Down-regulation of HDGF Inhibits the Growth of Hepatocellular Carcinoma Cells *In Vitro* and *In Vivo*

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Abstract. *Background: Hepatoma-derived growth factor (HDGF) is a growth factor of various malignant diseases. However, the in vivo effects of HDGF suppression targeting for hepatocellular carcinoma (HCC) have not been clarified to date. Materials and Methods: We stably transfected HDGF shRNA into SK-HEP-1 human HCC cells and investigated the effects of HDGF reduction on HCC growth using a cell proliferation assay and a murine xenograft model. The effects of HDGF reduction on VEGF expression and in vivo angiogenesis were also investigated with real-time PCR and immunostaining analyses, respectively. Results: HDGF reduction resulted in a decreased proliferative activity of SK-HEP-1 cells both in vitro and in vivo. The in vivo anti-tumor effects of HDGF were particularly higher than that expected in vitro. HDGF-reduction suppressed VEGF expression in SK-HEP-1 cells and in vivo angiogenesis of developed tumors. Conclusion: These findings suggest that targeted inhibition of HDGF may be a novel anti-HCC therapy.*

Hepatocellular carcinoma (HCC) is one of most common malignant diseases worldwide, and advanced HCC continues to have a poor prognosis despite recent development of anticancer therapies (1, 2). An oral multikinase inhibitor, sorafenib, is the first agent demonstrated to improve median survival and time to progression in patients with advanced HCC (3, 4). Sorafenib has been accepted as standard-treatment for advanced HCC; however, sorafenib therapy often provides only limited clinical effects on HCC patients. Although many molecules have been proposed as potential targets for anticancer therapy (5), no molecule other than sorafenib has been demonstrated to possess clinical significance on treatment of HCC, and new target molecules are, therefore, required.

Hepatoma-derived growth factor (HDGF) is a novel growth factor previously identified from the human hepatoma-derived cell line Huh-7 (6). HDGF stimulates the proliferation of hepatoma cells *in vitro* (7), and HDGF expression is significantly higher in human HCC tissues than in adjacent non-cancerous liver tissues (8). In addition, the high level of HDGF expression is related to several aggressive cancer phenotypes and correlated with the poor clinical outcomes of various malignant diseases (9-14), and HDGF has been suggested as a potential target for various malignant diseases, including HCC (14, 15). However, the *in vivo* effects of HDGF suppression targeting for HCC have not yet been clarified. In the present study, we investigated the potential of HDGF as a new target therapy for HCC.

Materials and Methods

Cell culture and establishment of stable transformants. The human HCC cell line, SK-HEP-1, was obtained from the American Type Culture Collection (Manassas, VA, USA). SK-HEP-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in 5% CO_2 at 37˚C. We previously generated several stably transformed clones in which the HDGF expression was down-regulated, and in the present study, two

Abbreviations: HCC: Hepatocellular carcinoma, HDGF: hepatoma-derived growth factor, DMEM: Dulbecco's modified Eagle's medium, FBS: fetal bovine serum.

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Key Words: Hepatoma-derived growth factor, hepatocellular carcinoma, targeted therapy, xenograft model, VEGF, angiogenesis.

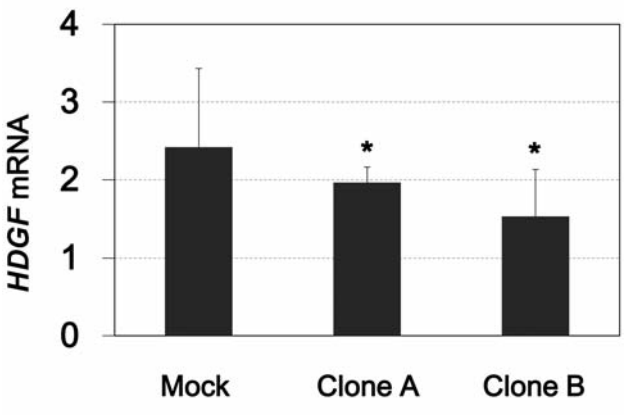

Figure 1. *Reduction of HDGF by introduction of shRNA plasmid. The expression levels of HDGF mRNA were measured by real-time PCR methods. The HDGF expressions levels in shRNA-transfected cells (clones A and B) were significantly lower compared to control (mock-transfected) cells (N=6 per each group). *p<0.05 control cells versus shRNA-transfected cells.*

