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Original Article

Heparanase promotes radiation resistance of cervical cancer by upregulating hypoxia inducible factor 1

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Abstract: Heparanase (HPSE1) is elevated in various types of cancers including cervical cancer, and correlated with poor prognosis. Current study is to investigate the effects of HPSE1 on radiation response in cervical cancer. Colony formation assays after radiation were performed to compare the radiation response among control, HPSE1 knockdown and HPSE1 overexpression HeLa cells. The mRNA and protein levels of HIF1, bFGF and VEGF were measured as indicators for the activity of HIF1 pathway. Xenograft mouse model were used to study the HPSE1 radiation regulator effects *in vivo*. Microvessel densities (MVD) were measured in xenograft tumor samples. The survival fractions were significantly lower in HPSE1 knockdown cells and higher in HPSE1 overexpression cells compared with control cells. The mRNA and protein levels of HIF1, VEGF and bFGF are decreased in HPSE1 knockdown cells and increased in HPSE1 overexpression cells. HIF1 inhibition eliminated the radiation protection effects by HPSE1 overexpression. Our results demonstrate HPSE1 is an important regulator of radiation response both *in vivo* and *in vitro*. Further studies are warranted to determine the underlie mechanism of how HPSE1 regulate HIF1 activity and the clinical effects of HPSE1 inhibitors in cervical cancer.

Keywords: Heparanase, HIF1, radiation sensitivity, cervical cancer, VEGF

Introduction

Cervical cancer is now the fourth common type of cancer in women [1], and most often attacks younger women with a mean diagnosis age at around 50 years old, comparing with other gynecologic cancer types [2]. For early stage cervical cancer patients (stage IA to IB1), the main treatment option is surgery with a 5-year survival of 88% to 96% [3-5]. While in cervical cancer patients with local advanced stage, the local recurrence rate with surgery treatment only is more than 30% [4, 6]. Nowadays, radiation therapy and chemotherapy have become the standard treatment options for local advanced stage cervical cancer. However, despite the great progress in radiotherapy and chemotherapy in recent years, the prognosis of local advanced cervical cancer patients is still not satisfied with a 5-year survival rates ranging from 80% for stage IB patients to 30% for stage III patients [7]. Thus, there is an urgent need to investigate and understand the mechanism of radio- or chemo-resistance to improve the therapeutic efficiency in cervical cancer patients.

Heparanase (HPSE1) is an endo-β-d-glucuronidase which is capable of cleaving heparan sulfate (HS) into shorter chain length oligosaccharides both at the cell-surface and within the extracellular matrix (ECM) [8]. Previous studies have found HPSE1 is elevated in various types of cancers including cervical cancer and correlated with poor prognosis [9]. Baraz L and his colleagues [10] identified that heparanase gene expression is transcriptional repressed by p53. Wild type p53 is an effective inhibitor of heparanase transcription in normal cells, while in tumor cells 50% of p53 are mutated which lead to the loss of inhibitory ability of heparanase expression. By cleaving HS, HPSE1 can release HS-bound growth factors (i.e., VEGF, FGF) residing in the ECM, stimulating the proliferation of surrounding cells [11]. Furthermore, clinical studies found the expression levels of

HPSE1 had correlated with tumor vascular densities [12] and hypoxia inducible factor 1 (HIF1) expression [13] in cancer patients.

High HIF1 expression has been shown to correlate with an increased risk of local recurrence and tumor-related death in patients with many cancer types treated with radiotherapy [14]. Radiation-induced tumor tissue reoxygenation leads to the surviving fraction hypoxic tumor cells become oxygenated. A few hours after the irradiation, intratumoral HIF1 activity decreases sharply as a result of von Hippel-Lindau-mediated HIF1 degradation under this reoxygenated status [15]. However, reoxygenation induced reactive oxygen species (ROS) contribute to improve HIF1 stabilization [16]. Consequently, 18 to 24 hours after radiotherapy, overall HIF1 expression increases in tumor tissue through a hypoxia-independent manner [15]. Elevated HIF1 pathway activity induces multiply HIF1 target gene expression including VEGF and other angiogenesis factors, which induce angiogenesis and protect tumor cells and the vasculature endothelial cells within tumor microenvironments from radiation-induced apoptosis [17]. In short, radiation induced HIF1 pathway activation is responsible for tumor cell and tumor associated vascular endothelial cell protection, recovery of tumor blood supply and tumor recurrence.

Since HPSE1 is correlated with HIF1 expression, and HIF1 pathway activation shows great effects on cancer radiation resistance, we hypothesize that HPSE1 could play a role on radiation sensitivity as well. Here, we investigated whether HPSE1 affected radiation sensitivity in cervical cancer both *in vitro* and *in vivo*.

Materials and methods

Cell culture and transfection

The human cervical cancer cell line HeLa was purchased from American Type Culture Collection (ATCC, VA). To generate stable HPSE1 or HIF1 knockdown cells, HPSE1 shRNA (sc-40685-SH), HIF1 shRNA (sc-35561-SH) and Control shRNA plasmid-A (sc-108060) were purchased from Santa Cruz, and performed following the manufacturer's instructions. To generate HPSE1 or HIF1 overexpression HeLa cells, the cDNA of human HPSE1 or HIF1 were synthesized by a SuperScript RT-PCR kit (Invitrogen) with total RNA from 293T cells as the templates and subcloned into the pCMV plasmids afterwards. HeLa cells were transfected with pCMV-HPSE1 or pCMV-HIF1 plasmids using Lipofectamine 2000 to generate HPSE1 or HIF1 overexpression cells respectively. All cells were cultured in Dulbecco's Modified Eagle high glucose Medium (DMEM) with 10% fetal bovine serum, penicillin and streptomycin at 37°C under 5% CO_2.

