Heparanase participates in the growth and invasion of human U-2OS osteosarcoma cells and its close relationship with hypoxia-inducible factor-1α in osteosarcoma

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Received May 19, 2010

Although the expression of heparanase is associated with invasion and metastasis of various human cancers, the effects of heparanase on human osteosarcoma have not been evaluated. We showed that down-regulating the expression of heparanase significantly reduced proliferation and invasion of human U-2OS osteosarcoma cells. Furthermore, heparanase silencing by short-hairpin RNA (shRNA) was associated with decreased hypoxia-inducible factor-1α (HIF-1α) level, implying that heparanase was associated with the expression of HIF-1α. This result was confirmed by immunohistochemistry analysis. In osteosarcoma tissues, immunohistochemical results revealed that heparanase expression had a close correlation with that of HIF-1α and they had a strong relation with presence of pulmonary metastasis (P<0.05). Heparanase-positive samples had higher microvessel density (MVD) than heparanase-negative samples. Similarly, compared with HIF-1α-negative samples, HIF-1α-positive samples had higher MVD. Therefore, heparanase and HIF-1α facilitated tumor angiogenesis and promoted pulmonary metastasis of osteosarcoma.

Key words: heparanase, HIF-1α, osteosarcoma, invasion, angiogenesis

Despite recent advances in treatments consisting of chemotherapy and tumor excision, however, few targets have demonstrated significant efficiency in the treatment of osteosarcoma. Tumor development and metastasis depend on the ability of cancer cells to break through the tissue barrier of the extracellular matrix (ECM) and basement membrane (BM). Heparan sulfate proteoglycan (HSPG) is a major component of the ECM and BM [1]. Heparan sulfate (HS) is a glycosaminoglycan chain present in HSPGs. Heparanase can degrade HS at several sites, thus facilitating the migration of tumor cells and playing an important role in invasion and metastasis of tumor [2–4]. Heparanase is involved in angiogenesis, primarily by means of releasing HS-binding angiogenic factors sequestered in the ECM and BM, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [5–7]. Protein and mRNA of heparanase are all increased in variety of human malignancies and in some xenografts of human tumors [8]. In a variety of cancers, the expression levels of heparanase are correlated with the metastasis of tumors and are predictive factors for prognosis [9–12]. Therefore, heparanase may be a promising cancer target.

RNA interference (RNAi) is a powerful specific gene silencing method [13]. Short hairpin RNA (shRNA) targeting mouse heparanase can effectively inhibit the metastatic and angiogenic potential of mouse melanoma cells [14]. However, to this time, no author has investigated the effects of heparanase in progression of osteosarcoma. In the present study, we used a vector-based heparanase shRNA expression system to suppress the expression of heparanase in human U-2OS osteosarcoma cells, and then evaluated the inhibitory effects of heparanase shRNA on the proliferation and invasion of human osteosarcoma cells.

Angiogenesis is an essential process required for tumor metastasis [15]. Heparanase release a number of active molecules that may relate to neovascularization. Overexpression of hypoxia-inducible factor-1α (HIF-1α) had relation with the poor prognosis of various cancers [16–20]. HIF-1α related to angiogenesis by inducing expression of VEGF and it played an important role in contribution of angiogenesis of tumor [21]. Therefore, both heparanase and HIF-1α are thought to play vital roles in angiogenesis of tumor. Naomoto [22] reported a hypothesis that heparanase promoted angiogenesis through
HIF-1α and cyclooxygenase-2 (Cox-2). Okawa [23] et al confirmed that heparanase was involved in angiogenesis through induction of Cox-2. However, the relationship of heparanase and HIF-1α in osteosarcoma has not been investigated.

Materials and methods

**Antibodies and reagents.** Anti-heparanase (sc-25825) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HIF-1α (bs-0737R), anti-CD34 (bs-0646R) and anti-GAPDH (bs-0834R) were purchased from Bioss Biotechnology (Bios, China). MTT (M2128) was the product of Sigma (St. Louis, USA). Matrigel (3432-005-01) was purchased from Trevigen (Gaithersburg, USA).

