Original Paper

Inhibition of HHIP Promoter Methylation Suppresses Human Gastric Cancer Cell Proliferation and Migration

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Key Words

Hhip • Hedgehog (HH) signaling • Gastric cancer • Methylation

Abstract

Background/Aims: Human hedgehog-interacting protein (HHIP) is a negative regulator of the hedgehog (HH) signaling pathway. It is deregulated in gastric cancer. The underlying molecular mechanism of HHIP-induced inhibition of HH signaling remains to be determined.

Methods: A lentiviral HHIP expression vector ("LV-HHIP") was established to exogenously over-express HHIP in gastric cancer cells. HHIP protein and *mRNA* were tested by Western blotting assay and quantitative real-time PCR assay, respectively. Cell survival was tested by the Cell Counting Kit-8 (CCK-8) assay. Cell proliferation was examined by the BrdU ELISA assay and [³H] Thymidine DNA incorporation assay. Cell invasion and migration were tested by the phagokinetic track assay and the "Transwell" assay. The bisulfite-sequencing PCR was applied to test HHIP promoter methylation. **Results:** In the established (AGS cell line) and primary human gastric cancer cells, LV-HHIP transfection increased HHIP expression and inhibited cancer cell survival and proliferation as well as cell migration and invasion. Furthermore, LV-HHIP significantly attenuated promoter methylation of the endogenous *HHIP* gene in AGS cells, causing it upregulation. Inhibition of methylation by 5-aza-dc similarly induced HHIP expression in gastric cancer cells, which inhibited cancer cell proliferation and migration.

Conclusions: Our results suggest that inhibition of HHIP promoter methylation can efficiently inhibit human gastric cancer cell proliferation and migration.

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Introduction

Gastric cancer (GC) causes significant human morbidities and mortalities [1]. Today, accompanied with the progresses in sanitation development [2], *H. pylori* control [3] and GC screening [4], the incidence of GC has been slightly decreased [5]. But, it is still the second-leading cause major fatal cancers [6, 7]. Molecularly-targeted therapy has drawn broad attention recently [8-14]. Identification of novel oncotarget proteins is important for GC diagnosis and treatment [8-11].

Hedgehog (HH) signaling pathway is an evolutionarily conserved cascade [15, 16]. It is vital in regulating cell growth and migration in embryonic development and adult tissue homeostasis [15, 16]. Deregulation of this pathway could induce birth defects [15, 16]. There are three HH ligands in the canonical HH gene family, including sonic hedgehog (SHH), Indian hedgehog (IHH), and desert hedgehog (DHH) [15, 16]. The hedgehog ligand binds to patched (PTCH), leading to the release and activation of smoothened (SMO), which in turn promotes the nuclear translocation of transcription factors of the Gli family, and dictates expression of targeted-genes [17-19]. Accumulative evidence has shown that HH signaling is deregulated in several cancers [15, 20-22] including GC [23, 24]. However, the underlying molecular mechanism of abnormal HH signaling in GC is largely unknown.

Hedgehog interacting protein (HHIP) binds to IHH, DHH and SHH to inhibit HH signaling pathway [25-27]. The upregulation of HH signaling pathway is associated with decreasing expression of HHIP in human cancers [28-30]. We have previously reported that HHIP was downregulated in GC [24, 31, 32]. However, the underlying molecular mechanism remains to be determined. In the current study, a lentiviral vector was established to over-express HHIP in human GC cells. This vector helps to investigate the effect of HHIP on GC cell survival, proliferation and migration.

Materials and Methods

Reagents and cell culture

The promoter methylation inhibitor 5-Aza-2'-deoxycytidine ("5-aza-dc") was purchased from Sigma-Aldrich (A3656, Shanghai, China). The Lenti-X HTX lentiviral packaging kit and the lentiviral vector PlvxDsRed-monomer-nl were purchased from Takara (Japan). AGS cells were obtained from the Shanghai Institute of Life Science Cell Information Center of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 U/mL penicillin at 37 °C in a humidified atmosphere containing 5% CO₂.

