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Original Article

LY6K promotes cervical cancer growth, invasion and migration through regulating VEGFA

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Abstract: Purposes: To investigate the effect of proliferation, migration and invasion after knockdown LY6K in cervical cancer HeLa cells, and then clarify the potential mechanism. Methods: The expression of LY6K in cervical cancer tissues was determined by S-P immunohistochemistry. The proliferation, migration and invasion ability were detected after silencing LY6K in cervical cancer HeLa cell lines. The expression of VEGFA, miR-15a and miR-24 was detected after knockdown LY6K in cervical cancer HeLa cells. The expression of LY6K and VEGFA was detected when transfected miR-15a mimics and miR-24 mimics. Then we determined the tumorigenicity of cervical cancer HeLa cells after knockdown LY6K. Results: The expression of LY6K was higher in cervical cancer tissues than normal cervical tissues ($P<0.05$). The proliferation, migration and invasion ability were decreased after silencing LY6K in cervical cancer HeLa cells ($P<0.05$). The expression of VEGFA was decreased after knockdown LY6K; however, the expression of miR-15a and miR-24 was increased after knockdown LY6K ($P<0.05$). The expression of LY6K was decreased after knockdown VEGFA; however, the expression of miR-15a and miR-24 was increased after knockdown VEGFA ($P<0.05$). The expression of LY6K and VEGFA was inhibited when transfected miR-15a and miR-24; however, the expression of LY6K and VEGFA was increased when transfected miR-15a and miR-24 inhibitors ($P<0.05$). Silencing LY6K prevented cervical cancer growth in vivo. Conclusion: Silencing LY6K can inhibited proliferation, migration and invasion ability in cervical HeLa cells by regulation VEGFA.

Keywords: Cervical cancer, VEGFA, LY6K, miR-24, miR-15a

Introduction

Cervical cancer is the second most common cancer in women worldwide and the fourth leading reason of cancer death especially in developing countries [1-3]. The common reason for cervical carcinogenesis was persistent infection of high-risk human papillomavirus (HPV) [4, 5]. Present studies suggest that inflammation play an important role in cervical cancer [6-8]. Therefore, the underlying mechanisms for cervical carcinogenesis and progression are still under investigation.

Ly6k is a relatively new member of the Ly-6-like protein superfamily, which is characterized by the presence of conserved cysteine residues and a putative GPI-anchoring site [9]. It has reported that an increased expression of LY6K in testis, neck squamous cell carcinomas and breast cancers. Ly-6K is regarded as potential target antigen for the diagnosis and therapy of HNSCC [9-11]. Gene expression profile analyses of non-small cell lung carcinomas (NSCLC) and esophageal squamous cell carcinomas (ESCC) revealed that lymphocyte antigen 6 complex locus K (LY6K) was specifically expressed in testis and transactivated in a majority of NSCLCs and ESCCs. Knocked down of LY6K expression resulted in growth suppression of the lung and esophageal cancer cells.

LY6K is regarded as a new type of tumor biomarker and probably as a target for the development of new molecular therapies for cancer treatment [12]. Overexpression of LY6K pro-

motes cell mobility of breast cancer cells [13]. However, the expression and function of LY6K in cervical cancer is still unknown.

In this study, we found an increased expression of LY6K in cervical cancer tissues. Silencing LY6K inhibited the proliferation, invasion and migration in HeLa cells. Mechanic study found that LY6K regulated VEGFA through miR-15a and miR-24. We also found that downregulation of LY6K abolished tumorigenicity of cervical cancer HeLa cells.

Materials and methods

Clinical data

The patients were admitted to our Hospital from January 2012 to January 2015. They were pathologically diagnosed cervical cancer patients. All patients were randomly selected. The study was approved by the Ethics Commission of our Hospital, and informed consents from the patients were obtained. Cervical cancer tissues from 160 patients were harvested. 40 cases of normal cervical tissues were harvested as the control group. The patients were ages 30 to 60 years, with a mean age of 45 years. All patients were confirmed by pathological examination.

Cell culture, transfection procedure and reagents

Human cervical cancer cells were cultured in RPM1 1640 containing 10% FBS and antibiotics in atmosphere of 5% carbon dioxide at 37°C. Lentiviral vector expressing shRNA targeting LY6K (named LV3-sh LY6K) was provided from Genepharma Co., Ltd. (Shanghai, China). miR-15a and miR-24 mimics and inhibitors were synthesized at Ruibo Biotech (Ruibo Biotechnology, Guangzhou, China).

