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Downregulating the expression of heparanase inhibits the invasion, angiogenesis and metastasis of human hepatocellular carcinoma

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Abstract

Invasion and metastasis are key features of human hepatocellular carcinoma (HCC). Heparanase is an endoglycosidase that can degrade extracellular matrix by cleaving heparan sulfate chains of heparan sulfate proteoglycan, thus playing important roles in the invasion and metastasis of human cancers. Heparanase has been detected in various human cancers and regarded as a prospective target in human cancer treatments. However, the effects of inhibiting the expression of heparanase on human HCC have not been fully evaluated. In this article we show that downregulating the expression of heparanase either by antisense oligodeoxynucleotide or by RNA interferencing can significantly reduce the expression of heparanase in SMMC7721 human HCC cells, leading to inhibition of the invasiveness, metastasis, and angiogenesis of HCC cells both *in vitro* and *in vivo*. Our results suggest that genetic downregulation of the expression of heparanase may serve as an efficient cancer therapeutic for human HCC.

Keywords: Heparanase; Antisense oligodeoxynucleotide; Small interfering RNA; Angiogenesis; Invasion; Metastasis; Human hepatocellular carcinoma

Human hepatocellular carcinoma (HCC) is one of the most common tumors in the world. Invasion and metastasis are key features and also predictors for poor prognosis of HCC [1]. In the process of invasion and metastasis, tumor cells must break through the barrier of the extracellular matrix (ECM) and the basement membrane (BM) [2]. Heparan sulfate proteoglycan (HSPG) is a major component of the ECM and BM, and is composed of a protein core, to which side chains of the complex glycosaminoglycan and heparan sulfate (HS) are attached [2]. Heparanase is an endoglycosidase that can degrade HS at several sites, thus facilitating the migration of many normal cell types and playing an important role in invasion and metastasis of tumor cells [3–5]. The heparanase gene is located on human chromosome 4q21.3, and its cDNA contains an open reading frame of 1629 bp that encodes for a 61.2 kDa polypeptide of 543 amino acids [6–8]. Expression of the human heparanase mRNA in normal tissues is restricted primarily to the placenta and lymphoid organs [6–8]. However, heparanase mRNA is increased in human malignancies and many xenografts of human tumors [6–8]. Preferential expression of the heparanase mRNA and protein in tumors is also evident in tissue specimens derived from adenocarcinoma of the ovary, metastatic melanoma, oral squamous cell carcinoma, hepatocellular carcinoma, and carcinomas of prostate, bladder, and pancreas [6–15]. In human colon carcinoma, pancreatic cancer and breast cancer, the expression levels of heparanase are even correlated with the differentiation and metastatic stages of tumors and are predictive indicators for prognosis [6–15].

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Due to its roles in cleavage of HS, degradation of ECM and subsequent release of HS-bound active fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), heparanase is widely involved in many aspects of tumor progression, including tumor cell invasion and metastasis as well as angiogenesis [3–5].

Heparanase may represent an important cancer target. Several groups are currently developing competitive heparin/HS-mimicking compounds, neutralizing anti-heparanase antibodies, antisense oligodeoxynucleotides (AS-ODN), and inhibiting small molecules [10,16–19]. It has been reported that inhibiting the expression or activity of heparanase can lead to inhibition of tumor invasion, metastasis and angiogenesis [10,16–19]. Non-anticoagulant species of heparin and several sulfated polysaccharides can inhibit the activity of heparanase, and therefore decrease the experimental tumor metastasis and has entered the clinical trials [3–5,18]. However, due to the potential non-specific activities of these inhibitors and the great difficulties in identifying efficient inhibitors, genetic approaches targeting heparanase have been regarded as a promising alternative [20–22]. We have shown previously that an AS-ODN against heparanase mRNA has inhibitory activity on heparanase expression and invasiveness of a human mammary cancer cell line *in vitro* [23]. However, the effects of downregulating the expression of heparanase in human HCC has not been fully evaluated. In the present study, we further evaluate the inhibitory effects of the heparanase AS-ODN and small interfering RNA (siRNA) on the invasion, angiogenesis and metastasis of human HCC.

