

HSG provides antitumor efficacy on hepatocellular carcinoma both *in vitro* and *in vivo*

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Abstract. Hyperplasia suppressor gene (HSG) is a novel gene that markedly suppresses the mitogenetic stimuli or injury mediated by vascular smooth muscle cell proliferation. Herein we provide experimental evidence to show that HSG can also play a key role in tumor proliferation. Down-regulation of HSG protein in hepatocellular carcinoma tissues compared to adjacent tissues. Overexpression of HSG suppressed the growth of liver cancer cell lines, resulted in cell cycle arrest in the G0/G1 phase, increased expression of the cyclin dependent kinase inhibitors (CKIs), and reduced expression of proliferating cell nuclear antigen (PCNA). It also showed that adenovirus-mediated HSG overexpression induced apoptosis. Up-regulation of HSG by adenovirus also significantly suppressed the growth of subcutaneous tumors in nude mice both *ex vivo* and *in vivo*. Collectively, our data suggest that HSG is a potential therapy for tumors and possibly other proliferative diseases as well and it has antitumor efficacy on hepatocellular carcinoma by using adenovirus vectors, which may be a new therapeutic target for liver cancer prevention.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide. Eighty-two percent of cases (and death) are in developing countries, especially in sub-Saharan Africa and southeastern Asia and the death rate is the second highest among males in China (1,2). Although there are many advances in HCC therapy, such as recent target therapies, and liver transplantation, the overall patient outcome has not been substantially improved. The five-year survival rate is limited to 25-39% after surgery and much lower elsewhere (3-5).

Therefore, novel treatments for liver cancer are urgently needed (6,7). For example, immunotherapy and potential novel gene therapy show a good prospect for the treatment of HCC (8,9).

HSG, a novel hyperplasia suppressor gene (later re-named mitofusin-2), was expressed at lower level in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats than those from Wistar-Kyoto rats. Overexpression of HSG inhibited mitogenetic stimuli- or injury-induced VSMC proliferation, and prevented balloon injury induced re-stenosis and reduced atherosclerosis (10). Reportedly the anti-proliferative effect of HSG is more potent than p53, a well known tumor suppressor (10,11). Furthermore, it was found that the HSG has apoptotic effect and this effect is mediated by the mitochondria apoptosis pathway (12-15).

However, whether the HSG contributes to the development of cancer is not very clear and needs further research. Moreover, whether altering the HSG expression could be used as a potential method for cancer therapy and its molecular basis are unknown. In this study, we established the HSG expression in HCC tissues and adjacent liver tissues. We employed the adenovirus-delivered HSG technique to study the effects of HSG on HCC both *in vitro* and *in vivo*.

Materials and methods

Tissue samples, cell lines and culture conditions. Twelve pair samples were obtained from HCC and adjacent benign liver tissues (>2 cm from tumor margin) of patients who underwent surgical treatment for HCC in the First Affiliated Hospital, College of Medicine, Zhejiang University. HepG2 and Hep3B, liver cancer cell lines, were preserved in our institute. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Biosciences). Hep3B cells were cultured in minimum essential medium (Gibco) supplemented with 10% fetal bovine serum (Biosciences). All cells were cultured at 37°C in a humidified chamber with 5% CO₂.

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Virus construction and adenovirus infection. Replication-defective adenoviruses encoding the complete HSG open reading frame (AdHSG) and an adenoviral vector encoding green fluorescent protein (AdGFP) which was made as a

control were constructed by Vector Gene Technology Company Ltd. (Beijing, China). Cells synchronization was achieved by culturing cells in DMEM or MEM with 0.2% FBS for 24 h. Cells were incubated with adenovirus at a multiplicity of infection (MOI) of 100 pfu per cell in a small volume of serum-free medium. After adsorption for 4 h, DMEM with adenovirus was discarded and fresh complete growth medium was added and cells were cultured for additional time for the following experiments. Infection was efficient showing almost 90% GFP-positive cells only 24 h after infection.