Primary culture of human GC cells

Culture of primary human cancer cells was described in detail in our previous studies [33, 34]. Briefly, the human gastric cancer tissues (from two independent GC patients at Zhangjiagang First People's Hospital, Soochow University) were thoroughly washed, minced and filtered. The single-cell suspension was obtained through digestion [33], and the primary cancer cells were cultured in the described medium [35]. A total of two lines of primary human GC cells were established. The written informed-consent was obtained from each patient. The protocol of using human cells is in line with the principles of Declaration of Helsinki, and is approved by the Soochow University's Ethics Review Board (ERB) and Internal Review Board (IRB).

Lentiviral vector construction and transduction

The 580-bp open reading frame (ORF) of the human *HHIP* gene was amplified by the polymerase chain reaction (PCR) and cloned into the lentiviral vector PlvxDsRed-monomer-nl to obtain the HHIP-overexpressing lentiviral vector, "LV-HHIP". Empty PlvxDsRed-monomer-nl was utilized as the negative control lentiviral vector ("LV-CON"). For lentiviral infection, cells were seeded onto six-well tissue culture plates (5 × 10⁴ cells/well) with the confluence of 60-70%. The appropriate volume of lentivirus was then added so that the multiplicity of infection was equal to one. After 72 hours, the cells were observed under a fluorescence microscope and were harvested for the following experiments.

Cell viability assay

The cell viability was tested by the routine Cell Counting Kit-8 (CCK-8) assay [36]. Briefly, cells were seeded at a density of 2×10^3 cells/well onto 96-well tissue-culture plate. After the described treatment, 10 μ L of CCK-8 solution (CCK-8, Dojindo, Japan) was added for another 2 hours. CCK-8 optical density (OD) of each well at 450 nm was measured.

The 5-bromo-2-deoxyuridine (BrdU) ELISA assay

The detailed protocol of BrdU incorporation ELISA assay was described in our previous study [37]. Briefly, cells were seeded onto the 48-well tissue culture plate at the density of 1×10^4 cells/well. Following the described treatment, the BrdU dye was added for another 6 hours. The BrdU ELISA colorimetric assay (Roche, Shanghai, China) was then applied to detect BrdU incorporation. The BrdU ELISA OD (at 450 nm) was recorded.

The [H^3] Thymidine DNA incorporation assay of cell proliferation

As described [37], cells were initially seeded onto the 48-well tissue-culture plate (1×10^4 cells/well). Cells with the applied treatment were also incubated with 1 μ Ci/mL of tritiated thymidine. To examine [H^3] Thymidine DNA incorporation, cells were washed, the DNA was precipitated, solubilized [37]. DNA aliquots were counted and quantified by liquid-scintillation spectrometry [H^3]. Thymidine DNA incorporation intensity in the treatment group was normalized to the untreated control group.

The phagokinetic track assay

The detailed protocol has been previously described in our previous studies [38-40]. In short, fibronectin (10 μ g/mL Sigma) was first plated onto the 12-well tissue-culture plate. Afterwards, microsphere suspension (Sigma) was coated onto each well. The plates were then centrifuged, to which the freshly-trypsinized cells (500 cells per well) were seeded. After another 24 hours, cell phagokinetic track was observed under the light microscope, showing cell migration. For each condition, at least 50 single cells in 10 random microscope views (1 : 100) were included to calculate average migration distance.

Transwell assay

The transwell chamber migration assay was performed using 24-well 8.0- μ m-pore Transwell plates (Fisher, Shanghai, China), according to the manufacturer's instructions. AGS cells were plated at 5×10^4 /mL in each well, and 500 μ L of culture medium containing 10% FBS was added to the bottom of the 24-well plate. After incubation for 24 hour, non-invading cells were removed from the upper surface using a cotton-tipped swab. Then, the invading cells were fixed in methanol for 10 min and stained with 0.1% crystal violet hydrate (Sigma, Shanghai, China) for 30 min. After air-drying, the membrane was mounted on a glass slide and examined under a microscope. The stained cells were counted as cells per field at 10 $\times$ magnification. For each condition, at least 10 random microscope views (1 : 100) were included to calculate average number of migrated cells.

