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Hepatocyte Nuclear Factor -1α stimulates cervical cancer cells to migrate and invade through regulating pyruvate kinase L/R.

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Key words: cervical cancer; hepatocyte nuclear factor -1α (HNF-1α); pyruvate kinase L/R(PKLR).

Abstract. This study was aimed to analyze the role of hepatocyte nuclear factor -1α (HNF-1α) in regulating migrative and invasive potentials in cervical cancer via the involvement of pyruvate kinase L/R (PKLR). The expression of HNF-1α and PKLR in cervical cancer tissues classified by tumor size and FIGO (Federation International of Gynecology and Obstetrics) stage were detected by qRT-PCR. The expression correlation between HNF-1α and PKLR in cervical cancer tissues was analyzed by Pearson correlation test. After intervening HNF-1α and PKLR levels in SiHa and Hela cells, their migratory and invasive abilities were examined by the Transwell assay. HNF-1α was upregulated in cervical cancer tissues, particularly those with large tumor size or advanced FIGO stage. PKLR was highly expressed in cervical cancer tissues as well, presenting a positive correlation with the HNF-1α level. Knockdown of HNF-1α attenuated migratory and invasive abilities in SiHa cells, whereas overexpression of HNF-1α enhanced migratory and invasive abilities in SiHa cells. PKLR was able to abolish the regulatory effects of HNF-1α on cervical cancer metastasis. HNF-1α and PKLR synergistically promote cervical cancer to migrate and invade.

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El factor nuclear del hepatocito-1α estimula las células de cáncer cervical para migrar e invadir a través de la regulación de la piruvato cinasa L/R.

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Palabras clave: cáncer de cuello uterino; factor nuclear del hepatocito -1α (HNF-1α); piruvato quinasa L/R (PKLR).

Resumen. Este estudio tuvo como objetivo analizar el papel del factor nuclear del hepatocito -1α (HNF-1α) en la regulación de los potenciales migratorios e invasivos en el cáncer de cuello uterino a través de la participación de la piruvato quinasa L/R (PKLR). Se detectó mediante qRT-PCR, la expresión de HNF-1α y PKLR en tejidos de cáncer de cuello uterino, clasificados de acuerdo al tamaño del tumor y su estadio FIGO (Federación Internacional de Ginecología y Obstetricia). La correlación de expresión entre HNF-1α y PKLR en tejidos de cáncer de cuello uterino se analizó mediante la prueba de correlación de Pearson. Después de intervenir los niveles de HNF-1α y PKLR en las células SiHa y Hela, se examinaron las capacidades migratorias e invasivas mediante el ensayo Transwell. HNF-1α se reguló positivamente en tejidos de cáncer de cuello uterino, en particular aquellos con tumores de gran tamaño o estadio FIGO avanzado. PKLR también se expresó en gran medida en los tejidos del cáncer de cuello uterino, presentando una correlación positiva con el nivel de HNF-1α. La eliminación de HNF-1α atenuó las capacidades migratorias e invasivas en las células SiHa, mientras que la sobreexpresión de HNF-1α aumentó las capacidades migratorias e invasivas en las células SiHa. La PKLR pudo abolir los efectos reguladores del HNF-1α sobre la metástasis del cáncer de cuello uterino. HNF-1α y PKLR promueven sinérgicamente la invasión y migración del cáncer de cuello.

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INTRODUCTION

Cervical cancer is a common malignancy of the female reproductive system (1). It is reported that in 2021, the diagnostic rate and mortality rate of cervical cancer in our country was 16.25/100,000, and 5.08/100,000, respectively (2). It is necessary to deeply understand the molecular mechanisms of cervical cancer. Seeking for effective cervical cancer biomarkers is conductive to stop the malignant phenotypes of cancer cells.

