

High expression of PYK2 is associated with poor prognosis and cancer progression in early-stage cervical carcinoma

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Abstract

Proline-rich tyrosine kinase-2 (PYK2), also known as calcium dependent tyrosine kinase, regulates different signal transduction cascades that control cell proliferation, migration, and invasion. However, the role of PYK2 in cervical cancer remains to be elucidated. The current study retrospectively included 134 patients with cervical cancer from December 2007 to September 2014. PYK2 expression was detected in tissue microarray and cervical cancer cell lines. Statistical analysis was performed to evaluate its clinicopathological significance. Small interfering RNA (siRNA) was employed to suppress endogenous PYK2 expression in cervical cancer cells to observe the biological function. PYK2 expression was up-regulated in cervical cancer specimens compared with paired adjacent normal cervical tissue samples. Statistical analyses indicated that PYK2 expression might be an independent prognostic indicator for patients with early-stage cervical cancer. A nomogram model was constructed based on PYK2 expression and other clinicopathological risk factors, and it performed well in predicting patients survival. In cellular studies, down-regulation of PYK2 remarkably inhibited cellular proliferation, migration and invasion. PYK2 expression possessed the potential to serve as a novel prognostic marker in cervical cancer patients.

Abbreviations: DFS = disease free survival, FIGO = international federation of gynecology and obstetrics, IHC = immunohistochemistry, OS = overall survival, PYK2 = proline-rich tyrosine kinase-2, siRNA = small interfering RNA.

Keywords: biomarker, early-stage cervical carcinoma, nomogram, prognosis, PYK2

1. Introduction

Cervical cancer is the most frequent gynecological cancer and the fourth leading cause of globally cancer-related mortality among women, especially in developing countries.^[1] As known, persistent infection of high-risk oncogenic types of human papillomavirus is the primary cause of cervical cancer. However, only a small percentage of patients infected develop into invasive cancer, indicating the carcinogenic effects of other factors.^[2] Although clinical factors, such as stage, squamous cell carcinoma antigen, lymph node metastasis, may serve as prognostic markers, more clinical trials are needed to confirm and validate the clinical sensitivity.^[3–5] Therefore, it is crucial to obtain a better understanding of the underlying molecular mechanism of cervical cancer development and progression, and novel biomarkers are required to investigate, which would provide reliable predictors as well as potential targets for cervical cancer.

Proline-rich tyrosine kinase-2 (PYK2) is a non-receptor tyrosine kinase, which belongs to the focal adhesion kinase family and regulates cell adhesion, morphology, migration and invasion via different signal transduction cascades.^[6–8] PYK2 consists of 3 domains associated with tumor progression and can be activated by multiple growth factors, neuropeptides, cytokines, hormones and chemokines.^[9–12] Overexpression of PYK2 has been reported to promote progression and lead to poor prognosis in many kinds of malignant tumors such as breast cancer, liver cancer, lung cancer and pancreatic cancer.^[7,13–17] PYK2 was only found to serve as an onco-suppressor gene in androgen-dependent prostate cancer cells and neuroblastoma cells so far.^[18,19] However, the role of PYK2 in cervical cancer is still unclear.

In our study, we evaluated the role of PYK2 in early-stage cervical carcinoma and found that the high expression of PYK2 is associated with poor clinical outcomes in cervical cancer patients. Furthermore, we gained insight into the important functions of this protein in cervical cancer lines.

CZ and XZ contributed equally to this work.

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2. Materials and Methods

2.1. Patients and clinical samples

The study was composed of 268 samples from 134 cervical cancer patients, who treated with radical hysterectomy plus pelvic lymphadenectomy without neoadjuvant chemotherapy or preoperative radiotherapy at Affiliated Tumor Hospital of Nantong University from December 2007 to September 2014. All patients had complete clinical and follow-up data. Patients who received preoperative neoadjuvant treatment, had other malignant tumors, or with missing follow-up or clinical data were excluded from the study. Tissue microarray was prepared with the 134 cervical cancer tissues and adjacent normal cervical tissue samples. The study was performed with the approval of the Ethics Committee of Affiliated Tumor Hospital of Nantong University and complied with the Helsinki Declaration. Informed consent was waived by the committee because of the retrospective nature of the study.

