (A) IHC detection of HOXB9 in the pancreatic tissues of normal subjects and those of patients with PDAC. The lower panel corresponds to the local zoom of the upper panel. Arrows indicate the nuclei localization of HOXB9. (B) Semiquantitative analysis of HOXB9 expression in the tissues of patients with PDAC and normal subjects ($n = 162$), ****$P < 0.0001$. (C) IHC detection of AcK27-HOXB9 in the pancreatic tissues of normal subjects and patients with PDAC. The lower panel corresponds to the local zoom of the upper panel. Arrows indicate cytoplasmic localization. (D) Semiquantitative analysis of AcK27-HOXB9 expression in the tissues of patients with PDAC and normal subjects ($n = 162$), **$P < 0.01$. (E) Western blot detection of HOXB9 and AcK27-HOXB9 expression levels in the fresh tissues of normal subjects and patients with PDAC.

increased, and AcK27-HOXB9 decreased in fresh samples from PDAC patients (Fig. 1E). These results suggested that HOXB9 mainly localized in nuclei as a transcription factor to promote PDAC, whereas AcK27-HOXB9 was translocated into cytoplasm and functioned as a tumor suppressor.

HOXB9 promoted and AcK27-HOXB9 inhibited PDAC progression

Given that HOXB9 and AcK27-HOXB9 are differentially expressed in PDAC and the normal pancreatic tissues, we

determined whether the variable expression was related to tumor pathological grades. Hence, we performed a correlative analysis on HOXB9 and AcK27-HOXB9 expression levels with pathological grades of PDAC conducted in 162 patients. Results showed that both HOXB9 and AcK27-HOXB9 were significantly associated with the pathological grades of PDAC (Fig. 2A and 2B and Tables 2 and 3). HOXB9 was upregulated in high-grade PDAC with poor differentiation compared with low pathological grade (P = 0.0115, Fig. 2A). However, AcK27-HOXB9 decreased in high-grade PDAC compared with the low-grade one (P = 0.0279, Fig. 2B).

