Manipulations in HIWI level exerts influence on the proliferation of human non-small cell lung cancer cells

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Abstract. Lung cancer is the leading cause of cancer-associated mortality worldwide, although molecular imaging techniques, including fludeoxyglucose positron emission tomography, have markedly improved the diagnosis of lung cancer. HIWI is a member of the human *piwi* family, members of which are known for their roles in RNA silencing. HIWI has been shown to serve a crucial function in stem cell self-renewal, and previous studies have reported HIWI overexpression in lung cancers. Furthermore, HIWI has been proposed to regulate the maintenance of cancer stem cell populations in lung cancers. The present study investigated the mRNA and protein expression levels of HIWI in non-small cell lung cancer (NSCLC) specimens harvested from 57 patients, using reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. Subsequently, the HIWI expression level was manipulated using gain-of-function and loss-of-function strategies, and the role of HIWI in the proliferation of human A549 NSCLC cells was investigated using Cell Counting Kit-8 and colony formation assays. The mRNA and protein expression levels of HIWI were significantly upregulated in the intratumor NSCLC specimens, as compared with the peritumor specimens. Furthermore, the mRNA and protein expression levels of HIWI in A549 cells were successfully manipulated using the two strategies. Overexpression and knockout of HIWI were associated with the promotion and inhibition of A549 cell proliferation, respectively. The results of the present study suggested that HIWI is overexpressed in NSCLC tissues and demonstrated that upregulation of HIWI may promote the growth of lung cancer cells; thus suggesting that HIWI may have an oncogenic role in lung cancer.

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Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide (1), among which non-small cell lung cancer (NSCLC) accounts for ~80% of all cases of lung cancer (2). The incidence of NSCLC in China has markedly increased over the past 5 years (3). At present, numerous clinical and molecular prognostic factors are considered in order to tailor therapies to particular subgroups of patients; however, treatment responses are routinely assessed by serial measurements of tumour sizes prior to and following treatment (4). Molecular imaging, which is a research and clinical tool that was developed >30 years ago (5), has markedly improved the diagnosis and the treatment of lung cancers. Previous studies have suggested the superior prognostic value of fludeoxyglucose positron emission tomography, as compared with computed tomography, and it has exhibited enhanced sensitivity for the evaluation of therapeutic effects (6-8). However, NSCLC is typically diagnosed at the advanced stages, leading to a poor prognosis in China (9).

The *hiwi* gene, which is a human homologue of the *piwi* family, is located at 12q24.33 and encodes the 98.5 kDa HIWI protein (10). The *piwi* family has been shown to have an important role in stem cell self-renewal in diverse organisms, including jellyfish, *Caenorhabditis elegans*, *Drosophila melanogaster*, zebrafish, mice and humans (11-13). HIWI is typically expressed in $CD34^+$ hematopoietic progenitor cells, although not in the more differentiated cell populations (14). Furthermore, HIWI has been shown to be highly expressed in numerous human cancer types (11,15), and abnormal HIWI expression levels have been associated with a poor prognosis in various human malignant tumors, including human esophageal squamous cell carcinoma (16), human adenocarcinoma of the pancreas (15), human gastric cancer (11), and colorectal cancer (17). In addition, a previous study demonstrated that silencing of the HIWI gene was able to decrease the proliferation and promote the apoptosis of lung cancer tumor stem cells (18), and it was suggested that the *hiwi* gene may be a key regulator in the maintenance of cancer stem cell (CSC) populations in lung cancers (19,20). Therefore,

HIWI has been implicated in the tumorigenesis of lung cancers and may be a potential target for novel cancer therapies.

The present study aimed to investigate whether HIWI is overexpressed in NSCLC specimens, and the effects of manipulating the expression levels of HIWI on the proliferation of a human A549 NSCLC cell line.