**Cell lines and tissue samples.** The U-2OS, MG63, and Saos-2 cell lines (Laboratory of Pathology Department, Sun Yat-sen university, Guangzhou, China) were maintained in RPMI1640 medium containing 10% fetal bovine serum (FBS), 100ug/ml penicillin and 100ug/ml streptomycin.

A total of 45 unselected cases (25 males and 20 females) with primary osteosarcoma were eligible for this study. The patients ranged in age from 7 to 49 years (average age: 18.6 years). Samples were recruited from the first affiliated hospital of Sun Yat-sen University. An approval for this study was given by the Medical Ethics Committee of Sun Yat-sen University.

**Construction of heparanase shRNA expression plasmids and transfection.** Sequences of three shRNA targeting human heparanase and a shRNA-scrambled sequence (negative control) were as follow (Table 1). Each insert contains a BamHI and BbsI-restriction site overhang, a 19-bp target sequence, a 9-bp loop sequence, a reverse complementary sequence of the 19-bp target, and an RNA polymerase III terminator sequence. These primers were annealed and inserted into pGPU6/GFP/Neo. The plasmids containing heparanase shRNA-1, 2, 3 and negative control sequences were named pSihHPA-1, pSihHPA-2, pSihHPA-3 and pNC respectively. U-2OS cells transfected with pSihHPA-1, pSihHPA-2, pSihHPA-3 and pNC were called SihHPA-1, SihHPA-2, SihHPA-3, and NC cells. By using western blot, we selected pSihHPA-1 as our target to transfect U-2OS cells. U-2OS cells were stably transfected with pSihHPA-1 and pNC using the Lipofactamine 2000 (Invitrogen). Cells were selected with G418 (600ug/ml) for 3 weeks. Surviving colonies were isolated and expanded. The cells of stable transfection of pSihHPA-1 and pNC were named SihHPA-1 and NC respectively.

**Western blot analysis.** Cells were lysed in a lysis buffer after washed twice by ice-cold PBS. Protein concentrations were determined by using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). 100ug total protein was boiled for 5 minutes before being loaded onto a 10% polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with primary antibody, followed by HRP-conjugated secondary antibody. At last, related proteins visualized by enhanced chemiluminescence (Pierce). GAPDH was served as the internal control.

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**Real-time RT-PCR analysis.** Total RNA was isolated by TRIzol (Invitrogen) from tumor cells. First Strand cDNA Synthesis Kit (GeneCopoeia, USA) was used for cDNA synthesis as recommended by the supplier. Primers included 5'-TTTGCAAGCTGCTTTATGTG-3' and 5'-TCCTGCTTCCAAGAATACTTGTC-3' for GAPDH (152bp); 5'-GTGGACCTGACCTGCTGCTCT-3' and 5'-GGAG-GAGTGAGGTGGTTGTCGCTGT-3' for heparanase (93bp). Real-time RT-PCR was performed by using 2×AllinOneTM Q-PCR Mix (GeneCopoeia, USA). The data were analyzed by a comparative threshold cycle (Ct) method. The level of GAPDH was used as internal reference to normalize the data; mRNA relative fold changes were calculated using the 2-ΔΔCt method.

**MTT and colony formation assay.** Cell proliferation was detected by the MTT assay. The U-2OS, SihHPA-1 and NC cells were seeding in 96-well plates at the density of 1×104 cells/well. 50μl of MTT was added to each well. Then the plates were incubated at 37°C for 4 hours. Each well added to 150μl dimethylsulfoxide and shaken for 10 minutes. The absorbance was measured at a wavelength of 492nm. Optical density is related with the cell numbers. Adjust the concentration of U-2OS, SihHPA-1 and NC cells to appropriate density. Then each culture dish inoculated 200 cells. Cells were incubated at 37°C and changed the medium every 4 days. After two weeks, cells were fixed with methanol and stained with trypan blue. Colonies were counted by a microscope.