RNA isolation and reverse transcription (RT)-PCR

Total cellular RNA was isolated via Trizol reagents (Shanghai Jingmei Bio Engineering, China) [41, 42] and converted to cDNA with the SuperScript III First-Strand Synthesis System (Invitrogen). The mRNA primers of HHIP were 5'-CTGCTTCTGTATTCAGGAGGTT-3' (forward) and 5'-GGGATGGAATGCGAGGCTTA-3' (reverse). The primer sequences of the internal control β -actin were 5'-AGAGCTACGAGCTGCCTGAC-3' (forward) and 5'-AGCACTGTGTTG GCGTACAG-3' (reverse). The PCR conditions and analyses were as described previously [24, 31].

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) assay

As described [33, 43], qRT-PCR was performed through the ABI-7600 fast system equipment using the SYBR Green PCR kit. Quantification of targeted mRNA was through the $2^{-\Delta\Delta C_t}$ method [44]. For the qRT-PCR assay, β -actin mRNA was tested as the internal control [33].

Methylation specific PCR and bisulfite-sequencing PCR for detection of HHIP gene methylation

The methylation primer sequences of HHIP promoter methylation for PCR analysis were described previously [24, 32]. The primers used to detect methylation of the CpG islands were designed to specifically

amplify bisulfite-converted DNA of the HHIP promoter transcriptional regulator region (Table 1). Aliquots of 2 µg of the bisulfite-modified DNA from AGS cells were subjected to the PCR analysis. The reaction was preheated at 95 °C for 5 min and amplified using a touch-down PCR program: 10 cycles of 95 °C for 30 s, 60 °C for 45 s (with touch-down of 1 °C per cycle), and 72 °C for 45 s; 30 cycles of 95 °C for 30 s, 50 °C for 45 s, and 72 °C for 45 s, and a final extension at 60 °C for 30 min [24, 32]. Ten clones from each sample were randomly selected for DNA sequencing by Sangon Co. (Shanghai, China).

Western blot analysis

Total proteins were extracted using the described cell lysis buffer [33, 43, 45] and quantified (Pierce). Equal amounts of protein were separated on 10% SDS polyacrylamide gels and transferred onto the polyvinylidene fluoride membrane (Millipore, Bedford, MA). The blot was then blocked with 10% milk (w/v) at room temperature for 2 hours and incubated overnight at 4 °C with the applied primary antibodies, followed by incubation with secondary antibodies. The bands were detected with the ECL plus kit (Beyotime Institute of Biotechnology, China). The same set of lysate samples were run in sister gels to examine different proteins.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). One-way ANOVA was employed to examine the significant difference between groups using SPSS 18.0 (SPSS, Chicago, CA). Values of $P < 0.05$ were considered statistically significant.

Results

Forced over-expression of HHIP in human GC cells

To assess the activity of HHIP in GC cells, a lentiviral HHIP expression vector ("LV-HHIP", GFP-tagged) was established, which was transfected to AGS cells. After 48 hours, over 90% of AGS cells express LV-HHIP, showing GFP green fluorescence (Fig. 1A). Western blotting assay results show that the basal HHIP protein expression is low in the parental control AGS cells ("Pare"), and in cells with lentiviral empty vector ("LV-CON") (Fig. 1B). Whereas significant exogenous HHIP protein over-expression was noticed in AGS cells with LV-HHIP (Fig. 1B). Endogenous HHIP level was also increased following LV-HHIP transfection (green "#", Fig. 1B). The qRT-PCR analysis results in Fig. 1C demonstrate that *HHIP* mRNA level is also significantly increased in LV-HHIP-expressing AGS cells, compared with the "Pare" cells and LV-CON cells. These results demonstrate that LV-HHIP induces HHIP expression in AGS cells.