Materials and methods

NSCLC specimens, cell lines and culture. The present study was approved by the Ethics Committee of the Zhongnan Hospital of Wuhan University (Wuhan, China). A total of 57 patients with NSCLC (average age, 64.3 years; 40 male and 17 female) at the Zhongnan Hospital of Wuhan University were enrolled in the present study between February 2008 and September 2013. Informed consent was obtained from all patients. A total of 57 intratumor specimens and 57 peritumor specimens (control; ≥10 mm from the tumor edge) were harvested during surgical resection (10 mm) from the patients prior to treatment with radiotherapy and chemotherapy. The specimens were immediately frozen in liquid nitrogen and stored at -80˚C. The clinico-pathological data of the patients was recorded prospectively, including the age at diagnosis, tumor size, axillary lymph node metastasis and histological grade (21). Human NSCLC A549 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Gibco Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Sijiqing Bio-technology, Co. Ltd., Hangzhou, China) at 37˚C in a humidified atmosphere containing 5% CO_2.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In order to evaluate the mRNA expression levels of HIWI, total RNA from the intratumor and peritumor specimens and A549 cells was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, and supplemented with 1 μl RNase inhibitor (Takara Bio, Inc., Otsu, Japan). RT-qPCR was conducted using a SYBR PrimeScript RT-qPCR kit (Takara Bio, Inc.) according to the manufacturer's instructions with a 1 μl RNA template in a LightCycler® 480 Instrument II thermal cycler (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions were as follows: 42˚C For 5 min, and 95˚C for 10 sec for the reverse transcription, and 40 cycles of 95˚C for 5 sec and at 60˚C for 20 sec for the PCR reaction. The primer sets for HIWI and β-actin genes were synthesized by Sangon Biotech, Co., Ltd. (Shanghai, China) according to previously reported sequences (22). The primer sequences were as following: HIWI forward, 5'-AGC ACTAACCATTTCCGGCTG-3' and reverse, 5'-TATAGT CAATGTGATACTGAT-3'; and β-actin forward, 5'-GTACCC TGGCATTGCCGACA-3' and reverse, 5'-CTCGTCATACTC CTGCTTGCT-3'. The mRNA expression levels of HIWI were normalized against those of β-actin, and were expressed as the fold-change over control, as determined using the $2^{-\Delta\Delta Cq}$ method (23).

Western blot analysis. NSCLC specimens were homogenized and subjected to protein isolation using a cell lysis reagent (V8571; Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. Protein samples (30 μg) were quantified using a BCA Protein Assay Reagent Kit (Pierce Biotechnology, Inc., Rockford, IL, USA) separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis [GenScript (Nanjing) Co., Ltd., Nanjing, China] with a loading quantity of 50-90 μg, and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA), which was blocked using Tris-buffered saline solution (Takara Bio, Inc.) containing 5% milk. Subsequently, the membranes were incubated with polyclonal rabbit anti-HIWI polyclonal antibody (1:300; 701177; Thermo Fisher Scientific Inc.) or anti-β-actin (1:00; 100162-RP02-100; Sino Biological, Inc., Beijing, China) at 37˚C for 1 h, followed by incubation with peroxidase-conjugated secondary antibody (1:500; PA1-096; Pierce Biotechnology, Inc., Rockford, IL, USA) at 37˚C for 45 min. Antibody complexes were visualized using the Enhanced Chemiluminescence Detection system (GE Healthcare Life Sciences, Chalfont, UK), according to the manufacturer's protocol.

Manipulation of HIWI expression levels in A549 cells. In order to overexpress HIWI in A549 cells, wild-type HIWI and enhanced green fluorescence protein (EGFP) coding sequences were amplified and cloned into the eucaryotic expression pcDNA3.1 (+) vector (Invitrogen; Thermo Fisher Scientific, Inc.). A self-cleaving 2A linker sequence was inserted between the HIWI and EGFP coding sequences, which enabled the genes to be transcribed into a single mRNA sequence, but translated into two separate proteins (24). The HIWI-2A-EGFP-pcDNA3.1 (+) or control CAT-2A-EGFP-pcDNA3.1 (+) vectors were transfected into A549 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The HIWI+EGFP positive cell clones [A549 HIWI (+)] and the CAT+EGFP positive cell clones (A549 control) were selected in the presence of 1.2 mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA), and maintained in DMEM containing 500 μg/ml G418. In order to downregulate HIWI expression levels, Stealth RNAi™ siRNA sequences (Thermo Fisher Scientific, Inc.) were used, according to the manufacturer's protocol. Briefly, small interfering (si)RNAs [Genscript (Nanjing) Co., Ltd.] specifically targeting HIWI, including siRNA-HIWI 1, siRNA-HIWI 2 or siRNA-Con (10 nM) were transfected into A549 HIWI (+) cells using Lipofectamine 2000.

