Hiwi overexpression does not affect proliferation, migration or apoptosis of liver cancer cells *in vitro* or *in vivo*

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Abstract. Piwi like RNA-mediated gene silencing 1 (Hiwi) is a human homolog of the Piwi gene family that has been reported to be upregulated in hepatocellular carcinoma (HCC). The present study aimed to investigate the role of Hiwi in the initiation and development of HCC *in vitro* and *in vivo*. Adenovirus-mediated Hiwi overexpression was established in primary murine hepatocytes and SMMC7721 HCC cells. Cell viability and proliferation were assessed using MTT and EdU assays, respectively. Cell migration was measured using a scratch migration assay. The cell cycle was assessed using flow cytometry, and the expression of genes associated with the epithelial mesenchymal transition (EMT) was assessed using reverse transcription-quantitative polymerase chain reaction. SMMC7721 cells that stably express Hiwi were also generated and injected subcutaneously into the nude mice, and tumor growth was examined. Recombinant adenovirus encoding green fluorescent protein or Hiwi was delivered by injection into the tail vein, and its effect on murine hepatocyte gene expression was studied. The present study revealed that the overexpression of Hiwi did not affect the proliferation or migration of liver cancer cells and failed to suppress perifosine- or doxorubicin-induced apoptosis *in vitro*. The tumors of mice that were injected with Hiwi-expressing SMMC7721 cells were not significantly larger compared with mice that were injected with control SMMC7721 cells. Hiwi overexpression did not noticeably alter the expression of genes involved in EMT, either *in vitro* or *in vivo*. The results of the present study indicate that although expression of Hiwi is associated with HCC development and progression in the clinic, it does not act as an oncogene in liver cancer cells.

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Abbreviation: RNAi, RNA interference

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Introduction

The piwi like RNA-mediated gene silencing 1 (Hiwi) gene is a human homolog of the P-element Induced wimpy testis (Piwi) gene family, which is a class of genes required for stem cell self-renewal in a range of organisms, including jellyfish, *Caenorhabditis elegans, Drosophila melanogaster*, *Danio rerio*, *Mus musculus* and humans (1). Previously, overexpression of Hiwi was reported to be associated with poor prognosis in patients with various human malignant tumors, including seminomas (2), esophageal squamous cell carcinoma (3), adenocarcinoma of the pancreas (4), gastric adenocarcinoma (5), colorectal cancer (6), soft-tissue sarcoma (7) and endometrial carcinoma (8), indicating that it may represent a promising biomarker and a potential target for anticancer treatment.

The function of Hiwi in tumorigenesis is unclear. Upregulation of Hiwi has been demonstrated to promote tumor cell growth in breast (9), cervical (10), endometrial (8) and colorectal cancer (6), as well as in mesenchymal stem cells (11), while its downregulation has been noted to suppress the growth, invasion and migration of glioma (12), gastric (5) and lung cancer (13). These observations indicate that Hiwi may act as an oncogene during carcinogenesis. However, Sharma *et al* (14) reported that overexpression of Hiwi suppressed proliferation and induced apoptosis of the acute myeloid leukemia-derived cell line KG1. Overexpression of Hiwi was reported to inhibit the growth of chronic myeloid leukemia K562 cells and enhance their chemosensitivity to daunomycin (15). These findings indicate that the biological functions of Hiwi may vary between types of tumor, necessitating its role in each cancer to be studied individually.

Hepatocellular carcinoma (HCC) is one of the most common malignancies in China (16). Elevated levels of Hiwi mRNA and protein have previously been observed in HCC, and that Hiwi expression is positively associated with tumor metastasis (17,18). It has been demonstrated that downregulation of Hiwi using RNA interference (RNAi) significantly suppressed the proliferation and invasion of HCC cell lines (18,19). However, whether Hiwi exerts a direct tumorigenic role in

HCC remains unknown. In the present study, an adenovirus vector was used to overexpress Hiwi in liver cells *in vitro* and *in vivo,* and the effect of Hiwi on cell growth and migration was evaluated in hepatocellular carcinoma SMMC7721 cells, primary hepatocytes and xenografts. Additionally, the effect of Hiwi on hepatotoxicant-induced apoptosis was examined.

Materials and methods

Cell culture. The effect of Hiwi overexpression on cell growth was evaluated in SMMC7721 cells and primary mouse hepatocytes, since these cells were reported to express low levels of endogenous Hiwi protein (18). 293 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). SMMC7721 cells were established in 1977 by the Second Military Medicine College (Shanghai, China). These cells were derived from the liver tissue of a 50-year-old male with a grade II-III (20) hepatocellular carcinoma. SMMC7721 cells are epithelial in morphology. The number of chromosomes varies between 44 and 107; 70% of the cells have 54-58 chromosomes. The cells are tumorigenic in nude mice but with a low metastatic potential. SMMC7721 and 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen; Thermo Fisher Scientific, Inc.) and penicillin/streptomycin.