Materials and methods

Cells and animals. The human HCC cell line SMMC7721, provided by the Central laboratory of the Second Military Medical University (Shanghai, China), was routinely grown in RPMI1640, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C in a 5% CO_2 incubator. Balb/c nu/nu mice (five weeks of age), provided by the Experimental Animal Center of Shanghai Institute of Life Science were maintained in a specific pathogen-free environment.

Antisense oligodeoxynucleotides and small interfering RNA. The AS-ODN and siRNA duplexes were designed according to the sequence of the heparanase gene (GenBank Accession No. AF155510). The sequences of ODNs used in the present study [23] were: AS-ODN, 5′-GGC TTC GAG CGC AGC AGC AT-3′; and NS-ODN (nonsense oligodeoxynucleotide), 5′-TCA GCT AGC GAG GCT GCG CA-3′. The ODNs were synthesized and phosphorothioated by the Shanghai Songon Biological Engineering Company (Shanghai, China). For RNA interferencing, 21-nucleotide sequences of human heparanase siRNA were chemically synthesized (Shanghai Songon Biological Engineering Company) as follows: 5′-AGC UUCGUACCUUGGCCAGtt-3′ (sense), and 5′-CUGGCCAAGGUAC GAAGCUtt-3′ (antisense). The sequence 5′- AGCAUCGUACGU AGGCCAGtt-3′ (sense), and 5′- CUGGCCUACGUACGAUGCUtt-3′ (antisense) were used as a scrambled RNA interference control (Ctrl siRNA). The ODNs were transfected by using cationic liposome Lipofectin (Gibco BRL, Gaithersburg, MD, USA), and siRNAs were transfected with Genesilencer Transfection Reagent (Genlantis, San Diego, CA), both of which were performed according to the manufacturer's instructions.

Real-time quantitative RT-PCR. Quantitative PCR was performed on a MJR Chromo4 Continuous Fluorescence detector (Bio-Rad, Hercules, CA) as described [24]. The following primers were used: 5′-TTC GAT CCC AAG AAG GAA TCA AC-3′ (sense) and 5′-GTA GTG ATG CCA TGT AAC TGA ATC-3′ (antisense) for heparanase; and 5′-TCC TGC ACC ACC AAC TGC TT-3′ (sense) and 5′-TCC ACC ACC CTG TTG CTG TA-3′ (antisense) for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH).

Western blotting. After transfection (24–48 h), the cells were lysed and subjected to Western blotting with a heparanase monoclonal antibody HP-130 (InSight Biopharmaceuticals, Rehovot, Israel) as described previously [25].

Matrigel invasion assay. Forty eight hours after transfection, the cells were suspended in serum-free RPMI 1640 (2×0^5 cells/ml). Two hundred microliters of cell suspension was placed onto the upper compartment of the matrigel invasion chamber (BD Biosciences, Bredford, MA) and 500 μl of RPMI1640 containing 10% FBS was added to the lower compartment. After a 24 h incubation period at 37 °C in 5% CO_2, membranes were fixed in methanol and stained with H&E. Cells on the upper surface of the filter were removed carefully with a cotton swab, and the cells that had migrated through the membrane to the lower surface of the filter were counted in nine different high fields under a light microscope.

Acute hematogenous metastasis assay. In this assay, each mouse was injected with 1×10^6 SMMC7721 cells through lateral tail vein ($n = 12$). Then ODN solution (100 μg in 100 μl PBS) or siRNA (10 nM in 100 μl RPMI1640) was administrated daily by a single intravenous injection through the tail vein. After two weeks of injections, the mice were sacrificed, the lungs were removed and fixed in Bouin's solution, and the number of metastatic nodules on the lung surface was then scored under a dissecting microscope.