Immunohistochemistry

The tissue slides were deparaffinized in xylene and rehydrated through graded ethanol. Antigen retrieval was performed in 10 mmol/l boiling sodium citrate buffer at pH 6.0 for 15 min by microwave irradiation. The slides were then incubated with 3% hydrogen peroxide (H_2O_2) for 10 min at room temperature. After rinsing, the non-specific binding site was blocked with 10% normal goat serum for 15 min at room temperature. Anti-LY6K Rabbit polyclonal antibody (1:100, ab117536, Abcam Company) was applied to slides in a moist chamber at 4°C overnight. After washing with phosphate-buffered saline (PBS), the slides were incubated with biotinylated secondary antibody for 15 min at room temperature (diluted 1:1,000, cat. no. SP-9002; Zhongshan Golden Bridge Inc., China). The slides were treated with ABC reagent for 15 min at room temperature, and stained with 3,3'-diaminobenzidine (DAB), followed by counterstaining with hematoxylin. All of the sections were observed by three independent pathologists using a light microscope. A total of 22 representative high power fields (40×) were chosen, and the positively-stained cells were counted for each sample. Slides were evaluated independently by 3 pathologists for distribution and intensity of signal as described by De Falco et al [14]. Intensity was scored from 0 to 3:0 (absent immunopositivity); 1 (low immunopositivity); 2 (moderate immunopositivity); 3 (intense immunopositivity). An average of 22 fields was observed for each tissue. All values were represented as the mean ± standard error (mean ± SEM).

The mRNA expression levels of miR-15a, miR-24, VEGFA and LY6K were detected by qPCR

Total RNA was isolated using TRIZOL (Invitrogen). The first-strand cDNA was generated using SuperScipt (Invitrogen). The primers used for amplifying miR-15a, miR-24, VEGFA, LY6K, U6 and GAPDH were synthesized by Guangzhou Funeng Co., Ltd. The real-time PCR kit was purchased from Guangzhou Funeng Co., Ltd. PCR conditions were 95°C for 10 s, 60°C for 20 s, 72°C for 10 s. The experiments were performed in triplicate for every sample.

Protein expression levels of LY6K were detected by Western blotting

Tissues were lysed with a RIPA buffer containing protease inhibitors. Aliquots of the lysates containing 25 μg of total protein were run on a SDS-polyacrylamide gel. The proteins were transferred to a PVDF membrane. The membrane was incubated overnight at 4°C in TTBS containing 5% non-fat milk powder (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). Anti-LY6K goat polyclonal antibody (1:500) or anti-VEGFA rabbit polyclonal antibody (1:500, ab136392, Abcam Company) was incubated

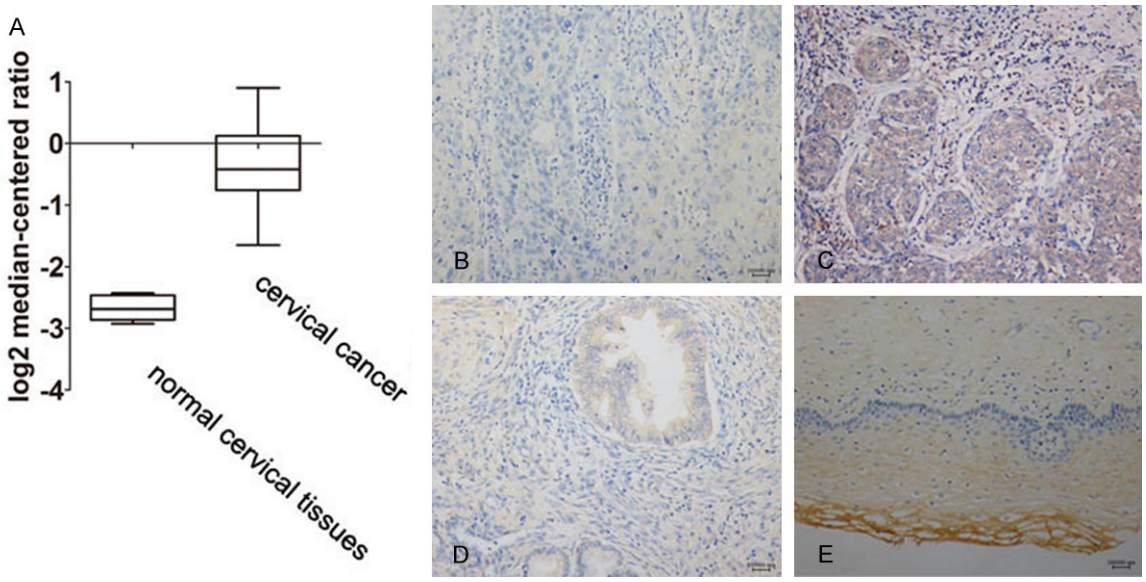

Figure 1. The expression of LY6K in cervical cancer tissues. A. LY6K expression in cervical cancer from oncomine database. B. Cervical squamous carcinoma (stage IA); C. Cervical squamous carcinoma (stage IIIA); D. Cervical adenocarcinoma (stage IB); E. Normal cervical tissues. ×200 magnification.

with the membrane for 2 h at 37°C. Secondary antibodies that were conjugated to horseradish peroxidase were incubated with the membrane for 1 h at 37°C. The proteins that were revealed by western blotting were visualized by chemiluminescence (Biyuntian Company). The densities of bands were analyzed by a gel imaging system and calculated compared to the internal control.

Cell proliferation assay

The proliferation ability was determined by CCK-8 and EdU assay. EdU assay was performed using the Cell-Light TM EdU imaging detecting kit according to the instructions in the kit described by previous study [15]. Cell proliferation was determined using the CCK-8 assay as described previously [16].