**Flow cytometry.** The U-2OS, SihHPA-1 and NC cells were washed twice with PBS, fixed with 75% ethanol and for 30 minutes at 4°C. Cells were incubated with the DNA-binding dye propidium iodide (50 μg/ml) and RNase (1.0 mg/ml) for 30 min at 37°C in the dark. Finally, cells were washed and red fluorescence was analyzed by a flow cytometer (BD, Heidelberg, Germany).
Figure 1. (A) Transfection of heparanase shRNA into U-2OS cells. The pGPU6 vector was used to construct plasmids containing heparanase shRNA and GFP. 48 hours after transfection, transfected U-2OS cells (NC, SihHPA-1, SihHPA-2 and SihHPA-3) could easily identified by fluorescence microscopy. The final two photofluorograms were shown the stable cell lines (NC, SihHPA-1) selected by G418. (B) Western blot examined expression of heparanase in U-2OS, MG-63 and Saos-2 cell lines. (C) Effects of heparanase shRNA on heparanase protein expression in U-2OS, NC, SihHPA-1, SihHPA-2, and SihHPA-3 cells. (D) The results of real-time RT-PCR indicated that the relative levels of heparanase mRNA in U-2OS, NC and SihHPA-1 were 0.91, 0.90, 0.61 respectively. *P < 0.05 vs. U-2OS or NC. (E) Representative western blot for heparanase protein expression displayed down-regulation of heparanase expression by shRNA. Moreover, expression of HIF-1α was suppressed following heparanase gene silencing. NC, negative control.
Migration and invasion assay. The U-2OS, SihHPA-1 and NC cells were seeded into 12-well plates at a density of 1×10^{5} cells/well. The cells were scraped with a 200μl sterile pipette tip when they formed monolayers. Then the cells were washed with PBS three times and incubated for 24 hours. Serum-free medium was used to culture medium and photographs were taken at 0 and 24 hours by microscope.

Invasion assay was performed using transwell (Costar). The U-2OS, SihHPA-1 and NC cells at a density of 1×10^{5} cells/well were added into the upper chamber with 0.2ml serum-free RPMI-1640. 0.5ml of 10% FBS medium was added into the lower chamber. The cells were allowed to invade for 24 hours at 37°C. After removing the cells on the upper surface of the membrane, cells on the lower were fixed in methanol and stained with trypan blue.

Immunohistochemistry. 5-μm sections were cut from the selected paraffin blocks and deparaffinized by routine techniques. The slides were microwaved in citrate buffer for 6 minutes for antigen retrieval. Sections were then incubated with primary antibodies at room temperature: heparanase (1:150 dilution), HIF-1α (1:100 dilution). Labeling was detected by adding biotinylated secondary antibodies (Maxim-Bio, Fuzhou, China), avidin-biotin complex (Maxim-Bio), and diaminobenzidine (Maxim-Bio). Sections were then counterstained with hematoxylin. Staining results for each antibody were examined by two authors independently. Heparanase staining was scored according to the intensity (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining) and the percentage (extent staining) of tumor cells that were stained (0, no positive cells; 1, <10% of tumor cells stained; 2, 10%-50% of tumor cells stained; 3, >50% of tumor cells stained; 4, >75% of cells staining positive). If the product of multiplication between staining intensity and the percentage of positive cells is ≥2, it is thought as immunoreaction positive (+). The number of CD-34-positive vessels was counted in five randomly selected areas of a 1-mm^{2} field, and the average was calculated.

Statistical analysis. Statistical analysis was done using the SPSS13.0. The correlation between heparanase or HIF-1α and clinicopathological parameters was evaluated by χ² test for comparison of two groups. A Fisher’s exact test was used to evaluate the association between the expressions of heparanase and HIF-1α in osteosarcoma biopsies by immunohistochemistry. The migration distance of SihHPA-1 cells was shorter than that of U-2OS (578±12, significantly lower than that of U-2OS cells (1276±25) (Fig. 2A). Furthermore, MTT assay confirmed that proliferation of SihHPA-1 cells was significantly inhibited compared with that of U-2OS and NC cells (P<0.05; Fig. 2B).

Effects of heparanase shRNA on U-2OS cell proliferation. The result of colony formation assay showed the number of colonies in SihHPA-1 cells (216±20.8) was significantly lower than that of U-2OS (374±12.8) and NC cells (351±9.6) (Fig. 1B). The result of colony formation assay showed the number of colonies in SihHPA-1 cells (216±20.8) was significantly lower than that of U-2OS (374±12.8) and NC cells (351±9.6) (Fig. 1B). Therefore, our experiments were focused on SihHPA-1 cells.