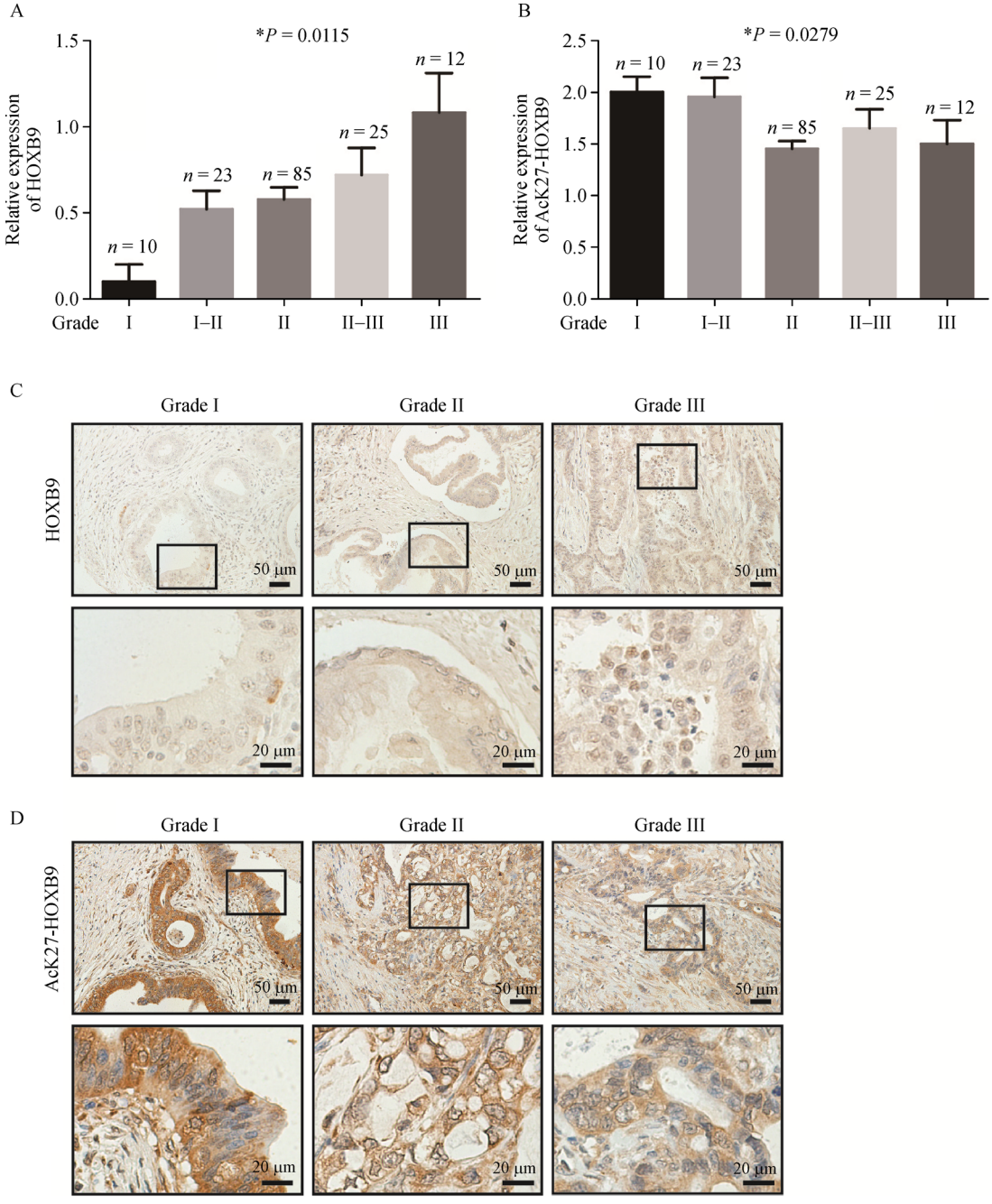

Fig. 2 HOXB9 and AcK27-HOXB9 are differently expressed during PDAC progression. (A) Semiquantitative analysis of the correlation between HOXB9 and tumor pathological grade, *$P < 0.05$. (B) Semiquantitative analysis of the correlation between AcK27-HOXB9 and tumor pathological grade, *$P < 0.05$. (C) Representative images of HOXB9 expression in pathological grades I, II, and III. The lower panel corresponds to the local zoom of the upper panel. (D) Representative images of AcK27-HOXB9 expression in pathological grades I, II, and III. The lower panel corresponds to the local zoom of the upper panel.

Table 2 Relationship between HOXB9 expression and various clinicopathological factors in patients with PDAC (n = 162)

Characteristics	HOXB9 expression						*P* value
	0		1		2		
Total	82	50.62%	62	38.27%	18	11.11%	
Age, year							0.9062
⩽60	45	27.78%	37	22.84%	10	6.17%	
>60	37	22.84%	25	15.43%	8	4.94%	
Sex							0.3853
Male	50	30.86%	38	23.46%	14	8.64%	