Establishment of SMMC7721 tumor models, evaluation of microvessel density (MVD) and in vivo tumor growth assays. SMMC7721 cells (6×10^6) were subcutaneously injected into the back of the Balb/c nu/nu mice ($n = 12$). Two weeks later, each mouse received daily a single injection of ODN solution (100 μg in 100 μl PBS) or only PBS as blank control into the tumor at multiple sites when the tumors grew to 2–3 mm in diameter. For *in vivo* interferencing of heparanase, 10 nM siRNA were incubated with Geneporter 2 Transfection Reagent (Genlantis) for 10 min, diluted to a total volume of 100 μl in RPMI1640 and injected daily into the tumor at multiple sites. 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 $= a \times b^2/2$. After three weeks of injections, the mice were sacrificed and the tumors were excised. The MVD was evaluated by immunostaining for von Willebrand factor. The tumor tissues ($n = 3$) were fixed in formalin, embedded in paraffin, cut into tissue sections (4 μm thick), and subjected to immunostaining using Envision$^+$ System (Dako Diagnostics, Zug, Switzerland) per the manufacturer's instructions. After microscopic screening for tumor areas of highest MVD under 40× magnification, the vessels in five randomly selected fields in these areas were counted under 200× magnification by two independent observers. The mean value of the vessels counted in five fields by the two observers was considered the MVD for the tumor [13].

Statistical analysis. Results are expressed as means ± SEM or means ± SD. Comparisons between two groups were conducted by Student's "*t*" test. Multiple comparisons were done with a one-way ANOVA followed by Fisher's least significant difference analysis. Pairwise comparisons were done by performing nonparametric Mann–Whitney *U* test. All of the experiments were repeated at least twice. *P* values of 0.05 or less were considered significant.

Results

Effects of the AS-ODN and siRNA against heparanase on the expression of heparanase in SMMC7721 cells

To examine the efficiency of AS-ODN and siRNA on the expression of human heparanase, SMMC7721 cells

were transfected with AS-ODN, siRNA and corresponding controls. Later (24–48 h), the mRNA and protein expression of heparanase were examined by both real-time quantitative RT-PCR and Western blot assays, respectively. We found that heparanase mRNA and protein could be detected in the parental cells, and the NS-ODN and Ctrl siRNA (Fig. 1) could not affect the expression level of heparanase. However, heparanase was significantly decreased in the AS-ODN- and siRNA- (Fig. 1) transfected cells. Therefore, the AS-ODN and siRNA specific for heparanase used in our study are specific and efficient in downregulating the expression of heparanase in SMMC7721 cells.

The heparanase AS-ODN and siRNA significantly inhibit the invasiveness and metastasis of SMMC7721 cells

Inhibiting the activity or the expression level of heparanase has been reported to impair the *in vitro* migration and invasion of tumor cells [16–23]. We first examined the effects of heparanase AS-ODN and siRNA on SMMC7721 cell invasiveness by using a matrigel invasion assay. We found that both the AS-ODN-transfected cells and the siRNA-transfected cells demonstrated an impaired transmigration capacity, when compared to the parental and control cells and as evidenced by reduction of migrated cell numbers (Fig. 2A).

We then examined the metastatic potential of SMMC7721 cells *in vivo* after the establishment of SMMC7721 tumors in nude mice. We performed the acute hematogenous metastasis assay by counting the lung metastases of SMMC7721 tumors after intravenous injection of SMMC7721 cells and then the administration of AS-ODN or siRNA. We found that the AS-ODN could inhibit the expression of heparanase in the lung metastases while the siRNA could minimally affect the heparanase expression level (Fig. 2B), which may be due to the differences in the *in vivo* stability of the AS-ODN and siRNA [26–28]. Therefore, we used the AS-ODN but not the siRNA to evaluate the effects of heparanase downregulation on *in vivo* metastasis of SMMC7721 cells. We found that AS-ODN against heparanase could significantly