Cell counting, Cell Counting Kit (CCK)-8 and colony formation assays. The cell counting assay was conducted using a method described in a previous study (25). Briefly, 1,000 cells/well (~85% confluence) were seeded into 6-well plates and were incubated for 24, 48, 72 or 96 h at 37˚C, followed by trypsinization with 0.25% trypsin (Amresco LLC, Solon, OH, USA). The number of viable cells was counted in a hemocytometer (Reichert Technologies, Buffalo, NY, USA) following Trypan blue staining (Sigma-Aldrich). For the proliferation assay, A549 HIWI (+) and A549 control cells were treated with 20 μM cisplatin (Sigma-Alrdich) for 12, 24, or 48 h, and were subsequently incubated with CCK-8 assay solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The absorbance of each well was detected at 450 nm following the development of coloration, as determined visually using a spectrophotometer (Crystaleye; Olympus Corporation, Tokyo, Japan). For the colony formation assay, 200 cells from each group were

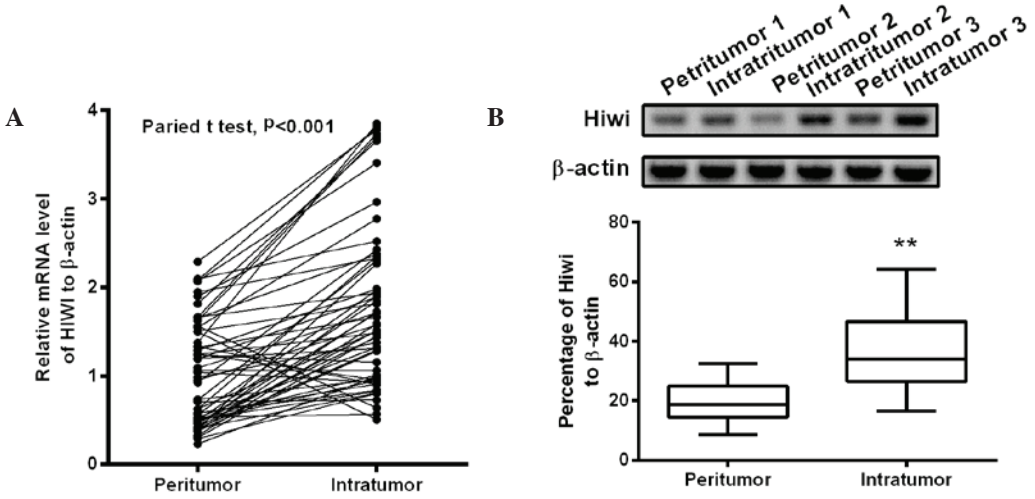

Figure 1. Overexpression of HIWI in non-small cell lung cancer (NSCLC) specimens from 57 patients. (A) Relative mRNA expression levels of HIWI to β-actin in the intra- (n=57) and peri-tumor (n=57) tissues were determined using the reverse transcription-quantitative polymerase chain reaction assay. (B) Relative protein expression levels of HIWI in the NSCLC intratumor specimens (n=15) vs. the peritumor specimens (n=15) were determined using western blotting. **P<0.01 vs. the peritumor specimens. ns, no significance.