Reverse transcription-PCR analysis. Detection of HSG mRNA expression was carried out by reverse transcription-PCR as described previously (10). The sequence primers were: 5'-GGAGCTGGACAGCTGGATTGAT-3' (forward), and 5'-AGCTCCAGCTGCTTGTCCATGA-3' (reverse) for HSG (301 bp); 5'-CTTAGTTGCGTTACACCCTTTC-3' (forward), and 5'-CACCTTCACCGTTCCAGTTT-3' (reverse) for β -actin (151 bp). The PCR reaction for HSG amplification was carried out with 1 cycle of 94°C for 10 min and 30 cycles of 94°C for 1 min, 58°C for 10 sec, 72°C for 10 sec, followed by a 1 cycle extension at 72°C for 10 min. The PCR reaction for β -actin amplification was carried out with 1 cycle of 94°C for 10 min and 30 cycles of 94°C for 1 min, 55°C for 10 sec, 72°C for 15 sec, followed by a 1 cycle extension at 72°C for 10 min.

Western blot analysis. The expression level of HSG protein and PCNA, p27, p21, cleaved caspase-3 and cleaved PARP were examined by Western blotting. Cells were infected and then fresh complete growth medium was added for an additional 48 h. Tissues were homogenized in nitrogen liquid. The total protein was isolated from cells and tissues using a lysis buffer (Cell Signaling) and subjected to Western blotting as described previously (10). The primary antibody were: against HSG (Sigma, at 1:1000 dilution); PCNA (Santa Cruz, at 1:500 dilution); p27 (Santa Cruz, at 1:250 dilution); p21 (Santa Cruz, at 1:250 dilution); cleaved caspase-3 (Cell Signaling, at 1:1000 dilution); cleaved PARP (Cell Signaling, at 1:1000 dilution) and β -actin (Sigma, at 1:1000 dilution).

Cell viability assay. Cell viability assay was achieved by using Cell Counting kit-8 (Dojindo Laboratories). Liver cancer cells were plated on 96-well plates at a density of 6×10^3 per well. When cells were grown to subconfluence, they were infected with AdGFP or AdHSG at a MOI of 100 pfu per cell, with the uninfected cells as control. We added 10 μ l of the Cell Counting kit-8 solution with 90 μ l growth medium into each well after 48 h. Two hours later, we read the absorbance at 450 nm to determine the cell viability in each well. Cell viability was calculated as follows:

$$\text{Cell viability} = \frac{\text{A 450 nm mean value of infected cells}}{\text{A 450 nm mean value of uninfected cells}} \times 100\%$$

Flow cytometry analysis for cell cycle distribution. HepG2 cells were synchronized and infected. After that, cells were kept in low serum DMEM (0.2% FBS) for 24 h and then stimulated by 10% FBS for 24 h. Then cells were collected

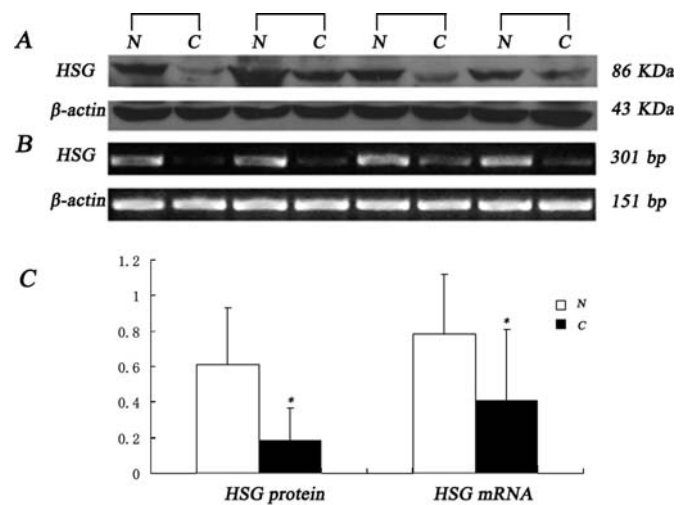


Figure 1. Expression pattern of HSG in HCC and corresponding normatic specimens. (A) Representative results of four matched pairs using Western blotting are shown. β -actin was used as a loading control. (B) Corresponding HSG mRNA expression using RT-PCR. (C) Densitometry analyses of HSG protein and mRNA level are presented as the mean \pm SD by compared with β -actin in HCC and corresponding normal liver samples. The expression of HSG was reduced in tumor tissue when compared with corresponding non-tumorous tissues. C, HCC; N, corresponding normatic tissues; n=12, *p<0.05.