Female	32	19.75%	24	14.81%	4	2.47%	
TNM category							
T1	4	2.47%	2	1.23%	0	0%	0.6695
T2	64	39.51%	46	28.40%	16	9.88%	
T3	13	8.02%	13	8.02%	2	1.23%	
Unknown	1	0.62%	0	0%	0	0%	
N0	37	22.84%	34	20.99%	9	5.56%	0.5732
N1	35	21.60%	22	13.58%	7	4.32%	
Unknown	9	5.56%	5	3.09%	2	1.23%	
M0	82	50.62%	61	37.65%	18	11.11%	0.4442
M1	0	0%	1	0.62%	0	0%	
AJCC							0.9566
I	30	18.52%	28	17.28%	8	4.94%	
I–II	6	3.70%	4	2.47%	1	0.62%	
II	45	27.78%	29	17.90%	9	5.56%	
IV	1	0.62%	1	0.62%	0	0%	
Pathological grade							0.0129*
I	9	5.56%	1	0.62%	0	0%	
I–II	11	6.79%	12	7.41%	0	0%	
II	44	27.16%	33	20.37%	8	4.94%	
II–III	12	7.41%	8	4.94%	5	3.09%	
III	3	1.85%	5	3.09%	4	2.47%	
Unknown	3	1.85%	2	1.23%	1	0.62%	
Vascular invasion							0.2920
Negative	35	21.60%	34	20.99%	10	6.17%	
Positive	47	29.01%	28	17.28%	8	4.94%	
Lymphatic							0.4588
Negative	81	50.00%	61	37.65%	17	10.49%	
Positive	1	0.62%	1	0.62%	1	0.62%	
Survival status							0.4510
Alive	13	8.02%	10	6.17%	1	0.62%	
Dead	30	18.52%	21	12.96%	8	4.94%	
Unknown	39	24.07%	31	19.14%	9	5.56%	