2.2. Data collection

We retrospectively collected clinical data such as demographic information, FIGO (International Federation of Gynecology and Obstetrics) stage, maximal tumor size, treatment programs and histopathological parameters for analysis. All patients were followed up every 3 months for the first 2 years, every 6 months for the next 5 years, and once per year thereafter. The last follow-up was July 9, 2019. The overall survival (OS) time was defined as the period from the initial treatment to the last contact or death. The disease free survival (DFS) time was defined as the time from the initial treatment to recurrence or death.

2.3. Cell culture and transfection

Hela cells was obtained from Medical College of Nantong University and Siha cells was purchased from the Cell bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Siha cells were cultured in minimum essential medium (HyClone Laboratories, Logan, UT) and Hela cells were cultured in DMEM medium (Biological Industries, Israel), which were all supplemented with 10% fetal bovine serum (Biological Industries, Israel) and 1% antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) in a 5% CO₂-humidified atmosphere at 37°C.

To silence endogenous PYK2 expression, the following PYK2 small interfering RNA (siRNA) were synthesized by GenePharma (Shanghai, China) with the sequence of 5'-GAUGUGGUCCUGAAUCGUATT-3'. A non-silencing siRNA oligonucleotide that does not recognize any known mammalian gene homolog (GenePharma) was used as control. Hela and Siha cells were transfected with PYK2 siRNA or control siRNA using LipoMaxTM transfection reagent (SUDGEN) according to the manufacturer's procedure. The transfection efficiencies were tested by Western blot analysis.

2.4. Immunohistochemistry (IHC) staining and evaluation

The expression of PYK2 was determined using IHC staining of tissue microarrays. A standard immunostaining procedure was performed using Rabbit molyclonal anti-PYK2 antibody (1:100 dilution; ab32571, Abcam, Cambridge, MA) at 4°C overnight. Two independent pathologists blind to the clinical or pathologic data assessed the IHC score. The staining intensity was scored according to 4 grades: 0 (No staining), 1 (weak), 2 (moderate), or 3 (intense). The percentage of tumor cells stained was in the scoring as follows: 1, 1% to 25%; 2, 26% to 50%; 3, 51% to 75%; or 4, 76% to 100%. The overall IHC score was finally obtained by multiplying the intensity score and percentage

scores (ranged from 0 to 12). The samples were considered to be high expression if the score was > 4, and defined as low expression if not.

2.5. Western blotting

Samples from cell lysates or tissues lysates were prepared using cold RIPA buffer and separated by sodium dodecyl sulfate-polyacrylamide. Then proteins were transferred onto polyvinylidene difluoride membranes and incubated with primary antibodies including anti-PYK2 (1:2000 dilution; ab32571, Abcam, Cambridge, MA), anti-GAPDH (1:5000 dilution; Santa Cruz, CA).

2.6. Cell proliferation assay

For CCK8 assay, Hela and Siha cells were seeded into 96-well plates at density of 3000 cells per well. At the specified time point, 10 μ L CCK8 solution was added and incubated for 2 hours. Cell viability was determined using enzyme-labeled instrument to measure absorbance at 450 nm.

2.7. Transwell assay

A total of 2×10^4 Hela and Siha cells were seeded into the upper part of a transwell chamber with a pore size of 8 μ m (BD, Franklin Lakes, NJ). For the invasion assay, the upper chamber was coated with 60 μ L prechilled Matrigel. The bottom chambers were filled with 0.5 mL of medium supplemented with 10% FBS and incubated for 24 hours. The transmigrated cells were fixed with 100% methanol and stained with 0.1% crystal violet for 15 minutes. Finally, the number of migrated and invading cells on the lower side of the membrane was counted under a light microscope.

2.8. Wound healing assay

Cells were inoculated into 6-well plates and grown in complete medium until 90% to 100% confluency. A 5 mm-diameter wound was made across the cells using a sterile plastic tip. The complete medium was replaced by serum-free medium then. The migration of the cells was observed under a microscope at 0, 24, and 48 hours, respectively.