Hepatocyte Nuclear Factors (HNFs) are transcription factors predominately expressed in the liver, including HNF-1, 3, 4, 6, CCAAT / enhancer binding-proteins and D-binding proteins. HNF-1 contains two members, that is, HNF-1α (also known as LFB1/TCF1) and HNF-1β. HNF-1α is a transcription factor containing a variant homology domain. It is located on human chromosome 12q24.2, and expressed in liver, kidney, intestinal and pancreatic tissues. HNF-1α is able to regulate expressions of multiple tissue-specific genes (3). A previous study has identified the close relationship between HNF-1α and hepatocellular carcinoma (HCC) progression, and HNF-1α is capable of maintaining hepatocyte differentiation phenotypes (3,4). In addition, HNF-1α mutation causes

maturity onset diabetes of the young type 3 (MODY3) (5). Some low-frequency mutations in HNF-1α (e.g. a single rare missense mutation) are associated with type 2 diabetes (6,7). The potential influence of HNF-1α on cervical cancer is rarely reported.

Pyruvate Kinase L/R (PKLR) is located on human chromosome 1q22, encoding pyruvate kinases (PKs). PKs are involved in anaerobic glycolysis and provide 50% of ATP to mature red blood cells. Dysfunctional PKLR can lead to PKs deficiency (PKD) and ATP deficiency, shortening the lifespan of red blood cells (8). The Warburg effect (aerobic glycolysis) is a well-defined metabolic change that is relevant to cancerous phenotypes, including the accelerated proliferative, invasive and migratory potentials (9). Nie *et al.* (10) pointed out that mineralocorticoid receptors (MRs) are able to alleviate the Warburg effect and cancer progression in HCC by targeting the miR-338-3p/PKLR axis. Therefore, PKLR is considered as a key regulator of glycolytic reprogramming and tumor cell functions. A recent research showed that HNF-1α drives the growth and anti-apoptosis capacity in pancreatic cancer via its target gene PKLR (11). This study aims to elucidate the role of HNF-1α and PKLR in the progression of cervical cancer, and to provide a novel guidance in clinical treatment.

MATERIALS AND METHODS

Collection of pathological tissues

A total of 50 cervical cancer tissues, confirmed by pathological examination and resected by surgical procedures, were collected. During the same period, 50 adjacent normal tissues that were 2 cm away from the cancer focus were collected as well. Tissues were immediately frozen in liquid nitrogen for RNA extraction. All patients did not receive neoadjuvant chemotherapy or radiotherapy before operation, and there was no surgical contraindication. The study was approved by the Ethics Committee of Hubei Provincial Maternal and Child Health Hospital, and informed consent was signed by patients and their families before operation.

Cell culture

HcerEpic, Hela and SiHa cell lines were provided by Fudan University (Shanghai, China). Cells were cultivated in Dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in a humidified incubator with 5% CO_2 at 37°C. For cell culture passages, cells were isolated and purified by pancreatin digestion and then were cultured and subculfured in DMEM with 20 % fetal bovine serum. Cells in the logarithmic growth phase were used for experiments.