Chi-squared test. * represents $P < 0.05$.

Representative IHC images were provided to show the opposite association between HOXB9 (Fig. 2C) and AcK27-HOXB9 (Fig. 2D) with PDAC pathological grades. Consistently, these data indicated that HOXB9 promoted PDAC progression, while AcK27-HOXB9 functioned as a suppressor of pancreatic cancer progression.

Enhanced AcK27-HOXB9 level predicted a favorable outcome in patients with PDAC

Given that HOXB9 and AcK27-HOXB9 were oppositely related to PDAC progression, we determined whether the varying HOXB9 and AcK27-HOXB9 levels were

Table 3 Relationship between AcK27-HOXB9 expression and various clinicopathological factors in patients with PDAC (n = 162)

Characteristics	AcK27-HOXB9 expression								*P* value
	0		1		2		3		
Total	16	9.88%	56	34.57%	72	44.44%	18	11.11%	
Age, year									0.2156
⩽60	6	3.70%	31	19.14%	43	26.54%	13	8.02%	
>60	10	6.17%	25	15.43%	29	17.90%	5	3.09%	
Sex									0.7431
Male	9	5.56%	34	20.37%	46	29.01%	13	8.02%	
Female	7	4.32%	22	13.58%	26	16.05%	5	3.09%	
TNM category									
T1	2	1.23%	1	0.62%	3	1.85%	0	0%	0.4287
T2	9	5.56%	47	29.01%	55	33.95%	16	9.88%	
T3	5	3.09%	8	4.94%	14	8.64%	2	1.23%	
Unknown	2	1.23%	7	4.32%	6	3.70%	2	1.23%	
N0	7	4.32%	23	14.20%	42	25.93%	8	4.94%	0.2373
N1	9	5.56%	33	20.37%	30	18.52%	10	6.17%	
Unknown	3	4.11%	8	10.96%	4	5.48%	1	0.62%	
M0	16	9.88%	55	33.95%	71	43.83%	18	11.11%	0.9013
M1	0	0%	1	0.62%	1	0.62%	0	0%	
AJCC									0.8980
I	6	3.70%	19	11.73%	33	20.37%	8	4.94%	
I–II	2	1.23%	4	2.47%	3	1.85%	2	1.23%	
II	8	4.93%	31	19.14%	35	21.60%	8	4.94%	
IV	0	0%	1	0.62%	1	0.62%	0	0%	
Pathological grade									0.0454*
I	0	0%	2	1.23%	7	4.32%	1	0.62%	
I–II	2	1.23%	4	2.47%	12	7.40%	5	3.08%	
II	10	6.17%	35	21.60%	47	29.01%	3	1.85%	
II–III	2	1.23%	9	5.56%	8	4.94%	7	4.32%	
III	1	0.62%	4	2.47%	6	3.70%	1	0.62%	
Unknown	2	1.23%	2	1.23%	2	1.23%	0	0%	
Vascular invasion									0.4983
Negative	7	4.32%	28	17.28%	38	23.46%	6	3.70%	
Positive	9	5.56%	28	17.28%	34	20.99%	12	7.41%	
Lymphatic									0.5461
Negative	15	9.26%	55	33.95%	71	43.83%	18	11.11%	
Positive	1	0.62%	1	0.62%	1	0.62%	0	0%	
Survival status									0.0121*
Alive	1	0.62%	7	4.32%	16	9.88%	0	0%	
Dead	7	4.32%	25	15.43%	18	11.11%	9	5.56%	
Unknown	8	4.94%	24	14.81%	38	23.46%	9	5.56%	

