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ORIGINAL ARTICLE

HHIP gene overexpression inhibits the growth, migration and invasion of human liver cancer cells

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Summary

Purpose: *Currently ranked as 5th most prevalent cancer type, liver cancer causes significant mortality across the globe. Additionally, the emergence of drug resistance and the alarming increase in the incidence of liver cancer has further worsened the situation. Therefore, development of effective chemotherapy, identification of molecular markers and therapeutic targets for proper treatment of liver cancer is the need of the hour. This study was undertaken to investigate the expression profile of hedgehog-interacting protein (HHIP) in liver cancer. Additionally, this study also investigated the effect of HHIP on the proliferation, migration and invasion of the human liver cancers.*

Methods: *The expression analysis was done qRT-PCR. The cell viability was determined by MTT assay. Apoptosis was detected by annexin V/propidium iodide (PI) assay. Wound healing and transwell assays were used to monitor cell migration and invasion. Protein expression was determined by western blot analysis.*

Results: *The results showed a significant (6.8-fold) down-regulation of HHIP in human liver cancer cells relative to the normal AML12 cells. Next, overexpression of HHIP resulted in significant ($p<0.05$) and time-dependent decrease in the growth of the HepG2 cells. The decrease in growth of the HepG2 cells was found to be mainly due to induction of apoptosis which was accompanied by increase in Bax and decrease in Bcl-2 expression. The wound healing assay showed that HHIP overexpression caused a remarkable decrease in the migration of the HepG2 cells. Furthermore, the transwell assay showed that the invasion of the HepG2 cells decreased by 65% upon HHIP overexpression.*

Conclusion: *Taken together, HHIP may serve as potential molecular marker and therapeutic target for liver cancer management.*

Key words: *liver cancer, apoptosis, hedgehog-interacting protein, migration*

Introduction

The hedgehog-interacting protein (HHIP) is located on the chromosome 4q31.21-31.2 and has been shown to play vital cellular and physiological roles via modulation of the hedgehog (HH) signalling pathway [1,2]. The expression of HHIP has been shown to be suppressed in several cancer types. For example, Tada et al reported significant suppression of HHIP in hepatocellular carcinoma [3]. Additionally, HHIP has also been shown to have an effect on the proliferation and metastasis of several cancers, for instance, the migration and proliferation of gastric cancer cells has been shown to be markedly suppressed upon blockage of methylation of HHIP promoter [4]. Given this

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background, this study was designed to determine the expression profile of HHIP on the human liver cancer cells. Additionally, the effects of HHIP were also examined on the growth, migration and invasion of the human liver cancer cells. Liver cancer is currently ranked as 5th most prevalent cancer type across the world [5]. This malignancy is believed to mainly result from the epithelial cells of liver where the control over their division and proliferation is lost, as it is true for human cancers [6]. The liver cancer exhibits very high mortality rates, and its aggressiveness is also evident from the very low overall survival rates [7,8]. The presently used anticancer strategies against liver cancer are based on surgery, chemo- and radiotherapies and these treatments are less effective and are associated with lots of adverse effects [9]. Therefore, development of effective chemotherapy, identification of molecular markers and therapeutic targets for proper treatment of liver cancer is the need of the hour. This study revealed significant downregulation of HHIP in human liver cancer cells. It also showed regulatory effect of HHIP on the proliferation, migration and invasion of the liver cancer cells. We strongly believe that this study will form a basis for the establishment of HHIP as therapeutic target for liver cancer

Methods

Cell lines and culture

The human liver cancer HepG2, SNU-182 and SNU-423 cell lines as well as normal THLE-2 cell line were acquired from the ATCC collection centre, USA. The cell lines were cultured using Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Scientific, Waltham, Mass, USA). The cell lines were maintained in a humidified incubator at 37°C with 5% CO_2/95% air.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The total RNA was extracted from the liver cancer cell lines and normal cell line with the assistance of RNeasy kits (Qiagen, Inc., Valencia, CA, USA). To reverse-transcribe the complementary DNA (cDNA), the Omniscript RT (Qiagen, Inc., Hilden, Germany) was employed using 1 µg of the extracted RNA. The cDNA was then used as a template for RT-qPCR analysis with the assistance of the *Taq* PCR Master Mix kit (Qiagen, Hilden, Germany, Inc.) according to the manufacturer's protocol. The reaction mixture consisted of 20 µl containing 1.5 mM $MgCl_2$, 2.5 units *Taq* DNA polymerase, 200 µM dNTP, 0.2 µM of each primer and 0.5 µg DNA. The cycling conditions were as follows: 95°C for 20 sec, followed by 40 cycles of 95°C for 15 sec, and 58°C for 1 min. GAPDH was used as internal control and the relative quantification ($2^{-\Delta\Delta Cq}$) method was used to evaluate the quantitative variation between the samples.