HHIP over-expression inhibits GC cell survival and proliferation

To assess the potential function of HHIP on GC cell proliferation, AGS cells were transfected with LV-HHIP or LV-CON for 1 to 5 days. The cell counting assay results in Fig. 2A demonstrate that forced expression of HHIP significantly inhibited AGS cell proliferation. The number of the growing AGS cells with LV-HHIP was decreased compared with the parental cells or cells with LV-CON (Fig. 2A). Furthermore, Cell Counting Kit-8 (CCK-8) cell viability assay results show that forced over-expression of HHIP decreases AGS cell viability (Fig. 2B). AGS cells with LV-HHIP showed decreased BrdU incorporation (Fig. 2C) [H^3]. Thymidine

Table 1. Primers used for methylation specific PCR and bisulfite-sequencing PCR. HHIP, hedgehog-interacting protein; BSP, bisulfite-sequencing PCR; MSP, methylation specific PCR

Method	Primers	Sequence	Length (bp)	Temp.
BSP	HHIP	5'-GGGGAGGAGAGAGGAGTTTG-3'	243	60 °C
		5'-CCCACACCTCCCTACTAC-3'		
	β-actin	5'-AGAGCTACGAGCTGCCTGAC-3'	184	60 °C
MSP		5'-AGCACTGTGTTGGCGTACAG-3'		
	Methylation	5'-GTAGTAGTCGGGTAGTTTCGGAATTTTC-3'	190	60 °C
		5'-AAAAACGACTAACCGGACG-3'		
	Non-methylation	5'-AGTAGTTGGGTAGTTTGGGAATTTTGG-3'	188	60 °C
		5'-AAAAACAATAACCAACA-3'		

DNA incorporation was also reduced after LV-HHIP transfection (Fig. 2D). These results demonstrate that forced over-expression of HHIP inhibits AGS cell survival and proliferation.

The experiments were also repeated in two lines of primary human GC cells: "Pri Can L1/2". qRT-PCR assay results confirmed that basal *HHIP* mRNA expression level was markedly reduced in both lines of the primary cancer cells (Fig. 2E). Infection of LV-HHIP significantly increased *HHIP* mRNA expression level (Fig. 2E). Notably, the proliferation of the primary cancer cells was largely inhibited after LV-HHIP transfection, which was tested by the simple cell counting assay (Fig. 2F) and the BrdU ELISA assay (Fig. 2G). Together, these results confirm that forced-expression of HHIP inhibits GC cell survival and proliferation.

HHIP over-expression inhibits GC cell migration and invasion in vitro

Previous studies have shown that HH signaling plays a pivotal role in regulating cell migration [46]. There, we examined the potential effect of HHIP over-expression on GC cell migration

Fig. 1. HHIP over-expression in AGS cells. The parental AGS cells ("Pare") or AGS cells with lentiviral LV-HHIP (GFP-tagged) or LV-CON were monitored by fluorescence microscopy (A); HHIP protein and HHIP mRNA were tested by Western blotting assay (B) and qRT-PCR assay (C), respectively. Bars stand for mean $\pm$ SD (all Figs). * $p < 0.05$ vs. "LV-CON" group (C). Bar = 50 μ m (A). Experiments in this Fig. were repeated three times, and similar results were obtained.

Fig. 2. Forced over-expression of HHIP inhibits GC cell survival and proliferation. The parental AGS cells ("Pare"), AGS cells infected with lentiviral LV-HHIP (Tagged with GFP) or LV-CON were subjected to cell counting assay (at day 1-5, A), Cell Counting Kit-8 (CCK-8) cell viability assay (B), BrdU ELISA assay (C) and [H^3] Thymidine DNA incorporation assay (D); The two lines of primary-cultured human GC cells, "Pri Can L1/2", were infected with lentiviral LV-HHIP or LV-CON for applied time; HHIP mRNA expression was tested by the qRT-PCR assay (E); Cell proliferation was assessed by the cell counting assay (F) and BrdU ELISA OD assay (G). * $p < 0.05$ vs. "LV-CON" group. Experiments in this Fig. were repeated three times, and similar results were obtained.

