

shRNA targeting HDGF suppressed cell growth and invasion of squamous cell lung cancer

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Hepatoma-derived growth factor (HDGF), a nuclear protein with both mitogenic and angiogenic activity, has been reported to be mainly involved in tumorigenesis and the progression of non-small cell lung cancer. In this study, the HDGF expression was knocked down by specific-shRNA with lentivirus expression vector targeting HDGF in lung squamous cell carcinoma 520 cells. HDGF knocked down by shRNA suppressed the cell proliferation significantly both *in vitro* and *in vivo* as indicated by MTT, plate clone and transplanted tumor model assays. In addition, the knocked-down expression of HDGF also inhibited cell migration and invasion as shown in transwell and Boyden experiments. We concluded that HDGF acts as an oncogene participating in the pathogenesis of squamous cell lung cancer, and HDGF may be a key therapeutic target for non-small cell lung cancer.

Keywords hepatoma-derived growth factor; squamous cell lung cancer; oncogene

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Introduction

Lung cancer is one of the main causes of cancer death worldwide. The disease is more common in countries with high tobacco consumption, including China. In spite of the recent advances in diagnostic techniques for early detection and the improvement in surgical procedures, lung cancer is ranked the fourth for males and the fifth for females as a cause of cancer death in China. Squamous cell carcinoma is the second commonest type of lung cancer, accounting for 29% of all cases of lung cancer and often metastasizes to other parts of the body because of the constant flow of fluids (blood and lymph) through the lungs. Patients with higher expression levels of malignant oncogenes, promoting cell proliferation and metastasis, will have a lower 5-year survival rate [1].

Hepatoma-derived growth factor (HDGF), a heparin-binding growth factor originally purified from media conditioned with the human hepatoma cell line HuH-7, can stimulate proliferation of NIH cells [2]. HDGF has a mitogenic function for various types of cells such as human hepatocellular carcinoma cells, fibroblasts, endothelial cells, vascular smooth muscle cells, and fetal hepatocytes. HDGF can translocate to the nucleus by nuclear localization signals, and its nuclear translocation is essential for the induction of cell growth activity.

Previous studies manifested that overexpressed HDGF is a metastatic and prognostic factor for lung cancer [3–5]. Downregulation of HDGF expression by chemically synthesized shRNA could suppress cell growth and invasion *in vitro* [6]. Previous results have indicated that HDGF is a unique nuclear/growth factor that plays an important role in the development and progression of lung cancer. In this study, the expression level of HDGF and its roles in cells were identified in squamous cell carcinoma 520 cell line.

Materials and Methods

Cell lines and animals

The human lung squamous cell carcinoma 520 cell line was grown in RPMI 1640 medium (Hyclone, Logan, USA) supplemented with 10% new born calf serum (PAA Laboratories Inc., Queensland, Australian). BALB/c nude mice, 4–6 weeks old, 18–22 g at the start of the study, were used.

Detection of HDGF mRNA expression in lung squamous cell carcinoma 520 cells by RT-PCR

Expression of HDGF mRNA in lung squamous cell carcinoma 520 cell lines was detected. Total RNA was prepared by using Trizol reagent (Takara, Dalian, China) according to the manufacturer's instructions. cDNA was reverse-transcribed from 2 µg of total RNA using oligo(dT)₁₈. RT-PCR was carried out to examine the mRNA levels of

HDGF, and *GAPDH* was used as a normalizing control. PCR primer sequences of two genes were designed as follows: *HDGF*, 5'-GAGGGTGACGGTGATAAGAA-3' (forward), 5'-GAAACATTGGTGGCTACAGG-3' (reverse); *GAPDH*, 5'-TTCGCTCTCTGCTCCTC-3' (forward), 5'-GATGATCTTGAGGCTGTTGT-3' (reverse). Cycling conditions were as follows: 95°C for 2 min; followed by 30 cycles of 95°C for 15 s, 55°C for 20 s, and 72°C for 25 s; 72°C for 10 min. Specificity of amplification products was confirmed by 1% agarose electrophoresis.