Wound healing assay and Matrigel invasion assays

Migration of HeLa cells were analyzed using the wound-healing assay in vitro. Cells were seeded into 6-well plates and cultivated until 90% growth confluence. Wounds were afflicted by scraping the monolayer cells with a sterile pipette tip. At 0 and 48 hours after the wounding, the distance between the 2 wounds were measured at each time point, and expressed as the average percent of wound closure as compared to that at zero time. Invasion of HeLa cells were evaluated by Matrigel invasion assays. Invasive cells on the lower side of the filter, were fixed in 4% paraformaldehyde, stained in 0.5% crystal violet (Beyotime), and counted using a microscope. A total of 5 fields were counted for each transwell filter. Each field was counted and photographed at 200× magnification.

In vivo tumor xenograft study

All procedures for animal experiments were approved by the Committee on the Use and Care on Animals, and performed in accordance with the institution guidelines. Cervical cancer HeLa cells were infected with lentiviral vectors and injected (5×10^6 cells per mouse in 100 μl) subcutaneously into the left armpit of 6-week-old BALB/c nude mice. 28 days later, animals were sacrificed to confirm the presence of tumors and weigh the established tumors.

Statistical analysis

All statistical analyses were performed using SPSS software, version 19.0. Each experiment was performed in triplicates. Statistical analysis was performed by Student's t-test or analysis of variance (ANOVA). The chi-square test was used to compare the associations between LY6K overexpression and clinicopath-

Table 1. Relationships between expression of LY6K and clinical pathology in 160 samples

Characteristics	No. of samples (n=160)	LY6K expression		*P* value
		Low no. (%)	High no. (%)	
Age (years)				
<50	102	65 (63.7)	37 (36.3)	>0.05
≥50	58	34 (58.6)	24 (41.4)	
Normal cervical	40	38 (95)	2 (5)	<0.05
Cancer tissues	160	35 (21.9)	125 (78.1)	
FIGO stage				<0.05
I/II	139	34 (24.4)	105 (75.6)	
III/IV	21	1 (4.8)	20 (95.2)	
Grade				
1	31	16 (51.6)	15 (48.4)	
2	109	10 (9.1)	99 (90.9)	
3	20	9 (45)	11 (55)	
	Grade 2-3	versus 1		<0.05
Tumor type				>0.05
Squamous cell carcinoma	120	20 (16.7)	100 (83.3)	
Adenosquamous carcinoma	32	12 (37.5)	20 (62.5)	
Adenocarcinoma	8	3 (37.5)	5 (62.5)	

ologic variables of cervical cancer samples. Data were presented as Mean ± standard deviation. Statistical significance was defined as a *p*-value less than 0.05.

Results

Increased expression of LY6K in cervical carcinoma tissues

The expression profile of LY6K in cervical cancer is not yet fully elucidated. To determine the relationship between LY6K expression and cervical carcinogenesis we firstly compared the expression of LY6K in normal and cervical carcinoma tissues from the data obtained from Oncomine (https://www.oncomine.org). Gene expression profile of normal and cervical carcinoma tissues showed elevated LY6K mRNA levels in cervical carcinomas than in normal cervical tissues ($P=3.51\times10^{-11}$; **Figure 1A**). Next, we examined the expression pattern of LY6K in normal cervical tissues and cervical cancer tissue samples using IHC. LY6K was predominantly localized on the plasma membrane, and cytoplasm (**Figure 1B-D**). The expression of LY6K was significantly higher in cervical carcinoma samples than that in normal cervical samples (**Figure 1** and **Table 1**).

The expression of LY6K was well-correlated to tumor stage (P<0.05), and histological grades (P<0.05) (**Table 1**). This data indicated that LY6K was associated with cervical carcinoma malignancy and progression.

Knockdown LY6K inhibited cellular proliferation

The CCK-8 and EdU assay showed that downregulation of LY6K using LV3-sh LY6K could inhibit cell proliferation in cervical cancer HeLa cells (P<0.05; **Figure 2A-G**).

Knockdown LY6K inhibited cellular migration and invasion

Migration of cancer cells and invasion of tissues are crucial prerequisites for tumor progression and metastasis. To investigate whether LY6K regulated cellular migration and invasion, we performed a wound healing test and matrigel invasion assay. Our data demonstrated that the migration and invasion abilities of HeLa cells were significantly suppressed when LY6K was silenced by LV3-shLY6K (**Figure 3A**, **3B**).

LY6K regulated VEGFA through miR-15a and miR-24

To elucidate the mechanism of LY6K in cervical cancer, we predicted the potential target molecular using bioinformatics (Starbase). We found that LY6K may regulated VEGFA through miRNAs (miR-125a-3p/1554/15abc/16/195/322/424/497/1907/24/24ab/24-3p/504/4725-5p/590-3p). Our data demonstrated that miR-15a and miR-24 were significantly increased when LY6K was silenced by LV3-shLY6K (**Figure 4A**; P<0.05). We also observed that miR-15a and miR-24 were significantly increased when VEGFA was silenced (**Figure 4B**; P<0.05). The expression of VEGFA was inhibited after silencing LY6K (**Figure 4C**; P<0.05). In turn, the expression of LY6K was inhibited after silencing VEGFA (**Figure 4D**; P<0.05).