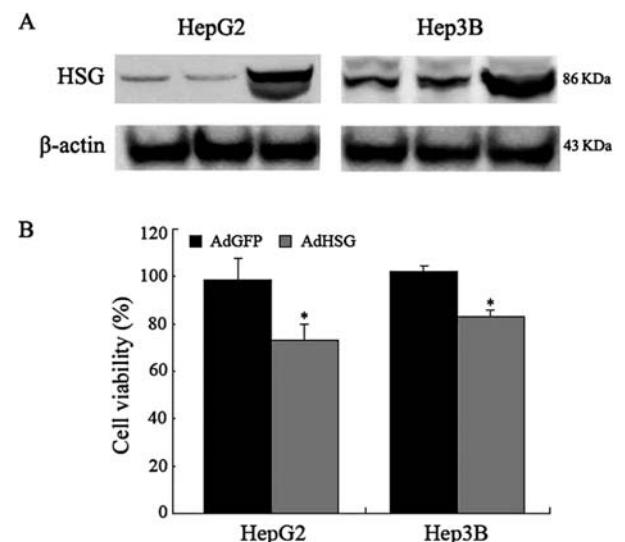


Figure 2. Adenovirus-mediated overexpression of HSG in HCC cells and its effect on cancer cells proliferation. (A) HCC cells were infected with AdGFP or AdHSG at a MOI of 100. After 48 h of incubation, the expression of HSG protein was analyzed by Western blotting. β -actin was used as the protein loading control. (B) To examine the HSG-induced cytotoxicity quantitatively, HepG2 and Hep3B cells were infected with AdHSG and AdGFP at a MOI of 100 pfu per cell and cell viability was analyzed at 48 h after infection by using Cell Counting kit-8. AdHSG infection led to a notable inhibition on proliferation of liver cancer cell lines compared with AdGFP infection (p<0.05, n=3).

and flow cytometric analysis (Beckman Coulter) was used to examine cell cycle distribution as described previously (10).

Ex vivo and in vivo treatments with adenoviruses. Male BALB/c nude mice at 4-5 weeks obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) were