Primary murine hepatocytes were isolated from male ICR mic (n=6, six weeks old, mean average weight 25 g, purchased from the experimental animal center of Nanjing medical University, Nanjing, China) by *in situ* liver perfusion with collagenase via the portal vein. Mice were allowed free access to drinking water and food at room temperature (25˚C) with an automatic 12 h light and 12 h dark cycle. Mice were anesthetized with pentobarbital sodium, and the livers were then perfused *in situ* with 45 ml calcium-free buffer (100 mM HEPES buffer at pH 7.4, 50 mM EGTA), followed by 8 ml liver digest medium (100 mM HEPES buffer at pH 7.6, with 0.5 mg/ml collagenase IV). Next, the liver was excised, minced and strained through a steel mesh. Hepatocytes were obtained by centrifugation 3 times at 50 x g for 5 min at 4˚C and washed twice with DMEM. The hepatocytes were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin for further experiments.

Cloning of human cDNA and construction of plasmids. Human Hiwi cDNA containing the full open reading frame was synthesized commercially (Shanghai GeneChem Co., Ltd., Shanghai, China). The human Hiwi cDNA was sub-cloned into the pcDNA3.1-myc vector, specifically into the myc epitope sequence with *Eco*RI and *Xho*I sites, which yielded the pcDNA3.1-myc-Hiwi plasmid (Fig. 1A). The following primers were used: Forward 5'-AAAAAGAATTCACTG GGAGAGCCCGAGCCAGAGCC-3'; and reverse, 5'-AAA AAACTCGAGTTAGAGGTAGTAAAGGCGGTTTG-3'. The length of the plasmid was confirmed by electrophoresis in 0.5x Tris-acetate EDTA buffer with a 1.5% agarose gel containing 0.1 μg/ml of ethidium bromide. The DNA bands were visualized and analyzed by Biosense 810 Gel Electrophoresis Image analytic system (Beyotime Institute of Biotechnology, Haimen, China). The full Hiwi coding region was verified by sequencing (performed commercially by Generay Co., Ltd., Shanghai, China).

Plasmid transfection. The 293 cells and SMMC7721 cells were transfected with pcDNA3.1-myc-Hiwi using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, and the controls were transfected with the control vector. Cells were used for subsequent experiments 48 h after transfection. For stable transfection, the cells were selected with G418 (400 μg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 2 weeks.

Construction of recombinant adenoviruses. Recombinant adenoviruses expressing Hiwi were constructed commercially using an AdEasy Adenoviral Vector system (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) and the pAdTrack cytomegalovirus vector (Shanghai GeneChem Co., Ltd.). To induce adenovirus-mediated overexpression *in vitro*, 24 h after plating, the cells were incubated at 37˚C for 4 h with adenovirus at a multiplicity of infection of 10-20 pfu per cell. Next, the virus was removed, and the cells were cultured at 37˚C for an additional 24-48 h in fresh DMEM supplemented with 10% FBS and penicillin/streptomycin. Under these conditions, infection efficiency routinely exceeded 90%.

Confirmation of Hiwi overexpression by western blotting. The cells were lysed with Radioimmunoprecipitation Assay buffer containing protease inhibitors (Roche Diagnostics, Basel, Switzerland) for 15 min on ice. Following centrifugation for 15 min at 20,000 x g (4˚C), the protein content of the samples was determined according to the Bradford method. The protein lysates (50 μg per lane) were analyzed by SDS-PAGE (8% gel) and transferred onto methanol-activated polyvinylidene fluoride membranes. Non-specific binding sites were blocked with 5% non-fat milk in Tris-buffered saline/0.1% Tween 20 for 2 h at room temperature. The membranes were incubated overnight with primary antibodies as follows: Hiwi (cat. no. ab12337; 1:1,000; Abcam, Cambridge, UK) or myc (cat. no. MABE282; 1:2,000; Sigma-Aldrich; Merck KGaA). Subsequent to being washed three times, the membranes were blotted with corresponding horseradish peroxidase-conjugated secondary antibody (cat. no. A0208 or A0216; 1:1,000; Beyotime Institute of Biotechnology) for 1 h at room temperature. The membranes were rinsed three times and then developed with enhanced chemiluminescence reagent using the ECL chemiluminescence kit (Thermo Fisher Scientific, Inc.). The two primary antibodies detected proteins with a molecular mass of ~98 kDa (Fig. 1B and C and data not shown), which is consistent with the reported molecular weight of human Hiwi (2). β-actin (cat. no. A5316; Sigma-Aldrich, USA) was used as the internal control.