RNAi sequence design against *HDGF* and construction of vectors expressing *HDGF* shRNA

HDGF-specific target sequence was chosen according to online shRNA tools of Invitrogen (<http://www.invitrogen.com/rnai>) using the *HDGF* reference sequence (GenBank accession No. NM_004494.2). The target sequence was designed as follows: top strand, *HDGF* 5'-CACCGCCGGCAGAAGGAGTACAAAACGTTTGTACTCCTTCTGCGG-3'; bottom strand, 5'-AAAACCGGCAGAAGGAGTACAAAACGTTTTTGTACTCCTTCTGCCGGC-3'. Then shRNAs were chemically synthesized and lentiviral vector was constructed as described previously [7]. The exact insertion of the specific shRNA was further confirmed by sequencing.

Plasmids and transfection

shRNA-*HDGF* and control plasmids were transfected into lung squamous cell carcinoma 520 cells by Lipofectamine 2000. To generate stable clones, transfected cells were selected with 5 µg/ml blasticidin. The medium was changed every 3–4 days until blasticidin-resistant colonies appeared. Single colonies were picked and grown in selection medium.

Detection of *HDGF* mRNA expression in cells by real-time qPCR

Total RNA of these cell clones was prepared and real-time qPCR was performed to examine the *HDGF* mRNA expression. *GAPDH* gene was used as a normalizing control. The designed paired primers were as follows: *HDGF*, 5'-CAGCCAACAAATACCAAGTCT-3' (forward), 5'-GTTCTCGATCTCCACAGC-3' (reverse); *GAPDH*, 5'-GAAGGTCCGAGTCAACGG-3' (forward), 5'-TGGAAGATGGTGTATGGGATT-3' (reverse). The PCR reaction was carried out in a volume of 20 µl using SYBR green mix (Takara) on MXP3000 instrument (Stratagene Laboratories, La Jolla, USA). The amplifying lengths of *HDGF* and *GAPDH* were 152 and 222 bp, respectively. Each sample was tested at least three times.

Western blot analysis of *HDGF* protein

Western blot was performed as described previously [7]. In brief, cells were washed twice with cold phosphate-buffered saline (PBS) and lysed on ice in RIPA buffer with protease inhibitors (Shanghai Biocolor BioScience & Technology Company, Shanghai, China). Protein lysates were resolved on 10% SDS polyacrylamide gel, electrotransferred to polyvinylidene fluoride membranes. Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline, pH 7.5, and then immunoblotted overnight at 4°C with primary antibodies: anti-*HDGF* polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA) (1:500) and anti-β-actin antibody (Santa Cruz Biotechnology) (1:1000), then with their respective horseradish peroxidase-conjugated secondary antibodies. Signals were detected by enhanced chemiluminescence (Pierce, Rockford, USA).

MTT assay

The cells were seeded into 96-well microtiter plates at a density of 1000 cells per well. The cells were incubated for 1, 2, 3, 4, 5, 6, and 7 days, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 µl of 5 mg/ml MTT (Promega, Madison, USA) for 4 h. When MTT incubation was completed, supernatants were removed. Then 150 µl of dimethyl sulfoxide (Sigma, St. Louis, USA) was added to each well. Fifteen minutes later, the absorbance value of each well was measured with a microplate reader set at 490 nm. Experiments were done in triplicate.

