

Heparanase overexpression participates in tumor growth of cervical cancer in vitro and in vivo

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Abstract Expression of heparanase is associated with invasion, metastasis and angiogenesis of a variety of human cancers. However, the roles of heparanase in cervical cancer are not clear. The aim of this study is to determine whether up-regulation of heparanase expression can promote growth of cervical cancer in vitro and in vivo. Heparanase protein expression was analyzed in cervical cancer patients using immunohistochemistry. In addition, expression of heparanase was also examined in cervical cancer cell lines. A series of in vivo and in vitro assays was performed to elucidate the role of heparanase in tumor growth of cervical cancer. Positive rate of heparanase was 63.3 % (38/60) in cervical cancer patients by immunohistochemistry, and it was significantly correlated with tumor size and clinical stage ($P < 0.05$). Overexpression of heparanase inhibited apoptosis of cervical cancer cells. Moreover, ectopic overexpression of heparanase in cervical cancer cells promoted proliferation of cervical cancer cells in vitro and tumor growth in vivo. These results suggest that heparanase participates in tumor growth of cervical cancer by influencing the proliferation and apoptosis of

cervical cancer cells, and heparanase could be used as an effective therapeutic target for cervical cancer.

Keywords Heparanase · Cervical cancer · Proliferation · Apoptosis

Introduction

Cervical cancer is the second most common cancer among women worldwide, with 400,000 new cases diagnosed each year [1]. As cervical cancer is characterized by few specific symptoms, it has great difficulty detected in early stage. Therefore, there is a high mortality rate in cervical cancer. Despite the etiology of cervical cancer is initiated by persistent infection with high-risk human papillomavirus (HPV), the molecular mechanisms underlying progression of this disease still poorly understood.

Tumor invasion and metastasis depend on the ability of cancer cells to degrade the tissue barrier of the extracellular matrix (ECM) and basement membrane (BM). Heparan sulfate proteoglycan (HSPG) and Heparan sulfate (HS) are the major components of the ECM and BM [2]. HS is a glycosaminoglycan chain in HSPG, which bind a large variety of molecules, such as growth factors and chemokines. Accumulating research found heparanase contributed to invasion and metastasis of in many cancers [3–6]. Heparanase, a mammalian endo- β -D-glucuronidase, has the ability in cleavage of HS, degradation of ECM and subsequent release of HS-bound active vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [7–9]. Increased expression of heparanase has been reported in a variety of human tumor tissues and cancer cell lines [10–15]. Moreover, inhibiting the expression of heparanase can lead to inhibition of tumor invasion,

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metastasis and angiogenesis [16, 17]. Retrospective study showed that up-regulation of heparanase correlated with increased lymph node and distant metastasis, higher microvessel density and reduced post-operation survival of cancer patients [13, 14].

Taking into account the important biologic role of HSPG and HS, heparanase participated in many aspects of tumor development, including migration, invasion, angiogenesis and metastasis. We have reported previously that short-hairpin RNA (shRNA) against heparanase has inhibitory activity on migration, adhesion and invasiveness of osteosarcoma cell line in vitro [18]. In contrast, few studies investigate the roles of heparanase in proliferation and apoptosis of cancer cells. In this study, we investigated the roles of heparanase in cervical cancer. To date, the effects of heparanase in human cervical cancer have not been evaluated. In the present study, we focus on effects of the heparanase on the apoptosis and proliferation of cervical cancer cells in vitro and in vivo.

Materials and methods

Cervical cancer specimens

In this study, the paraffin-embedded pathologic specimens from 60 patients with cervical cancer were obtained from the Department of Pathology, the First Affiliated hospital of Sun Yat-sen University. The details of cervical cancer

Table 1 Clinicopathological features of patients with cervical cancer and heparanase expression

Variable	N	Heparanase expression		P value
		–	+	
Age (years)				
≤40	28	11	17	0.791
>40	32	11	21	
Tumor size				
≤3 cm	33	18	15	0.003*
>3 cm	27	4	23	
Differentiation grade				
High/moderate	42	14	28	0.560
Low	18	8	10	
Clinical stage				
≤Ib	31	19	12	0.000*
>Ib	29	3	26	
Lymph node				
Metastasis				
Absent	45	15	30	0.372
Present	15	7	8	

* <0.05

patient characteristics are in Table 1. Informed consent was obtained from each patient, and the study was approved by the Institutional Research Ethics Committee. Hysteromyoma as normal control cervical tissues ($n = 4$) that obtained from surgically removed uteruses.