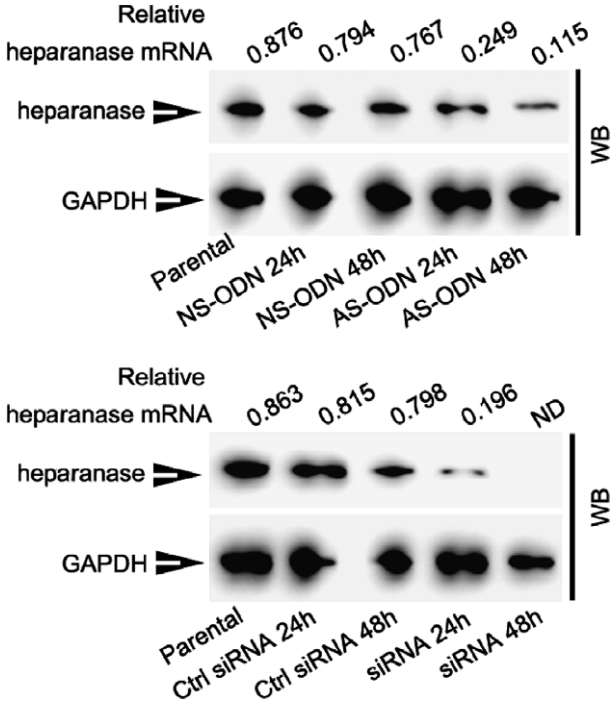

Fig. 1. Expression of heparanase in SMMC 7721 cells after AS-ODN or siRNA transfection. 1×10^5 SMMC7721 cells cultured in 6-well plates were transiently transfected with ODNs (upper panel) or siRNA duplexes (0.1 nM, lower panel). At indicated time points, the expression of heparanase was evaluated by both Sybr-green-based real-time quantitative RT-PCR and Western blot. The relative expression of heparanase mRNA to that of GAPDH was presented as means ± SEM of three independent experiments, and was shown at the top of each panel. GAPDH was used as quantitative control. WB, Western blotting; ND, not detected.

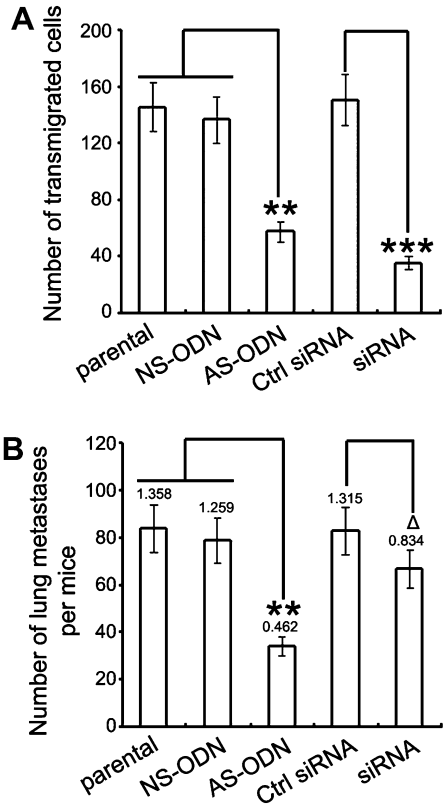

Fig. 2. The heparanase AS-ODN and siRNA significantly inhibit the invasiveness and metastasis of SMMC7721 cells. (A) *In vitro* matrigel assays. Forty eight hours after transient transfection of SMMC7721 cells with indicated ODNs or siRNAs, the ability of SMMC7721 cells to trans across the matrigel was evaluated after a 24 h incubation in the matrigel invasion chamber. The results were expressed as means ± SD of triplicate chambers. (B) *In vivo* metastasis assay. An acute hematogenous lung metastasis model was established by injection of 1×10^6 SMMC7721 cells through lateral tail vein. 100 μg ODNs in 100 μl PBS or 10 nM siRNA in 100 μl RPMI1640 was administrated daily through the tail vein. Two weeks later, the number of metastatic nodules on the lung surface was scored and presented as means ± SD of 12 mice. The relative heparanase mRNA expression in metastases was shown at the top of each bar. $^{\Delta}p > 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

decrease the lung metastases after administration while NS-ODN and blank control (parental) could not (Fig. 2B).