Cell transfection

HNF-1α siRNAs were constructed by Ambion (Austin, TX, USA) based on the HNF-1α sequences in GeneBank (Accession No. NM 000545). Primer sequences were as follows: siHNF-1α-#1 forward primer, 5′-CCGGTGCTAGTGGAGGAGTGCAATTTCAAGAGAATTGCACTCCTCCACTAGCTTTTTG-3′ and reverse primer, 5′-AATTCAAAAAGCTAGTGGAGGAGTGCAATTCTCTTGAAATTGCACTCCTCCACTAGCA-3′; siHNF-1α-#2 forward primer, 5′-CCGGTGCAGAAGTACCCTCAAGCATTCAAGAGATGCTTGAGGG TACTTCTGCTTTTTG-3′ and reverse primer, 5′-AATTCAAAAAGCAGAAGTACCCTCAAGCATCTCTTGAATGCTTGAGGGTACTTCTGCA-3′. Cells were prepared to suspension after 0.25% trypsin digestion, and inoculated in the 6-well plate with 1.0×10^5 cells/well. Once cell confluence reached about 70%, transfection was conducted using Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced at 6-8 h, and medium containing 2 μg/mL puromycin was applied at 48 h. After 72 h cell culture, cells were passaged to a new 6-well plate and cultivated for 1-2 weeks. Visible colonies were picked up and inoculated in a 96-well plate for extended culture. Their growth was regularly observed and passaged to 6-well plates and culture bottles two days later.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) was reversely transcribed to complementary deoxyribose nucleic acid (cDNA) using the First Chain cDNA Synthesis Kit in a system containing 1 μL of Oligo dT Primer, 1 μL of dNTP Mixture, 5 μL of RNA template and 3 μL of ddH_2O. The system was incubated at 65°C for 5 min and immediately frozen on ice. Reverse transcription solution was prepared as follows: 4 μL of 5×Prime Script II Buffer, 0.5 μL of RNase Inhibitor, 1 μL of Prime Script II RTase and 4.5 μL of RNase free ddH_2O. The reaction conditions were 45 min at 42℃, 5 min at 95℃ and cooling on ice. PCR primers were: HNF-1α forward primer: 5′-TCTACAGCCACAAGCCCGAG-3′ and reverse primer: 5′-GAGGTGAAGACCTGCTTGGT-3′; PKLR forward primer: 5′-TGGGAAAACTGGGTGGGATGGATG-3′ and reverse primer: 5′-GAAGGAAGCAGCCGGGGATTTGAC-3′; β-actin forward primer: 5′-TTGGCCTTAGGGTTCAGAGGGG-3′, and reverse primer: 5′-CGTGGGCCGCCCTAGGCACCA-3′. PCR system in a total of 25 μL was then prepared, including 12.5 μL of SYBR Fast qPCR Mix (2×), 1 μL of PCR Forward Primer (10 μmol/L), 1 μL of PCR Reverse Primer (10 μmol/L), 0.5 μL of ROX Reference Dye (50×), 1 μL of cDNA and 8 μL of ddH_2O. The system was amplified at 95°C for 30 s, 40 cycles at 95°C for 5 s and 60°C for 32 s, followed by 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and 60°C for 15 s. The expression level was calculated by $2^{-\Delta\Delta Ct}$.

Western blot

Cells were digested in 0.25% trypsin, washed in phosphate buffered saline (PBS) for three times and lysed in radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China). The isolated protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blockage of non-specific antigens in 5% skim milk for 1 h, membranes were reacted with primary antibodies (1: 1000) at 4°C overnight, and horseradish peroxidase (HRP)-labeled sheep anti-mouse antibody (1:5000) for 1 h. Band exposure and grey value analyses were finally conducted by Image-Pro Plus.

Transwell assay

Until cells were cultured to 75% confluence, serum-free medium was replaced. 100 μL of serum-free suspension (1.0×10^5 cells/mL) and 600 μL of serum-containing medium were applied to the top and bottom Transwell chamber, respectively, and cultured overnight. Cells in the bottom were subjected to methanol fixation for 15 min, and crystal violet staining for 20 min. Migratory cells were counted in five randomly selected fields per sample. Transwell invasion assay was conducted in chambers pre-coated with 50 μL of Matrigel diluted in serum-free medium.

Statistical analysis

Data processing was conducted by Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA). Linear relationship of genes was analyzed by Pearson correlation test. Differences between groups was compared by the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). Percentage (%) was used to express the enumeration data and chi-square test was used for data analysis. A significant difference was set at $p<0.05$.

RESULTS

Correlation between HNF-1α and clinical features in cervical cancer

Based on the median level of HNF-1α expression in recruited cervical cancer patients, they were classified into high HNF-1α level group (n=25) and low HNF-1α level group (n=25), respectively. No significant differences in age and histology were observed be-

tween groups ($p>0.05$). Notably, tumor size, FIGO stage and lymphatic metastasis incidence were significantly different ($p<0.05$) (Table I). It is indicated that HNF-1α was closely linked to tumor size, FIGO grade and lymphatic metastasis in cervical cancer patients, and might be a potential biomarker.