Cells and transfection

Human cervical cancer cell lines, HeLa, CaSki and SiHa, were obtained from American Type Culture Collection (ATCC). Cervical cancer cells were transfected with the full-length human heparanase cDNA or a control pcDNA3 vector (provided by Kai Nuo biotechnology company, Guang Zhou, China) using Lipofectamine 2000. After selected with G418 (600μg/ml) for 3 weeks and expanded, the cells with overexpression of heparanase were labeled as “HeLa-Heparanase group” and “SiHa-Heparanase group” in this manuscript, and the pcDNA3.0 transfected cells were referred to “HeLa-Vector group” and “SiHa-Vector group” as the control group.

Western blot analysis

Cervical cancer cells were lysed in a lysis buffer, and lysates were obtained by centrifugation at 4 °C. Forty μg total protein was subjected to SDS-PAGE and was transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with human heparanase antibody (sc-25825, Santa Cruz, CA), followed by HRP-conjugated secondary antibody. ECL substrate was used for the detection of heparanase expression.

MTT and colony formation assay

Cervical cancer cells were seeding in 96-well plates at the density of 1×10^4 cells/well in MTT assay. The next procedure was performed as described previously [18]. In colony formation assay, each culture dish inoculated 200 cells and incubated the cells at 37 °C. The medium changed every 4 days. Two weeks later, cells were fixed with methanol and stained with trypan blue. Colonies were counted by a microscope.

Hochest33258 staining

Cervical cancer cells were seeding in 6-well plates at the density of 4×10^5 cells/well. After incubated 24 h, culture medium was absorbed and added to 0.5 ml fixative solution. Fixing 10 min, removed the fixing solution and washed three times, each time lasted 5 min. Lastly, 0.5 ml Hochest33258 was added and stained of 5 min. The results were observed under fluorescence microscopy.

Immunohistochemistry

Five micrometre sections were cut from the selected paraffin blocks and deparaffinized by routine techniques. The slides were microwaved in 10 mM citrate buffer (pH 6.0) for 10 min and incubated with anti-heparanase (1:100 dilution, Santa Cruz, CA) and anti-Ki-67 (1:50 dilution, Maxim-Bio, Fuzhou, China), respectively, and stored overnight at 4 °C. The slides were sequentially incubated with a secondary antibody (Maxim-Bio). Labeling was detected by adding diaminobenzidine and hematoxylin (Maxim-Bio). Heparanase staining was scored according to the intensity (0, negative; 1, weak; 2, moderate; 3, strong) and the percentage of cancer cells that were stained (0, 0 %; 1, 1–10 %; 2, 11–50 %; 3, 51–75 %; 4, >75 %). If the product of multiplication between staining intensity and the percentage of positive cancer cells are ≥ 2 , it is thought as immunoreaction positive (+). Staining results for each antibody were examined by two pathologists.

In vivo tumor growth assay

Nude mice (BALB/cA nu/nu) aged 4–5 weeks were obtained from the Medical Experimental Animal Center of Guang Dong. HeLa-Heparanase ($n = 5$) and SiHa-Heparanase cells ($n = 5$) were injected into the back of the nude mice. Simultaneously, HeLa-Vector ($n = 5$) and SiHa-Vector cells ($n = 5$) were injected into the back of

mice as control. The maximum diameter (a) and the minimum diameter (b) of the tumors were measured, with tumor volume being calculated according to the formula: Tumor volume (cm^3) = $a \times b^2/2$. After 5 weeks of injections, the mice were killed and the tumors were excised.

Statistical analysis

Statistical analysis was done using the SPSS13.0. The correlation between heparanase and clinicopathological parameters was evaluated by χ^2 test. Results are expressed as mean \pm SD. Comparisons between two groups were conducted by Student's *t* test. *P* value of less than 0.05 was considered significant.