2.9. Statistical analysis

The relationship between PYK2 expression and the clinicopathological characteristics was assessed using the Chi-squared test or Fisher's exact test. OS and DFS were calculated using the Kaplan–Meier method and were compared using the log-rank test. Univariate and multivariate Cox proportional hazards regression models were performed to identify the independent prognostic factors. Results were reported as hazard ratio and 95% confidence interval. A 2-sided $P < .05$ was considered to indicate statistical significance. All statistical analysis were obtained by using IBM SPSS software (Statistical Package for the Social Sciences; Version 25.0). Nomograms and calibration plots for OS and DFS based on multivariable analysis were established with R software (version 3.6.1).

3. Results

3.1. PYK2 expression with clinicopathological features

PYK2 was mainly localized in the cytoplasm of cervical cancer cells as shown in (Fig. 1). Negative or low PYK2 protein expression was detected in adjacent normal cervical tissue samples. Among the 134 cervical cancer specimens, 62 samples (46.3%) showed high PYK2 protein expression and low expression of

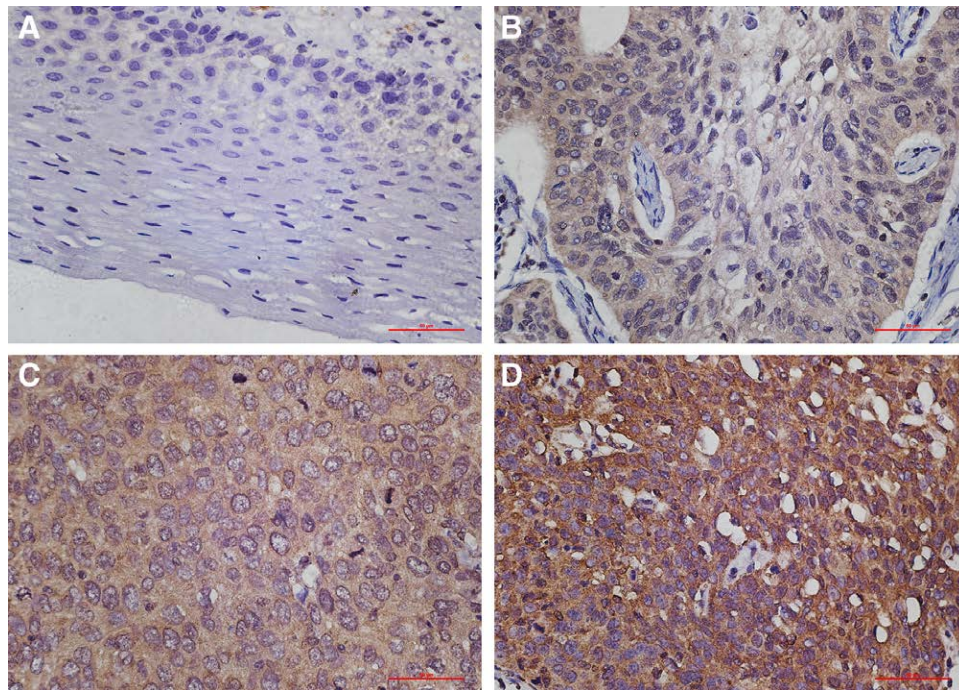


Figure 1. Representative images from immunohistochemistry analysis of PYK2 expression in cervical cancer tissues and adjacent noncancerous tissues (ANT). Positive PYK2 staining was observed mainly in cervical cancer cell cytoplasm (original magnification $\times 400$). (a) Negative PYK2 staining in ANT. (b) Weak PYK2 staining in cervical cancer tissues. (c) Moderate PYK2 staining in cervical cancer tissues. (d) Strong PYK2 staining in cervical cancer tissues. PYK2 = proline-rich tyrosine kinase-2.

Table 1

Association between PYK2 expression and clinicopathological variables in cervical cancer patients.