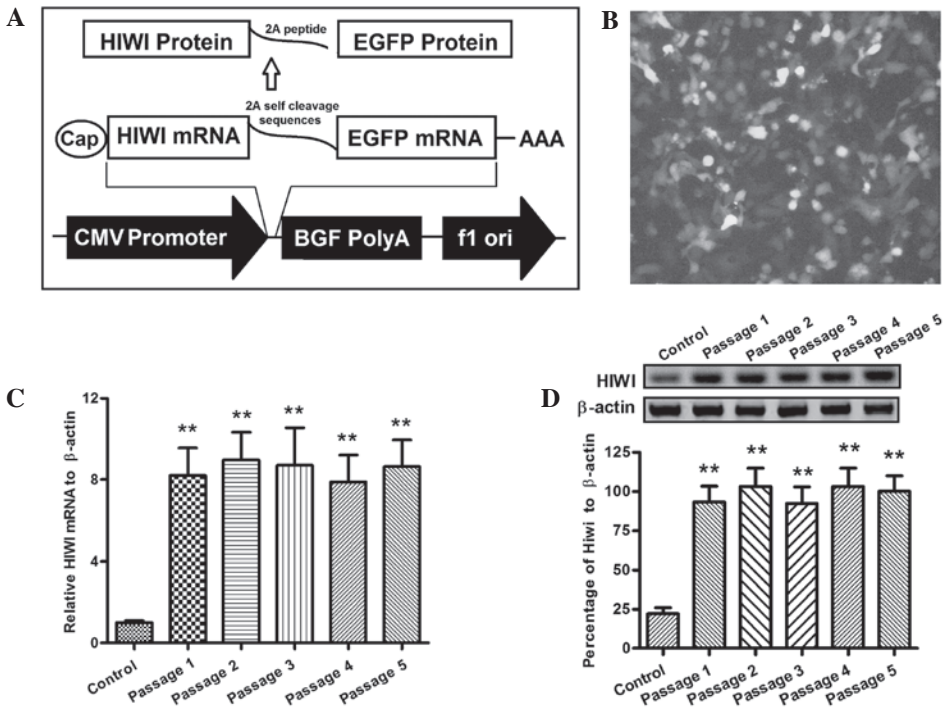

Figure 2. HIWI overexpression was stabilized in the HIWI and EGFP co-expressing A549 HIWI (+) cells. (A) A sketch map of the HIWI and EGFP genes joined with a self-cleaving 2A linkage sequence. (B) The EGFP-positive A549 HIWI (+) cells were selected under G418 pressure. The (C) mRNA and (D) protein expression levels of HIWI in the A549 HIWI (+) cells were stabilized following serial passages, as demonstrated using reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. **P<0.01 vs. the A549 control cells. Each result was the average of three independent results. EGFP, enhanced green fluorescent protein; CMV promoter, cytomegalovirus promoter.

seeded into a 12-well plate, with or without siRNA transfection. Following 48 h, the cells were stained with 0.5% crystal violet solution (Sigma-Aldrich). The number of colonies were counted directly on the plate.

Statistical analysis. Statistical analyses were conducted using the GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). The mRNA and protein expression levels of HIWI and the cell and colony numbers were compared between the two groups using the Student's *t*-test. P<0.05 was considered to indicate a statistically significant difference.

Results

HIWI is upregulated in NSCLC specimens. The mRNA and protein expression levels of HIWI in NSCLC tissues were determined using RT-qPCR and western blot analysis, respectively. The mRNA expression levels of HIWI were significantly