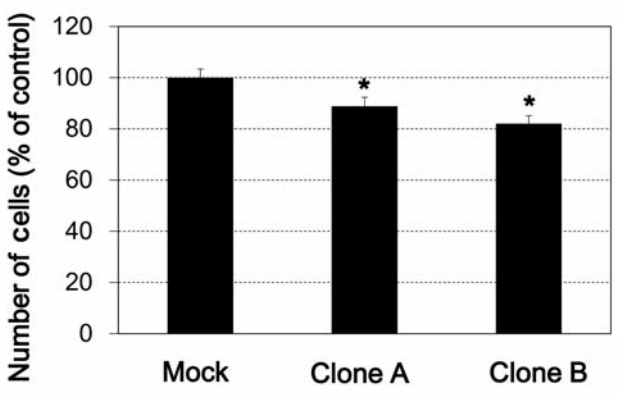

Figure 2. *In vitro growth suppression of SK-HEP-1 cells by HDGF reduction. The cells were plated onto 96-well plates at a density of 3×10^3 cells/well. After 24 h, the cells were given fresh medium supplemented with 10% FBS. The cells were cultured for an additional 48 h, and cell numbers were measured using colorimetric assay methods. The absorbance level of the control, clone A and clone B were 1.24±0.12, 1.10±0.10, and 1.02±0.08, respectively. The cell numbers of HDGF-reduced cells (clones A and B) were significantly lower than those of control (mock-transfected) cells (N=8 per each group). *p<0.05 control cells versus shRNA-transfected cells.*

new stable transfectants of HDGF-reduced SK-HEP-1 cell lines were established according to the methods previously reported (16). In brief, SureSilencing shRNA plasmid for human HDGF (SABiosciences) and negative control shRNA plasmids were transfected using the lipofection method, and stable transformants were selected and maintained with Geneticin (16).

Cell proliferation assay. For cell proliferation assay, cells were plated onto 96-well plates at a density of 2.5×10^3 cells/well in DMEM supplemented with 10% FBS. After 24 h, cells were administered fresh medium supplemented with 10% FBS. The cells were cultured in the medium for an additional 48 h, and cell numbers were measured using colorimetric assay methods with the Cell Counting Kit (Dojindo, Kumamoto, Japan) (7).

Quantitative analysis of HDGF mRNA levels using real-time PCR. The *HDGF* mRNA expression was measured using quantitative real-time PCR according to a method reported previously (16). In brief, total RNA was extracted through the AGPC method using Isogen (Nippon Gene, Tokyo, Japan). After reverse transcription, the obtained complementary DNA was amplified using the TaqMan PCR Reagent Kit (Applied Biosystems, Foster City, CA, USA) under the following conditions: 50˚C for 2 min, 95˚C for 10 min, and 40 cycles at 95˚C for 15 s and 60˚C for 1 min. The forward primer 5'-AAGTTT GGCAAGCCCAACA-3', reverse primer 5'-GGCTCTTCCA CACAG CTCTTT-3', and probe 5'-FAM-AACC CTACTGTCAAGGCTTCC GGCTTAMRA-3' were used for HDGF (16). The VEGF mRNA expression was also measured using the TaqMan® Gene Expression Assay (Cat. # 4331182) (Life Technologies Japan, Tokyo, Japan) according to the manufactures' instructions. Expression of *beta-actin* mRNA was used as an internal control.

Mouse xenograft model. A xenograft model was generated according to methods previously described (17). Briefly, three transfected SK-Hep1 cell lines (mock-transfected cells and two HDGF-reduced cells) were used, and control (mock-transfected) or HDGF-reduced cells were inoculated into both flanks (1×10^7 cells/flank) of four-week-old BALB/c nu/nu mice. The tumor size was measured with a caliper once per week, and the volumes were estimated according to the following formula: volume=length×$(\text{width})^2\times0.52$. The tumors that developed in the mouse xenograft model were used for the histological evaluation. Sections of the tumors were immunostained with anti-mouse CD31 antibody (Clone SZ31), which was able to detect mouse endothelial cells without reacting with human cells (Dianova, Hamburg, Germany), and positive cells were imaged with DAB. All experimental procedures were approved by the Animal Care Committee of Hyogo College of Medicine (Nos. 22009 and 28011) and performed according to the "Guide for the Care and Use of Laboratory Animals" by the National Academy of Science of Japan.