Upregulation of HNF-1α in cervical cancer

Compared with adjacent normal tissues, HNF-1α was upregulated in cervical cancer ones (Fig. 1A). In addition, collected cervical cancer tissues were classified into two groups based on the tumor size (4 cm as the cut-off value). Higher level of HNF-1α was detected in cervical cancer tissues with ≥ 4 cm in tumor size compared with those < 4 cm (Fig. 1B). In particular, we detected higher abundance of HNF-1α in FIGO stage III-IV cervical cancer patients than those FIGO stage I-II patients (Fig. 1C). It is suggested that HNF-1α may function as an oncogene driving the progression of cervical cancer.

HNF-1α overexpression and knockdown models

HNF-1α level was first detected in cervical cancer cell lines (Fig. 2A). Based on the differential level of HNF-1α, we constructed HNF-1α knockdown and overexpression models in SiHa and Hela cells, respectively. Transfection of either si-HNF-1α 1# or si-HNF-1α 2# could effectively decrease HNF-1α level in SiHa cells (Fig. 2B, 2C). Besides, HNF-1α level was markedly upregulated in Hela cells transfected with pcDNA-HNF-1α (Fig. 2D, 2E).

HNF-1α promoted cervical cancer cells to migrate and invade

Cell migration and invasion was a vital process of tumor metastasis. Transwell assay was conducted to assess migratory and

TABLE I
CORRELATION BETWEEN HNF-1A AND CLINICAL FEATURES IN CERVICAL CANCER PATIENTS (N=50).

Clinico-pathologic features	Number of cases	HNF-1α expression		*p*
		Low (n=25)	High (n=25)	
Age (years)				
<50	31	16	15	0.7708
≥50	19	9	10	
Histology				
Squamous	23	12	11	0.7766
Adenocarcinoma	27	13	14	
Tumor size				
<4CM	26	19	7	**0.0007***
≥4CM	24	6	18	
FIGO stage				
I~II	22	16	6	**0.0044***
III~IV	28	9	19	
Lymph node metastasis				
No	26	17	9	**0.0235***
Yes	24	8	16	

The high and low expression was classified based on the median level of HNF-1α expression in recruited cervical cancer patients, *$p<0.05$.