Soft agar clone formation

Cells were planted in 24-well plastic plates spread with low-melting point agar (0.35% in the upper layer and 0.6% in the low layer). Each type of cell was planted in five wells, with 100 cells in each well. The cells were cultured at 37°C, with 5% CO₂ and under saturation humidity for 14 days. Cells clones with more than 50 cells were counted under an invert microscope. Clone form rate was calculated as follows.

$$\text{Clone form rate} = \left(\frac{\text{the clone form number}}{\text{inoculation cell number}} \right) \times 100\%$$

In vitro migration and invasion assay

Total of 1×10^5 cells were seeded on a fibronectin-coated polycarbonate membrane insert with a pore size of 8 µm in a transwell apparatus (Chemicon, Temecula, USA). In the lower chamber, 600 µl RPMI 1640 with 15% FBS was added as chemoattractant. After the cells were incubated for 16 h at 37°C with 5% CO₂, the insert was washed with

PBS, and cells on the top surface of the insert were removed by a cotton swab. For the Matrigel invasion assay, the procedure was similar with the cell migration assay, except that the transwell membrane was pre-coated with 20 $\mu\text{g}/\mu\text{l}$ Matrigel (Chemicon) and the cells were incubated for 16 h at 37°C with 5% CO₂. Cells adhered to the lower surface were fixed by methanol, stained by Giemsa, and counted under a microscope in five predetermined fields (magnification, 200 \times). All assays were independently repeated in triplicate.

Tumor transplantation in nude mice

The 1×10^6 logarithmically growing cells were injected subcutaneously into 4–6 weeks old male BALB/c nude/nude mice. Each experimental group consisted of three mice. After 4 weeks of observation, the mice were sacrificed and tumors were stripped. Tumor weight was measured and tumor volume was calculated according to the formula

$$\text{Tumor volume} = \text{length} \times \text{width} \times \text{height}$$

Finally, the HDGF expression was examined again by real-time PCR in implanted nude mice of shRNA-HDGF and control cell groups.

Statistic analysis

SPSS13.0 package (Abbott Laboratories, North Chicago, USA) was used for statistical analysis. Differences among groups in *in vitro* cell growth and *in vivo* tumor growth were tested for statistical significance using analysis of variance (ANOVA). Soft agar clone formation assay, *in vitro* migration, and invasion assays were tested using one-way ANOVA. A result is statistically significant when the *P*-value is <0.05.

Results

HDGF mRNA is highly expressed in lung squamous cell carcinoma 520 cells and shRNA sequence is confirmed by sequencing

HDGF is highly expressed in lung squamous cell carcinoma 520 cells, and its expression density is nearly equal to that of housekeeping gene *GAPDH* [Fig. 1(A)]. shRNA sequence inserted into lentivirus expression vector was identified, which was consistent with the designed sequence [Fig. 1(B)].

ShRNA targeting HDGF suppressed the expression of HDGF at mRNA and protein levels in lung squamous cell carcinoma 520 cells

shRNA targeting *HDGF* was used to decrease the expression of *HDGF* mRNA in lung squamous cell carcinoma 520 cells. Ten single cell clones with the integration

of shRNA-HDGF lentivirus vector was selected to examine the silencing efficiency compared with control 520 cells by real-time quantitative PCR, and cell clones shRNA-HDGF-2 and -5 revealed decreased *HDGF* mRNA by up to 73.1% and 76.7% respectively [Fig. 1(C)]. To further confirm the specificity of shRNA-mediated silencing of HDGF, the expression of HDGF protein was also determined by western blot. As shown in Fig. 1(D), HDGF protein of both cell clones, shRNA-HDGF-2 and -5, was decreased by 71% and 73%, respectively, compared with the control shRNA-Ctr 520 cells. shRNA-HDGF-5 cell clone was used in subsequent experiments as an HDGF knockdown cell model.