Quantitative RT-PCR

Total RNA was obtained and cDNA was synthesized using a SuperScript RT-PCR kit (Invitrogen). The Quantitative RT-PCR was performed as described previously [18]. The primers used in the current study included: *HIF1α* forward 5'-CTC AAA GTC GGA CAG CCT CA-3', reverse 5'-CCC TGC AGT AGG TTT CTG CT-3'; *HPSE1* forward 5'-CAC AAA CAC TGA CAA TCC AAG G-3', reverse 5'-CCA TTG AGT TGG ACA GAT TTG G-3'; *bFGF* forward 5'-AGG AGT GTG TGC TAA CCG TTA C-3', reverse 5'-ACT CAT CCG TAA CAC ATT TAG AA-3'; *VEGF* forward 5'-AAG GAG GAG GGC AGA ATC AT-3', reverse 5'-CAG AAG GAG AGC AGA AGT CC-3'; *GAPDH* forward 5'-TGC ACC ACC AAC TGC TTA GC-3', reverse 5'-GGC ATG GAC TGT GGT CAT GAG-3'.

Western blot analysis

All cell lysates were prepared using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA, 1% TritonX-100, 0.1% SDS, and protease inhibitor cocktail). 25 μg of each sample was separated by a SDS-PAGE gel and blotted to nitrocellulose membrane. Blots were incubated with anti-HPSE1 antibody, anti-HIF1, anti-bFGF, anti-VEGF, or anti-actin antibody overnight at different dilution rates following the antibody instructions. All antibodies were purchased from Santa-Cruz CA. The bands were detected using chemiluminescence (ECL Plus; Amersham Biosciences, Piscataway, NJ).

Clonogenic assays

Clonogenic assay was used to determine the radiation effect on different cells. Briefly, 1,000 cells per well were seeded onto 6-well plate incubated at 37°C, 5% CO_2 overnight. Then cells were radiated with different doses of radiation (0, 4 and 8 Gy) with a 210 kVX-ray source at 2.02 Gy/min (RS-2000 Biological irradiator, Rad Source Technologies, GA). After incubated at 37°C, 5% CO_2 for 14 days, cells were fixed

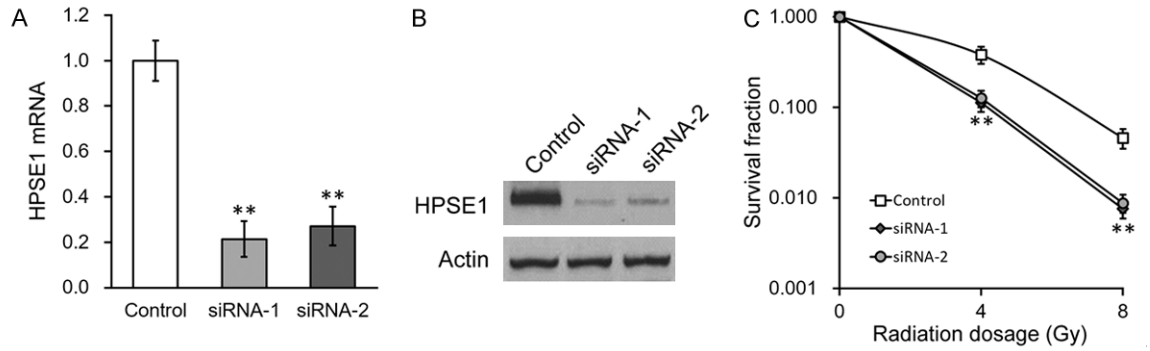

Figure 1. HPSE1 knock-down repressed radiation resistance of cervical cancer cells. HPSE1 knock-down was performed in HeLa cells using two different siRNA constructs, as well as control siRNA. (A) and (B) Levels of HPSE1 mRNA (A) and protein (B) were examined. (C) The above treated cells were then subjected to radiation dosage as indicated, and viability was examined after HPSE1 knock-down. Values were expressed as mean ± SD from at least three independent experiments. **P<0.01, siRNA-1 and siRNA-2 both compared to control siRNA.

with 95% ethanol and stained with 0.5% crystal violet. Colonies were counted in each well, plating efficiency (PE) and survival fraction (SF) were calculated following formula: PE = colony numbers in control/1000, SF = (colony numbers/1000)/PE. Survival curves were fitted in a linear-quadratic model [$S = \exp(-\alpha D - \beta D^2)$] using Graphpad Prism 4.0 (GraphPad Prism, San Diego, CA).

Xenograft mouse model and treatment

6-8 weeks old, female BALB/C nude mice were purchased from the Shanghai Experimental Animal Center (Shanghai, China). Each mouse was injected with 10^6 indicated cells indicated by each experiment subcutaneously, 10 mice for each group. The injection day was marked as day-7. At day 0 and day 10, 10 Gy dosage of radiation was given to each mouse by an X-ray generator (PCM 1000; Pantak) respectively. During the radiation, each mouse was covered by a lead cover to protect normal tissue. Tumor volumes were measured for each mouse every three days. All mice were scarified at day 14 and tumors were collected for further analysis.