The heparanase AS-ODN and siRNA significantly inhibit the angiogenesis of SMMC7721 tumor

Angiogenesis is one of the most important features of human HCC [1]. In the established SMMC7721 tumors, the intratumoral administration of both AS-ODN and siRNA could significantly downregulate heparanase expression (Fig. 3). More importantly, we found that the MVDs in the AS-ODN- or siRNA-treated tumors were significantly decreased (Fig. 3), as evidenced by immunohistochemistry assays and counting of the von Willebrand factor positive vessels. Therefore, we suggest that AS-ODN and siRNA used in our study are efficient in inhibiting the angiogenesis process of the established SMMC7721 human HCC *in vivo*.

The heparanase AS-ODN and siRNA significantly inhibit the in vivo growth of SMMC7721 tumor

Next we evaluated the effects of the AS-ODN- and siRNA-mediated effects *in vivo* by measuring the growth of SMMC7721 tumors. After the establishment of SMMC7721 HCC in nude mice, the tumors were treated with either AS-ODN or siRNA by intratumoral injections at multiple sites. We found that the administration of both AS-ODN and siRNA could significantly inhibit the expression of heparanase *in vivo*, resulting in inhibition of the growth of SMMC7721 tumors (Fig. 4), while the NS-ODN and the Ctrl siRNA could not affect the growth of the established tumors, suggesting that the AS-ODN and siRNA against heparanase were efficient in inhibiting tumor growth *in vivo*.

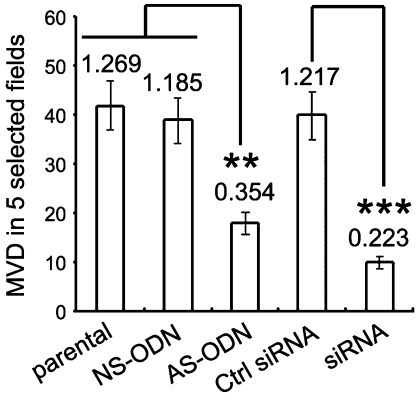

Fig. 3. The heparanase AS-ODN and siRNA significantly inhibit the *in vivo* angiogenesis of SMMC7721 tumor. 6×10^6 SMMC7721 cells were subcutaneously injected into the back of the Balb/c nu/nu mice. Two weeks later, ODNs (100 μg in 100 μl PBS) or siRNAs (10 nM in 100 μl RPMI1640) were intratumorally injected daily at multiple sites for weeks. The MVD was evaluated by counting the vessels in five randomly selected fields (200×) after immunohistochemistry of von Willebrand factor. The results were presented as means ± SD of three randomly selected mice that showed regressed tumor growth *in vivo*. The relative mRNA expression of heparanase in tumor nodules was shown at the top of each bar. $^{**}p < 0.01$; $^{***}p < 0.001$.

Discussion

The roles of heparanase in tumor invasion, metastasis and angiogenesis have been extensively established [2–5]. Inhibiting the activity or the expression of heparanase has been shown to be efficient in the regression of tumor progression both *in vitro* and *in vivo* [16–23]. In our study, we have evaluated the effects of heparanase AS-ODN and siRNA on the invasion, metastasis and angiogenesis of human SMMC7721 cells. Our study suggests that down-regulating the expression of heparanase by either AS-ODN or siRNA can impair the tumor progression of human HCC possibly by inhibiting the invasion, metastasis and angiogenesis of HCC.

By virtue of its HS-degrading activity, heparanase plays an important role in the process of tumor progression, and has been considered a key target for drug development [2–5]. Potent inhibition of heparanase activity have been obtained with sulphated polysaccharides and heparin-mimicking polyanionic molecules. One representative of these inhibitors, PI-88, is being evaluated in a multicentre phase II clinical trial [18]. However, due to the potential non-specific activity of these inhibitors and the insufficient knowledge in the functional mechanisms of the heparanase, the application of heparanase inhibitors in clinical trials may require further investigations, such as identification of the sugar residues in HS adjacent to the heparanase cleavage site and the crystallization and analysis of the three-dimensional structure of the enzyme [2–5]. Therefore, genetic blockade of the expression of heparanase, such as AS-ODN and siRNA, may be a promising alternative, given the highly specificity of these methods. Antisense oligodeoxynucleotides (AS-ODNs) are potential therapeutic