Reduction of HDGF expression inhibited cell growth

The growth curves determined by MTT assays showed significant proliferation suppression in shRNA-HDGF cells compared with control shRNA-Ctr 520 cells [Fig. 2(A)]. shRNA-HDGF cells yielded significantly fewer colonies than control cells ($P < 0.01$) according to soft agar clone formation assay ($n = 3$) (Table 1). Anchorage-dependent growth in cells treated with shRNA-HDGF transfection was also reduced compared with control cells. Weight and volume of tumors were dramatically lighter and smaller ($P < 0.05$) in shRNA-HDGF cells-implanted nude mice than those implanted with control cells ($n = 3$) [Fig. 2(B)]. Real-time quantitative PCR showed that HDGF expression was obviously reduced in implanted nude mice of shRNA-HDGF cell groups compared with control cell groups [Fig. 2(C)].

Reduction of HDGF expression suppressed cell migration and invasion *in vitro*

In vitro studies were performed to detect the effects of HDGF repression on both migration and invasion in 520 cells. As shown in Fig. 2(D), inhibition of HDGF expression resulted in decreased migration capacity of 520 cells ($P < 0.05$). As compared with control cells, the shRNA-HDGF cells showed a significant decrease in invasion capacity ($P < 0.05$) [Fig. 2(E)].

Discussion

In this study, we determined the expression of HDGF in 520 cell line, and constructed lentivirus vector that could stably repress HDGF expression in these cells. We observed that the decreased expression of HDGF by sequence specific shRNA can inhibit the growth of 520 cells both *in vivo* and *in vitro*. In addition, we observed that inhibition of HDGF expression by shRNA suppressed the migration and invasion capacity of 520 cells.

HDGF encodes a member of the hepatoma-derived growth factor family. The encoded protein has a wide