Results

Expression of heparanase in cervical cancer tissues and cervical cancer cell lines

In the present study, we examined heparanase expression in 60 cervical cancer samples. Using immunohistochemistry, we found heparanase immunoreactivity in the cytoplasm of 38/60 (63.3 %) of the cervical cancer samples. Immunohistochemical staining results for heparanase are shown in Fig. 1. Interestingly, no heparanase expression was observed in the normal cervical tissues. As shown in

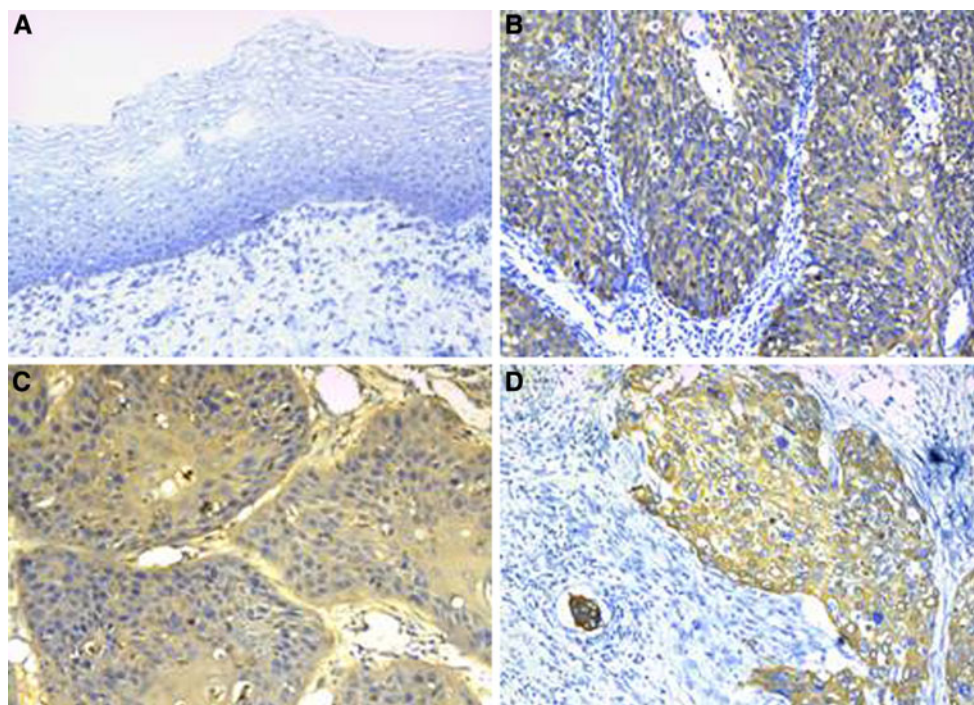


Fig. 1 Representative immunostaining of heparanase in normal cervical and cervical cancer tissues **a** Negative staining of heparanase in normal cervical tissue. **b–d** Positive expression of heparanase in cervical cancer tissues

Fig. 2 Heparanase overexpression enhanced cervical cancer cell growth. **a** Both HeLa and SiHa exhibited low levels of heparanase expression. Increased heparanase expression was observed in HeLa-Heparanase and SiHa-Heparanase cells. **b** HeLa-Heparanase and SiHa-Heparanase cells showed increased cell proliferation. **c** HeLa-Heparanase and SiHa-Heparanase cells developed more and bigger colonies than HeLa-Vector and SiHa-Vector cells. **d** The results of colony formation assay. **<math>P < 0.01</math>, *<math>P < 0.05</math>