Variables	Total (n = 134)	PYK2		P value
		Low expression (%)	High expression (%)	
Age				
≤ 52 yrs	64 (47.8%)	31 (48.4%)	33 (51.6%)	.240
> 52 yrs	70 (52.2%)	41 (58.6%)	29 (41.4%)	
FIGO stage				
IB	121 (90.3%)	65 (53.7%)	56 (46.3%)	.993
IIA	13 (9.70%)	7 (53.8%)	6 (46.2%)	
Pathological type				
SCC	115 (85.8%)	61 (53.0%)	54 (47.0%)	.694
No-SCC	19 (14.2%)	11 (57.9%)	8 (42.1%)	
Tumor size				
≤ 2 cm	33 (24.6%)	15 (45.5%)	18 (54.5%)	.272
> 2 cm	101 (75.4%)	57 (56.4%)	44 (43.6%)	
Histologic grade				
I-II	110 (80.3%)	64 (58.2%)	46 (41.8%)	.027*
III	24 (17.9%)	8 (33.3%)	16 (66.7%)	
Depth of invasion				
$< 2/3$	99 (73.9%)	51 (51.5%)	48 (48.5%)	.387
$\geq 2/3$	35 (26.1%)	21 (60.0%)	14 (40.0%)	
LVI				
No	113 (84.3%)	60 (53.1%)	53 (46.9%)	.733
Yes	21 (15.7%)	12 (57.1%)	9 (42.9%)	
LNM				
No	110 (82.1%)	57 (51.8%)	53 (48.2%)	.342
Yes	24 (17.9%)	15 (62.5%)	9 (37.5%)	
Adjuvant therapy				
No	49 (36.6%)	23 (46.9%)	26 (53.1%)	.278
Chemoradiotherapy	42 (31.3%)	21 (50.0%)	21 (50.0%)	
Chemotherapy	39 (29.1%)	26 (66.7%)	13 (33.3%)	
Radiotherapy	4 (3.0%)	2 (50.0%)	2 (50.0%)	

Notes: Chi-squared test was employed for categorical variables.

* $P < .05$.

FIGO = international federation of gynecology and obstetrics, LNM = lymph node metastasis, LVI = lympho-vascular space invasion, PYK2 = proline-rich tyrosine kinase-2, SCC = squamous cell carcinoma.

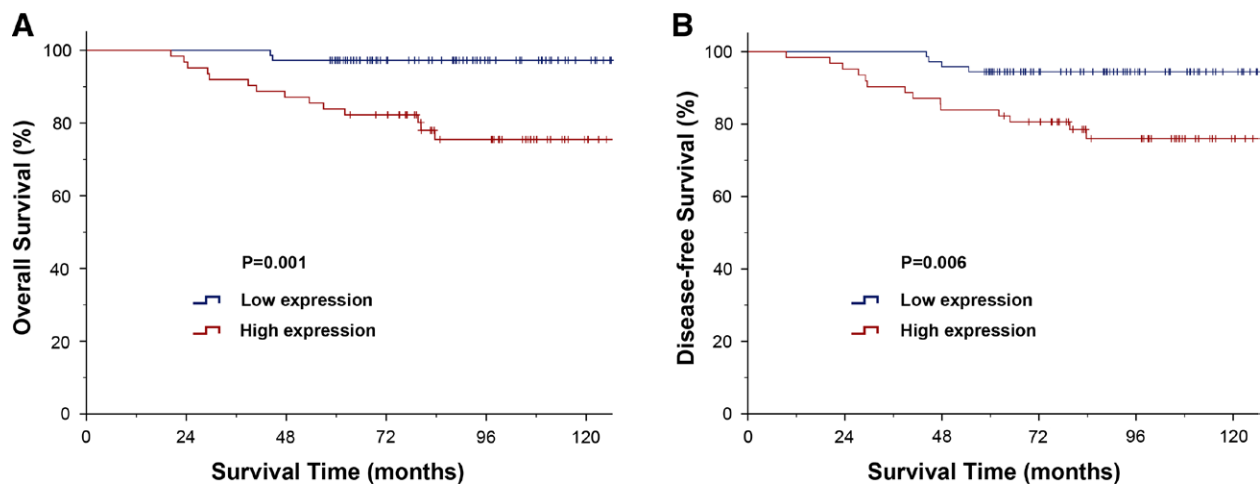


Figure 2. Kaplan–Meier survival curves for overall survival (a) and disease-free survival (b) according to PYK2 expression levels. *P* values were determined using the log-rank test. PYK2 = proline-rich tyrosine kinase-2.

PYK2 was observed in 72 tumor samples. The clinicopathologic analysis exhibited that PYK2 expression was strongly correlated with differentiation grade ($P = .027$). However, PYK2 expression did not correlate with other clinical features, including age, FIGO stage, pathological type, tumor size, depth of invasion, lympho-vascular space invasion, lymph node metastasis and adjuvant therapy (Table 1).