Immunohistochemistry

Mice tumor tissues were fixed by formalin and embedded in paraffin. Immunohistochemistry (IHC) was performed to assess the microvessel density (MVD) following the standard IHC protocol. Briefly, 4-μm sections were cut from paraffin-embedded tumor tissues and incubated with AR-10 Solution (EDTA buffer) (Biogenex San Ramon, CA) at 121°C 10 minutes for Antigen retrieval. 0.3% H_2O_2 was used to eliminate endogenous peroxidase activity afterwards. Then the Sections were incubated with CD34 antibody (Abcam; Cambridge, UK) at the concentration 1:100 at 4°C overnight. The sections were further incubated with an anti-rabbit secondary antibody and developed with a diaminobenzidine (DAB) detection kit (BioGenex, San Ramon, CA). To quantify the MVD, CD34-positive intratumoral microvessels were counted using a microscope field (×400 magnification). 10 fields per section randomly were selected and analyzed. Single CD34-positive cell without visible lumen structure was excluded.

Statistical analysis

Data are represented as means ± SD. One-way ANOVA was used for comparison among multiply groups and student's t-test was performed for statistical analysis between groups. All tests were two-tailed and *P*-values <0.05 were considered as statistically significant. All statistical analysis was performed using SPSS17.0 (SPSS, Inc., Chicago, IL, USA).

Results

HPSE1 regulate radiation sensitivity in cervical cancer cells

To test whether HPSE1 could affect radiation sensitivity in cervical cancer, HPSE1 knock-down HeLa cells were made using shRNA (**Figure 1A**, **1B**). Both control and HPSE1 knock-down HeLa cells were radiated with 4 Gy and 8 Gy, respectively. Colony formation assay was used to evaluate the radiation sensitivity. As

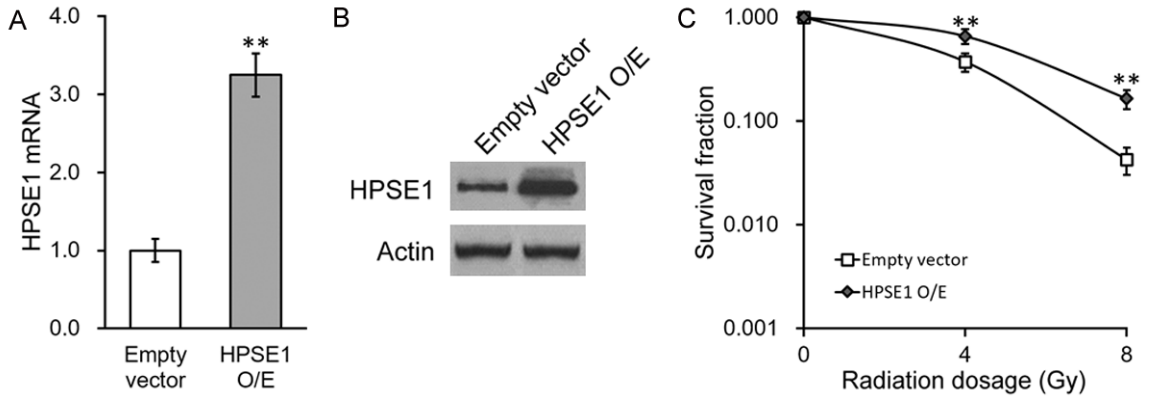

Figure 2. HPSE1 overexpression enhanced radiation resistance of cervical cancer cells. Empty vector and plasmid overexpressing HPSE1 (HPSE1 O/E) were transfected into HeLa cells, respectively. (A) and (B) Levels of HPSE1 mRNA (A) and protein (B) were examined. (C) The above treated cells were then subjected to radiation dosage as indicated, and viability was examined after HPSE1 overexpression. Values were expressed as mean ± SD from at least three independent experiments. **P<0.01, compared to empty vector.

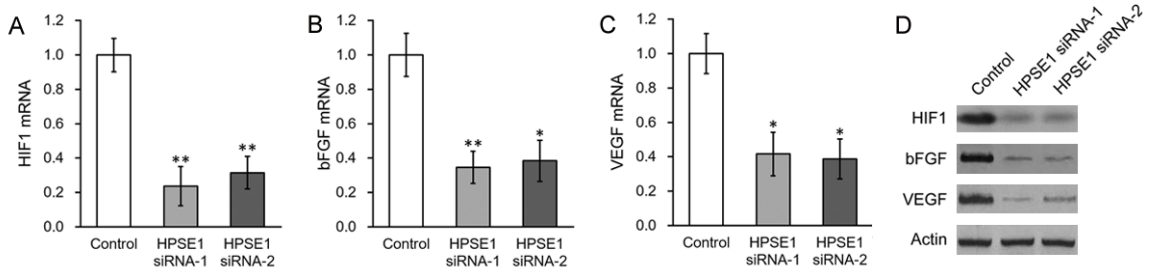

Figure 3. HPSE1 knock-down inhibited expressions of HIF1, bFGF and VEGF in cervical cancer cells. HPSE1 knock-down was performed in HeLa cells using two different siRNA constructs, as well as control siRNA. Levels of HIF1 mRNA (A), bFGF mRNA (B), VEGF mRNA (C), as well as their protein levels (D) were examined. Values were expressed as mean ± SD from at least three independent experiments. *P<0.05, **P<0.01, compared to control siRNA.

shown in **Figure 1C**, the survival fractions were significantly lower in both HPSE1 knockdown cells than control cells. Furthermore, HPSE1 overexpression cells (**Figure 2A**, **2B**) showed increased radiation resistance (**Figure 2C**).