Effects of heparanase shRNA on U-2OS cell cycle. Analysis of cell cycle showed that SihHPA-1 cells at the G1 phase reached 58.3±1.9%, obviously higher than U-2OS (43.5±7.1%) and NC cells (44.9±2.8%), indicating that the heparanase shRNA arrested cells in the G1 phase. However, cells in the S phase decreased from 43.9±6.7% of NC group to 25.2±1.5% of SihHPA-1 group, suggesting that the heparanase shRNA induced a significant decrease of the S phase fraction (P<0.05; Fig. 2C).

Effects of heparanase shRNA on U-2OS cell invasion. We found the number of SihHPA-1 cells on the membrane was 578±12, significantly lower than that of U-2OS cells (1276±25) and the NC cells (1183±15) (P<0.05), whereas there was little difference between U-2OS and NC cells (P>0.05; Fig. 3A). These data indicated the role of heparanase in the migration of U-2OS cells.

Effects of heparanase shRNA on U-2OS cell invasion. We found the number of SihHPA-1 cells on the membrane was 578±12, significantly lower than that of U-2OS cells (1276±25) and the NC cells (1183±15) (P<0.05), whereas there was little difference between U-2OS and NC cells (Fig. 3B; P>0.05). Our results indicated that down-regulating the expression of heparanase inhibited the invasion of human osteosarcoma cells.

Relationship between heparanase and HIF-1α expressions. Through western blot, we detected that decreased expression of HIF-1α was observed following heparanase gene silencing by means of shRNA in osteosarcoma cells (Fig. 1E). Then we further demonstrated the relationship between heparanase and HIF-1α in osteosarcoma biopsies by immunohistochemistry. Forty-five osteosarcoma biopsies were performed for heparanase and HIF-1α staining. 18 of 45 (40%) cases were positive for both heparanase and HIF-1α, whereas neither heparanase nor HIF-1α was expressed in 33% (15/45) cases. In addition, 5 of 45 (11%) cases were heparanase positive but HIF-1α negative, whereas only HIF-1α was expressed in 16% (7/45) cases (Table 2). These results indicated that heparanase was apparently link to HIF-1α expression (P=0.003).
Figure 2. Effects of heparanase shRNA on tumor cell proliferation. (A) Colony formation of osteosarcoma cells in vitro. U-2OS, NC and SihHPA-1 cells in flat plates were cultured for 3 weeks and stained with Giemsa. (B) Proliferation of osteosarcoma cells. U-2OS, NC and SihHPA-1 at 10^4/well were cultured in 10% FBS RPMI1640 and their proliferation ability were determined by MTT. Data are the OD (492 nm) values. (C) Effects of heparanase shRNA on tumor cell cycle distribution. *P <0.05 vs. U-2OS or NC.

Figure 3. Effects of heparanase shRNA on migration, invasion of U-2OS cells in vitro. (A) Cell wound healing assay of U-2OS, NC and SihHPA-1 cells treated as indicated and the migration distance was shown in bar chart. (B) U-2OS, NC and SihHPA-1 cells were seeded into the upper chamber of the Transwell respectively. After 24 hours of incubation at 37℃, the cells through the pores to the lower surface of the membrane were stained with Giemsa and counted under a microscope. *P <0.05 vs. U-2OS or NC.
HEPARANASE IN THE GROWTH AND INVASION OF HUMAN U-2OS

Relationship between heparanase/HIF-1α expression and clinicopathological factors.

Heparanase expression was observed in 23 (51%) of the 45 cases. Positive heparanase staining appeared the tumor cell nuclei and cytoplasm (Fig. 4A, B, C). Nearly all tumor cells were heparanase positive, when tumors were stained by heparanase.

The relationship between heparanase and clinicopathological factors was examined and the results were presented in Table 3. Heparanase expression significantly correlated with tumor size ($P=0.002$). Similar results were found for the correlation between heparanase and the patients who had pulmonary metastasis ($P=0.000$). Heparanase expression did not differ significantly depending on age, gender, anatomic location and histologic type (Table 3).