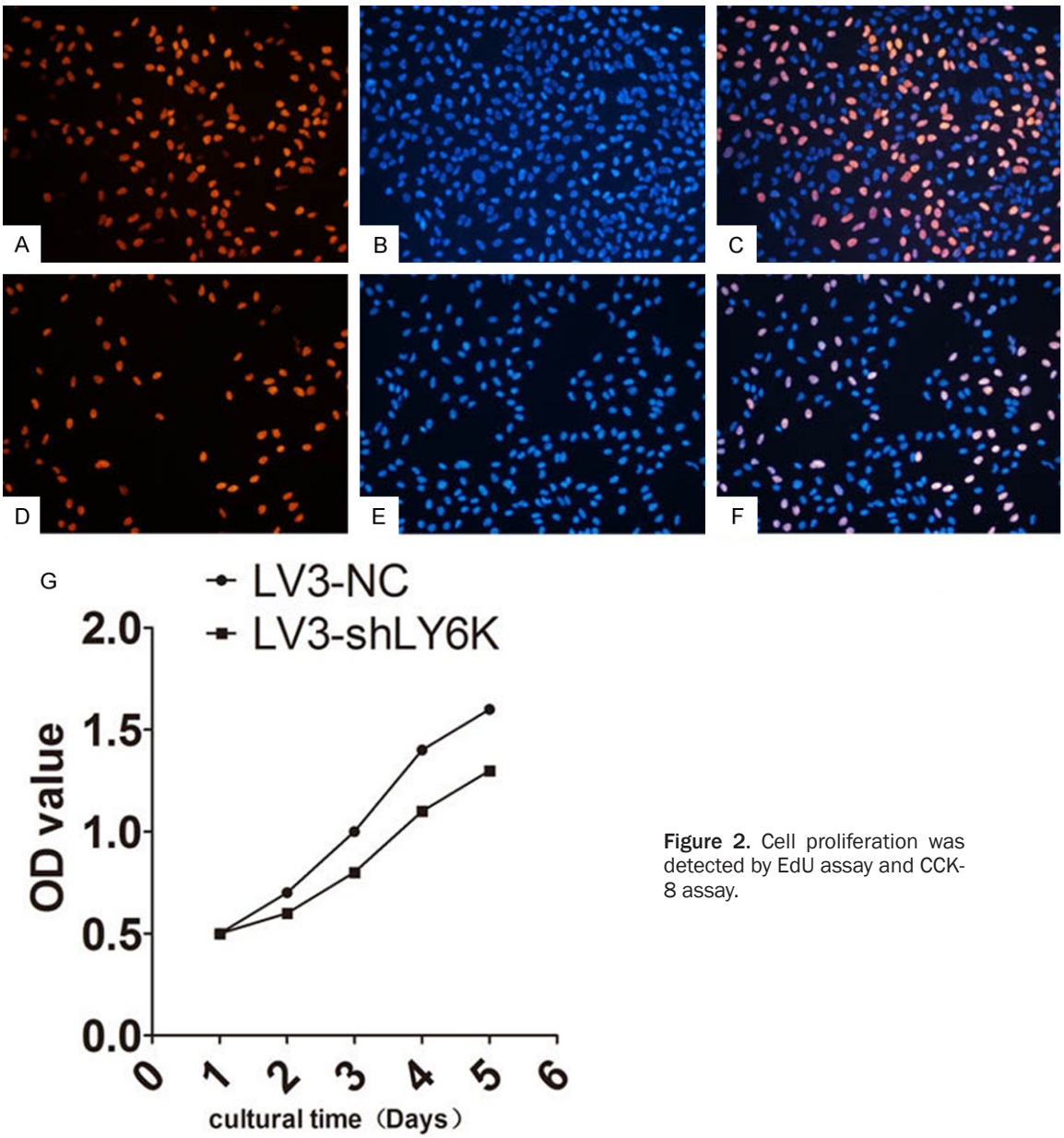

Figure 2. Cell proliferation was detected by EdU assay and CCK-8 assay.

LY6K and VEGFA are direct targets of miR-15a and miR-24

MiR-15a/24 mimics and inhibitors were transfected into cervical cancer Hela cells. The expression of LY6K and VEGFA was decreased when transfection with miR-15a or miR-24 mimics (**Figure 5A**, **5B**; $P<0.05$). In contrary, the expression of LY6K and VEGFA was increased when transfection with miR-15a or miR-24 inhibitors (**Figure 5C**, **5D**; $P<0.05$). Luciferase assays confirmed the existence of specific crosstalk between the LY6K and VEGFA mRNA through competition for miR-15a or miR-24 binding (**Figure 5E**, **5F**).