Assessment of cell viability by MTT assay. An MTT assay was performed in 96-well plates. Briefly, $1x10^4$ primary mouse hepatocytes were seeded per well and cultured overnight prior to infection with the adenovirus. At 0, 20, 40, 60, 80 h, 10 μl MTT (5 mg/ml; Sigma-Aldrich, Merck KGaA) was added to each well, and after 4 h of incubation at 37˚C, the medium was gently decanted, and dimethysulfoxide (100 μl/well) was added to dissolve the formazan product. The absorbance at

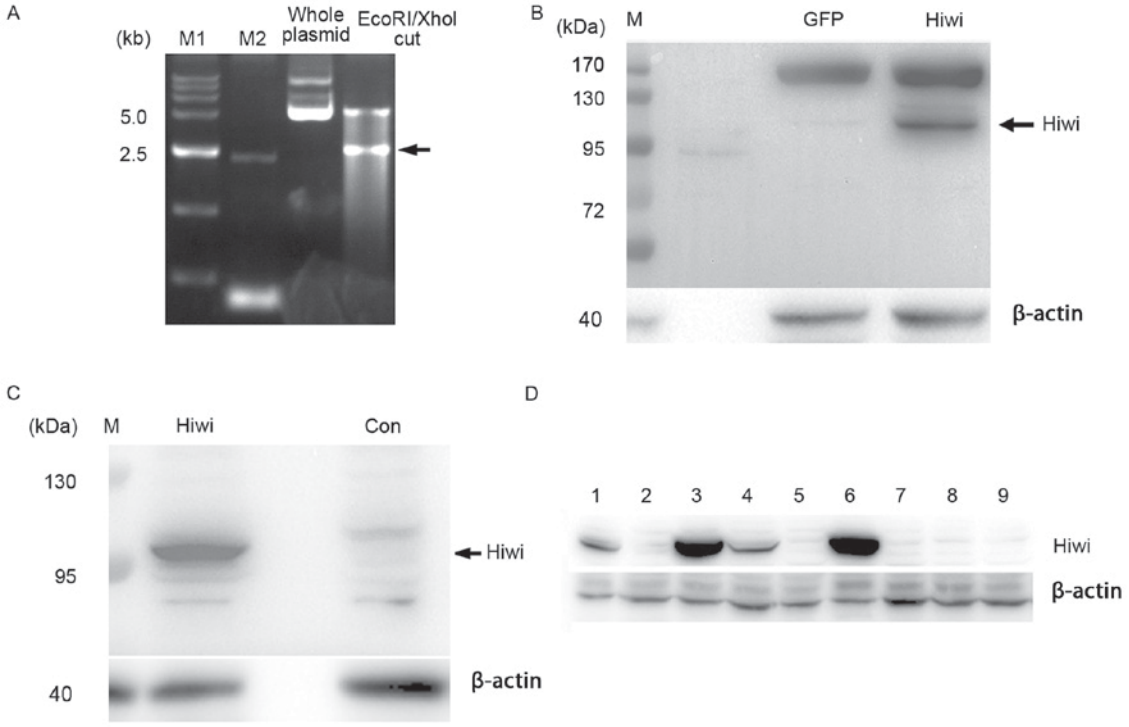

Figure 1. Generation of human Hiwi-expressing plasmid. (A) PcDNA3.1-myc-Hiwi plasmids were cut using *Eco*RI and *Xho*I restriction enzymes and separated by agarose gel electrophoresis. The arrow indicates inserts of ~2.5 kb. (B) 293 cells were transfected with PcDNA3.1-myc-Hiwi plasmid, and the cell lysates were subjected to western blotting using a Hiwi-directed antibody. (C) At 24 h after plating, primary mouse hepatocytes were incubated for 4 h with recombinant adenoviruses expressing Hiwi at a total multiplicity of infection of 10 pfu per cell. The cells were then cultured for an additional 24 h in fresh medium, and the cell lysates were subjected to western blotting using a myc-directed antibody. The arrow indicates the Hiwi protein band. (D) SMMC7721 cells were transfected with pcDNA3.1-myc-Hiwi plasmid and were selected in 400 μg/ml G418 for 2 weeks. The cell lines that stably express Hiwi at high (lanes 3 and 6) or low (lanes 1 and 4) levels were identified by western blot analysis using a Hiwi-directed antibody. The cell lines that do not express Hiwi were used as controls. Hiwi, piwi like RNA-mediated gene silencing 1.

490 nm was measured with a microplate reader according to the manufacturer's protocol.