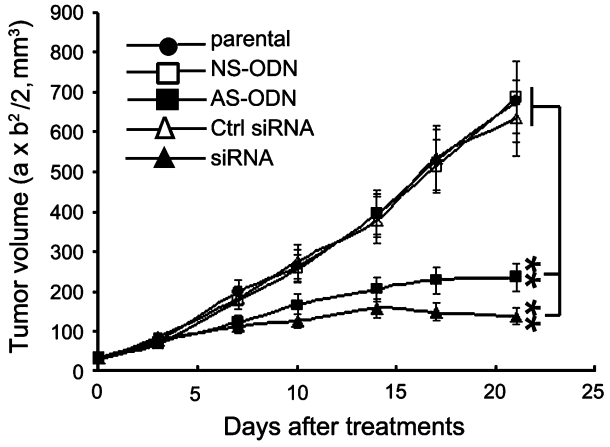

Fig. 4. The heparanase AS-ODN and siRNA significantly inhibit the *in vivo* growth of SMMC7721 tumor. After the establishment of SMMC7721 tumors, they were treated with ODNs or siRNAs as described in Fig. 3 (day 0). Tumor volume was monitored on days indicated, and results presented as means ± SD of 12 mice. $^{**}p < 0.01$.

agents, and can inhibit gene expression in a sequence-specific manner [26,27]. RNA interference (RNAi) is a post-transcriptional mechanism of gene silencing through chromatin remodeling, inhibition of protein translation or direct mRNA degradation [28]. Our previous study demonstrated that AS-ODN inhibited heparanase expression and *in vitro* invasiveness of mammary cancer cells in a dose-dependent manner [23]. Uno et al. constructed an adenoviral vector carrying an antisense full-length human heparanase cDNA, and they found that the heparanase expression, the *in vitro* invasiveness and *in vivo* metastasis of human lung cancer A549 cells were specifically inhibited [20]. A similar study also found that the inhibition of heparanase enzymatic activity was efficacious in the prevention and treatment of melanoma metastasis [21]. Downregulation of heparanase by stable overexpression of either anti-heparanase ribozyme or RNA silencing vector had been shown previously to not only inhibit heparanase activity but also impair tumor invasion and metastasis, resulting in improved survival of tumor-bearing mice [22]. Data from our present study have shown that heparanase AS-ODN and siRNA inhibit the invasion and metastasis of human HCC. More importantly, we show that *in vivo* administration of AS-ODN and siRNA against heparanase can inhibit the *in vivo* tumor growth, which may possibly be contributed to inhibition of cell proliferation, impairment of *in vivo* invasion and decreased angiogenesis of the established HCC tumors. However, the *in vitro* examination of SMMC7721 proliferation by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method demonstrated that AS-ODN and siRNA could not affect the SMMC7721 cell proliferation (data not shown). Therefore, the inhibitory effects of AS-ODN and siRNA for heparanase on HCC *in vivo* may result from inhibition of the invasion and angiogenesis of human HCC. Additionally, it should be noted that the *in vivo* administration of siRNA via intravascular injection can not efficiently decrease the expression of heparanase, indicating that the stability of siRNAs may be lower than the phosphorothioated AS-ODN despite the fact that siRNA is more efficient than AS-ODN in inhibiting the expression of heparanase and impairing the *in vitro* invasion and *in vivo* angiogenesis of SMMC7721 cells/tumors.

In sum, we have demonstrated that downregulation of the expression of human heparanase can efficiently inhibit the invasion, metastasis and angiogenesis of human HCC. It is likely that the inhibition of heparanase expression possibly depresses the degrading of ECM and BM, thus inhibiting the invasion and metastasis of HCC. Therefore, our study suggest that AS-ODN and siRNA specific for human heparanase may be of potential values as a novel therapeutic agent for human HCC.

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