Downregulation of LY6K abolished tumorigenicity of cervical cancer HeLa cells

The role of LY6K in tumor formation of cervical cancer Hela cells was investigated in an animal model. LV3-shLY6K and LV3-NC infected HeLa cells formed tumors in nude mice. The average weight of tumors was significantly lower in LV3-shLY6K infected group than that in LV3-NC infected group ($P<0.05$; **Figure 6A**). The average volume of tumors in LV3-shLY6K infected group was significantly lower than that of the LV3-NC infected group ($P<0.05$; **Figure 6A**). IHC revealed that the expression of LY6K and VEGFA in tumors from LV3-shLY6K infected

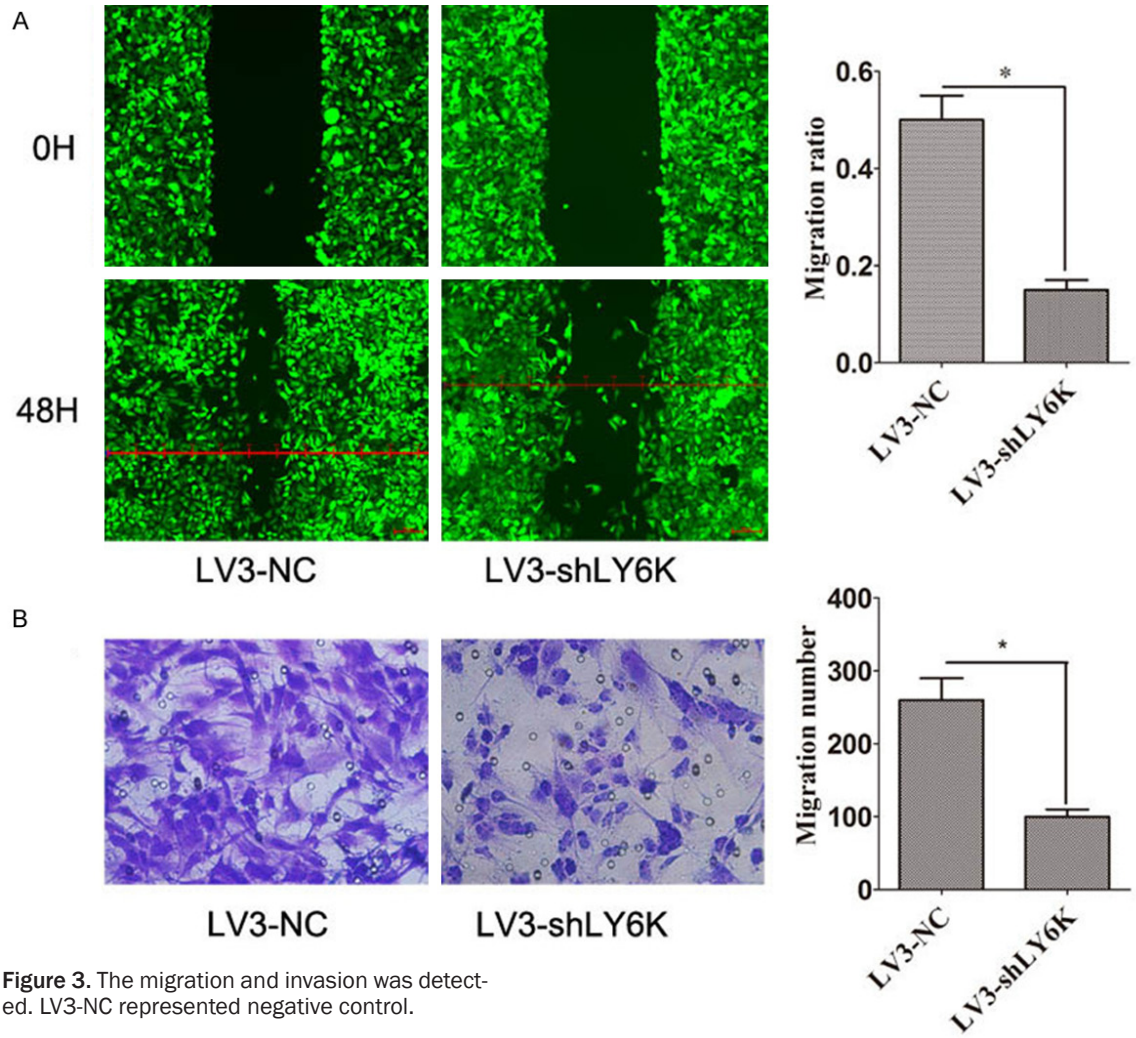

Figure 3. The migration and invasion was detected. LV3-NC represented negative control.

group was lower than that in the LV3-NC infected group ($P<0.05$) (**Figure 6B**). These data show that knockdown LY6K inhibited tumor formation in vivo. LY6K can regulate the expression VEGFA in vivo.

Discussion

In this study, we observed that LY6K expression was higher in cervical cancer tissues compared with normal cervical tissues. We also observed that the aberrant expression of LY6K in cervical cancer was associated with tumor stage and histological grades. Our results also showed that increased expression of LY6K in cervical cancer promoted cellular migration, invasion, and cell proliferation. These results suggest that elevated expression of LY6K promotes the aggressive behavior. Our data also demonstrates that LY6K can regulate VEGFA through miR-15a and miR-24.

LY6K is a member of LY6 family. It is either a cell surface receptor or a secreted granule involved in the cell signaling pathway [11]. It is highly expressed in cancer cells but not in normal cells. previous study has reported that elevated expression of LY6K in human head and neck squamous cell carcinomas, lung cancer, esophageal and breast cancers [10-12]. In human breast cancer, LY6K can promote the ability of invasion and metastasis. Here, our result found knockdown LY6K gene significantly inhibited proliferation, migration and invasion in cervical cancer HeLa cells. The increased expression of LY6K in cervical cancer may be associated with the malignant behavior of cervical cancer.