HPSE1 regulate the activity of HIF1 pathway

Pervious study has shown that the expression of HPSE1 is correlated with HIF1 expression in pancreatic ductal adenocarcinomas patients [13]. Then we examined whether HPSE1 regulated HIF1 expression in cervical cancer cells. The mRNA and protein expression of HIF1 was measured in both HPSE1 knockdown and overexpression HeLa cells. Indeed, both mRNA (**Figure 3A**) and protein (**Figure 3D**) level of HIF1 were decreased in HPSE1 knockdown cells and increased in HPSE1 overexpression cells (**Figure 4A**, **4D**). Furthermore, the mRNA and protein level of VEGF and bFGF which are the targets of HIF1 pathway decreased in HPSE1 knockdown cells (**Figure 3B-D**) and increased in HPSE1 overexpression cells (**Figure 4B-D**). Together, our results showed HPSE1 can regulate HIF1 expression and activate HIF1 pathway.

HPSE1 regulate the radiation sensitivity through HIF1 pathway

The above data suggested that HPSE1 can regulate the radiation sensitivity and the activity of HIF1 pathway. Since HIF1 pathway is a powerful regulator of radiation sensitivity, we next investigated whether the effects of HPSE1 on radiation sensitivity was mediated by HIF1 pathway. We overexpressed HIF1 in HPSE1 knockdown cells (**Figure 5A**, **5D**). The mRNA and protein level of bFGF and VEGF were increased in HIF1 overexpression HPSE1 knock down cells (**Figure 5B-D**), suggesting HIF1 pathway re-activation in HPSE1 knockdown cells. As shown in **Figure 5E**, the colony formation assay after

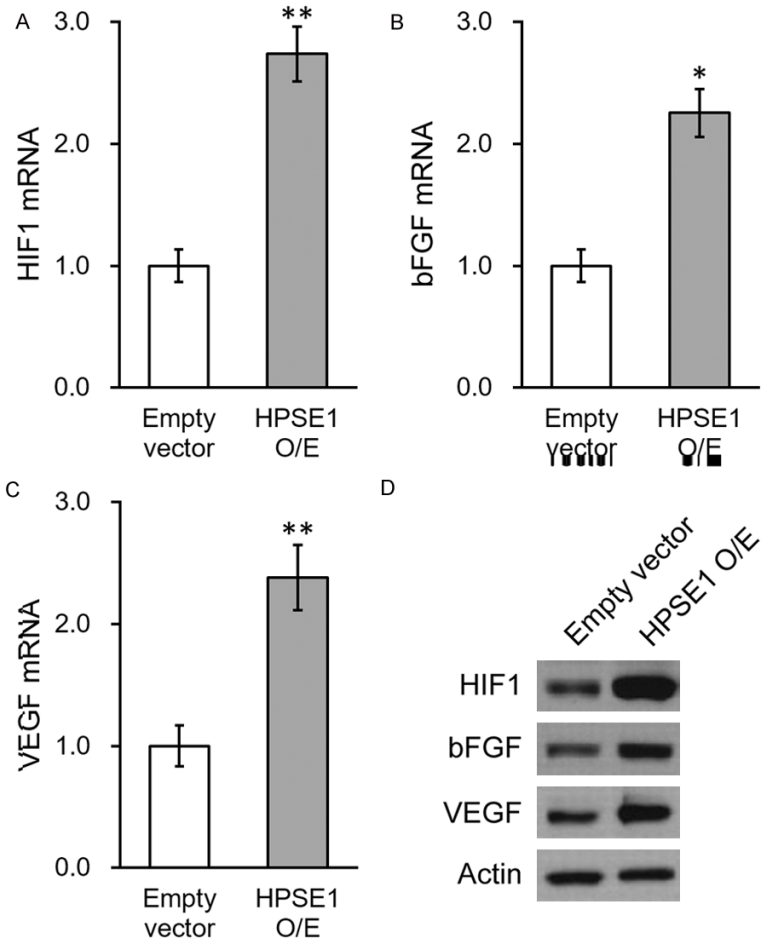

Figure 4. HPSE1 overexpression elevated expressions of HIF1, bFGF and VEGF in cervical cancer cells. Empty vector and plasmid overexpressing HPSE1 (HPSE1 O/E) were transfected into HeLa cells, respectively. Levels of HIF1 mRNA (A), bFGF mRNA (B), VEGF mRNA (C), as well as their protein levels (D) were examined. Values were expressed as mean ± SD from at least three independent experiments. *P<0.05, **P<0.01, compared to empty vector.

radiation showed the decreased radiation resistance by HPSE1 knockdown was restored by activation of HIF1 pathway. Furthermore, we knocked down the HIF1 in HPSE1 overexpression cells (**Figure 6A-D**) and found the inhibitory of HIF1 pathway can eliminate the radiation protection effects by HPSE1 overexpression as well (**Figure 6E**). Taken together, our data suggested the regulation effects of HPSE1 on radiation response were mediated by HIF1 pathway.