Fig. 3. Over-expression of HHIP inhibits GC cell migration and invasion. The parental AGS cells ("Pare"), AGS cells infected with lentiviral LV-HHIP or LV-CON (for 24 hours) were subjected to "Transwell" assay (A) and the phagokinetic track assay (C). The primary cultured GC cells, "Pri Can L1/2", were infected with lentiviral LV-HHIP or LV-CON for 24 hours, cell migration was tested by "Transwell" assay, the number of transfected cells in 10 random views was shown (B). * $p < 0.05$ vs. "LV-CON" group. Experiments in this Fig. were repeated three times, and similar results were obtained.

Fig. 4. HHIP over-expression inhibits HHIP promoter methylation. In the parental AGS cells ("Pare"), AGS cells infected with lentiviral LV-HHIP or LV-CON, the endogenous HHIP promoter methylation status was analyzed by methylation specific PCR (A); HHIP CpG island methylation proportion was shown (B); The endogenous HHIP promoter CpG island methylation was determined by bisulfite-sequencing PCR (C). * $p < 0.05$ vs. "LV-CON" group. Experiments in this Fig. were repeated three times, and similar results were obtained.

with the "Transwell" assay. Results in Fig. 3A show that forced over-expression of HHIP by LV-HHIP inhibits AGS cell migration. The number of migrated AGS cells was significantly reduced after transfection of LV-HHIP (see quantified results in Fig. 3A). Similarly, in the two lines of primary human GC cells, infection of LV-HHIP decreased the number of migrated cells (Transwell assay, Fig. 3B). The phagokinetic track assay is an established method to test cell invasion ability [39]. Results demonstrate that the mobility of AGS cells with LV-HHIP is significantly decreased (Fig. 3C). As compared to the control AGS cells, the migration distance per cell was sharply reduced in the LV-HHIP-AGS cells (Fig. 3C). Taken together, these results show that over-expression of HHIP inhibits GC cell migration and invasion *in vitro*.

HHIP over-expression inhibits HHIP promoter methylation

Our results show that the basal HHIP expression level is low in AGS cells (Fig. 1). Previous studies have indicated that gene methylation plays an important role in the decreased gene expression [47-49]. Therefore, HHIP promoter methylation status in AGS cells was tested. As shown in Fig. 4A, the basal HHIP promoter methylation level was high in AGS cells, while after transfection of LV-HHIP for 72 hours, its level significantly decreases (Fig. 4A). The methylation specific PCR results show that the PCR products from the non-methylated primers increased significantly after transfection of LV-HHIP, while the PCR products of the methylated primers were decreased (Fig. 4A). The methylation PCR results further show that the HHIP CpG island methylation proportion accounted for 99.67% in the cells infected with LV-CON, while it was only 13.85% in the cells with LV-HHIP (Fig. 4B). The bisulfite-sequencing PCR results show that LV-HHIP reduces methylation of the HHIP promoter (Fig. 4C). The sequencing results confirmed that the HHIP promoter region had a large number of CpG islands (each circle represents a CpG island) (Fig. 4C). Again, the HHIP gene promoter CpG island methylation loci were decreased after transfection of LV-HHIP (Fig. 4C). Collectively, these results suggest that HHIP over-expression decreases endogenous HHIP promoter methylation in AGS cells. These results also explain why endogenous HHIP expression level is increased after transfection of LV-HHIP (Fig. 1B).