3.2. Prognostic significance of PYK2 expression in early-stage cervical patient

The median follow-up was 87.5 (20.3–141.1) months. The survival time was markedly different between the 2 groups based on the log-rank test. Kaplan–Meier analysis demonstrated that patients in the high-PYK2 group exhibited significantly shorter OS ($P = .001$) and DFS ($P = .004$) than those in the low-PYK2 group (Fig. 2).

Table 2

Univariate and multivariate of survival for cervical cancer patients by Cox proportional hazards model.

Variables	Overall survival				Disease-free survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95%CI)	<i>P</i> value	HR (95%CI)	<i>P</i> value	HR (95%CI)	<i>P</i> value	HR (95%CI)	<i>P</i> value
Age								
≤52 yrs	1	.919			1	.729		
>52 yrs	0.950 (0.357-2.532)				1.179 (0.465-2.986)			
FIGO stage								
IB	1	.205			1	.273		
IIA	2.254 (0.641-7.920)				2.001 (0.579-6.918)			
Pathological type								
SCC	1	.142			1	.242		
No-SCC	2.337 (0.753-7.256)				1.942 (0.639-5.903)			
Tumor size								
≤2 cm	1	.998			1	.785		
>2 cm	0.999 (0.322-3.098)				1.167 (0.384-3.547)			
Histologic grade								
I-II	1	<.001*	1	.001	1	<.001*	1	<.001*
III	7.004 (2.602-18.853)		5.461 (1.939-15.385)		7.018 (2.764-17.815)		5.982 (2.255-15.874)	
Depth of invasion								
<2/3	1	.006*	1	.001*	1	.017*	1	.005*
≥2/3	4.006 (1.490-10.766)		5.430 (1.978-14.907)		3.076 (1.220-7.754)		3.859 (1.515-9.828)	
LVSI								
No	1	.432			1	.144		
Yes	1.423 (0.591-3429)				2.158 (0.768-6.063)			
LNM								
No	1	.027*			1	.062		
Yes	3.138 (1.137-8.662)				2.544 (0.953-6.790)			
Adjuvant therapy								
No	1	.506			1	.392		
Chemoradiotherapy	0.374 (0.078-1.804)				0.328 (0.070-1.543)			
Chemotherapy	1.274 (0.447-3.632)				1.287 (0.483-3.430)			
Radiotherapy	-	-			-	-		
PYK2 expression								
Low	1	.004*	1	.015*	1	.009*	1	.047*
High	8.643 (1.964-38.037)		6.711 (1.457-30.907)		4.371(1.438-13.283)		3.237 (1.017-10.298)	

CI = confidence interval, FIGO = international federation of gynecology and obstetrics, HR = hazard rate, LNM = lymph node metastasis, LVSI = lympho-vascular space invasion, SCC = squamous cell carcinoma. * $P < .05$.

Univariate and multivariate Cox proportional hazard models were performed to identify whether PYK2 protein expression could serve as a prominently independent prognostic factor in cervical cancer (Table 2). Several potential prognosis-related factors were tested, including age, FIGO stage, pathological type, tumor size, histologic grade, depth of invasion, lymphovascular space invasion, lymph node metastasis, adjuvant therapy and PYK2 expression. The result showed that PYK2 expression was indicated as an independent prognostic factor for OS ($P = .015$) and DFS ($P = .047$) eventually. Furthermore, histologic grade and depth of invasion were also significantly associated with OS and DFS.

3.3. Prognostic nomogram for 5-year OS and DFS in patients with cervical cancer

The nomogram was built on the basis of independent prognostic factors obtained from multivariate analysis of OS and DFS, including PYK2 expression, histologic grade and depth of invasion (Fig. 3A and B). The calibration plots for the prognostic accuracy were established and certified high consistencies between nomogram-predicted values and the actual observed outcome (Fig. 3C and D). The Harrell's concordance index (C-index) were 0.849 and 0.799 for 5-year OS and DFS respectively, which were calculated to validate the result of nomogram.