Statistical analysis. Data for comparisons among the three groups were analyzed using non-repeated ANOVA measurements and was subsequently evaluated with the Bonferroni correction. A *p*-value of <0.05 was considered statistically significant.

Results

Effects of HDGF reduction on HCC growth in vitro. We generated two new stable clones in which HDGF expression was down-regulated and assessed its effects on the proliferation of SK-HEP-1 cells. Real-time PCR methods revealed that the expression levels of HDGF in two shRNA-introduced clones (clones A and B) were significantly lower than those of the mock-transfected clone, although the degrees of HDGF reduction were mild (Figure 1). In

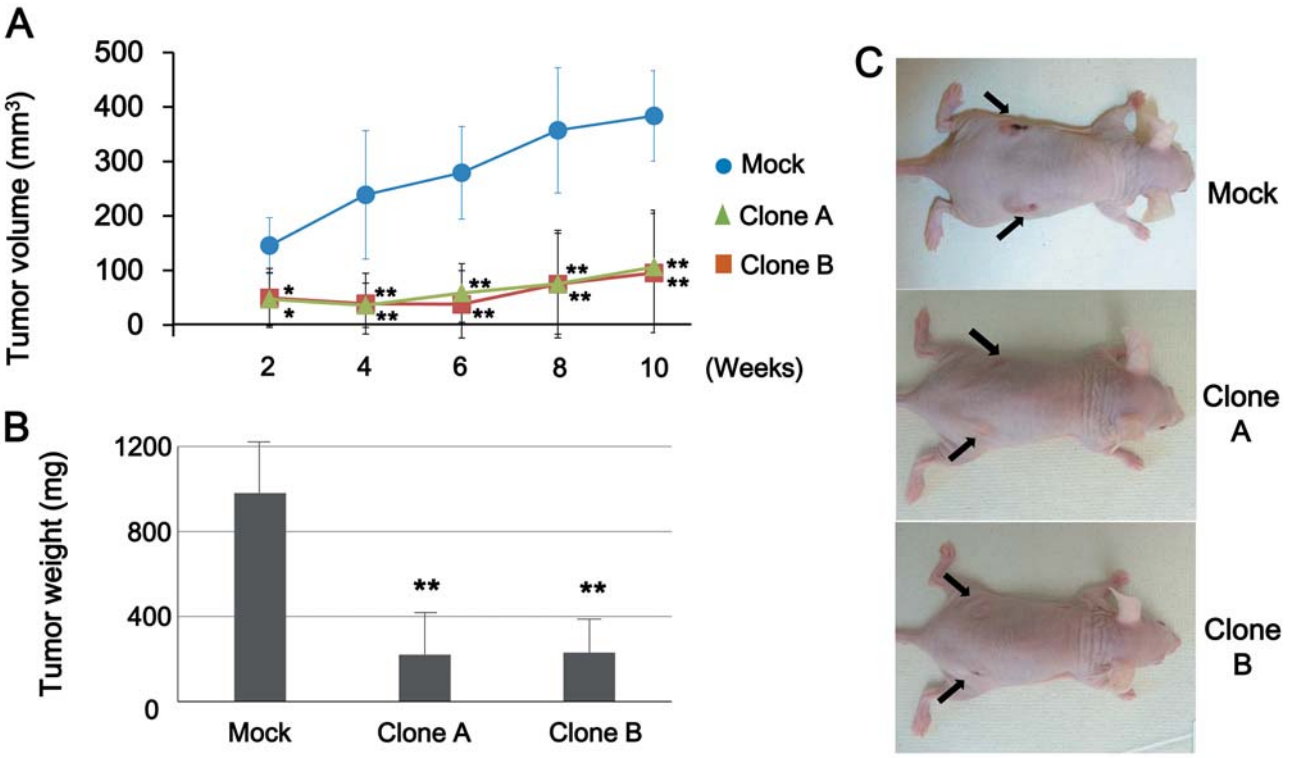

Figure 3. *In vivo growth suppression of SK-HEP-1 tumors by HDGF reduction. Three transfected SK-HEP-1 cell lines (mock-transfected cells and two HDGF-reduced cells) were inoculated into both flanks (1×10^7 cells/flank) of four-week-old BALB/c nu/nu mice (N=10 per each group. (A) The tumor sizes derived from the HDGF-reduced cells (clones A and B) were significantly smaller than those derived from the control (mock-transfected) cells. (B) The tumor weights derived from the HDGF-reduced cells (clones A and B) were also significantly lower than those derived from the control (mock-transfected) cells. **p<0.05 and **p<0.01 control cells versus shRNA-transfected cells. Representative photos of the tumors are shown in (C).*

addition, the *in vitro* proliferations of HDGF-reduced clones were significantly suppressed when compared to that of the mock-transfected control clone (Figure 2).