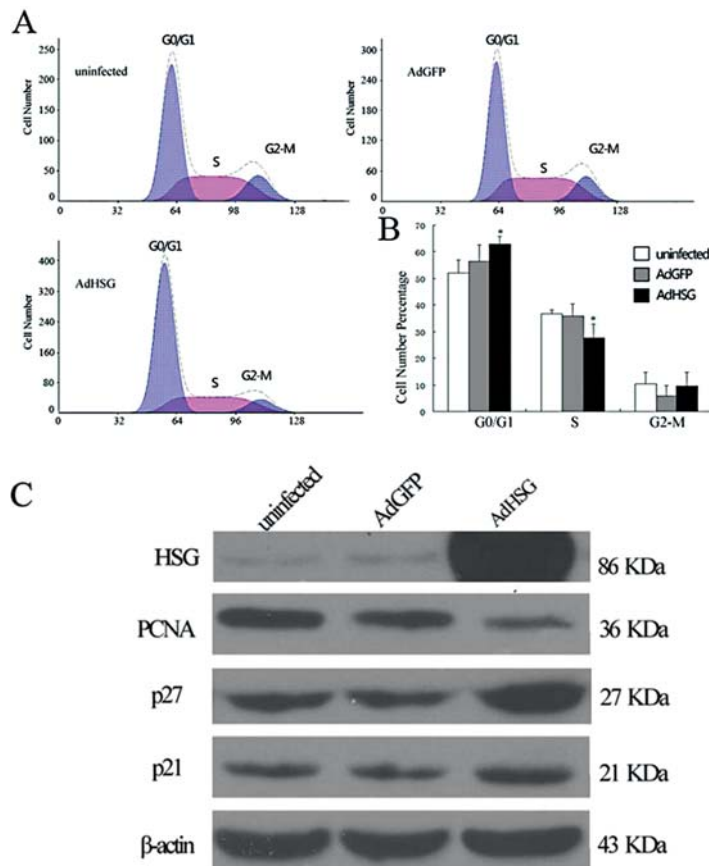


Figure 3. HSG-induced cell cycle arrest. (A) Typical examples of cell cycle distribution in the uninfected group, AdGFP group and AdHSG group. HepG2 were first synchronized and then infected with either AdHSG or AdGFP at 100 pfu per cell. After infection, cells were kept in low serum medium (0.2% FBS) for 24 h and then stimulated by normal serum medium. Flow cytometric analysis was used to examine cell cycle distribution. (B) The average data of cell cycle distributions (n=3; *p<0.01, compared with uninfected or AdGFP). (C) HepG2 cells were infected with AdGFP or AdHSG at a MOI of 100. After incubation for 48 h, the expression of HSG, PCNA, p27 and p21 protein was analyzed by Western blotting. β-actin was used as the protein loading control.

used in all of the experiments and kept in a dedicated animal facility with 6 mice per cage. The experiments were performed according to the institutional ethics guidelines. For the *ex vivo* experiment, mice were divided randomly into three groups with 6 mice each. HepG2 cells, AdGFP-infected HepG2 cells and AdHSG-infected HepG2 cells were injected subcutaneously into the armpit of mice in a total volume of 100 μ l (2×10^6 cells in PBS). For the *in vivo* experiment, HepG2 cells were injected subcutaneously into the armpit of mice (2×10^6 cells in PBS/ 100 μ l). When majority of the tumor volume reached 100 mm³ in size, mice were randomly allocated into three groups (6 mice each). These mice were treated with multiple-point intratumor injection 100 μ l of AdHSG or AdGFP at 2×10^8 pfu per animal or PBS as a control every other day for 4 times. After cell inoculation, tumor volumes for each mouse were monitored with a caliper every 5 days by measuring in two directions (length and width). The volume was calculated as length x (width)²/2. Then mice were sacrificed. Tumors were removed and weighed. HSG expression in tumors of different groups was analyzed by Western blotting as described above.

Statistical analysis. Data are displayed as mean \pm SD. Densitometry analysis of protein and mRNA levels was achieved by using Image-Pro Plus 5.0.2 software (Media Cybernetics, USA). The statistical significance of difference between the

three groups was analyzed by the Student-Newman-Keuls test for multiple comparisons. Also, all the statistical analysis was performed by the SPSS 16.0 program for Windows (SPSS, Chicago, IL, USA). p<0.05 was considered as statistically significant.

Results

HSG expression is decreased in liver tumor tissue. Compared to its adjacent normal tissues, the expression of HSG was decreased in hepatocellular carcinoma samples. The density value ratio of cancer and corresponding normal tissue (β-actin expression as a loading control) was evaluated in all patients, as shown in Fig. 1A and B. The density value of tumor was significantly lower than corresponding normal tissue (p<0.05, Fig. 1C).