Chi-squared test. * represents $P < 0.05$.

associated with the prognosis of patients with PDAC. We performed K-M plot analysis in this cohort. The representative IHC images of the HOXB9 (Fig. 3A) and AcK27-HOXB9 levels (Fig. 3B) with variable scores were shown. The results showed that the HOXB9 level was insignificantly correlated with the overall survival of patients with PDAC (Fig. 3C). However, increased AcK27-HOXB9 level displayed a significant correlation with the overall survival of patients with PDAC (Fig. 3D). High AcK27-HOXB9 level indicated a favorable outcome in patients, while a low AcK27-HOXB9 leveled predicted poor outcome ($P = 0.0412$, Fig. 3D). Hence, AcK27-HOXB9 is an indicator in predicting prognosis in patients with PDAC.

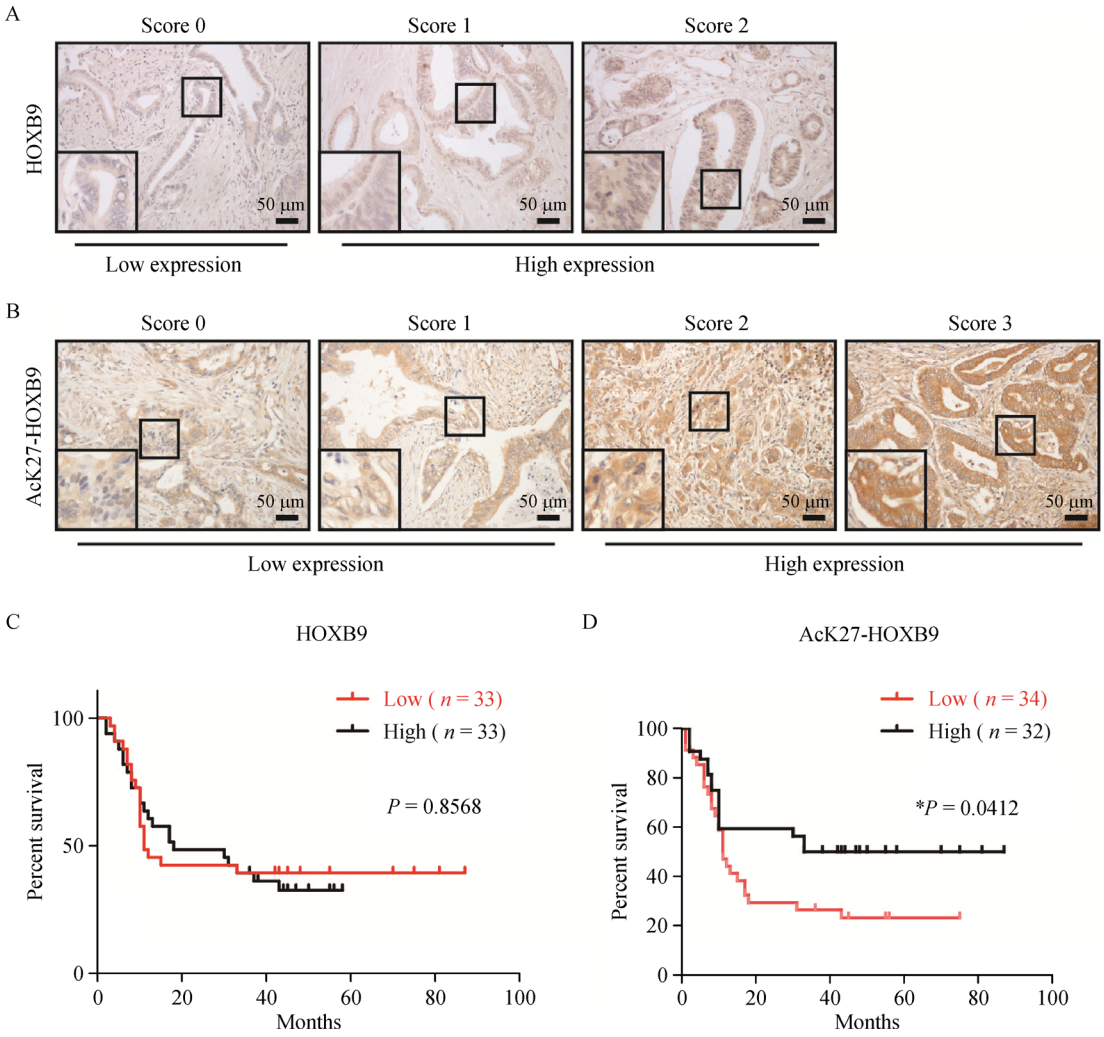

Fig. 3 AcK27-HOXB9 is correlated with prognosis of patients with PDAC. (A) Representative images of low (score of 0) and high HOXB9 expression (scores 1 and 2) in the tissues of patients with PDAC. The picture zoom is located at the lower left corner. (B) Representative images of low (scores 0 and 1) and high AcK27-HOXB9 expression (scores 2 and 3) in the tissues of patients with PDAC. The picture zoom is located at the lower left corner. (C) Kaplan–Meier analysis of the overall survival grouped by high and low HOXB9 expression levels. $P = 0.8568$. (D) Kaplan–Meier analysis of the overall survival grouped by high and low AcK27-HOXB9 expression levels. $*P = 0.0412$.

AcK27-HOXB9 inhibited PDAC cell migration

To explore the physiologic functions of HOXB9 and AcK27-HOXB9 further, we investigated their effect on PDAC cell migration. We first transfected ASPC-1 cells with FLAG-tagged HOXB9-WT, HOXB9-K27Q (a mimic of hyperacetylated HOXB9), and HOXB9-K27R (a mimic of acetylation-deficient HOXB9) transiently. Overexpressed HOXB9 significantly increased ASPC-1 cell migration. HOXB9-K27R expression promoted ASPC-1 cell migration significantly, whereas that of HOXB9-K27Q considerably decreased ASPC-1 cell migration compared with HOXB9-WT cells (Fig. 4A and 4B).

These results suggested that HOXB9 promoted PDAC progression, while AcK27-HOXB9 suppressed PDAC progression.

Discussion

HOX family proteins function as monomers or homodimers to regulate the transcription of downstream genes directly, thereby controlling a series of cell functions, including differentiation, apoptosis, cell motility, and angiogenesis [16–18]. In particular, as a member of HOX family transcription factors, HOXB9 plays a crucial role in thoracic skeletal element specification and mammary gland development [19,20]. In addition to the