Cell transfection

The HepG2 cells at 80% confluence were transfected with negative control (NC) and pcDNA-HHIP from Shanghai GenePharma (Shanghai, China; 10 pmol), with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Waltham, Mass, USA, Inc.) as per the manufacturer's protocol.

MTT cell viability assay

The seeding of the HepG2 cells was done in 96-well plates with 4500 cells per well. The HepG2 cells were then transfected with NC or pcDNA-HHIP and cultured for 24 h. After replacing the media, the cells were treated with 10 µL of 5 mg/mL MTT solution at 37°C for 4 h. Finally, 100 µL of DMSO were added and absorbance was taken by spectrophotometer at 570 nm at different time intervals to determine cell viability.

AO/EB dual staining

The NC and pcDNA-HHIP transfected HepG2 cancer cells were cultured for 24 h. The cells were collected by centrifugation and cell pellets were washed with phosphate buffered saline (PBS) and then fixed using 70% ethanol. The cells were then stained with acridine orange (AO) and ethidium bromide (EB) dual staining mix and analyzed morphologically under fluorescent microscope.

Annexin V- FITC/PI fluorescent staining

The annexin V-FITC/PI staining assay was performed to assess the level of cell apoptosis in transfected HepG2 cells. The HepG2 cancer cells were fixed using methanol and stained with dual annexin V-FITC/PI staining solution. Then, the cells were examined for nuclear morphology under fluorescent microscope and the percentage of apoptotic cells was determined.

Wound healing assay

The NC and pcDNA-HHIP transfected HepG2 cells were cultured till 80% confluence was reached. A wound was scratched on the plates and photographs were taken. The plates were then incubated at 37°C for 24 h and photographs were taken again.

Transwell cell invasion assay

The transwell chambers containing polycarbonate members with a pore size of 8 µm were put into 6-well plates. I-type collagen (10 µg/mL) was utilized for the coating of the lower transwell compartment and subsequently dried. About 200 mL transfected HepG2 cells were inoculated in the Matrigel coated upper compartment at a density of 1.5×10^5 cells/mL. The lower compartment was filled with 800 µL Roswell Park Memorial Institute 1640 (RPMI-1640) containing fetal bovine serum (FBS) (20%). The whole system was incubated at 37°C for 24 h. The cells that invaded through the membrane with Matrigel were stained with crystal violet and photographed under an inverted microscope.

Western blot analysis

To extract the total proteins the HepG2 cells were fractioned with RIPA lysis buffer (Thermo Scientific,

Waltham, Mass, USA). The Bradford method was used to determine the total protein concentrations of the cell lysates. Equal protein concentrations were loaded on 8% SDS-PAGE gel. The page gel was then blotted to transfer the contents to nylon membranes which were exposed to primary and secondary antibodies designed for the respective proteins of interest which were then visualized and their expression levels were assessed by the chemiluminescence method.

Statistics

The experiments were performed in triplicate. The values are shown as mean ± SD. The differences between groups were analysed by one-way ANOVA followed by Tukey's test, using SPSS software. The statistically significant difference was set at $p<0.05$.

Results

HHIP is downregulated in liver cancer

The expression of HHIP was examined in normal AML12 cells and the HepG2, SNU-182 and SNU-423 liver cancer cell lines. The results showed that HHIP was significantly ($p<0.05$) suppressed in all the liver cancer cell lines relative to the normal THLE-2 cells. The downregulation was upto 6.8-folds higher expression of HHIP in HepG2 cells (Figure 1). Hence, these cells were used in further experimentation.