Effects of HDGF reduction on HCC growth in vivo. Next, we transplanted the transformants into nude mice and evaluated the effects of HDGF reduction *in vivo*. Unlike the relatively mild inhibition of HCC cell proliferation observed in the *in vitro* experiments, the transplanted HDGF-reduced SK-HEP-1 cells formed remarkably smaller tumor volumes when compared to the mock-transfected control cells (Figure 3A). Additionally, the developed tumors from HDGF-reduced SK-HEP-1 clones had significantly lower weight than the tumors derived from the control clone (Figure 3B). These findings suggest that HDGF reduction had a significant anti-tumor effect on the development of HCC tumors *in vivo*.

Effects of HDGF reduction on VEGF expression and in vivo angiogenesis. Since HDGF is considered to be both a growth stimulating factor and an angiogenic factor (21), the significant growth decrease of HDGF-reduced cells *in vivo* may depend on reduced angiogenic activity, in addition to its

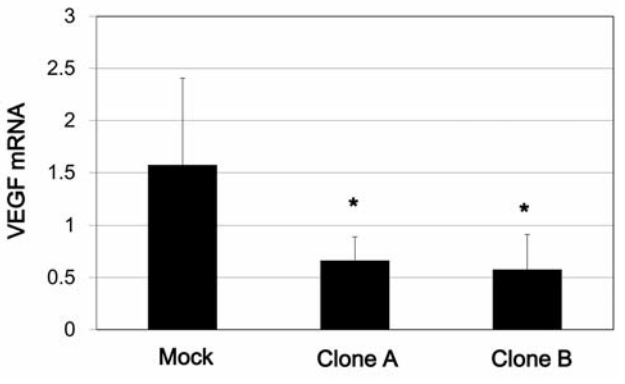

Figure 4. *Effects of HDGF-reduction in VEGF expression in SK-HEP-1 cells. The expression levels of VEGF mRNA were measured by real-time PCR methods. The VEGF expressions levels in shRNA-transfected cells (clones A and B) were significantly lower than the control (mock-transfected) cells (N=6 per each group). *p<0.05 control cells versus shRNA-transfected cells.*