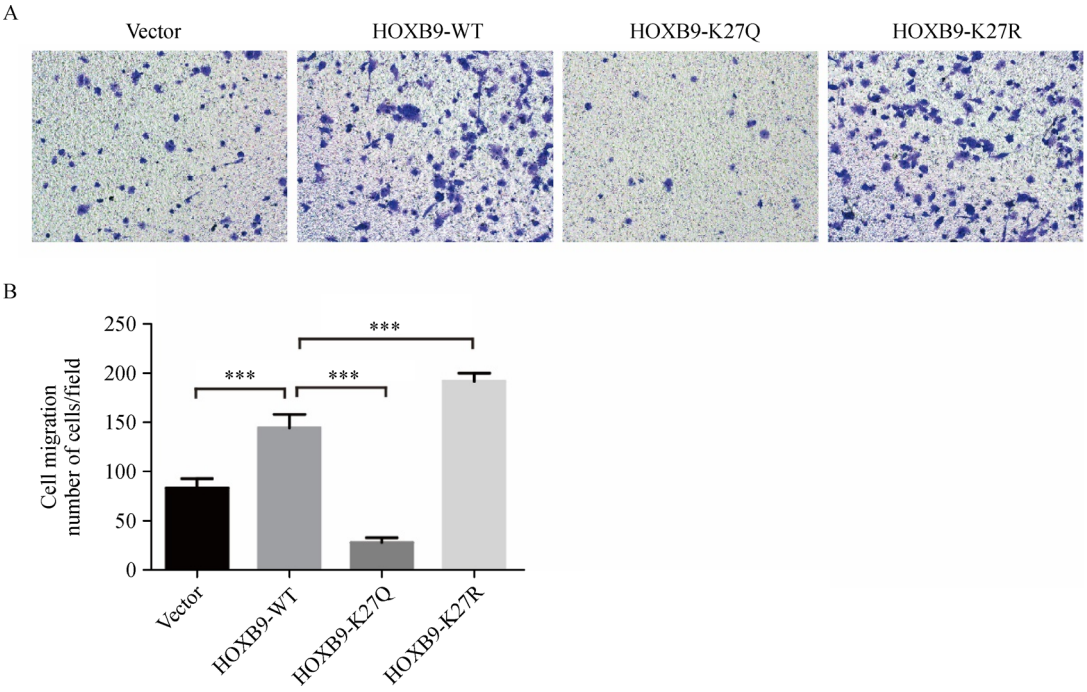

Fig. 4 AcK27-HOXB9 inhibited PDAC cell migration. (A) Migration assay was performed to detect the roles of HOXB9-WT, HOXB9-K27Q, and HOXB9-K27R in ASPC-1 cells compared with vector transfection. Representative images were shown. (B) Semiquantitative analysis of migration cell number per field. Data were presented as mean ± SEM from five independent experiments. The statistical analyses were performed by Student's *t*-test. ***$P < 0.001$.

important function in development, HOXB9 is also considerably involved in the progression of human cancers. In most cancers reported to date, HOXB9 is frequently overexpressed in tumor tissues, including ovarian cancer, breast cancer, lung cancer, and endometrial cancer. Increased HOXB9 expression in lung adenocarcinoma patients also predicts poor outcome [11]. By contrast, HOXB9 downregulation is also related to a poor overall survival in patients with colon adenocarcinoma and gastric carcinoma [7,14,21]. These contradictory findings indicated the diverse role of HOXB9 and the complexity of HOXB9-regulated cancer progression. The results of this work showed that the post-translational modification of HOXB9 accounted for its functional diversity, that is, acetylated HOXB9 functions only opposite to that of HOXB9 in PDAC.

As previously reported, HOXB9 acetylation in K27 inhibits HOXB9 function in promoting lung cancer cell migration and tumor growth by downregulating JMJD6 and EZH2. In PDAC, AcK27-HOXB9 functioned as a tumor suppressor. High AcK27-HOXB9 level predicted a favorable outcome in patients with PDAC. These findings suggested that AcK27-HOXB9 can be used as a universal tumor suppressor.

We hypothesized a dynamic balance between HOXB9 and AcK27-HOXB9. HOXB9 acetylation may represent an important control for HOXB9 transcriptional activation and precisely regulate target gene expression both in physiologic and pathological processes. Hence, HOXB9 promotes or suppresses tumor progression depending upon the acetylation level, which requires further confirmation. High AcK27-HOXB9 level counteracted the tumor promotion effect of HOXB9. The ratio of HOXB9 and AcK27-HOXB9 levels may be pivotal in predicting patient outcomes. Therefore, HOXB9 is a potential valuable marker in predicting the outcome of multiple cancer types.

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Compliance with ethics guidelines

Xiaoran Sun, Jiagui Song, Jing Zhang, Jun Zhan, Weigang Fang, and Hongquan Zhang declare that they have no conflicts of interest. All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and the *Helsinki Declaration* of 1975, as revised in 2000

(5). Informed consent was obtained from all patients that were included in the study.

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