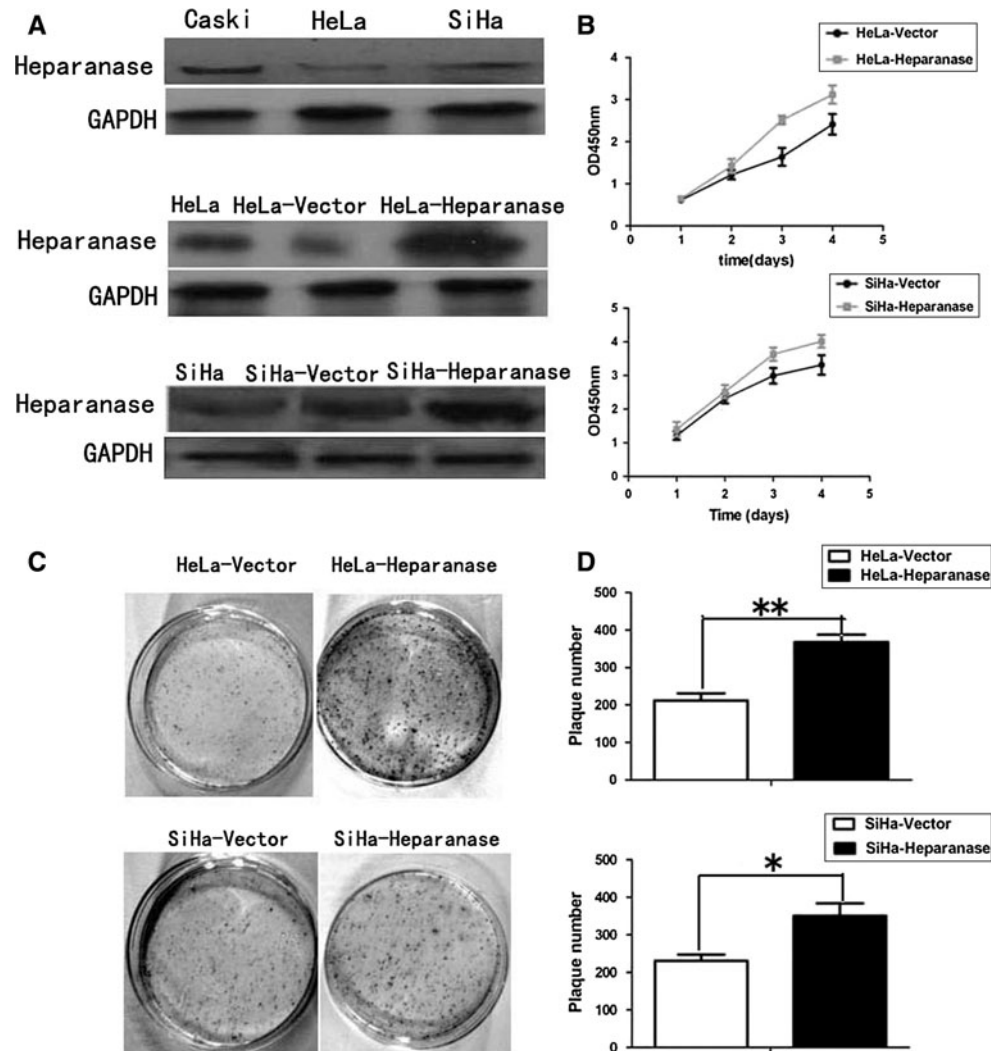


Table 1, tumor size had statistically significant correlation with heparanase expression ($P = 0.003$). Moreover, heparanase expression was associated with clinical stage ($P = 0.000$). However, heparanase expression was not associated with age, differentiation grade and lymph node metastasis (Table 1).

As shown in Fig. 2a, heparanase could be detected in all of protein that was isolated from HeLa, CaSki and SiHa cells. However, heparanase protein level of CaSki cells was 3-fold higher than that of HeLa and 2-fold higher than that of SiHa (1.288 ± 0.12 vs. 0.401 ± 0.09 and 0.624 ± 0.08 , respectively).