Analysis of cell proliferation by 5-ethynyl-2'-deoxyuridine (EdU) assay. A total of $2x10^4$ cells per well were seeded in 48-well plates and cultured overnight prior to infection with the adenovirus. At the indicated time, 150 μl of EdU (50 μM; Guangzhou RiboBio Co., Ltd., Guangzhou, China) was added to each well. After 4 h of incubation at 37˚C, the cells were fixed and EdU incorporation was visualized using a Leica microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Cell cycle analysis by flow cytometry. SMMC7721 cells were collected and fixed with ice-cold 70% ethanol at 4˚C for 2 h. Next, the cells were washed twice with PBS and incubated with DAPI in the presence of RNase A at 37˚C for 30 min in the dark. The DNA contents were then measured using a flow cytometer (Applied Biosystems; Thermo Fisher Scientific, Inc.) and were analyzed by Modifit software (version 4.0; Verify Software House, Inc., Topsham, ME, USA) for the proportions of cells in the phases of the cell cycle.

Assessment of cell migration in culture. Two-dimensional cell migration was analyzed using a scratch wound assay. SMMC7721 cells were cultured in 6-well plate. The scratch was performed by using a 200 μl pipette tip to press firmly against the top of the tissue culture plate and swiftly make a vertical wound down through the cell monolayer. SMMC7721 cells were fixed with 70% ethanol and captured using a light microscope at 48 h after the scratch was made. The farthest distance that the cells migrated from the wound edge was measured by calculating the mean of three independent microscope fields in each of the three independent experiments.

Three-dimensional cell migration was determined using a Transwell migration assay as per the manufacturer's instructions (Corning Life Science, Corning, NY, USA). A total of 5,000 cells in serum-free DMEM were added into the top chamber. The bottom chamber contained 1% or 10% FBS in DMEM as a chemoattractant. After 24 h, the cells that had not penetrated the filters were scraped from the upper surface. The membrane was fixed with formalin, and the cells that migrated to the bottom surface of the filter were stained with Giemsa at room temperature for 5-10 min (Beyotime Institute of Biotechnology) and counted using a light microscope (magnification, x200). An average of four randomly chosen fields were counted in each of the three independent experiments.

Effect of Hiwi on genes involved in the epithelial-mesenchymal transition (EMT) by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total cellular RNA was extracted using TRIzol reagent (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed at 50˚C for 50 min, and 70˚C for 10 min using the PrimeScript RT reagent kit (Takara Bio, Inc.). qPCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher

Table I. Primer sequences for polymerase chain reaction.

Gene	GeneBank accession number	Species	Primer sequence (5'-3')
Hiwi	AF104260.2	Human	
Forward	-	-	GAAGCAGCCTGTCTTGGTCAGC
Reverse	-	-	GAATCAAAGCTCAAACCCCAGTCTC
E-cadherin	AB025106.1	Human	
Forward	-	-	GACACCAACGATAATCCT
Reverse	-	-	TTTCAGTGTGGTGATTACGACGTTA
N-cadherin	M34064.1	Human	
Forward	-	-	GGTGGAGGAGAAGAAGACCAG
Reverse	-	-	GGCATCAGGCTCCACAGT
α-catenin	D13866.1	Human	
Forward	-	-	AGCGAAGATTGCGGAACAGGT
Reverse	-	-	GCCTTGACCTTGCTGCAGATG
Vimentin	NM_003380.3	Human	
Forward	-	-	GAGAACTTTGCCGTTGAAGC
Reverse	-	-	TCCAGCAGCTTCCTGTAGGT
GAPDH	AJ005371.1	Human	
Forward	-	-	TGTTCGTCATGGGTGTGAACC
Reverse	-	-	GCAGTGATGGCATGGACTGTG
E-cadherin	NM_009864.2	Mouse	
Forward	-	-	TCGGAAGACTCCCGATTCAAA
Reverse	-	-	CGGACGAGGAAACTGGTCTC
N-cadherin	NM_007664.4	Mouse	
Forward	-	-	CTCCAACGGGCATCTTCATTAT
Reverse	-	-	CAAGTGAAACCGGGCTATCAG
Bcl2	NM_009741.5	Mouse	
Forward	-	-	ATGCCTTTGTGGAACTATATGGC
Reverse	-	-	GGTATGCACCCAGAGTGATGC
Bax	NM_007527.3	Mouse	
Forward	-	-	AGACAGGGGCCTTTTTGCTAC
Reverse	-	-	AATTCGCCGGAGACACTCG
P53	AB021961.1	Mouse	
Forward			GTCACAGCACATGACGGAGG
Reverse			TCTTCCAGATGCTCGGGATAC
Mcl1	NM_008562.3	Mouse	
Forward			TCCAAGGACTCGAAGCCTCT
Reverse			CCAGTTTGTTACGCCATCTTTG
GAPDH	AY618568.1	Mouse	
Forward			AGGTCGGTGTGAACGGATTTG
Reverse			GGGGTCGTTGATGGCAACA

Hiwi, piwi like RNA-mediated gene silencing 1; Bcl-2, B-cell lymphoma 2; Bax, Bcl-associated X; p53, tumor protein p53; Mcl1, induced myeloid leukemia cell differentiation protein.