Increased expression of HSG mediated by adenovirus vector in liver cancer cell lines and its effect on cancer cell proliferation. Fig. 1 shows that down-regulated expression or loss-of-function of HSG in HCC might play a role in the development of HCC. We hypothesized that overexpression of HSG mediated by adenovirus may inhibit the growth of HCC. The cells infected with AdHSG expressed much decreased HSG protein than those infected with AdGFP (Fig. 2A), which indicated that AdHSG infection resulted in

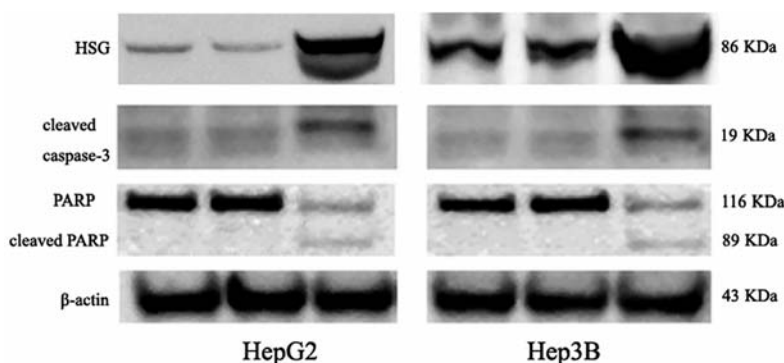


Figure 4. Overexpression of HSG triggers apoptosis in liver cancer cells. HepG2 and Hep3B cells were infected with AdHSG and AdGFP. Cleaved caspase-3 and cleaved PARP increased after 48 h infection.

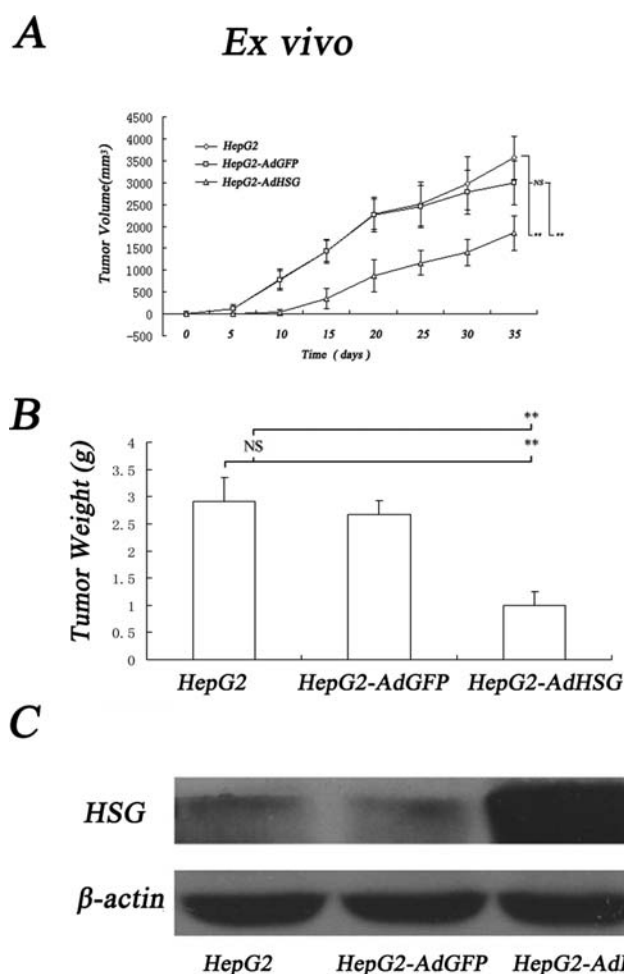


Figure 5. The antitumor activity of HSG *ex vivo* in nude mice. (A) The effect of AdHSG on HepG2 tumor growth *ex vivo*. HepG2 cells, AdGFP-infected HepG2 cells and AdHSG-infected HepG2 cells were injected subcutaneously into the armpit of mice in a total volume of 100 μ l (2×10^6 cells). Tumor volume was recorded. Data are expressed as mean volumes of tumors over time (\pm SD), $n=6$. NS, $p>0.05$; ** $p<0.001$. (B) Mice were sacrificed and tumors were removed and weighed after 35 days. Data are expressed as weights of tumors (\pm SD), $n=6$. NS, $p>0.05$; ** $p<0.001$. (C) Tumor sections were excised and analyzed for HSG expression of the three groups by Western blotting.

an efficient overexpression of HSG. In order to examine the cytotoxicity induced by overexpression of HSG, HepG2 and Hep3B cells were infected with AdHSG or AdGFP and cell viability was analyzed at 48 h after infection. As shown in

Fig. 2B, AdHSG infection caused a significant inhibition on proliferation of liver cancer cell lines compared with AdGFP infection ($p<0.05$).