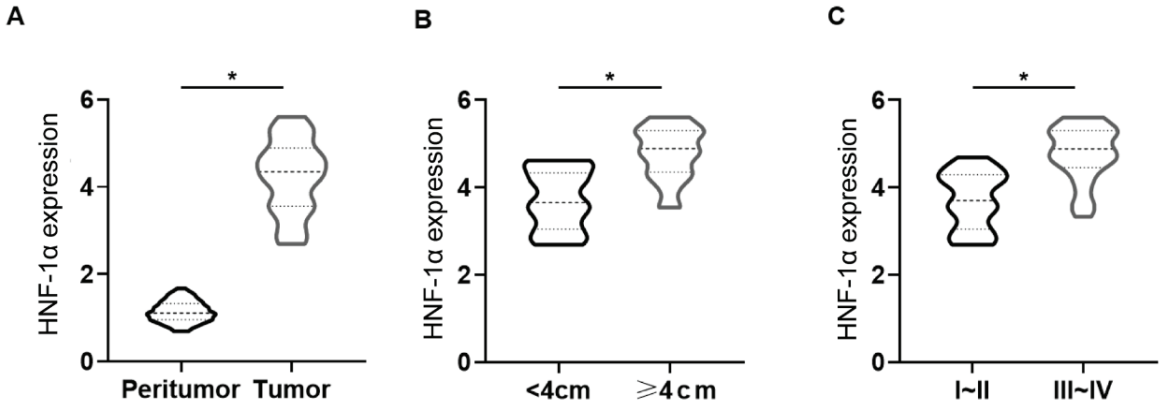

Fig. 1. Upregulation of HNF-1α in cervical cancer tissues. (A) Higher level of HNF-1α was detected in cervical cancer tissues (Tumor) (n=50) than adjacent normal ones (Peritumor) (n=50); (B) Higher level of HNF-1α was detected in cervical cancer tissues larger than 4 cm in tumor size (≥ 4 cm) than those smaller than 4 cm (<4 cm); (C) Higher level of HNF-1α was detected in FIGO grade III-IV(III-IV) cervical cancer patients(III-IV) than those FIGO grade I-II patients(I-II). *$p<0.05$.

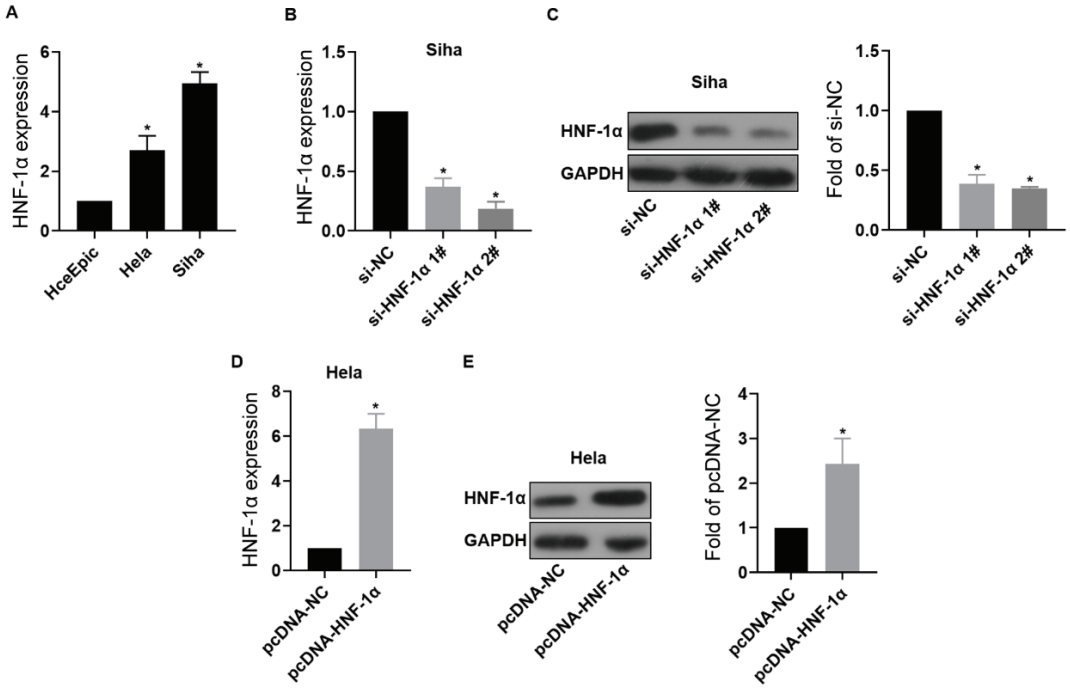

Fig. 2. HNF-1α overexpression and knockdown models. (A) HNF-1α levels in HcerEpic, Hela and SiHa cells detected by qRT-PCR. The comparison was conducted between HcerEpic and Hela, and another comparison was conducted between HcerEpic and SiHa; (B) The mRNA level of HNF-1α was downregulated by transfection of either si-HNF-1α 1# or si-HNF-1α 2#. The comparison was conducted between si-NC and si-HNF-1α 1#, and another comparison was conducted between si-NC and si-HNF-1α 2# (C) The protein level of HNF-1α was downregulated by transfection of either si-HNF-1α 1# or si-HNF-1α 2#; The comparison was conducted between si-NC and si-HNF-1α 1#, and another comparison was conducted between si-NC and si-HNF-1α 2# (D) The mRNA level of HNF-1α was upregulated by transfection of pcDNA-HNF-1α in Hela cells; The comparison was conducted between pcDNA-NC and pcDNA-HNF-1α.(E) The protein level of HNF-1α was upregulated by transfection of pcDNA-HNF-1α in Hela cells. The comparison was conducted between pcDNA-NC and pcDNA-HNF-1α.*$p<0.05$.