Scientific, Inc.) using a 7500 Real-Time PCR system. The thermal cycling conditions of the PCR were 94˚C for 5 min, followed by 22-35 cycles for 20 sec at 94˚C, 20 sec at 64˚C, 1 min at 72˚C, and the final extension at 72˚C for 7 min. The primer sequences used are listed in Table I. The relative quantities of mRNA were determined using the comparative cycle threshold method (21) and normalized against GAPDH mRNA.

Analysis of the effect of Hiwi or green fluorescent protein overexpression on apoptosis. Perifosine, an inhibitor of RAC serine/threonine-protein kinase (22) and doxorubicin were used to induce apoptosis in primary murine hepatocytes *in vitro*. A total of 5 μM Perifosine and 2 μM doxorubicin (DOX) were separately added to the primary murine hepatocytes 24 h following infection with recombinant adenovirus that express Hiwi or green fluorescent protein (GFP). Cell

viability was evaluated at different time points by MTT assays.

Xenograft model to study tumor growth. Six-week-old male BALB/C nude mice (n=18, purchased from the experimental animal center of Nanjing medical University, Nanjing, China) were used to study the effect of Hiwi overexpression. They were allowed free access to drinking water and food at room temperature (25˚C) with an automatic 12 h light and 12 h dark cycle. All animal protocols and experimental procedures were approved by the Animal Care and Use Committee of Nanjing Medical University (Nanjing, China). SMMC7721 cells were transfected with pcDNA3.1-myc-Hiwi plasmid and selected with 400 μg/ml G418 for 2 weeks, as aforementioned. A total of 9 colonies were selected. The colonies (1,3,4,6) that stably expressed Hiwi were verified by western blot analysis (Fig. 1D). The colonies (2,5,7-9) that did not express Hiwi were used as blank controls. Hiwi-expressing or blank control SMMC7721 cells ($2x10^7$ for each sample) were suspended in 0.2 ml serum-free DMEM media and implanted into the flank of the nude mice mentioned above. After 70 days, the tumors were isolated, and the volumes were calculated from caliper measurements of tumor diameter.

Effects of Hiwi on genes associated with apoptosis and EMT in vivo. Adenoviruses expressing Hiwi or GFP ($5x10^9$ pfu) were injected into the tail vein of male BALB/c mice (n=10). Anesthesia was achieved by the intraperitoneal administration of freshly prepared sodium pentobarbital (60 mg/kg) 5 days later. The mice were sacrificed by cervical dislocation, and the liver tissues were harvested. RT-qPCR was used to examine the expression of Hiwi, B-cell lymphoma 2 (Bcl-2), myeloid cell leukemia 1, Bcl-associated X, p53, E-cadherin and N-cadherin expression as aforementioned.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical significance was assessed using one-way analysis of variance and Scheffe test. In all statistical comparisons, $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of Hiwi overexpression on the proliferation and migration of liver cells. The growth curve of primary mouse hepatocytes that were infected with Hiwi-expressing adenovirus did not differ significantly from the control cells (Fig. 2A). Next, the functional role of Hiwi in cell division and cell cycle of liver cells was analyzed. As indicated in Fig. 2B, overexpression of Hiwi did not alter the percentage of SMMC7721 cells in G_1, S and G_2/M phases. No significant difference was observed between the Hiwi-overexpressing and control SMMC7721 cells in the EdU incorporation assays (Fig. 2C). These results indicate that overexpression of Hiwi did not affect liver cell proliferation.

Subsequently, the effect of Hiwi on the invasion of liver cells was examined. As indicated by the scratch migration assay, the average distance that SMMC7721 cells overexpressing Hiwi migrated at 48 h after the cell scratch did not differ significantly from the control cells (Fig. 3A). The