The role of HSG in regulating the cell cycle. By using flow cytometry, the alteration of cell cycle distribution in response to increased expression of HSG was observed (Fig. 3A). After 10% FBS stimulation for 24 h, a significant increase of G0/G1 arrest in AdHSG infected HepG2 cells was found, compared with the uninfected and AdGFP groups. Overexpression of HSG also caused alterations in key components of the cell-proliferation regulatory machinery. An increased expression of the cell proliferation inhibitors p27 and p21 and a significant reduction of PCNA were observed in the AdHSG group (Fig. 3C).

Overexpression of HSG triggers apoptosis in liver cancer cells. To explore the potential role of HSG in regulating liver cancer cell apoptosis, HepG2 and Hep3B cells were infected with AdHSG and AdGFP. After 48 h, AdHSG induced liver cancer cell apoptosis, as manifested by activation of caspase-3 (Fig. 4) by Western blotting. Also PARP, as a representative substrate of caspase-3, was cleaved in AdHSG infected cells (Fig. 4).

AdHSG mediates inhibition of tumorigenicity ex vivo and in vivo. We next sought to determine whether exogenous HSG affected tumorigenicity both *ex vivo* and *in vivo*. Cells were treated with PBS or with 100 MOI of AdGFP and AdHSG and injected into nude mice 12 h later. AdHSG-treated tumor cells suppressed tumor growth *ex vivo*, whereas AdGFP-treated and PBS-treated cells resulted in tumor growth ($p<0.001$; Fig. 5A). At the end of our experiments, the average weight of tumors in the AdHSG-treated group was significantly lighter compared with AdGFP-treated group or in the PBS-treated group ($p<0.001$, Fig. 5B), and there was no difference in the AdGFP-treated group and PBS-treated group ($p>0.05$). It indicated that HSG protein may have therapeutic efficacy. The HSG expression of the three groups is showed in Fig. 5C. The therapeutic potential of AdHSG was further examined using an *in vivo* tumor model. PBS, AdGFP, or AdHSG was injected into HepG2 tumors. Also intratumoral injection of AdHSG resulted in significant tumor growth delay compared with injection of AdGFP or PBS ($p<0.001$, Fig. 6A), whereas the AdGFP treated group has a similar tumor volume to the