HIF-1α expression was observed in 25 (56%) of the 45 cases. In positive cases, HIF-1α protein immunoreactivity was present in the tumor cell nuclei (Fig. 5A, B, C). The relationship between HIF-1α and clinicopathological factors was displayed in Table 3. HIF-1α expression significantly correlated with pulmonary metastasis ($P=0.000$). HIF-1α expression did not differ significantly depending on age, gender, anatomic location, tumor size and histologic type (Table 3).

Seventy-three percent and 62% of patients with osteosarcoma negative for heparanase and HIF-1α survived, respectively, whereas the survival rate was 39% and 33% in patients with

| Table 2. Correlations of heparanase with HIF-1α and MVD |
|-----------------|-----------------|---------|---------|
| Heparanase (+)  | HIF-1α (+)      | $<0.05$ | $48.61\pm6.32$ | $<0.0001$ |
| Heparanase (+)  | HIF-1α (–)      | $>0.05$ | $27.18\pm3.99$ |          |

*, Mean±SD.
+ and –, positive and negative, respectively.

Figure 4. Immunohistochemical staining of heparanase in osteosarcoma specimens. (A) Nuclear localization of heparanase in osteosarcoma specimens. (B, C) Cytoplasm localization of heparanase in osteosarcoma specimens. (D) Kaplan-Meier survival curve of patients in osteosarcoma for the presence and absence of heparanase expression. Heparanase-positive patients exhibited significantly poorer survival rate compared with heparanase-negative patients. (A-C, IHC staining with original magnification of $\times400$).
Table 3. Relationship between heparanase or HIF-1α expression and clinicopathological factors in osteosarcoma samples

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Abbreviation: (+) and (-), presence and absence of pulmonary metastasis respectively.

*< 0.05

Figure 5. Immunohistochemical staining of HIF-1α in osteosarcoma specimens. (A, B, C) Nuclear localization of HIF-1α in osteosarcoma specimens. (D) Kaplan-Meier survival curve of patients in osteosarcoma for the presence and absence of HIF-1α expression. HIF-1α-positive patients exhibited significantly poorer survival rate compared with HIF-1α-negative patients. (A-C, IHC staining with original magnification of ×400).
positive for heparanase and HIF-1α 5 years after operation ($P=0.0001$ and 0.002, respectively; Fig. 4D and Fig. 5D).

Expressions of heparanase and HIF-1α predict neovascularization of osteosarcoma.

Microvessels in osteosarcoma tissues were examined by using anti-CD34 antibody. Fig. 6 showed microvessels in osteosarcoma tissues according to the expressions of heparanase and HIF-1α. Compared with both heparanase-negative and HIF-1α-negative samples (25.87±1.85; Fig. 6A), both heparanase-positive and HIF-1α-positive samples were related with higher MVD (68.56±2.81; Fig. 6D; $P<0.0001$). Heparanase-positive samples had a significantly higher MVD (48.61±6.32; Fig. 6B) than heparanase-negative samples (27.18±3.99; Table 2; $P<0.0001$). Tumors with positive HIF-1α expression also had a significantly higher MVD (50.57±7.12; Fig. 6C) compared with those negative for HIF-1α (28.91±4.10; $P<0.0001$).

Combination of heparanase-positive and HIF-1α-positive expression was correlated with higher MVD (Fig. 6D). These data strongly suggested the expressions of heparanase and HIF-1α facilitated angiogenesis of osteosarcoma.

Discussion

It is well accepted that heparanase plays a significant role in tumor invasion, metastasis, and angiogenesis [24–25]. Overexpression of heparanase was associated with distant metastasis and poor prognosis in various cancers, such as pancreatic cancer [26], gastric cancer [27], bladder cancer [28] and glioma [29]. The aim of this study was to explore the role of heparanase in progression of osteosarcoma.