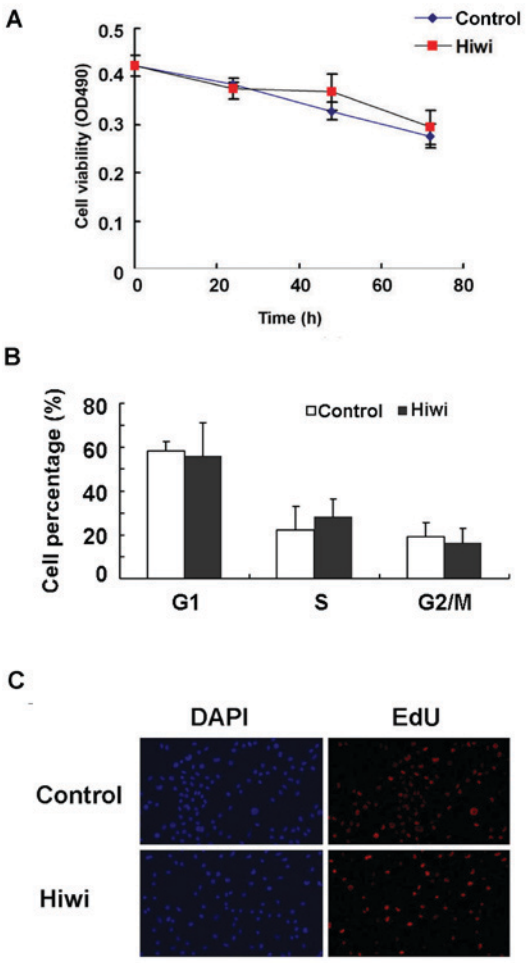

Figure 2. Effect of Hiwi overexpression on liver cell proliferation. (A) Primary mouse hepatocytes were infected with Hiwi-expressing or control adenovirus 24 h following plating. Cell viability was assessed by an MTT assay (n=3) at the indicated times. (B) SMMC7721 cells were incubated with recombinant adenoviruses for 48 h. The cell cycle was analyzed by flow cytometry (n=3). (C) The proliferative rate of Hiwi-overexpressing and control SMMC7721 cells was assessed by EdU incorporation assay (magnification, x200). Hiwi, piwi like RNA-mediated gene silencing 1; OD, optical density.

effect of Hiwi expression on SMMC7721 cell migration was also assessed by Transwell assay using a modified Boyden chamber. The fraction of SMMC7721 cells overexpressing Hiwi that migrated through a gelatin-coated membrane in response to 1% or 10% FBS did not differ significantly from the control cells (Fig. 3B). These data indicated that overexpression of Hiwi did not affect the migration of liver cells.

To assess the effect of Hiwi on genes involved in EMT, levels of α-catenin, E-cadherin, N-cadherin and vimentin mRNA were measured by RT-qPCR. Consistently, levels of α-catenin, E-cadherin, N-cadherin and vimentin mRNA did not differ significantly between Hiwi-overexpressing cells and control cells (Fig. 4).

Effect of Hiwi on drug-induced apoptosis in vitro. Oncoproteins, such as Bcl-2, contribute to neoplastic cell growth primarily by promoting cell survival via interfering with apoptosis (23). Therefore, it was hypothesized that Hiwi might promote tumor growth by inhibiting apoptosis. Mouse primary hepatocytes

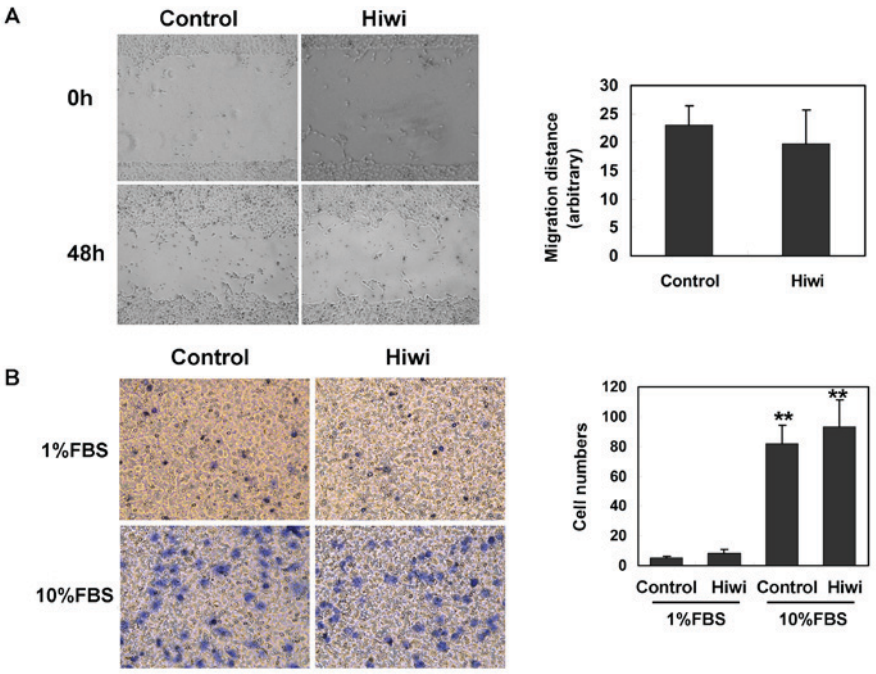

Figure 3. Effect of Hiwi overexpression on the migration of liver cells. (A) SMMC7721 cells were incubated with recombinant adenoviruses expressing Hiwi at a total multiplicity of infection of 20 pfu per cell. Cell migration was observed 48 h after the scratch was made (magnification, x100; n=3). (B) The migration of SMMC7721 cells was assessed in a Transwell migration assay using a modified Boyden chamber in response to 1 or 10% FBS (magnification, x200, n=3). **P<0.05 vs. the 1% FBS group. Data are presented as the mean ± standard deviation from three independent experiments. FBS, fetal bovine serum; Hiwi, piwi like RNA-mediated gene silencing 1.