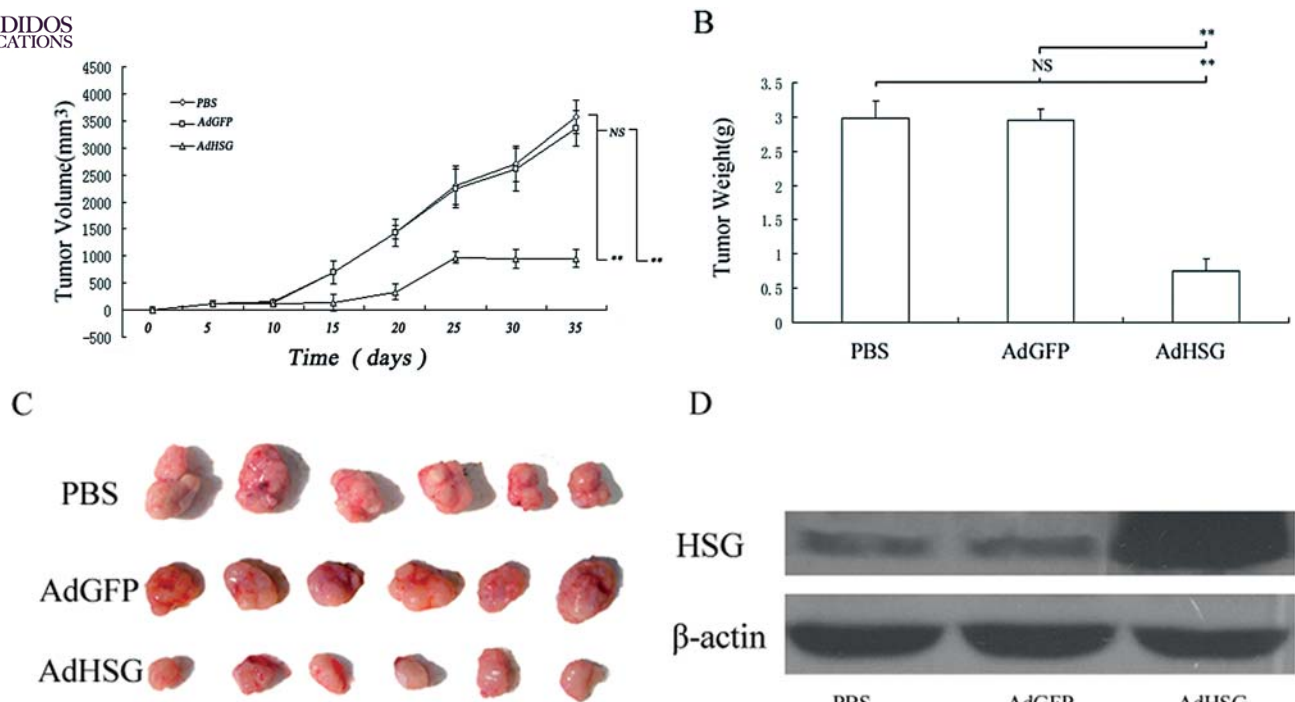


Figure 6. The antitumor activity of HSG *in vivo* in nude mice. (A) The effect of Adv-HSG on HepG2 tumor growth *in vivo*. Mice were inoculated subcutaneously with HepG2 cells (2×10^6 in PBS/100 μ l). When most of the tumor volume reached 100 mm³ in size, mice were divided randomly into three groups (six mice per group) and were treated every other day for 4 times by way of multiple-center intratumor injection 100 μ l of AdHSG or AdGFP at 2×10^8 pfu per animal or PBS as a control. Tumor volume was recorded. Data are expressed as mean volumes of tumors over time (\pm SD), $n=6$. Arrow, start of adenovirus injection; NS, $p>0.05$; ** $p<0.001$. (B) Mice were sacrificed and tumors were removed and weighed after 35 days. Data are expressed as mean weights of tumors (\pm SD), $n=6$. NS, $p>0.05$; ** $p<0.001$. (C) Tumors from mice after the treatments as above were removed and weighed. (D) Tumor sections were excised and analyzed for HSG expression of the three groups by Western blotting.

PBS treated group ($p>0.05$). The tumor weight of the three groups is shown in Fig. 6B. The tumor image and HSG expression of the three groups are shown in Fig. 6C and D.

Discussion

Liver cancer is the third most common cause of death and China alone accounts for 55% of all liver cancer cases worldwide (1). Novel treatments for liver cancers are urgently needed. In recent years gene therapy has shown great potential for the treatment of liver cancer (8,9). HSG is a novel hyperplasia suppressor gene. Overexpression of HSG markedly prevented cell proliferation through the Ras-Raf-MEK-ERK1/2 signaling cascade and resulted in cell cycle arrest in the G0/G1 phases. It also prevented balloon injury induced re-stenosis and reduced atherosclerosis (10). HSG was also found to be involved in the mitochondrial fusion reaction. Furthermore, it was found that the HSG has apoptotic effect and this effect is mediated by the mitochondria apoptosis pathway. The level of mitochondria anti-apoptotic protein Bcl-2 was decreased, while Bax mitochondrial accumulation was increased by overexpression of HSG, which resulted in mitochondrial cytochrome c releasing and activation of caspase-9 and caspase-3 (12-14). The anti-proliferative effect of HSG may be more effective than p53, a well known tumor suppressor (10,11). However, whether the HSG contributes to cancer development remains unclear. Moreover, its potential effect for cancer therapy is completely unknown. In this study, we first confirmed the

antitumor activity of hyperplasia suppressor gene (HSG) in hepatocellular carcinoma.