HPSE1 mediate radiation response and angiogenesis in xenograft mouse model

We next studied whether HPSE1 have similar effects on radiation response *in vivo*. Control, HPSE1 knockdown or HPSE1 overexpression HeLa cells were injected to female BALB/C nude mice subcutaneously, with 10 mice for each group. Each mouse received radiation with a total dosage of 20 Gy. The procedure was shown in **Figure 7A**. Tumor volumes were measured for each mouse every 7 days to compare the radiation response effects among cells with different HPSE1 expression levels. We found the tumor sizes were significantly smaller in HPSE1 knockdown tumors (**Figure 7B**) and larger in HPSE1 overexpression tumors (**Figure 7C**), comparing with control tumors after radiation. Next we validated the role of HIF1, on top of HPSE1, in affecting radiation response of the xenograft tumors. We found that size of tumors was increased when HIF1 was overexpressed in HPSE1 knockdown tumors (**Figure 7B**), whereas reduced when HIF1 was knocked down in HPSE1 overexpression tumors. These above *in vivo* animal experiments further confirmed our earlier result that, the promotional effects of HPSE1 on radiation resistance were mediated by HIF1 pathway.

Furthermore, we took the tumor samples from different experimental groups of tumors 14 days after radiation and analyzed the microvessel density (MVD) of each samples. As shown in **Figure 8A** and **8B**, the MVD in tumors derived from HPSE1 cells is significantly lower than those from control cells, while overexpressing HIF1 in HPSE1 knock-down background can eliminate the effects. Meanwhile, MVD in tumors derived from HPSE1 overexpression cells is higher than those from control cells, which can be reduced by HIF1 knock-down (**Figure 8C**, **8D**). These findings were consistent with the data from our *in vitro* experiments suggesting HPSE1 is an important enhancer of radiation resistance, which functions to promote angiogenesis in the xenograft tumors *in vivo*.

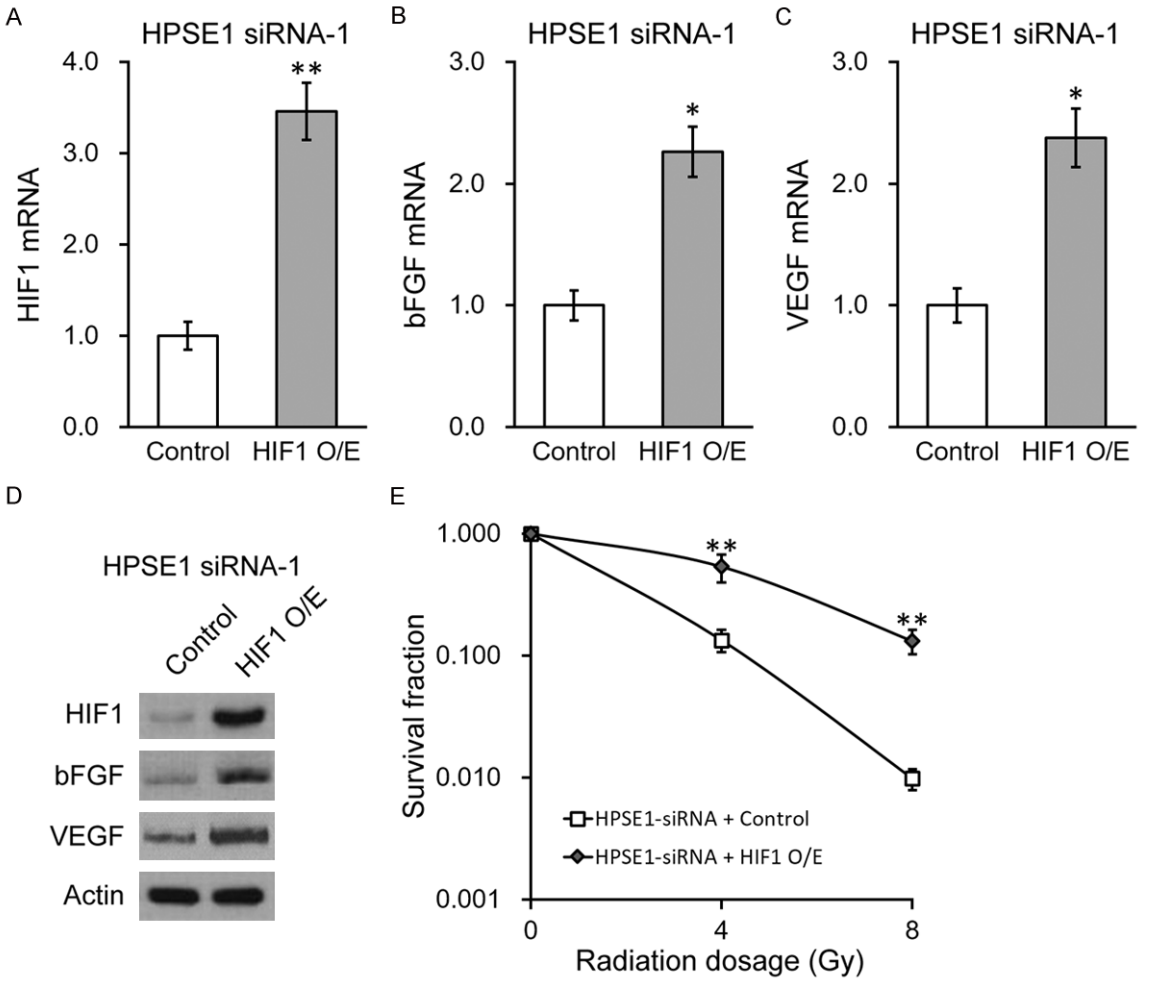

Figure 5. HIF1 overexpression restored radiation resistance of HPSE1 knock-down cervical cancer cells. Empty vector (control) and plasmid overexpressing HIF1 (HIF1 O/E) were transfected into HPSE1 knock-down HeLa cells, respectively. (A-D) Levels of HIF1 mRNA (A), bFGF mRNA (B), VEGF mRNA, as well as their protein levels (D) were examined. (E) The above treated cells were then subjected to radiation dosage as indicated, and viability was examined after HIF1 overexpression. Values were expressed as mean ± SD from at least three independent experiments. *P<0.05, **P<0.01, compared to control.