VEGFA plays an important role in invasion and metastasis of cervical cancer. VEGFA can regulate cervical cancer growth and invasion [17]. The expression of VEGFA predicts early recur-

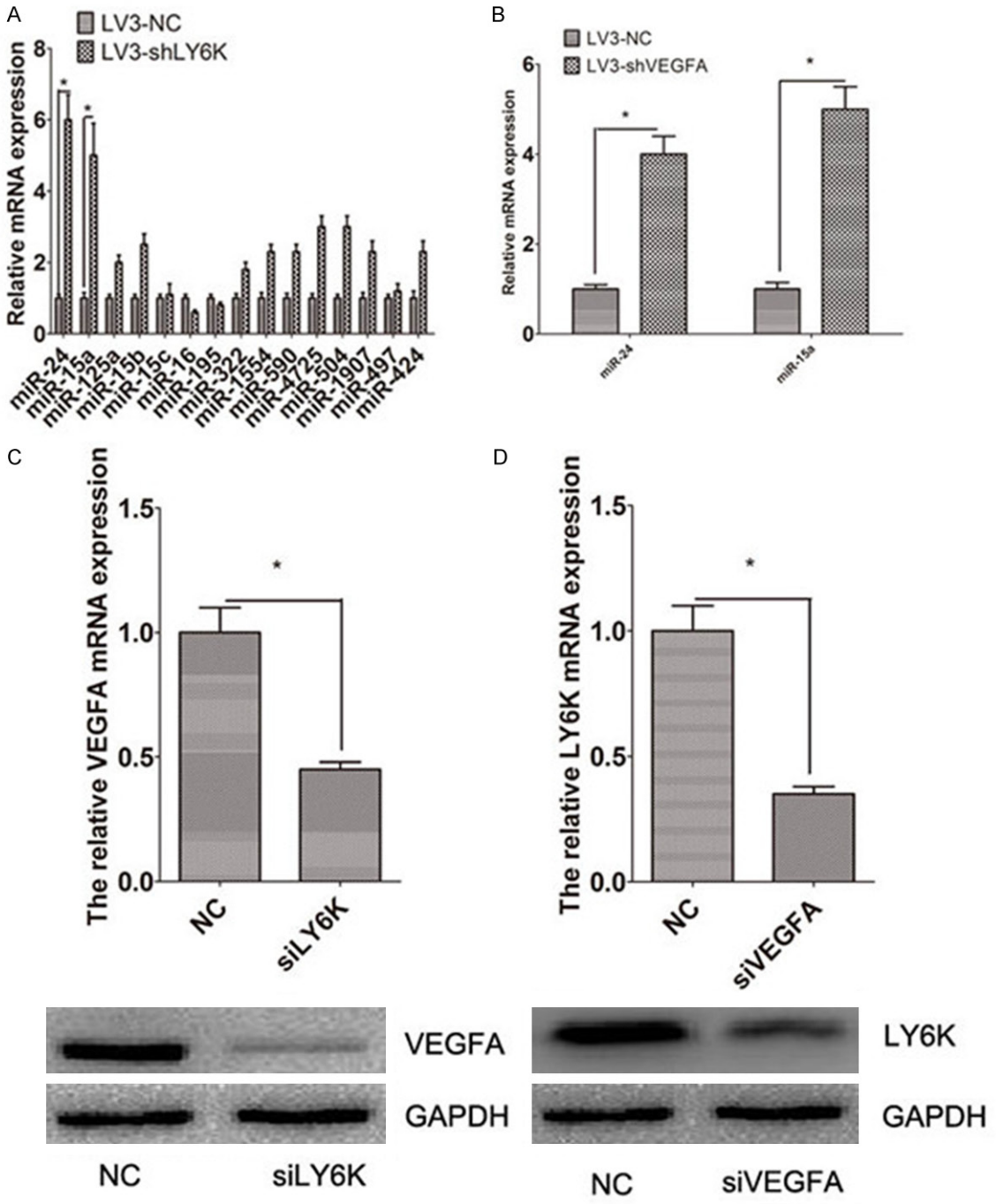

Figure 4. LY6K and VEGFA regulated the expression of miR-15a and miR-24. A. miR-15a and miR-24 were up-regulated after silencing LY6K. B. miR-15a and miR-24 were up-regulated after silencing VEGFA. C. VEGFA was reduced when silencing LY6K. D. LY6K was reduced when silencing VEGFA. *P<0.05 (versus control group); error bars, SEM.

rence in cervical cancer patients undergoing radiochemotherapy [18]. miR-203 suppressed cervical cancer cell proliferation, tumor growth, and angiogenesis in nude mice by targeting VEGFA [19]. In this study, we found that LY6K can regulate VEGFA. Silencing LY6K inhibited the expression of VEGFA. This data indicated LY6K was involved in the malignant behavior by regulating VEGFA.