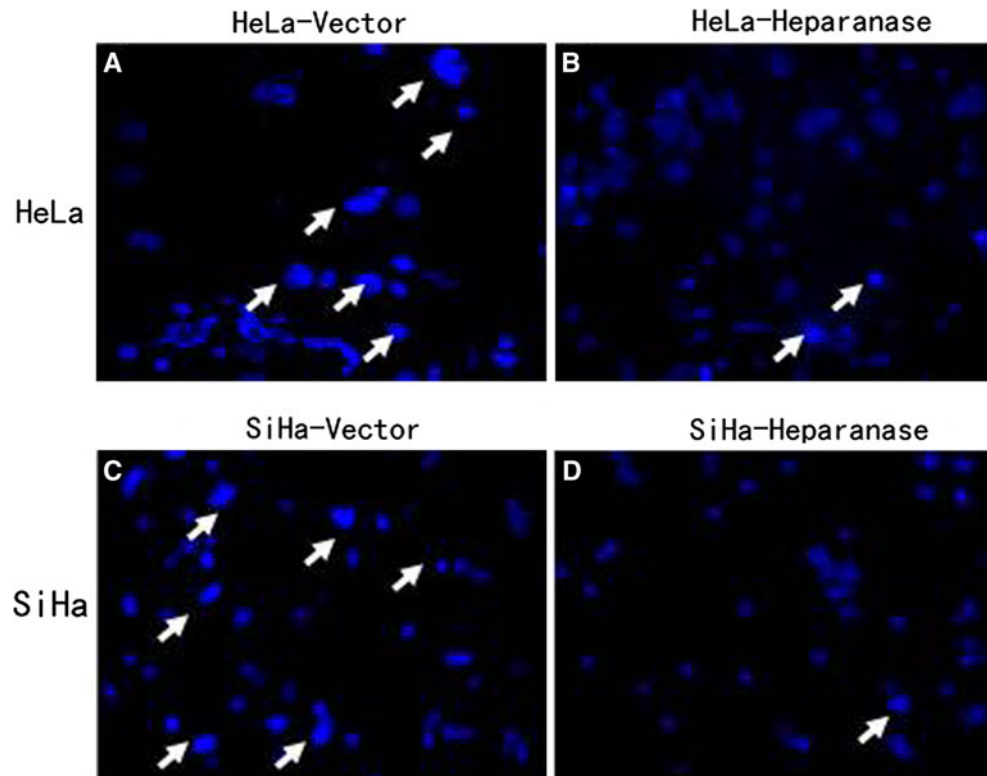
Heparanase overexpression enhanced cervical cancer cell growth

Taking into account the weak endogenous expression of heparanase in HeLa and SiHa cells (Fig. 2a), we used them

as models to study the effect of heparanase on cell growth. Expression of heparanase was increased in HeLa and SiHa cells after transfected with heparanase cDNA, as shown in Fig. 2a.

In MTT assay, heparanase overexpression resulted in an enhanced proliferation rate. Four days after seeding, the cell number of the HeLa-Heparanase group was significantly higher than that of the HeLa-Vector group ($P < 0.05$) (Fig. 2b). Subsequently, the proliferation-promoting effect of heparanase was confirmed in another cervical cancer cell SiHa. Colony formation assay showed that the number of colonies of HeLa-Heparanase group was significantly higher (368 ± 20.2) than that of HeLa-Vector group (212 ± 19.3) (Fig. 2c) ($P < 0.05$). Similar results were obtained in SiHa-Heparanase group (349 ± 26.4) and SiHa -Vector group (228 ± 18.6), suggesting that the findings were not idiosyncratic to HeLa cells (Fig. 2d).

Fig. 3 Heparanase overexpression inhibited apoptosis of cervical cancer cells. **a, b** Apoptosis of HeLa-Heparanase cells were significantly decreased as compared with HeLa-Vector cells. **c, d** Compared with SiHa-Vector cells, apoptosis of SiHa-Heparanase cells were significantly decreased



Heparanase overexpression inhibited cervical cancer cell apoptosis

After heparanase expression was enhanced, apoptosis cells of HeLa and SiHa were significantly decreased by using Hoechst33258 staining. Apoptotic index (AI) of HeLa-Heparanase group was less than that in HeLa-Vector group (3.23 ± 0.98 , 10.82 ± 1.78 , respectively) (Fig. 3a, b) ($P < 0.01$). Likewise, apoptosis cells in SiHa-Heparanase group showed a dramatic reduction compared with SiHa-Vector group (2.18 ± 0.71 , 11.36 ± 1.54 , respectively) (Fig. 3c, d) ($P < 0.01$). These results indicated that overexpression of heparanase inhibited apoptosis of cervical cancer cells.