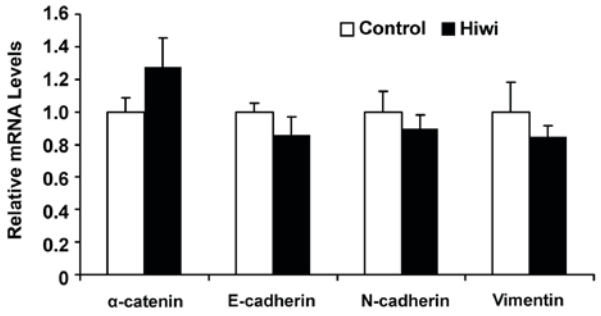

Figure 4. Effect of Hiwi on genes involved in the epithelial-mesenchymal transition. Primary mouse hepatocytes were incubated with Hiwi-expressing or control adenovirus for 24 h after plating. The mRNA levels of the indicated genes were analyzed by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation from three experiments. Hiwi, piwi like RNA-mediated gene silencing 1.

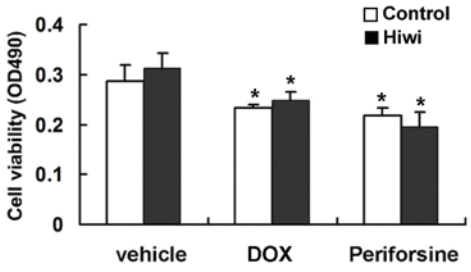

Figure 5. Effect of Hiwi overexpression on the apoptosis of liver cells. Primary mouse hepatocytes were infected with Hiwi-expressing or control adenovirus 24 h after plating. Then cells were treated with 5 μM perifosine or 2 μM DOX. After 24 h, cell viability was assessed by MTT assay (n=3). *P<0.05 vs. the vehicle. Data are presented as the mean ± standard deviation from three experiments. Hiwi, piwi like RNA-mediated gene silencing 1; DOX, doxorubicin; OD, optical density.

were treated with perifosine and subsequently a significant reduction in cell viability was observed (Fig. 5). However, the overexpression of Hiwi did not enhance the survival of perifosine-treated cells (Fig. 5). DOX is a widely used anticancer drug that induces apoptosis (24). Overexpression of Hiwi had no detectable effect on DOX-induced hepatotoxicity and apoptosis (Fig. 5). These results indicated that overexpression of Hiwi did not inhibit apoptosis.

Effect of Hiwi overexpression on the growth of HCC xenografts. Although exogenous expression of Hiwi did not affect liver cell growth *in vitro*, the growth of Hiwi-overexpressing HCC cells was examined *in vivo*. Hiwi-expressing SMMC7721 cells were implanted into the flank of 6-weeks-old BALB/c nude mice. After 70 days, tumor volume was measured. Consistently, the mean size of tumors derived from Hiwi-expressing cells did not differ significantly from the tumors that derived from control cells (Fig. 6). These results indicated that overexpression of Hiwi did not alter tumor growth *in vivo*.

Role of Hiwi overexpression in altering the expression of genes that are involved in EMT or apoptosis. The mice were injected with adenovirus expressing Hiwi or GFP ($5x10^9$ pfu) in the tail vein. After 5 days, Hiwi was detected in hepatocytes via RT-qPCR and western blot analysis. Expression of genes that are involved in EMT or apoptosis expressed by these hepatocytes did not differ significantly between mice that were injected with the Hiwi-expressing vector or the control adenoviral vector (Fig. 7).

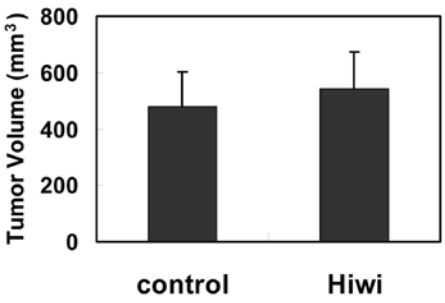

Figure 6. Effect of Hiwi overexpression on tumor growth *in vivo*. An equal number ($2x10^7$) of Hiwi-expressing (no. 3 in Fig 1D) stable cells or controls were injected subcutaneously into the flank of nude mice. At 70 days later, the tumors derived from parental cells were isolated, and the tumor volume was calculated. The diameter of the tumors were measured using a caliper. Colonies 6, 1 and 4 in Fig 1D were also tested and similar results were observed. Data are presented as the mean ± standard deviation (n=8 in control group, n=10 in Hiwi group). Hiwi, piwi like RNA-mediated gene silencing 1.