We examined the mRNA and protein expression of HSG in hepatocellular carcinoma samples and corresponding normal tissues. Compared with paired normal tissues, the decreased expression of HSG was found in hepatocellular cancer samples. HSG is located in human chromosome 1p36.3. Many types of human malignancies, from solid tumors to leukemias and myeloproliferative disorders, have shown nonrandom abnormalities on chromosome 1p36 (16,17). In the past few decades, many tumor suppressor genes have been found in this region (18,19), showing that down-regulated expression or loss-of-function of HSG in HCC might have a role in the development of HCC.

Consequently, we hypothesized that overexpression of HSG mediated by adenovirus may inhibit tumorigenic growth of HCC. Our results show that AdHSG can up-regulate HSG expression effectively in liver cancer cells, indicating that the adenovirus-based HSG may be an effective strategy for cancer therapy. Additionally, the prevention of proliferation, the promotion of apoptosis in liver cancer cells and the tumor suppression effect in nude mice support the efficiency of the treatment. Also HSG mediates inhibition of tumorigenicity both *ex vivo* and *in vivo*, which shows the antitumor activity of AdHSG is independent of the state of endogenous HSG, making it even more effective than P53.

To date, the mechanism of the hepatogenesis is not very clear, but it has been shown that disruption of the G1/S and G2/M check points leads to uncontrolled cell growth, resulting

in the development and progression of cancers. Many studies have reported that genetic and epigenetic alterations of cell cycle regulators in hepatocellular carcinoma are involved in the carcinogenesis of many types of human cancer. In hepatocellular carcinoma, cyclin dependent kinase inhibitors (CKIs) have an important role in the early stage of carcinogenesis and in disease progression (20,21).

The outcome of CKI induction in most cells is the cessation of cell proliferation, differentiation or even cell death. In tumor cells, the regulation of the CKIs is altered leading to either lack of function or expression. Hence, if the CKIs, such as p27 and p21, could be induced consistently in tumor cells and their induction leads to G1 arrest, the goal of controlling the proliferation of cancer cells could be achieved. Particularly p27, reduced p27 expression relates with poor prognosis in most tumor types, making it a novel and powerful prognostic marker (22). High p27 expression, foreshowing long survival, is a favorable independent prognostic index for HCC (23,24).

In this study, after HepG2 cells were treated with AdHSG for 48 h, a significant decrease of S-phase population in HepG2 cells was observed, compared with the uninfected group and AdGFP group. The effect of HSG on cell cycle arrest was also proved by HSG-mediated alterations in key components of the cell cycle regulatory machinery. An increased expression of the CKIs p21 and p27 was caused by overexpression of HSG. These results showed the great antitumor efficacy of HSG on hepatocellular carcinoma. On the other hand, we also found overexpression of HSG caused a significant reduction of proliferating cell nuclear antigen (PCNA), which plays a key role in DNA repair, cell proliferation and cell cycle control (25). These results validated our hypothesis that overexpression of HSG mediated by adenovirus may inhibit tumorigenic growth in HCC.

In conclusion, we provide experimental evidence to confirm HSG as a novel tumor suppressor gene at least in hepatocellular cancer. HSG was down-regulated in HCC tissue compared to adjacent normal tissue. Overexpression of HSG remarkably suppressed the growth of liver cancer cell lines, resulted in cancer cell cycle arrest in the G0/G1 phase, increased expression of the CKIs, and reduced expression of PCNA. Up-regulation of HSG by adenovirus also induced liver cancer cell apoptosis and significantly suppressed the growth of subcutaneous tumors in nude mice both *ex vivo* and *in vivo*. The observations show that HSG has a previously unappreciated function in cancer development, thereby revealing an important and novel therapeutic target for the treatment of tumors and possibly also other hyper-proliferative diseases.

Acknowledgements

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