invasive abilities. The number of migrated and invading cells assessed the ability of migration and invasion. Knockdown of HNF-1α markedly weakened the migratory and invasive abilities in SiHa cells (Fig. 3A). On the contrary, they were enhanced in Hela cells overexpressing HNF-1α (Fig. 3B).

PKLR abolished the regulatory effects of HNF-1α on cervical cancer metastasis

The expression of PKLR was detected by qRT-PCR and was found upregulated in cervical cancer tissues compared with the normal ones (Fig. 4A). Pearson correlation test identified a positive linear correlation between HNF-1α and PKLR in cervical cancer tissues (r^2=0.4141, Fig. 4B). Interestingly, overexpression of PKLR could reverse the attenuated migratory and invasive abilities in SiHa cells with HNF-1α knockdown (Fig. 4C). It is suggested that PKLR abolished the regulatory effects of HNF-1α on cervical cancer metastasis, which might be a vital process during cervical cancer metastasis.

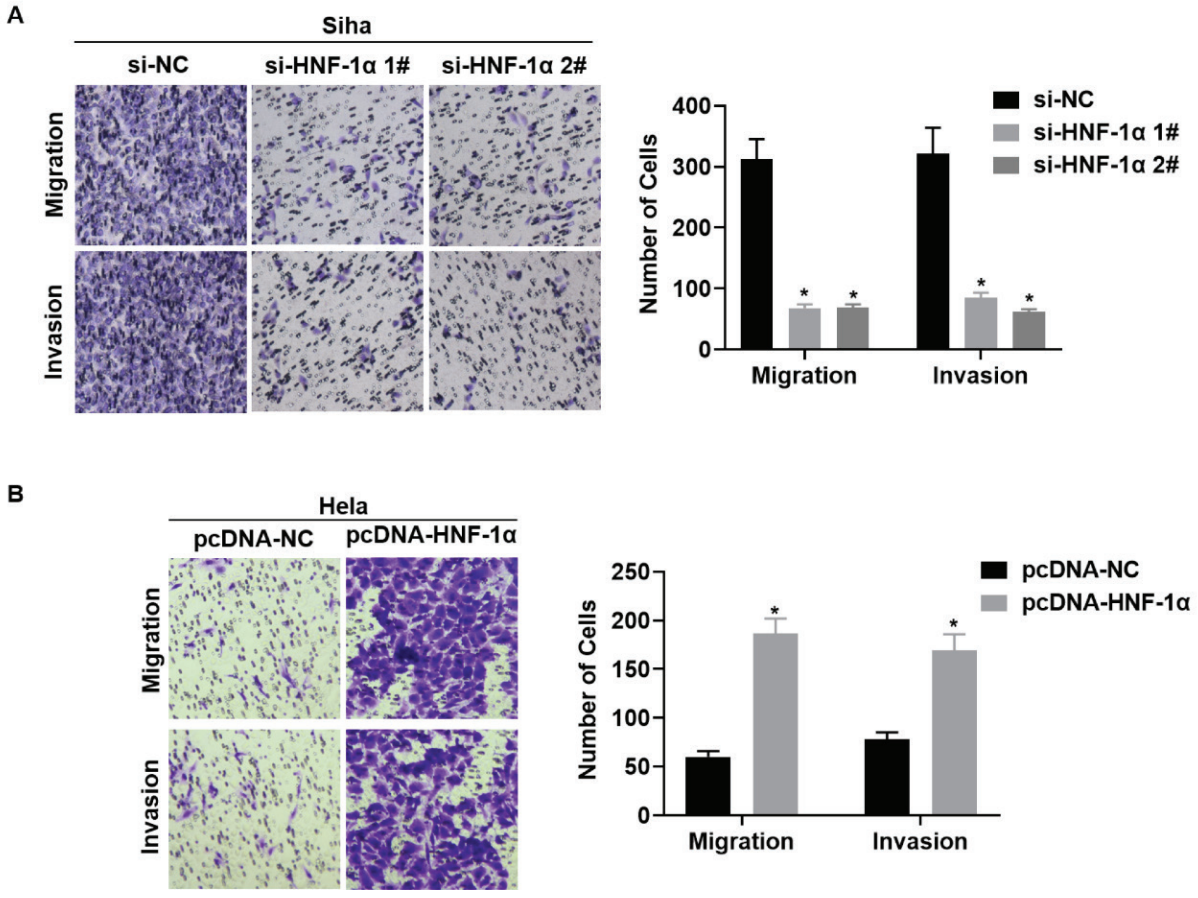

Fig. 3. HNF-1α promoted cervical cancer cells to migrate and invade. (A) Knockdown of HNF-1α weakened migration and invasion in SiHa cells; The comparison was conducted between si-NC and si-HNF-1α 1#, and another comparison was conducted between si-NC and si-HNF-1α 2# (B) Overexpression of HNF-1α promoted migration and invasion in Hela cells. The comparison was conducted between pcDNA-NC and pcDNA-HNF-1α. *$p<0.05$.