Discussion

Heparanase (HPSE1) is an endoglucuronidase and is elevated in numerous types of cancers correlating with the poor prognosis [19]. HPSE1 is responsible for extracellular heparan sulfate (HS) cleavage which results in the ECM remodeling and release of bioactive saccharide fragments and HS-bound cytokines and growth factors [12]. In addition to its well-recognized extra cellular effects, recent studies showed heparanase upregulates the expression of a variety of genes [20-22] that involving in cancer metastasis, angiogenesis, glucose metabolism, immune response and inflammation, suggesting HPSE1 play a significant role in regulating transcription within cancer cells. Our current study suggested through transcriptional regulating HIF1 expression, HPSE1 can affect radiation response in cervical cancer cells both *in vivo* and *in vitro*.

Our data showed HPSE1 overexpression lead to radiation resistant in HeLa cells. This type of radiation protection effects by HPSE1 in cervical cancer is consistent with other researchers' finding in pancreatic carcinoma. Meirovitz and his colleague [23] found that clinical relevant doses of irradiation promoted the invasive ability in pancreatic carcinoma cells by upregulating HPSE1 *in vitro* and *in vivo*. HPSE1 upregulation after irradiation is mediated by radiation

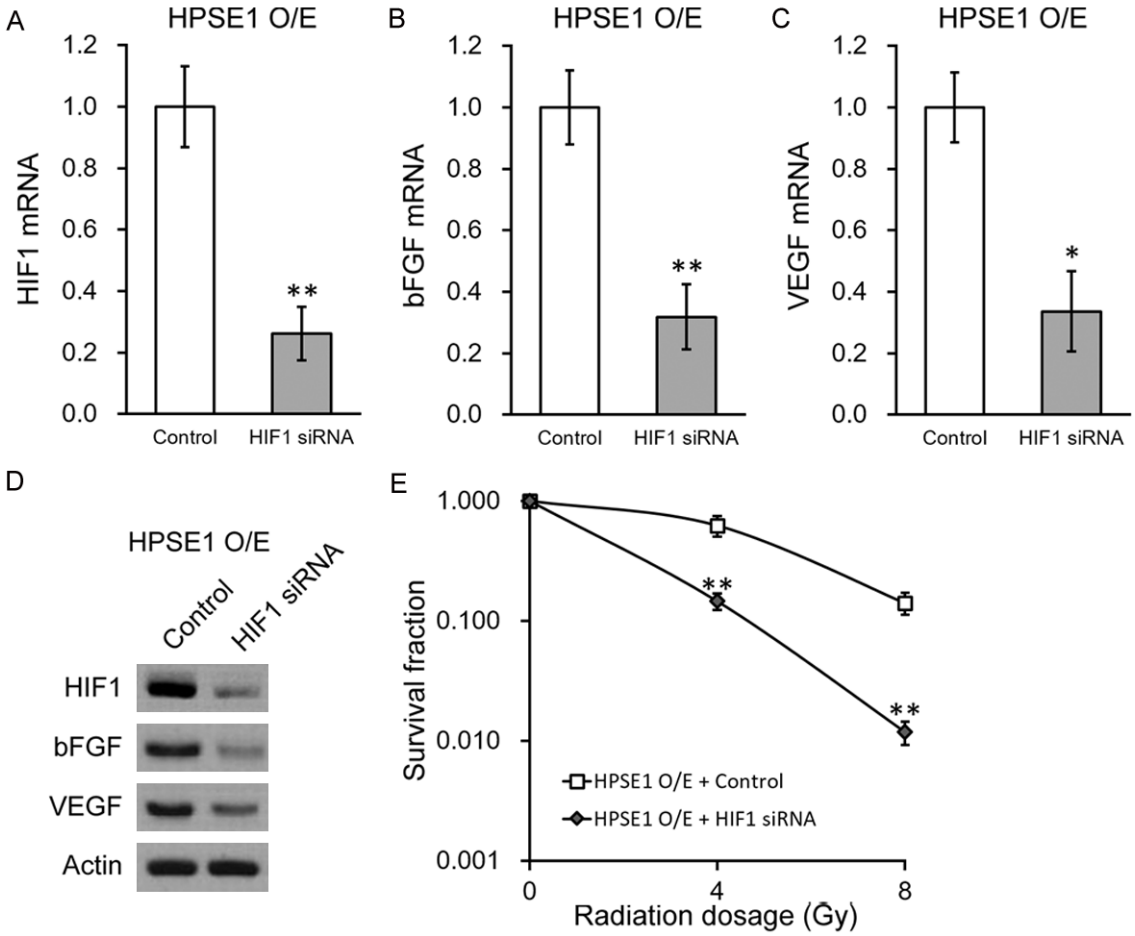

Figure 6. HIF1 knock-down inhibited HPSE1 overexpression-induced radiation resistance of knock-down cervical cancer cells. HIF1 knock-down, as well as control siRNA, was performed in HPSE1 overexpressing (HPSE1 O/E) HeLa cells, respectively. (A-D) Levels of HIF1 mRNA (A), bFGF mRNA (B), VEGF mRNA, as well as their protein levels (D) were examined. (E) The above treated cells were then subjected to radiation dosage as indicated, and viability was examined after HIF1 knock-down. Values were expressed as mean ± SD from at least three independent experiments. *P<0.05, **P<0.01, compared to control.

induced changes in the levels of the transcription factor Egr-1. Furthermore, their found heparanase inhibitor SST0001 abolished IR-enhanced invasiveness of pancreatic carcinoma cells *in vitro* and attenuated orthotopic pancreatic tumors metastasis *in vivo*.