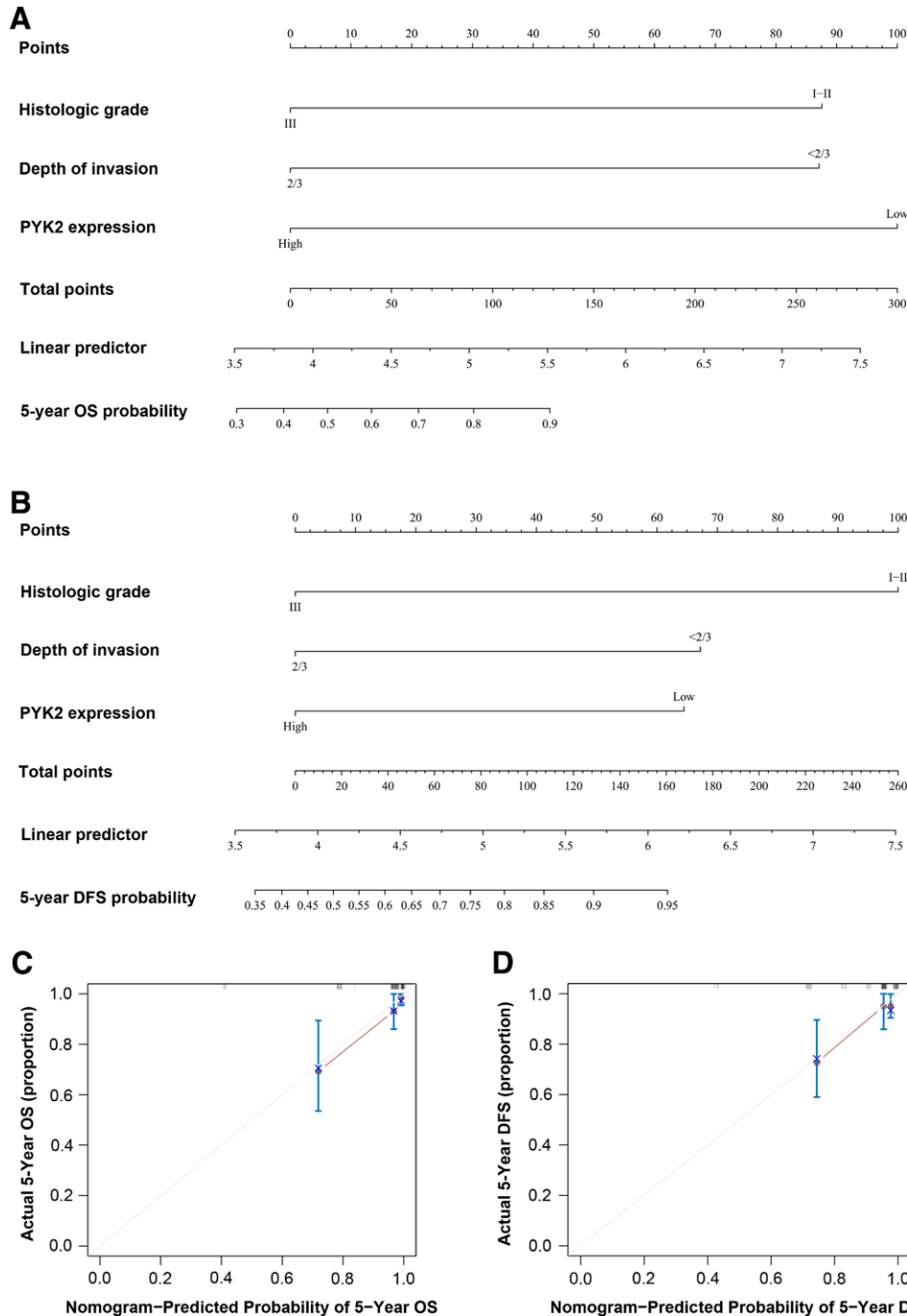


Figure 3. Nomogram and calibration plot for OS and DFS prediction in patients with cervical cancer. (a-b) Three independent prognostic factors were integrated into the nomogram to predict 5-year OS survival. (c-d) The calibration plots for predicting OS and DFS at 5 years. DFS = disease-free survival, OS = overall survival.