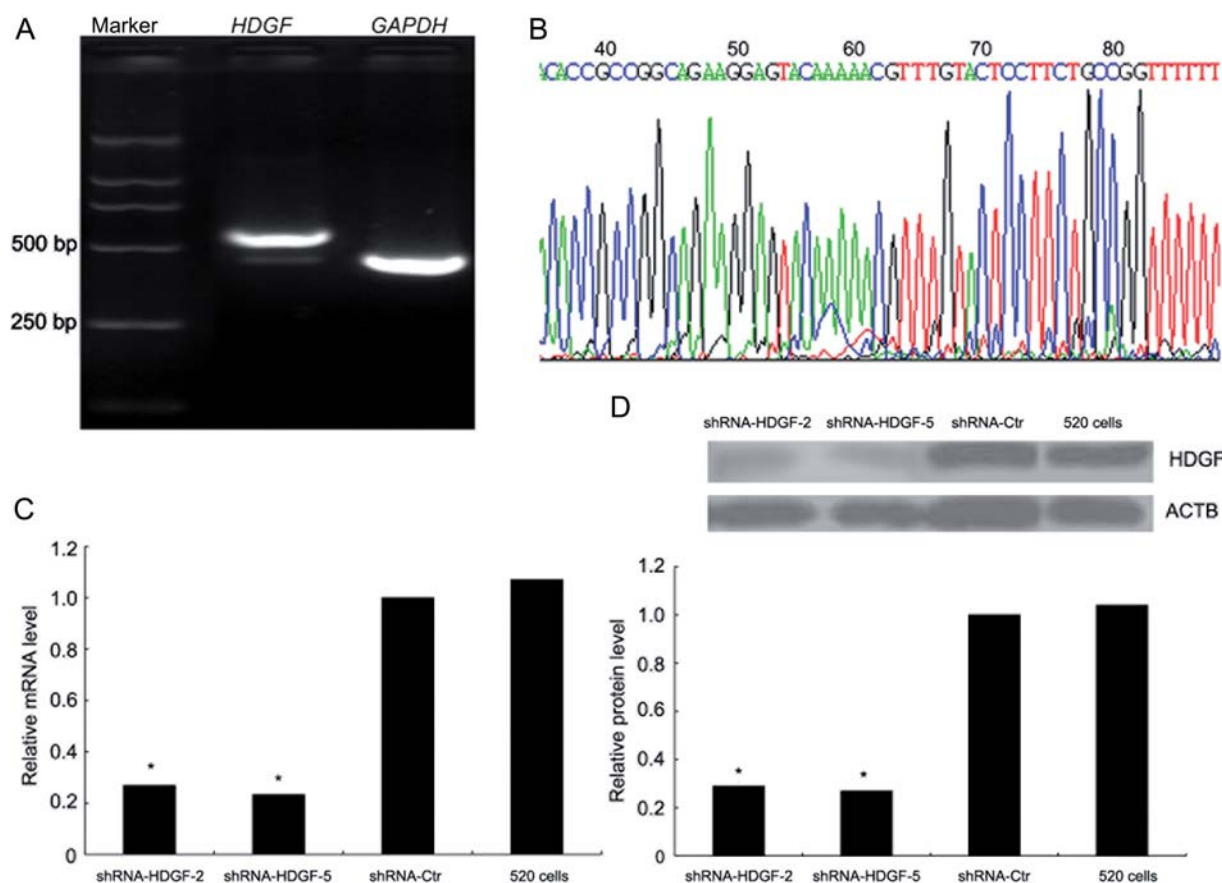


Figure 1 Expression of *HDGF* mRNA and the suppression of mRNA and protein expression of *HDGF* by specific shRNA targeting *HDGF* in lung squamous cell carcinoma 520 cells (A) Detection of expression of *HDGF* mRNA in 520 cells by RT-PCR. $n = 3$. *HDGF* expression density was nearly equal to *GAPDH* mRNA expression. (B) Sequence identification of shRNA targeting *HDGF*. (C) The expression of *HDGF* mRNA was detected by real-time quantitative RT-PCR, normalized to *GAPDH* mRNA expression. (D) The expression of *HDGF* protein was detected by western blot, normalized to *ACTB* protein expression. Data were expressed as the mean \pm SEM value. $n = 3$. * $P < 0.05$ vs. shRNA-Ctr.