RNA interference is an effective and widely used tool for functional genomic analyses and silencing specific genes. Zhang et al [30] reported down-regulation the expression of heparanase by RNAi could inhibit the invasiveness, metastasis,
and angiogenesis of human hepatocellular carcinoma. However, in their study, they showed that siRNA for heparanase could not influence the proliferation of SMMC7721 cells. In our study, we have confirmed that inhibition of heparanase expression significantly reduce the proliferation ability of U-2OS by MTT and colony forming assay. By analysing cell cycle, we found that the number of G1 phase U-2OS cells transfected with shRNA increased, while the number of S phase cells decreased significantly compared with the control. This result clarified the reason that the proliferation ability of SiHPE-1 cells was suppressed. Furthermore, our data suggested that the heparanase shRNA not only inhibited the proliferation of U-2OS cells, but also suppressed their ability of migration and invasion. This was consistent with Vlodavsky’s report [31] that silencing of endogenous heparanase gene expression effectively inhibited the invasive and metastatic potential of B16-BL6 melanoma cells. Our findings demonstrated an important role of heparanase in proliferation activity of human osteosarcoma.

Because heparanase and HIF-1α have been known as bad prognostic markers in various tumors, we hypothesized that heparanase and HIF-1α expressions might link to each other in osteosarcoma. Moreover, decreased HIF-1α level was detected following heparanase gene silencing by means of shRNA, implying that heparanase might be related to the expression of HIF-1α (Fig. 1E). In order to clarify the effects of heparanase and HIF-1α in osteosarcoma, we further examined their expression in osteosarcoma specimens. In a series of 45 osteosarcoma specimens, positive expression of heparanase was detected in 23 (51%) cases, correlating with larger tumor size, presence of pulmonary metastasis (Table 3) and poor survival (Fig. 4D). This was in accordance with previous reports in several other human tumors [12, 28, 29]. Our findings revealed that heparanase participated in the growth of osteosarcoma cells in vitro assays. This result provided a basis for correlation between heparanase and larger tumor size. Twenty-five (56%) patients qualified for the positive HIF-1α expression, correlating with presence of pulmonary metastasis (Table 3) and poor survival (Fig. 5D). Of the 45 cases, 18 showed expressions of both heparanase and HIF-1α, whereas 15 cases showed neither heparanase nor HIF-1α expression. Five cases showed expression of heparanase alone, whereas 7 cases showed expression of HIF-1α alone. Consequently, expression of heparanase had a close correlation with that of HIF-1α in vivo. However, the particular transcriptional mechanisms that are involved in regulation of HIF-1α need to be further investigated.

From our results, both heparanase and HIF-1α had a strong correlation with presence of pulmonary metastasis. As is known to all, pulmonary metastasis occurs in 40-50% of patients with osteosarcoma [32]. Angiogenesis facilitated the procession of pulmonary metastasis of osteosarcoma. Heparanase induced angiogenesis by releasing some growth factors, such as VEGF, bFGF and so on. HIF-1α expression was significantly associated with microvessel density and surgical stage in human osteosarcoma [33]. VEGF was regulated by HIF-1α. Hence, both heparanase and HIF-1α were key regulators in the angiogenesis of tumor. MVD reflected the number of microvessel in angiogenesis. In our study, heparanase and HIF-1α independently had higher MVD and they were tightly correlated with MVD. This might explain why patients who had positive expression of heparanase or HIF-1α were more likely to have pulmonary metastasis of osteosarcoma compared with those patients who had negative expression of heparanase or HIF-1α. Interestingly, the positive of both heparanase and HIF-1α showed the highest MVD, suggesting their important role on angiogenesis and these molecules might interact with each other. Therefore, heparanase and HIF-1α expressions are predictive of poor outcome in osteosarcoma patients.

In summary, we showed that down-regulating the expression of heparanase could significantly inhibit proliferation and invasion of human osteosarcoma cells. A significant correlation was noted between heparanase and HIF-1α expressions, and they were closely correlated with pulmonary metastasis of osteosarcoma through angiogenesis. Heparanase should be evaluated prospectively as a potential prognostic factor. Furthermore, our findings provide a new clue concerning metastasis of osteosarcoma and will help the development of new therapy for osteosarcoma treatment.

Acknowledgments. Our research was supported by National Natural Science Foundation of China (No. 30900650/C140403).

References