3.4. PYK2 downregulation inhibits cervical cancer cell proliferation, invasion and migration ability

We performed cellular studies by knocking down PYK2 in Siha and HeLa cells, respectively. The downregulation efficiency of PYK2 siRNA was verified using western blotting (Fig. 4A). As shown in Figure 4B, the CCK8 assay demonstrated that cells transfected with PYK2 siRNA displayed significantly decreased cell growth rate compared with cells transfected with control siRNA. Additionally, the wound healing assay and transwell assay revealed that co-transfection of PYK2 siRNA attenuated cell migration and invasion ability in vitro (Fig. 4C and D).

4. Discussion

In this study, we elucidated the clinical significance of PYK2 in early-stage cervical cancer patients for the first time. The high expression level of PYK2 was significantly associated with unfavorable outcomes. We built nomogram models for OS and DFS depend on 3 prognostic factors, which includes PYK2 expression. Furthermore, by using loss-of-function approaches, we found that PYK2 downregulation hampered the cellular capabilities of proliferation, migration and invasion in an obvious manner.

Our study showed that differentiation grade was positively correlated with PYK2 expression, indicating a probability that tumor with higher expression of PYK2 was associated with a greater progressive and invasive risk. PYK2 is found to involved in the biological processes of tumorigenesis, tumor development

and invasion via several processes.^[20,21] In hepatocellular carcinoma, PYK2 can promote cancer progression by activating PI3K/AKT signaling pathways.^[14] It was reported that PYK2 can stabilize TAZ protein and negatively regulate the Hippo pathway to promote cell growth and prevent apoptotic cell death in triple-negative breast cancer.^[13] Additionally, overexpressed PYK2 is uncovered to modulate Wnt/b-catenin pathway by phosphorylating GSK3b^{Y216}, which initiates and reinforces intestinal tumorigenesis.^[22] PYK2 was also found to take part in metastasis or recurrence in different tumors. As reported in breast cancer, a signaling network between EGFR, c-Met, PYK2 and STAT3 can potentiate EMT and contribute to cancer metastasis.^[7] Moreover, miR-23b may regulate migration by targeting PYK2 via regulation of EMT in hepatocellular carcinoma.^[23] Nevertheless, no significant correlation was found between PYK2 expression and lymph node metastasis in this study with a possibility that the samples included were all relatively early-stage patients with a small probability of lymph node metastasis.

As a novel and high-value therapy for cancer, molecule inhibitors of PYK2 obtain continuous attention recent years. PF-00562271 is a widely-concerned PYK2 inhibitor and has been reported to produce robust anti-tumor effects in different cancer cells and xenograft tumor models, such as pancreatic cancer, prostate cancer, bone tumors and hepatocellular carcinoma.^[24-27] As a second-generation inhibitor of PYK2, VS-6063 was rationally designed to have less potential for drug-drug interaction than PF-00562271.^[28] Both PF-00562271 and VS-6063 were well-tolerated in patients in Phase 1 studies and