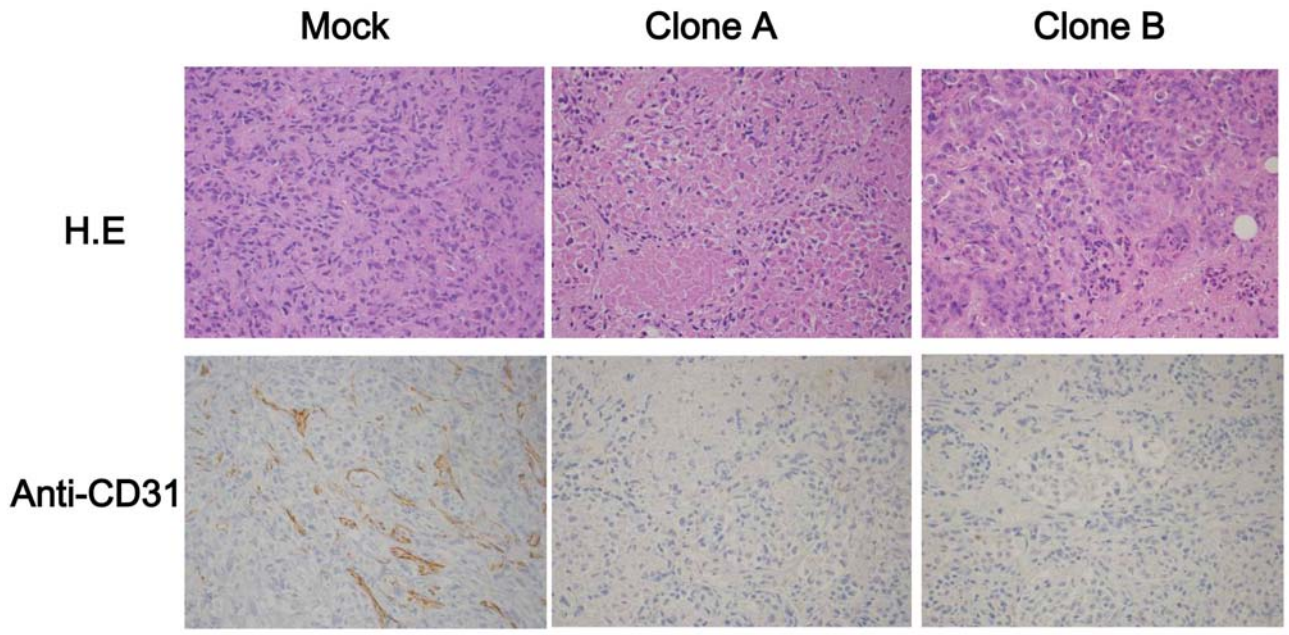

Figure 5. *Immunostaining of CD31 in SK-HEP-1 tumors. In tumors developed from control cells and HDGF-reduced cells, endothelial cells were detected through immunostaining analyses for CD31. CD31-positive cells were rarely detected in tumors derived from the HDGF-reduced cells (clones A and B). (H.E; Hematoxylin and eosin staining).*

growth inhibitory effects on SK-HEP-1 cells. We, therefore, evaluated the effects of HDGF-reduction on the VEGF expression and *in vivo* angiogenesis. The expression of VEGF was decreased in HDGF-reduced SK-HEP-1 cells (Figure 4) and CD31-positive endothelial cells were rarely detected in the developed tumors from HDGF-reduced SK-HEP-1 clones (Figure 5), suggesting that a higher *in vivo* anti-tumor effect of HDGF-reduction than that expected from the *in vitro* study may be caused by the anti-angiogenic effects.

Discussion

HCC is a major health concern worldwide. Sorafenib, that targets VEGF, is the only currently established drug for treatment of HCC, and the discovery of new molecular targets is clinically important for further HCC therapy. It was previously reported that HCC patients with a higher HDGF expression show earlier recurrence and poor overall survival rates than those with a lower HDGF expression. Moreover, HDGF expression was shown to be an independent prognostic factor for the disease-free and overall survival in patients treated with curative resection for HCC (12, 18, 19). In addition, previous reports have shown that HDGF promotes the proliferation of HCC cells *in vitro* (7, 20). These findings strongly suggest that HDGF plays a significant role in the progression of human HCC.

In the present study, we found that down-regulation of HDGF showed significant anti-tumor effects on HCC *in vivo*. Interestingly, the *in vivo* anti-tumor effects of HDGF were particularly high (Figure 3), despite the relatively mild anti-proliferative effects on HCC cells *in vitro* (Figure 2). In our previous experiments, HDGF-overexpressing cells developed large tumors in a murine xenograft model, although the proliferative activity of these cells was only moderately increased *in vitro* (20). In addition, HDGF-overexpressing NIH/3T3 cells showed a limited transformation capacity in soft agar, while these cells formed large tumors in nude mice, thus indicating that HDGF-overexpressing cells had a more prominent growth stimulating activity *in vivo* than that expected *in vitro* (17). Our *in vivo* results in the present study, which showed notable antitumor effects of HDGF-reduction, are consistent with these previous findings. HDGF is considered to be not only a growth stimulating factor, but also an angiogenic factor (21) and our findings showed that the VEGF expression in HCC cells and angiogenesis in xenograft tumors decreased by the reduction of HDGF (Figures 4 and 5). Therefore, the significant growth decrease of HDGF-reduced cells *in vivo* may depend on reduced angiogenic activity, in addition to its growth inhibitory effects on SK-HEP-1 cells. The high antitumor effects of HDGF reduction *in vivo* suggest the potential of HDGF as a target molecule for HCC treatment. Further investigations to clarify the regulation and signal transduction of HDGF may provide important information to establish new treatments for HCC.

Conflicts of Interest

The Authors declare there exist no conflicts of interest.

Acknowledgements

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