HPSE1 may have stronger effects on radiation response *in vivo*, since it can both induce VEGF expression mediated by HIF1 pathway within cancer cells after radiation and release the HS-bounded VEGF from ECM. Our *in vivo* experiments using a xenograft mouse model showed tumor derived from HPSE1 overexpression HeLa cells is more resistant to radiation therapy and have a high MVD suggesting a high VEGF level in HPSE1 overexpression tumors. Angiogenesis is an important regulator of radiation response, and bevacizumab, a VEGFR inhibitor was tested for clinical effects in combination with standard pelvic chemoradiation therapy for locally advanced cervical cancer. Schefter and his colleagues [24] performed a phase II study evaluated the safety and efficacy of the combination of bevacizumab with chemoradiation therapy for locally advanced cervical carcinoma. The primary results from the trial shows promising efficacy of this combination with 81.3% of 3-years overall survival rate, suggesting the inhibitory of angiogenesis is efficient to improve the therapeutic benefits for local advance cervical cancer patients. Since our data showed tumors derived from HPSE1 knockdown cells have significantly lower MVD

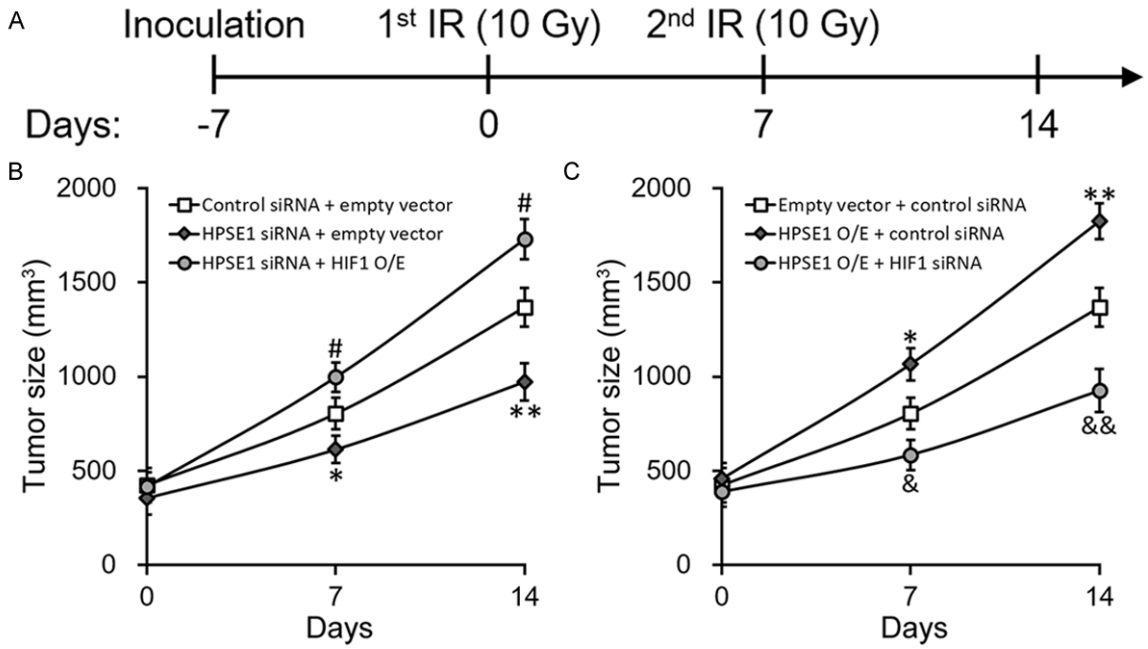

Figure 7. HPSE1 enhanced radiation resistance of xenograft cervical tumor through HIF1. A: Scheme of the xenograft mouse model design, with inoculation performed on day-7 and irradiation (IR) on day 0 and 7. B and C: Tumor growth curve of HeLa cell tumors in each group of xenograft mice (n = 10 each). Values were expressed as mean ± SD. *P<0.05, **P<0.01, compared to control siRNA+ empty vector. #P<0.05, compared to both control siRNA+ empty vector and HPSE1 siRNA+ empty vector. &P<0.05, &&P<0.01, compared to both empty vector + control siRNA and HPSE1 O/E+ control siRNA.

and lower proliferation rate after irradiation, HPSE1 became a very interesting target to improve radiation sensitivity through inhibition angiogenesis.

Our data also showed the HPSE1 effects on radiation response and angiogenesis is mediated by HIF1 pathway. HIF1 is a heterodimer composed of two subunits, HIF1α and HIF1β. The major regulation mechanism of HIF1 is the oxygen concentration. Under normoxic conditions, HIF1α can be hydroxylated by PHD and ubiquitinated by a pVHL-containing E3 ubiquitin ligase, resulting in rapid proteolysis [25, 26]. While under hypoxic conditions, HIF1α is stabilized and associated with HIF1β. The HIF1 heterodimer then binds to the hypoxia-responsive element (HRE) and induces the transcriptional expression of various genes responsible for adaptation to hypoxia. Another known HIF1 regulator is ROS [16]. In our current study, we showed HPSE1 regulated HIF1 expression in HeLa cells. The underlie mechanism seems very interesting since HIF1 plays an important part in tumorigenesis and progression by regulating target genes that control metastasis, angiogenesis and resistance to microenvironment stress.