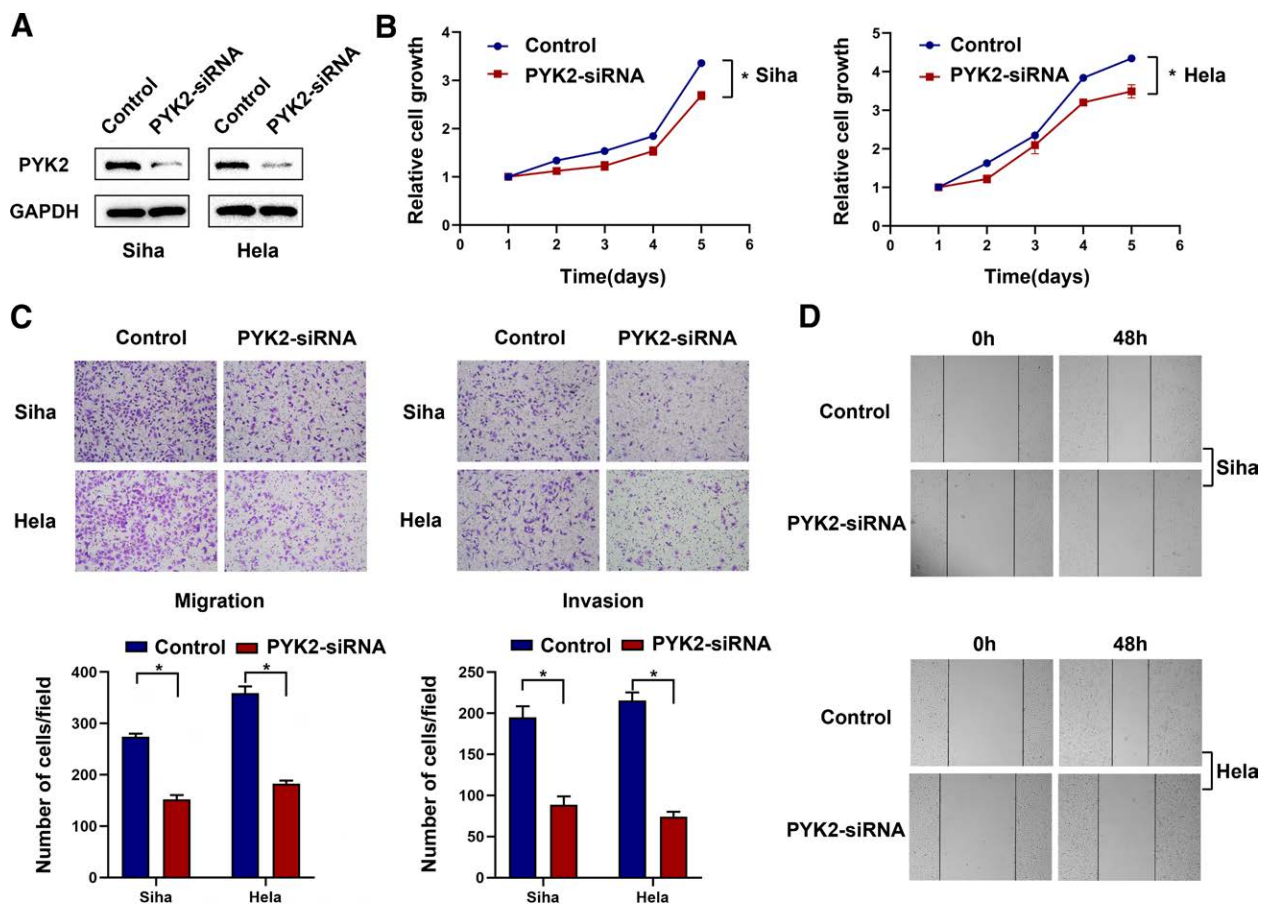


Figure 4. Down-regulation of PYK2 repressed cellular proliferation, motility, and invasion. (a) Western blot analysis of PYK2 expression in SiHa and HeLa cells transfected with siRNA. (b) CCK8 assay showed that PYK2 knockdown suppressed proliferation abilities. (c-d) The migration and invasion abilities were inhibited in PYK2-knockdown cells, which were evaluated by transwell assays, matrigel invasion assays and wound healing assays. Error bars represent the mean \pm SD values of 3 independent experiments. * $P < .05$. PYK2 = proline-rich tyrosine kinase-2, si, small interfering.

need further investigation as promising therapeutic drugs.^[29,30] We revealed the tumor-promoting role of PYK2 in cervical cancer and further exploration of PYK2 inhibitors are needed to confirm the effect of it on cervical cancer cells. Furthermore, we constructed a prognostic nomogram model to predict the survival rates by integrating PYK2 expression and other clinicopathological prognostic factors. The Harrell's concordance index indicated a good prognostic ability of the model, which makes it available for clinical use.

However, our study had several limitations. Firstly, the clinical data analysis were conducted on a limited cohort size. Secondly, the patients involved were all in the early-stage of cervical cancer with relatively favorable prognosis. Therefore, a larger cohort of patients with high-risk pathological factors are needed in the subsequent study. Thirdly, the underlying molecular mechanisms and signaling pathways of PYK2 involved in the development and progression of cervical cancer remain unknown, which needs further exploration.

5. Conclusion

In summary, our study identified that PYK2 was overexpressed in cervical cancer and is associated with its progression. PYK2 may represent a prognostic indicator of cervical cancer, which can identify patients at high-risk and serve as a relevant biomarker for predicting the patient outcome. However, further investigation is needed to verify the possibility of applying PYK2 as a potential therapeutic target in clinical practice.

Author contributions

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