HHIP suppresses the growth of liver cancer cells

To gain insights about the effect of HHIP on the growth, the HepG2 liver cancer cells were transfected them with pcDNA-HHIP. Next, qRT-PCR analysis was performed to authenticate the overexpression of HHIP (Figure 2A). The HepG2 cells were then cultured and subjected to MTT assay. The results showed that overexpression of HHIP in HepG2 cells caused significant ($p<0.05$) decline in the viability of the HepG2 cells in a time-dependent manner (Figure 2B). All these finding indicate that overexpression of HHIP was responsible for the inhibition of liver cancer cell proliferation.

HHIP suppresses apoptosis of HEPG2 cells

The NC and PcDNA-HHIP transfected HepG2 cells were stained with AO/EB to gain insights about the impact of HHIP overexpression on apoptosis. The results showed that overexpression of HHIP caused significant increase in red and orange colored cells, suggestive of apoptosis (Figure 3A). The annexin V/PI staining showed 1.1% early and 6.2% late apoptosis in relation to NC 13.0% early and 27.8% late apoptosis in pcDNA-HHIP (Figure 3B). The HHIP overexpression also caused suppression of Bcl-2 and upregulation of Bax, further

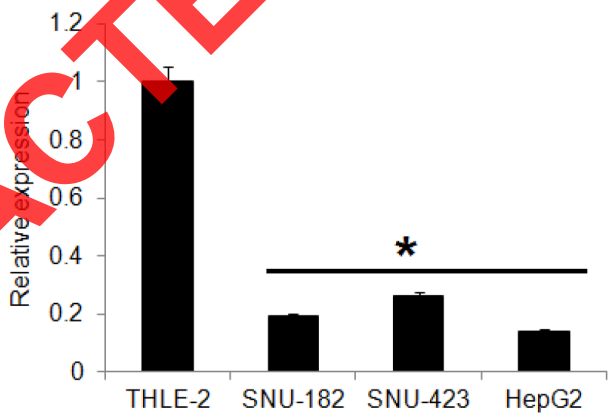

Figure 1. Expression analysis of HHIP in normal THLE-2 and liver cancer cell lines. The experiments were performed in triplicate and expressed as mean ± SD (*$p<0.05$).

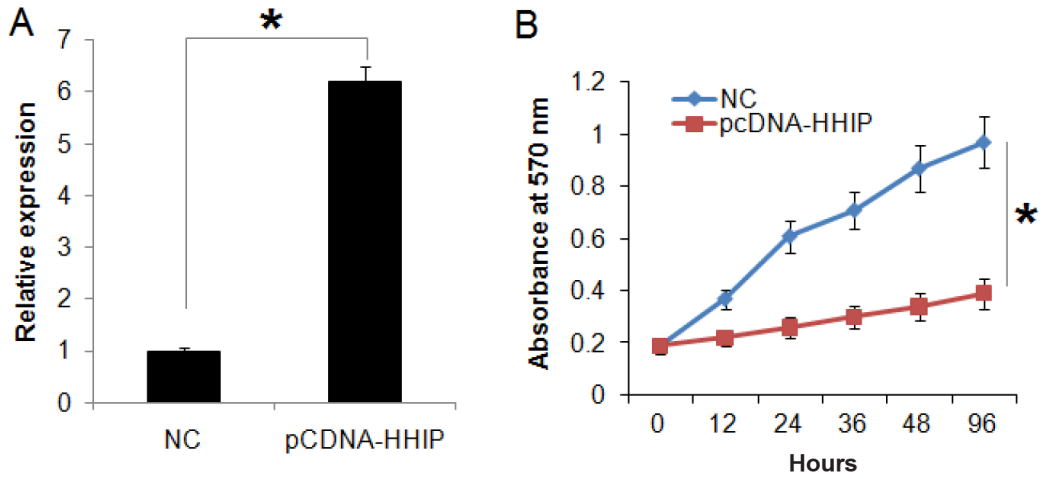

Figure 2. A: Expression analysis of HHIP in NC and pcDNA-HHIP transfected HepG2 cell lines. **B:** Cell viability of NC and pcDNA-HHIP transfected HepG2 liver cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*$p<0.05$).

confirming apoptosis in HEPG2 cells (Figure 4). These findings indicate that HHIP overexpression promoted apoptosis in liver cancer cells.