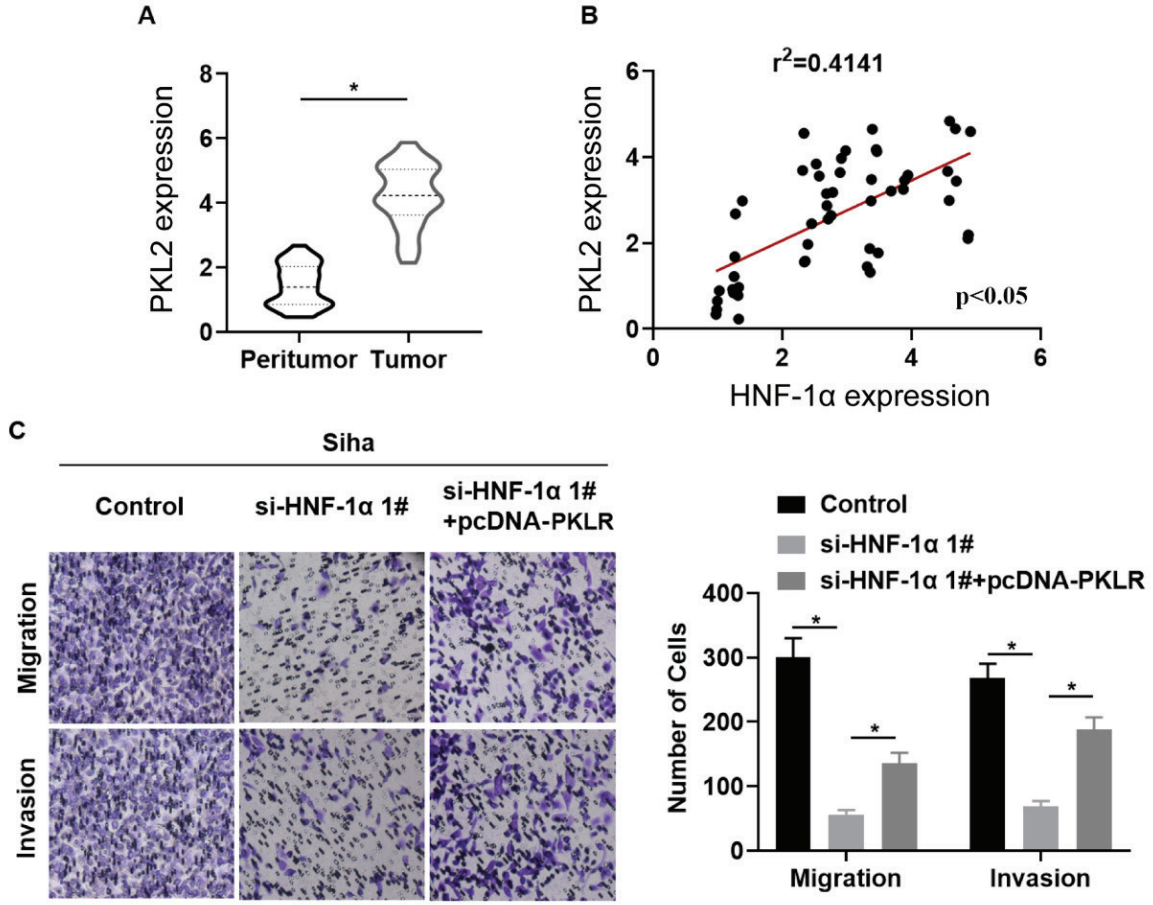

Fig. 4. PKLR abolished the regulatory effects of HNF-1α on cervical cancer metastasis. (A) Higher level of PKLR was detected in cervical cancer tissues (Tumor) (n=50) than adjacent normal ones (Peritumor) (n=50); (B) Pearson correlation test identified a positive linear correlation between HNF-1α and PKLR; (C) Overexpression of PKLR abolished the inhibited migration and invasion in SiHa cells with HNF-1α knockdown. The comparison was conducted between control and si-HNF-1α 1#, and another comparison was conducted between si-HNF-1α 1# and si-HNF-1α 1#+pcDNA-HNF-1α. *$p<0.05$.

DISCUSSION

Cervical cancer is one of the common malignant tumors in gynecology, and its morbidity and mortality have remained high, which seriously threatens the physical and mental health of women worldwide (12). With the continuous advancement of medical technologies, the diagnosis and treatment strategies of cervical cancer have greatly improved (13). However, many patients are diagnosed in advanced stages with distant metastases, which leads to an unsatisfactory prognosis (13). Therefore, it is of great significance to explore the mechanism underlying the occurrence and progression of cervical cancer, and to find targets for the diagnosis and treatment.

Dysfunctional tumor-related genes can affect tumorigenesis and tumor progression (14). Transcription factors regulate the initiation, extension, and termination of gene transcription (15). As a transcription factor, HNF-1α is not only involved in regulating the metabolism and expressions of immune genes (16,17), but