Previous studies reported that miR-15a is located on chromosome13. In many cancers,

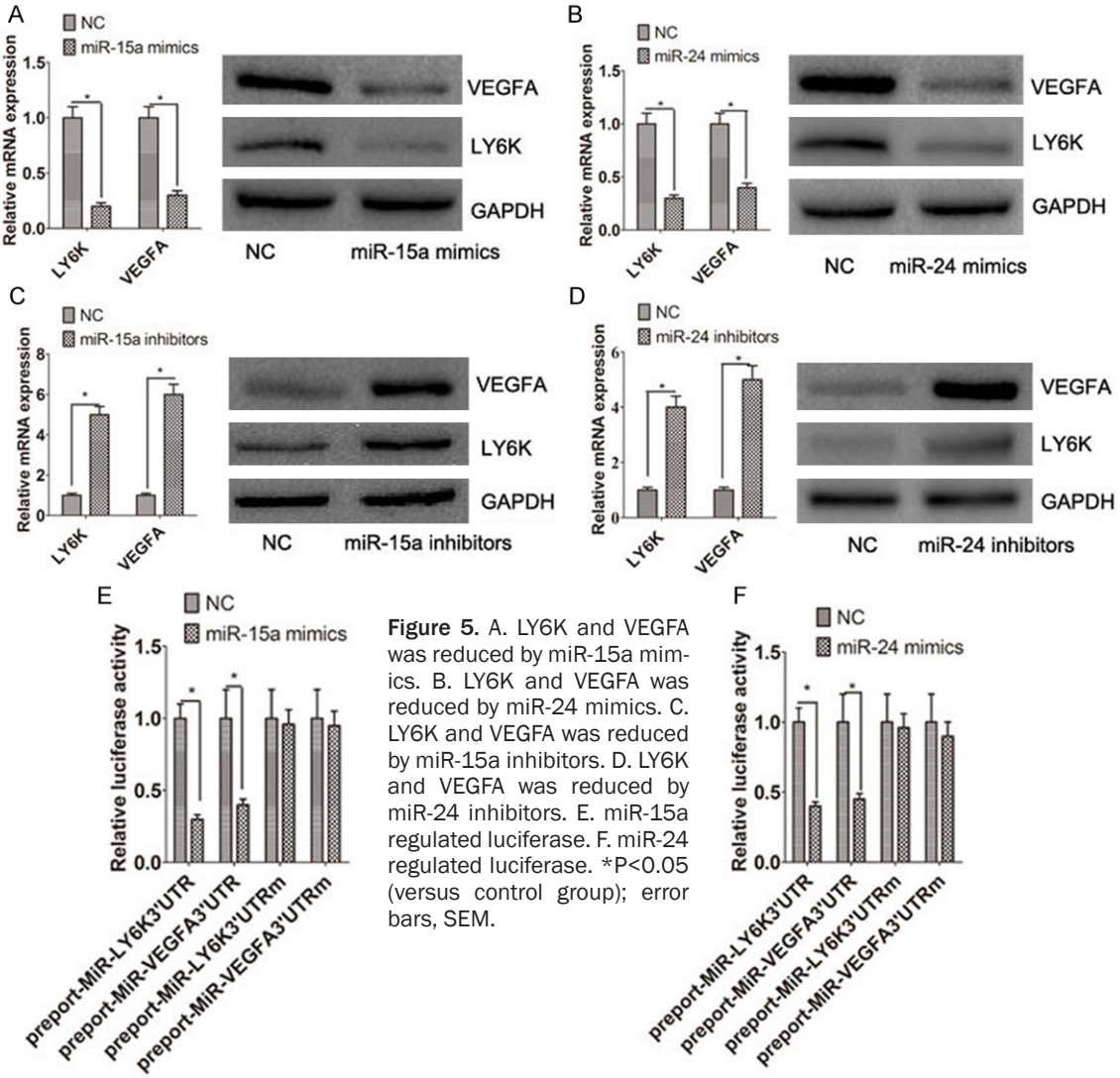

Figure 5. A. LY6K and VEGFA was reduced by miR-15a mimics. B. LY6K and VEGFA was reduced by miR-24 mimics. C. LY6K and VEGFA was reduced by miR-15a inhibitors. D. LY6K and VEGFA was reduced by miR-24 inhibitors. E. miR-15a regulated luciferase. F. miR-24 regulated luciferase. *P<0.05 (versus control group); error bars, SEM.

miR-15a plays a role of tumor suppressor function [20]. In colorectal cancer, miR-15a regulates epithelial mesenchymal transition and metastasis [21]. miR-15a was decreased expression in cervical cancer, and was associated with the metastasis [22]. The down-regulation of miR-24 in colorectal cancer is associated with malignant behavior in colorectal cancer [23]. In small-cell lung cancer, downregulation of miR-24 contributes to VP16-DDP resistance by targeting ATG4A [24]. In this study, we observed that miR-15a and miR-24 inhibited the expression of LY6K and VEGFA. We also confirmed that LY6K and VEGFA is direct target of miR-15a and miR-24. Our data indicated LY6K is an oncogene in cercal cancer.

ceRNA hypothesis is initially studied for the tumor-suppressor gene PTEN, which is regulated by its pseudogene PTENP1 in a miRNA-dependent manner [25]. Recent studies have demonstrated lncRNA HOTAIR or MEG3 promotes or inhibits gastric cancer progression acted as ceRNAs [26, 27]. Hmga 3'UTR as potent ceRNAs is related to the transformation of lung cancer [28]. In breast cancer, the pseudogene CYP4Z2P 3'UTR promoting angiogenesis through acting as a ceRNA for CYP4Z1 [29, 30]. FOXO1 3'UTR inhibiting breast cancer metastasis through increasing E-cadherin levels by sharing miRNA miR-9 [31]. In human non-small cell lung cancer, AEG-1 3'UTR induced EMT by its ceRNA activity [32]. In this study, LY6K regulated VEGFA required miR-15a and miR-24. In turn, knockdown VEGFA inhibited LY6K expression dependent miR-15a and miR-24. LY6K regulated VEGFA acting ceRNA through sharing miR-15a and miR-24.