HHIP suppresses the migration and invasion of HepG2 cells

The effects of HHIP overexpression were firstly examined on the migration of the HepG2 cells. The results showed that the migration of the pcDNA-HHIP transfected cells was significantly inhibited relative to NC transfected cells (Figure 5). The effects of HHIP overexpression were also examined on the invasion of HepG2 cells (Figure 6) which revealed that the invasion of these cells was inhibited by 65% relative to control cells.

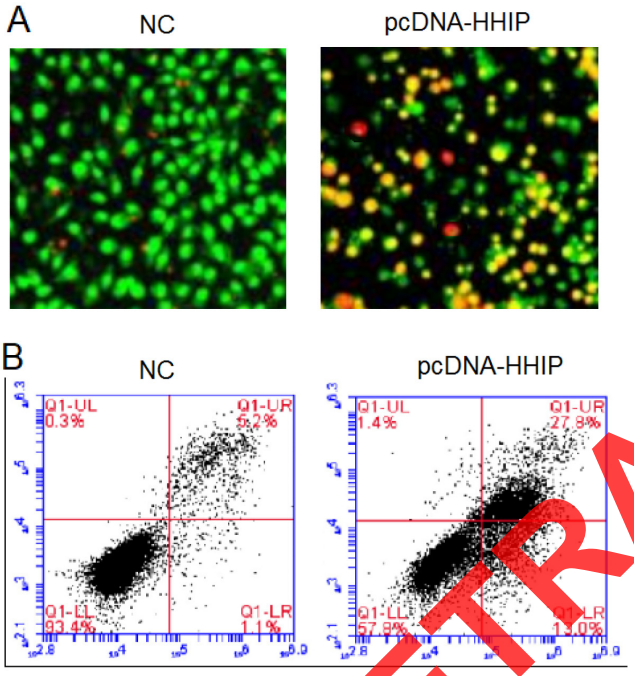

Figure 3. A: AO/EB showing induction of apoptosis in HepG2 cells (green depicts normal, orange early apoptotic and red late apoptotic cells). **B:** Annexin V/PI staining showing HHIP overexpression induces apoptosis in the HepG2 liver cancer cells. The experiments were performed in triplicate.

Discussion

Liver cancer is one of the devastating cancer types and causes significant number of deaths in humans. The need of the hour is to develop reliable molecular markers for early diagnosis and identify novel molecular therapeutic targets for targeted therapy of this disease [10]. In this study, we explored the therapeutic implications of HHIP in liver cancer. Over the years, researches carried out on HH signalling cascade have proved the involvement of HH pathway in the progression and tumorigenesis of different cancer types [11]. The HHIP has been shown to negatively regulate this HH pathway and consequently silencing of HHIP expression leads to the over-activation of HH sig-

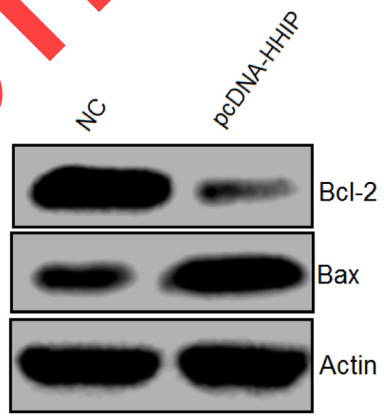

Figure 4. Western blot analysis showing that the expression of Bax increases and that of Bcl-2 decreases upon HHIP overexpression. The experiments were performed in triplicate.