Heparanase overexpression promoted tumor growth in vivo

As shown in Fig. 4a, heparanase staining was enhanced in tumors of HeLa-Heparanase and SiHa-Heparanase groups compared with that in HeLa-Vector and SiHa-Vector groups. Proliferation index (PI) detected by means of Ki-67 was greatly increased in the HeLa-Heparanase group compared with the HeLa-Vector group (23.59 ± 0.16 , 8.56 ± 0.12 , respectively; $P < 0.01$; Fig. 4a). Likewise, PI was 20.46 ± 0.21 in SiHa-Heparanase group, obviously higher than that in SiHa-Vector group (7.89 ± 0.17) ($P < 0.01$; Fig. 4a). Moreover, in animals with

HeLa-Heparanase and SiHa-Heparanase cells xenograft, tumor volume was significantly larger than that of animals receiving HeLa-Vector and SiHa-Vector cells ($P < 0.05$; Fig. 4b). Collectively, these results suggested that up-regulation of heparanase in cervical cancer cells promoted tumor growth in vivo.

Discussion

Heparanase, owing to its important function in cleaving HS chains, is considered to play a key role in invasion and angiogenesis during cancer progression [7]. Current studies show that heparanase contribute to cancer invasion by degrading the extracellular matrix, releasing of angiogenic factors. Apart from its traditional function, heparanase shows potential effects in regulating migration, growth and colony formation of cancer cells which seem to be independent of the degradation of ECM and BM. These biological properties make heparanase a potential target for cancer therapy.

In the present study, we show that heparanase is over-expressed in cervical cancer patient samples. Interestingly, heparanase expression is correlated with tumor size and clinical stage. Therefore, it is interesting to speculate that heparanase may be participating in tumor growth. To date, the correlation between heparanase and growth of cervical cancer cell has not been fully evaluated. In breast cancer,