also in the carcinogenesis (18,19). It is reported that HNF-1α is the characteristic of malignant gliomas (20) and childhood T-cell acute lymphoblastic leukemia (21). Hellerbrand *et al.* (22) found that HNF-1α is downregulated in HCC cells and tissues. Overexpression of HNF-1α induces the expression of tumor suppressor MIA2, thus alleviating the malignant growth of HCC. Our findings showed that HNF-1α was upregulated in cervical cancer tissues, and was closely linked to tumor size, FIGO grade and lymphatic metastasis. In cervical cancer cells, HNF-1α was able to stimulate the migratory and invasive abilities, further supporting its carcinogenic role.

As the target gene of HNF-1α, PKLR catalyzes the transphosphorylation of phosphoenolpyruvate to pyruvate and ATP, a rate-limiting step in glycolysis (23). The role of PKLR in tumor progression has been previously reported. PKLR promotes breast cancer progression by regulating glycolytic reprogramming (24). By inducing glutathione synthesis, PKLR triggers liver colonization of colorectal cancer (25). In our experiment, PKLR and HNF-1α level was dysregulated in cervical cancer tissues. Notably, PKLR was able to abolish the regulatory effects of HNF-1α on cervical cancer metastasis, which played a vital role in the malignant progression of cervical cancer. This result may provide a new intervention target for the clinical treatment of cervical cancer metastasis. We can conclude that HNF-1α and PKLR synergistically promote cervical cancer to migrate and invade.

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