Besides the angiogenesis effects, HPSE1 is involved in multiple regulatory events related to tumor growth and metastasis by modulating growth factor or cytokines-mediated signaling, ECM remodeling and cell-cell interactions in the tumor microenvironment. Thus, by targeting heparanase, the chronic inflammation conditions, angiogenic status within the tumor microenvironments and the proliferation and invasive ability within tumor cells could be affected at the same time [19], which makes heparanase a very interesting target for cancer therapy. Current approaches for targeting HPSE1 inhibition include development modified heparins, small molecule inhibitors and neutralizing antibodies. Some of the inhibitors were undergoing clinical trials and primary data showed promising therapeutic benefits when combined with other drugs [27]. Thus, further studies are needed to evaluate the therapeutic effects of HPSE1 inhibition on cervical cancer patients.

In conclusion, our study here demonstrates HPSE1 has a radiation protection effects in cervical cancer cells both *in vivo* and *in vitro*. This type of radiation regulator effects is mediated by activation of HIF1 pathway. Further studies

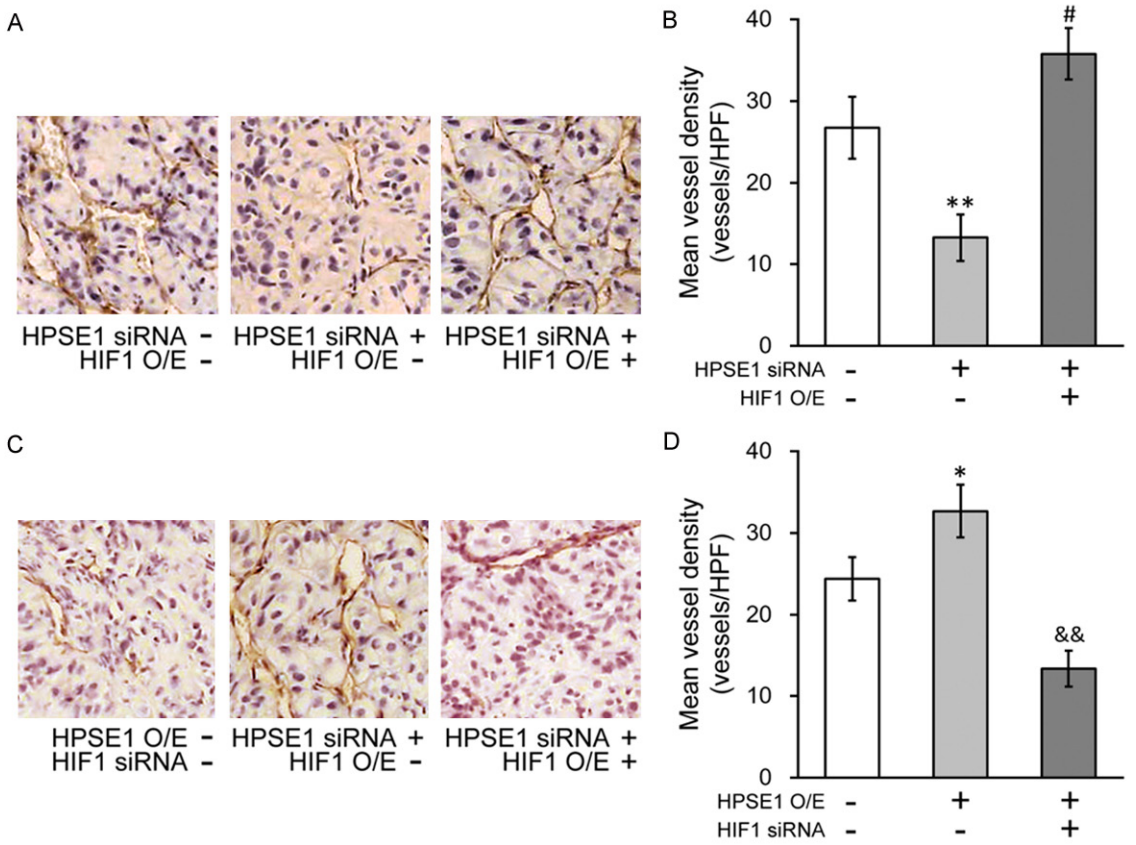

Figure 8. HPSE1 enhanced angiogenesis of xenograft cervical tumor through HIF1. A and B: Representative images of CD34-positive intratumoral microvessels after various treatments as indicated below. C and D: Mean vessel density as quantified from the CD34-positive intratumoral microvessels, after various treatments as indicated below. Values were expressed as mean ± SD (n = 10 each group). *P<0.05, **P<0.01, compared to control siRNA+ empty vector. #P<0.05, compared to both control siRNA+ empty vector and HPSE1 siRNA+ empty vector. &&P<0.01, compared to both empty vector+ control siRNA and HPSE1 O/E+ control siRNA.

are warranted to determine the underlie mechanism of how HPSE1 regulate HIF1 activity and the clinical effects of HPSE1 inhibitors combined with chemoradiotherapy in cervical cancer patients.

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Disclosure of conflict of interest

None.

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