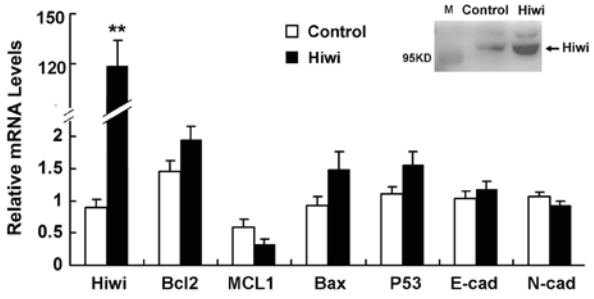

Figure 7. Effect of Hiwi overexpression on hepatocyte gene expression *in vivo*. Adenovirus expressing Hiwi or green fluorescent protein ($5x10^9$ pfu) were injected via the mouse tail vein. The livers were harvested 5 days after injection. The mRNA levels of Hiwi, Bcl2, MCL1 and Bax were analyzed by reverse transcription-quantitative polymerase chain reaction. $^{**}P<0.01$ vs. the control. Insert: The livers were harvested 5 days after adenovirus injection. Hiwi expression was detected by western blotting using a myc-directed antibody. M, marker; Hiwi, piwi like RNA-mediated gene silencing 1; Bcl-2, B-cell lymphoma 2; Bax, Bcl-associated X; p53, tumor protein p53; Mcl1, induced myeloid leukemia cell differentiation protein; E-cad, E-cadherin.

Discussion

Hiwi has been suggested to have a role in several human malignant tumors (2,3,5,7), and increased expression of the Hiwi gene has been demonstrated in seminoma cell hyperplasia (2), esophageal squamous cell carcinoma (3), gastric carcinoma (5), and pancreas adenocarcinoma (4). In the majority of cases, increased expression of Hiwi was detected in tumors and found to be associated with poorer patient outcomes (2-8,17,18). These prior studies indicated that Hiwi expression might be a useful clinical marker of germ cell malignancies and solid tumors of epithelial or mesenchymal origin.

Previous studies revealed that significant decreases in Hiwi expression were observed in renal cell carcinoma, and Hiwi expression was found to be inversely associated with overall survival (25,26). Functional analysis of Hiwi protein has also generated conflicting results. Although overexpression of Hiwi has been demonstrated to promote the growth of breast or cervical tumors (9,10), in acute or chronic myeloid leukemia cell lines Hiwi proteins may suppress proliferation (14,15). These findings indicate that the function of Hiwi differs in different cellular contexts, and the biological function of Hiwi remains poorly understood.

Since the downregulation of Hiwi suppressed proliferation and invasion of HCC cells (18,19), we hypothesized that the overexpression of Hiwi may contribute to the development and progression of HCC. However, overexpression of Hiwi in SMMC7721 HCC cell lines, primary mouse hepatocytes, xenografts and an adenovirus-mediated mouse hepatic gene-expression model did not alter the proliferation, migration or apoptosis of liver cells *in vitro* or *in vivo*. Although Hiwi expression was elevated in HCC (17,18), it did not appear to function as an oncoprotein. The RNAi-mediated downregulation of Hiwi was previously revealed to suppress the proliferation and invasion of HCC cell lines (18). Therefore, we hypothesize that Hiwi may be necessary but not sufficient for tumor genesis of liver cells.

Similar results were previously obtained in gastric cancer cells. Overexpression of Hiwi in AGS cells did not alter their proliferative rate, whereas suppression of Hiwi expression using antisense RNAs or RNAi inhibited cell growth and induced cell cycle arrest (5). One possible explanation for this discrepancy would be that, in certain types of tumors, other cellular factors might be required to interact with Hiwi to promote tumorigenesis. The existence of additional signaling pathways that counteract the function of Hiwi in these tumors cannot be ruled out. Additionally, the findings of the present study do not necessarily reflect the role of Hiwi in humans *in vivo*, and other unknown mechanisms may well affect tumor growth in humans.

In summary, the findings of the present study indicate that although expression of Hiwi is associated with the development and progression of HCC, it does not act as an oncogene in liver cancer cells. To characterize the contribution of Hiwi to the progression of HCC in humans further, other factors or pathways that interact with Hiwi require elucidation.

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Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CXS and HaL performed the cloning and transfection of plasmids, and generated SMMC7721 cells stably expressing Hiwi. HuL and HaL performed the apoptosis assays and generated xenograft model. HHZ and HMS assisted in the flow cytometry

analysis. MDS, JLC and SFX studied the expression of genes and analyzed the data. HaL and JXJ designed the study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal protocols and experimental procedures were approved by the Animal Care and Use Committee of Nanjing Medical University (Nanjing, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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