5-aza-dc induces HHIP expression in GC cells

Based on the results above, we speculated that pharmacological inhibition of gene methylation could increase HHIP expression. Thus, 5-aza-dc, a well-established promoter methylation inhibitor [31, 50], was employed. As shown, 5-aza-dc (1 μ M) significantly inhibited HHIP promoter methylation in AGS cells (Fig. 5A). Consequently, protein (Fig. 5B) and mRNA (Fig. 5C) expression of HHIP were significantly increased. Furthermore, AGS cell survival (CCK-8 OD, Fig. 5D), proliferation (BrdU ELISA OD, Fig. 5E) and migration ("Transwell assay", Fig. 5F) were largely inhibited by 5-aza-dc. In the primary GC cells, treatment with 5-aza-dc (1 μ M) inhibited HHIP promoter methylation (Fig. 5G), increased HHIP expression (Fig. 5H and I), and inhibited cancer cell proliferation (BrdU ELISA assay, Fig. 5J). Collectively,

Fig. 5. 5-aza-dc induces HHIP expression in GC cells. AGS cells (A-F) or the primary-cultured GC cells, "Pri Can L1/2" (G-J), were either left untreated ("C") or treated with 5-Aza-2'-deoxycytidine ("5-aza-dc", 1 μ M), cells were further cultured for described time, HHIP promoter methylation level (A and G), HHIP protein (B and H) and mRNA (C and I) expression were shown; Cell viability (CCK-8 assay, D), cell proliferation (BrdU ELISA assay, E and J) and migration ("Transwell" assay, F) were also tested. * $p < 0.05$ vs. "C" group. Experiments in this Fig. were repeated three times, and similar results were obtained.

these results suggest that inhibition of promoter methylation by 5-aza-dc induces HHIP expression, and inhibits GC cell proliferation and migration.

Discussion

The *HHIP* gene encodes a transmembrane glycoprotein that binds to HH proteins, and inhibits HH signaling pathway [51, 52]. *HHIP* gene is located at the chromosome 3q31.21-31.3 [51, 52]. The loss of *HHIP* is correlated with the incidence of gastrointestinal tumors [53]. In addition, previous studies have shown that HHIP is downregulated in GC tissues [24]. These findings suggest that HHIP might function as a tumor suppressor protein of GC. However, the underlying molecular mechanism of HHIP functioning in GC tumorigenesis remains to be determined. In this study, we found that forced over-expression of HHIP significantly inhibited GC cell survival, proliferation migration and invasion. Our results provide further evidence to support the tumor-suppressing function of HHIP.

HH signaling pathway is involved in cancer cell survival, proliferation, invasion, and differentiation, which is upregulated in GC [53, 54]. However, the underlying molecular mechanism is largely unknown [53, 54]. As HHIP is a negative regulator of HH signaling [53]. Downregulation/inhibition of HHIP might be the reason for HH upregulation in GC. Our results show that HHIP over-expression by LV-HHIP significantly decreases the HHIP promoter methylation, which leads to inhibition of GC cell survival, proliferation, migration and invasion. Our results show that there is a positive feed-back loop that regulates the expression of HHIP via controlling HHIP promoter methylation. Similarly, inhibition of gene methylation by 5-aza-dc induced HHIP expression, which inhibited GC cell proliferation and migration.

The constructed lentiviral HHIP-expressing vector demonstrated high efficiency in increasing HHIP expression in the tested GC cells. Importantly, forced over-expression of HHIP by the construct led to significant inhibition of GC cell survival, proliferation, migration and invasion. These results suggest that the lentiviral HHIP vector might be a promising method for the treatment of GC with over-activated HH signaling.

Conclusion

In summary, forced over-expression of HHIP significantly inhibits proliferation and migration of GC cells, which is accompanied with decreased promoter methylation of the endogenous HHIP gene. Pharmacological inhibition of methylation by 5-aza-dc similarly induces HHIP expression to inhibit GC cell proliferation and migration.

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All authors listed in the current study carried out the experiments, participated in the design of the study and performed the statistical analysis, conceived of the study, and helped to draft the manuscript.

Disclosure Statement

No potential conflicts of interest were disclosed.

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