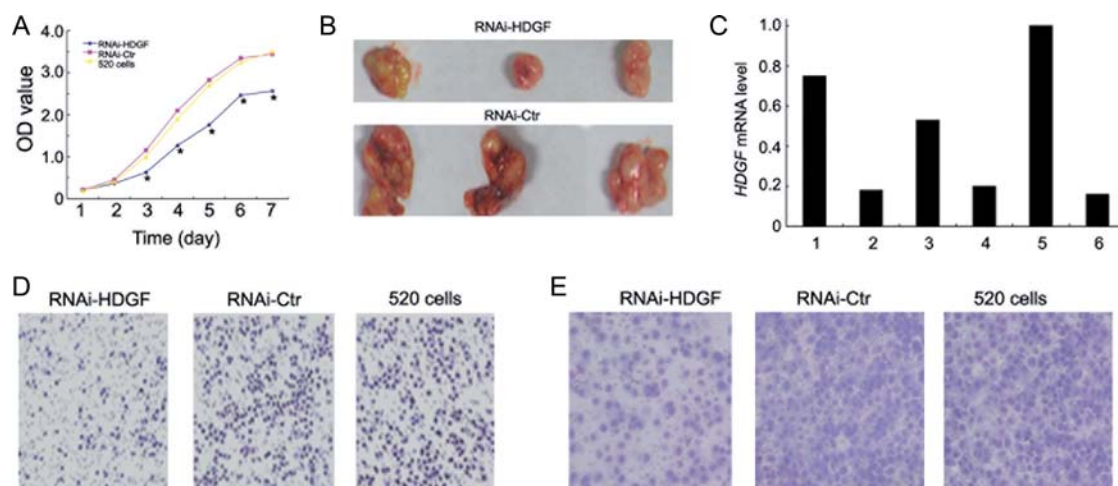


Figure 2 *HDGF* knock-down repressed the growth, migration and invasion of lung squamous cell carcinoma 520 cell *in vitro* and *in vivo* (A) Growth curves of different treated 520 cells determined by MTT assay. The values shown were the mean of three determinations. * $P < 0.05$. (B) Tumorigenesis in nude mice. Tumor weight and volume in RNAi-*HDGF* group were markedly lighter and smaller than that of RNAi-Ctr ($P < 0.05$). (C) *HDGF* mRNA expression determined by real-time quantitative PCR. Compared with RNAi-Ctr groups, the expression of *HDGF* was markedly decreased in RNAi-*HDGF* groups. 1, shRNA-Ctr1; 2, shRNA-*HDGF*-1; 3, shRNA-Ctr2; 4, shRNA-*HDGF*-2; 5, shRNA-Ctr3; 6, shRNA-*HDGF*-3. (D) Cell migration determined by transwell experiment. (E) Cell invasion determined by Boyden chamber experiment. Compared with RNAi-Ctr 520 cells and 520 cells, the capacity of cell migration and invasion was decreased markedly in RNAi-*HDGF* 520 cells ($P < 0.05$). Each assay was performed in triplicate, and the results were expressed as the mean \pm SD.

Table 1 Soft agar clone formation ratio among RNAi-HDGF 520 cells, RNAi-Ctr 520 cells and 520 cells

Group	Clone formation ratio
Control	50.21 ± 3.27
RNAi-Ctr	49.47 ± 2.67
RNAi-HDGF	23.96 ± 2.53*

Data were represented as the mean ± SD. * $P < 0.01$, compared with RNAi-Ctr 520 cells and 520 cells.

range of biological functions in cellular biology, such as mitogenic activity [8], promoting angiogenesis [9] and lung remodeling [10]. It is reported that HDGF is overexpressed in lung cancer tissues [3–5]. In agreement with these studies, we also observed high level of *HDGF* expression in 520 cell line. This indicated that HDGF might play a role in the development of lung cancer.

Clinical studies have shown that overexpression of HDGF is associated with poor outcome of tumors such as gastric [11,12], lung cancer [3–5], liver cancer [13], colorectal stromal tumors [14], and pancreatic cancer [15] in human. It is also reported that HDGF transfection significantly activated ERK1/2 in gastric cancer AGS cells and promoted anchorage-independent growth. Evidence has also shown that the expression of HDGF is increased gradually in the process of gastric carcinogenesis [16]. HDGF knock-down by antisense oligos not only repressed the growth of human hepatocellular carcinoma HepG2 cells, but also induced apoptosis in HepG2 and other human cancer cell lines. Transfection with chemically synthesized HDGF-specific small interfering RNA resulted in down-regulation of HDGF expression in lung cancer cell lines. Down-regulation of HDGF significantly reduced the ability of cell proliferation and invasion *in vivo* and *in vitro* experiments [6]. While in our study, we observed that knock-down HDGF expression by vector-mediated HDGF shRNA suppression could markedly decrease cell proliferation, as well as migration and invasion capacity of 520 cells. We also observed that down-regulation of HDGF could suppress tumorigenicity of 520 cells in nude mice. Our results added new insight into the involvement of HDGF in the development of lung cancer. HDGF is a potential target for lung cancer.

However, it remains unclear the mechanisms of HDGF contributing to the proliferation and invasive capacity of 520 cells. There are reports showing that HDGF knock-down can lead to the induction of the expression of the pro-apoptotic protein Bad and inactivation ERK and Akt, the latter in turn led to dephosphorylation of Bad and activation of the intrinsic apoptotic pathway [17]. It is deserved to determine whether HDGF contributes to the development of lung cancer in similar pathways in future.

In conclusion, HDGF may play a key role in promoting the growth and invasion of lung squamous cell carcinoma 520 cells. Lentivirus-mediated shRNA vector can stably down-regulate HDGF expression and inhibit the proliferation and invasion capacity of 520 cells. HDGF may serve as a potential target for the treatment of lung cancer in patients with HDGF overexpression.

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