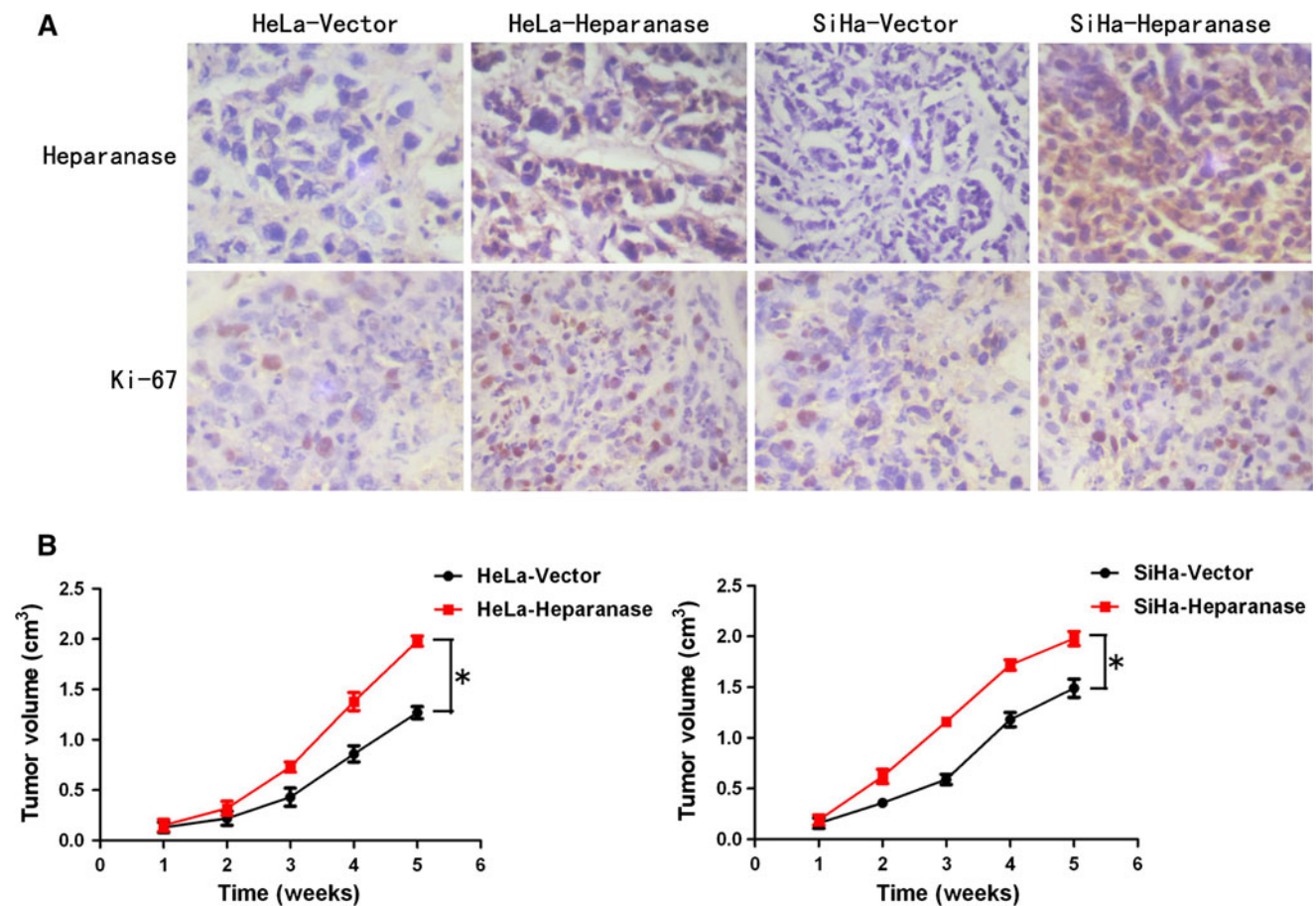


Fig. 4 Overexpression of heparanase promoted tumor growth in vivo. **a** Compared with HeLa-Vector and SiHa-Vector groups, the expressions of heparanase and Ki-67 were up-regulated in

HeLa-Heparanase and SiHa-Heparanase groups. **b** Tumor volume in HeLa-Heparanase and SiHa-Heparanase groups were significantly larger than those in control groups. * <0.05

silencing of heparanase by small interfering RNA (siRNA) inhibited the proliferation of MDA-MB-435 cells in vitro [16]. Similarly, when heparanase was impaired by anti-sense oligodeoxynucleotides (AS-ODN) and siRNA in a hepatocellular carcinoma cell line, SMMC7721, growth of tumor implanted with SMMC7721 was significantly inhibited in vivo [17]. Conversely, heparanase overexpression significantly increased cell proliferation, anchorage-independent colony formation in glioma cells [19]. Consistent with previous report, in the present study, we provide evidence that enhanced heparanase significantly promote cervical cancer cells' growth in vitro and in vivo. At cell level, heparanase overexpression promoted cell proliferation and colony formation. In cervical cancer cell xenograft model, tumors implanted with HeLa-Heparanase and SiHa-Heparanase cells were larger than those treated with HeLa-Vector and SiHa-Vector cells. Likewise, PI reflected by Ki-67 in heparanase overexpression groups was higher than that in control groups. The correlation of enhanced levels of heparanase and altered tumorigenic properties in cells indicate that heparanase is important in

growth of cervical cancer. Paradoxically, Edovitsky et al. reported that inhibition of the mouse heparanase by shRNA had no significant effect on the proliferation of B16-L6 cells [20]. The discrepancy might be explained by different types of tumor.

Because heparanase has been shown to participate in tumor progression by regulating apoptosis, we further examined the effect of heparanase on apoptosis of cervical cancer cells. Our results showed that overexpression of heparanase significantly inhibited apoptosis of HeLa cells and SiHa cells. Zetser et al. reported that heparanase overexpression in U87 glioma, correlated with enhanced Akt/PKB phosphorylation levels [21]. The ability of heparanase to stimulate Akt suggests that heparanase may protect tumor cells from apoptosis.

In summary, we have demonstrated that up-regulation of heparanase expression can efficiently promote growth of cervical cancer in vitro and in vivo. Moreover, overexpression of heparanase inhibited apoptosis of cervical cancer cells. To elucidate the molecular mechanism of heparanase how heparanase regulates growth of cancer

cells, further studies are essential *in vitro* and *in vivo*. Therefore, heparanase may be of potential values as a novel therapeutic target for cervical cancer.

Conflict of interest None.

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