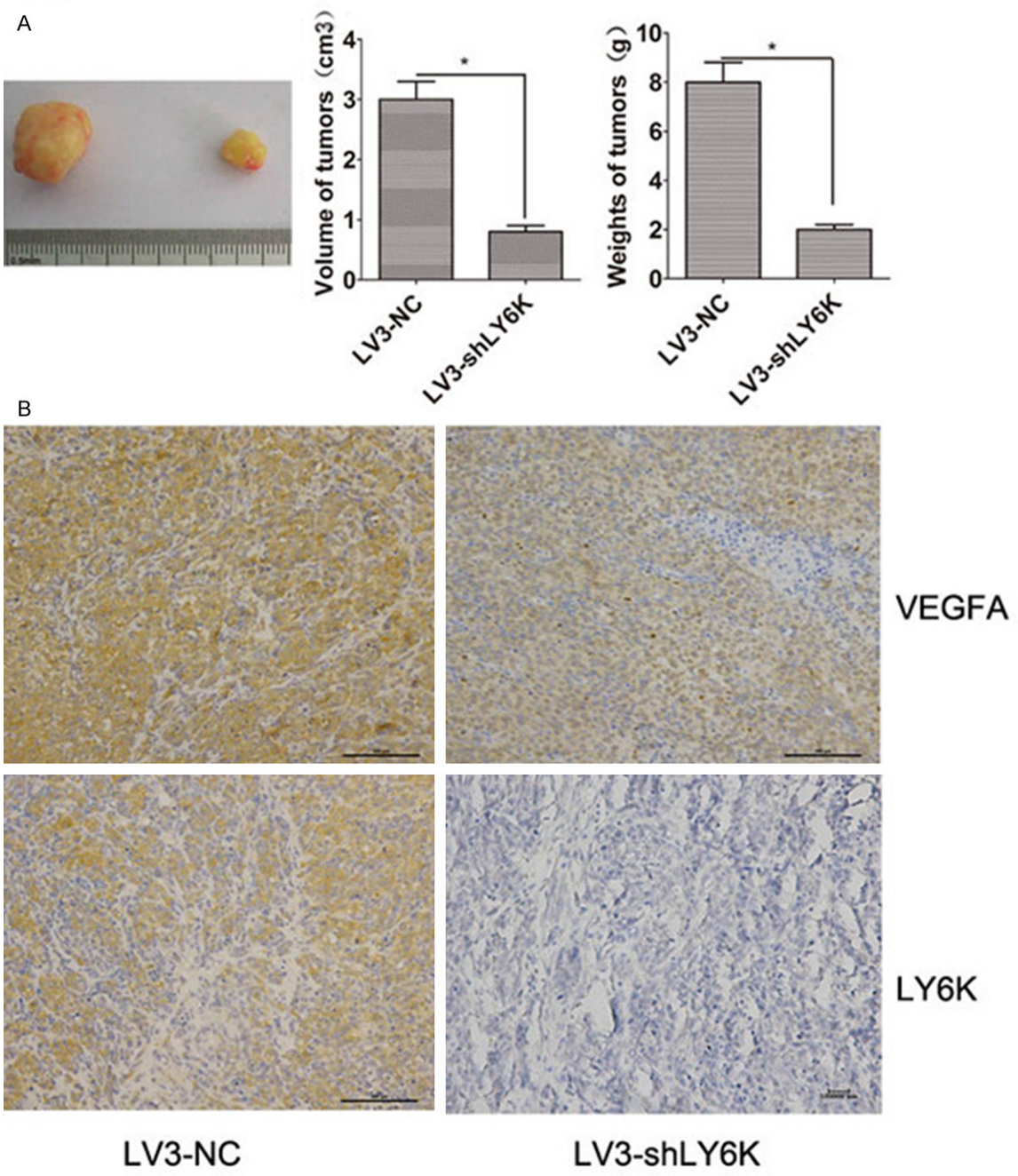

Figure 6. LY6K regulated tumorigenesis in nude mice model. A. Mean tumor volume and weight on day 21 after tumor cell injection. LV3-shLY6K-1 or LV3-NC infected HeLa cells were implanted s.c. into the left armpit. B. Immunohistochemical analysis of LY6K and VEGFA expression were performed on tumor xenografts. Representative images are shown (original magnification ×200). *P<0.05 (versus control group); error bars, SEM.

In conclusion, we found LY6K regulated the malignant behavior in cervical cancer. mechanical study found that LY6K regulated VEGFA acting ceRNA through sharing miR-15a and miR-24. In the future study, we will further study the function and mechanism of LY6K in cervical cancer.

Disclosure of conflict of interest

None.

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