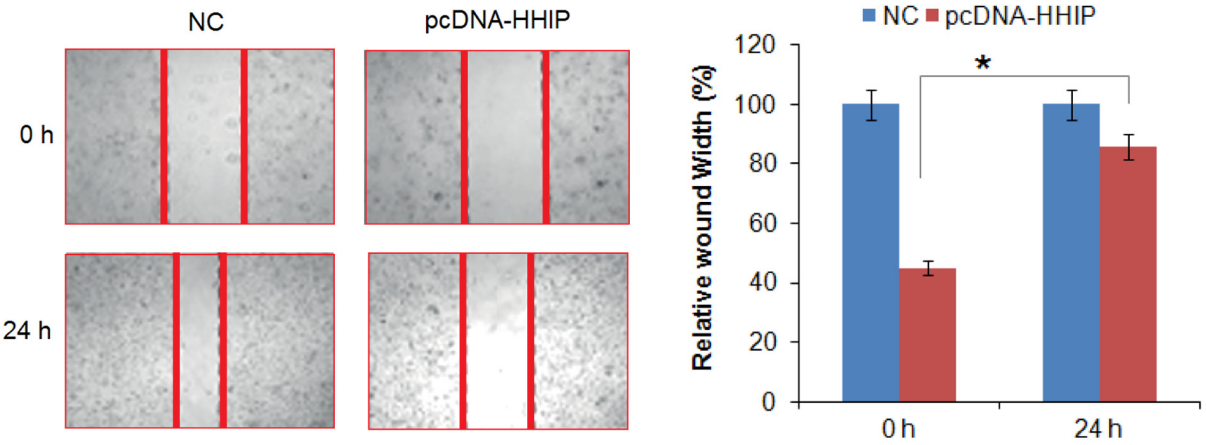

Figure 5. Wound healing assay showing HHIP overexpression inhibits the migration of HepG2 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

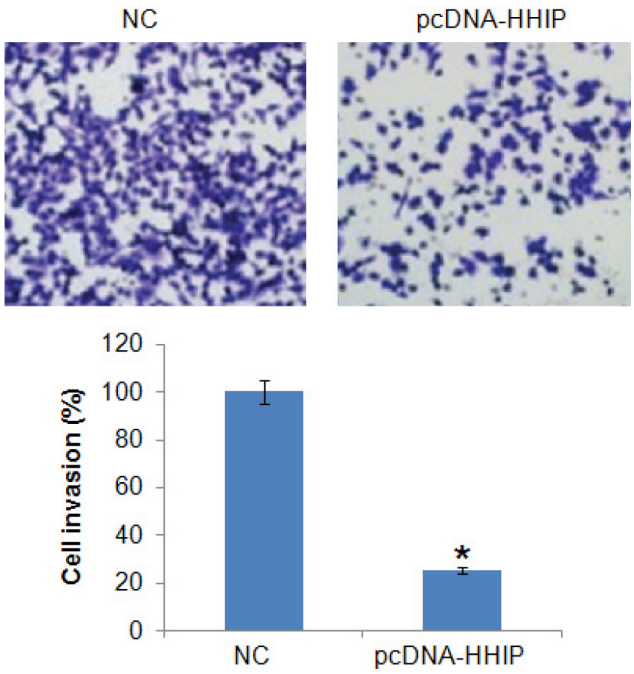

Figure 6. Transwell assay showing HHIP overexpression inhibits the invasion of HepG2 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

nalling pathway [12]. Yue et al reported the suppression of HHIP in human colorectal cancer tissues [13]. Similarly, the expression of HHIP has also been shown to be significantly suppressed in ovarian cancer tissues relative to the normal adjacent tissues [14]. In the current study, we examined the expression of HHIP in human liver cancer cells and found significant downregulation relative to the normal cells. This confirms earlier investigations wherein HHIP has been shown to be significantly suppressed in hepatocellular carcinoma [3,15]. The overexpression of HHIP suppressed the viability of the liver cancer cells via activation of apoptotic cell death. Apoptosis is an important process that allows the elimination of cancer cells. There are different biomarker proteins for apoptosis such as Bax and Bcl-2 [16]. In the current study we found that HHIP overexpression caused a remarkable increase in Bax and decrease in the Bcl-2 expression, further confirming the induction of apoptosis. Cell migration and invasion are the critical steps for the metastasis of the cancer cells [17]. In this study, we found that HHIP overexpression caused suppression of migration and invasion of the HepG2 cancer cells.

Conclusion

Taken together, the findings of the present study revealed a significant downregulation of HHIP in human liver cancer cells. Overexpression of HHIP caused a remarkable decrease in the growth, migration and invasion of the human liver cancer cells via induction of apoptosis. This study points towards the therapeutic implications of HHIP in the treatment of liver cancer.

Conflict of interests

The authors declare no conflict of interests.

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