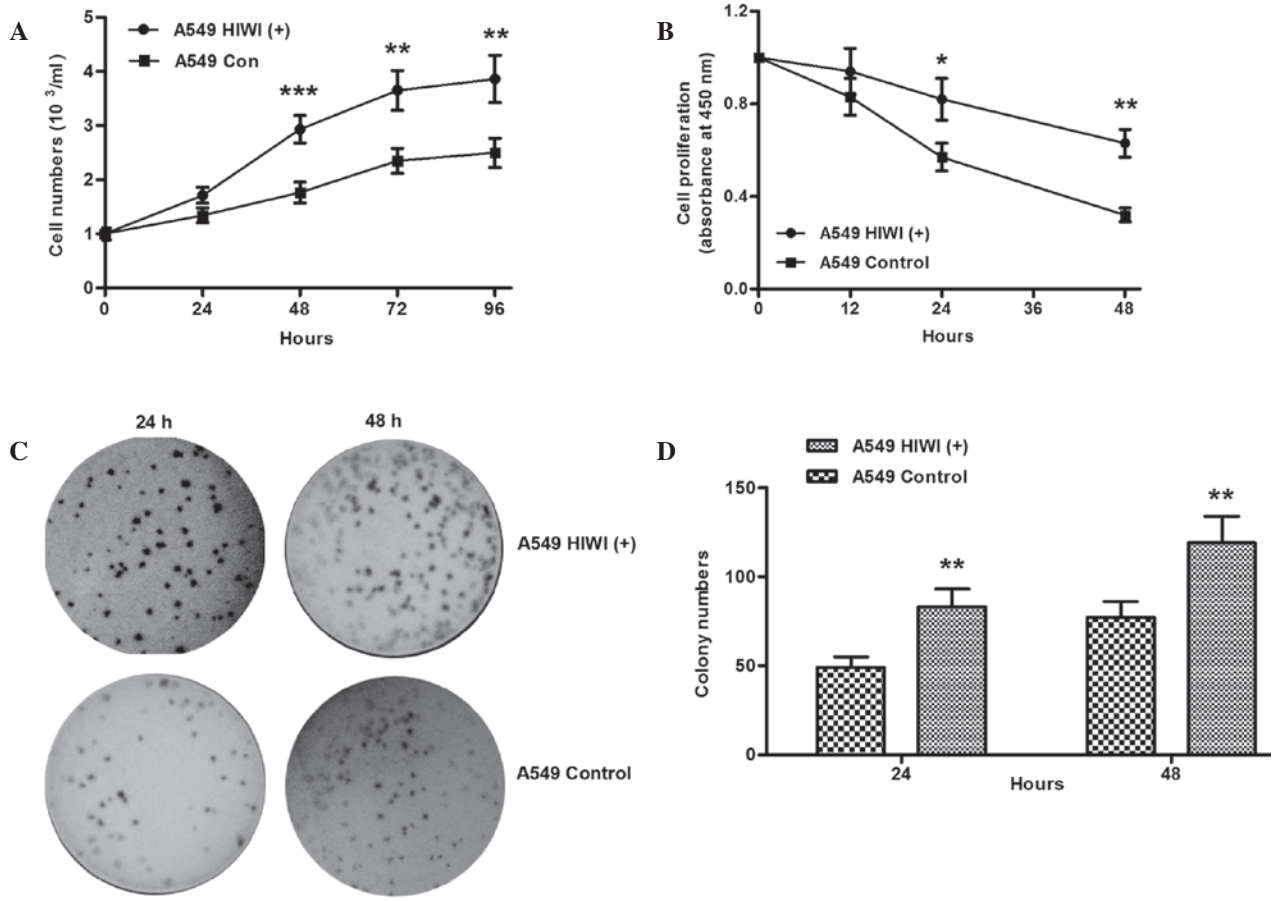

Figure 3. Overexpression of HIWI promotes the proliferation of human non-small cell lung cancer A549 HIWI (+) cells. (A) A significantly higher proliferation rate was detected for the A549 HIWI (+) cells, as compared with the A549 control cells, at 24, 48, 72 and 96 h post-inoculation (10^3 cells/ml). (B) The rate of cell death was significantly reduced in the A549 HIWI (+) group following 20 μM cisplatin treatment for 12, 24 and 48 h, as compared with the A549 control group (C and D). Differences in colony formation between the A549 HIWI (+) and A549 control cells. Each value is an average of three independent tests. *P<0.05, **P<0.01 and ***P<0.001 vs. the A549 control group.

upregulated in the intratumor specimens from 57 patients with NSCLC, as compared with the peritumor specimens from the same patients [mean difference=0.7642; 95% confidence interval (CI), 0.5695-0.9590; R^2, 0.5249; P<0.01; Fig. 1A]. Similarly, the protein expression levels of HIWI in 15 intratumor specimens were significantly increased, as compared with 15 peritumor specimens (P<0.01; Fig. 1B). These results suggest that HIWI is upregulated in NSCLC specimens.

HIWI promotes A549 cell proliferation. In order to evaluate the effects of HIWI overexpression on the proliferation of A549 NSCLC cells, growth curves for A549 HIWI (+) cells and A549 control cells were constructed as indicated by Fig. 2A. The GFP-positive cell clone was selected (Fig. 2B) and high expression levels of HIWI mRNA (Fig. 2C) and protein (Fig. 2D) were detected. The CCK-8 assay indicated that the proliferation rate of A549 HIWI (+) cells was significantly higher between 24 and 96 h post-inoculation, as compared with the A549 control cells (P<0.001 and P<0.01, respectively; Fig. 3A). Furthermore, in the presence of cisplatin, the proliferation rate was significantly higher in the A549 HIWI (+) group, as compared with the A549 control group (P<0.05 and P<0.01 at 24 and 48 h post-inoculation, respectively; Fig. 3B). The growth difference between the A549 HIWI (+) and A549 control cells was reconfirmed by the colony formation assay; significantly more colonies were formed by the A549 HIWI (+) cells at 24 and 48 h post-inoculation, as compared with the A549 control cells (P<0.01; Fig. 3C and D). These results suggest that overexpression of HIWI *in vitro* promotes the proliferation of human A549 NSCLC cells.

HIWI knockdown by siRNAs blocks the HIWI overexpression-induced promotion of A549 NSCLC cell proliferation. The ability of HIWI overexpression to promote the proliferation of A549 cells was re-evaluated using *hiwi*-specific siRNA transfection. Briefly, HIWI expression was knocked-down by transfecting *hiwi*-specific siRNAs into the A549 HIWI (+) cells. Significantly reduced mRNA and protein expression levels of HIWI were detected in siRNA-HIWI 1- or siRNA-HIWI 2-transfected A549 HIWI (+) cells, as compared with the siRNA-con transfected cells (P<0.01; Fig. 4A and B). Subsequently, the effects of HIWI knockdown on the rate of proliferation of A549 NSCLC cells was analyzed. The CCK-8 assay indicated that cell proliferation was significantly reduced in the siRNA-HIWI 1- and siRNA-HIWI 2-transfected cells (P<0.05 and P<0.01 at 24 and 48 h post-transfection, respectively; Fig. 4C). In addition, the number of colonies formed was significantly reduced in the siRNA-HIWI 1- and siRNA-HIWI 2-transfected groups, as compared with the siRNA-con transfected group (P<0.01; Fig. 4D and E). These results suggest that HIWI overexpression may promote the proliferation of NSCLC A549 cells.

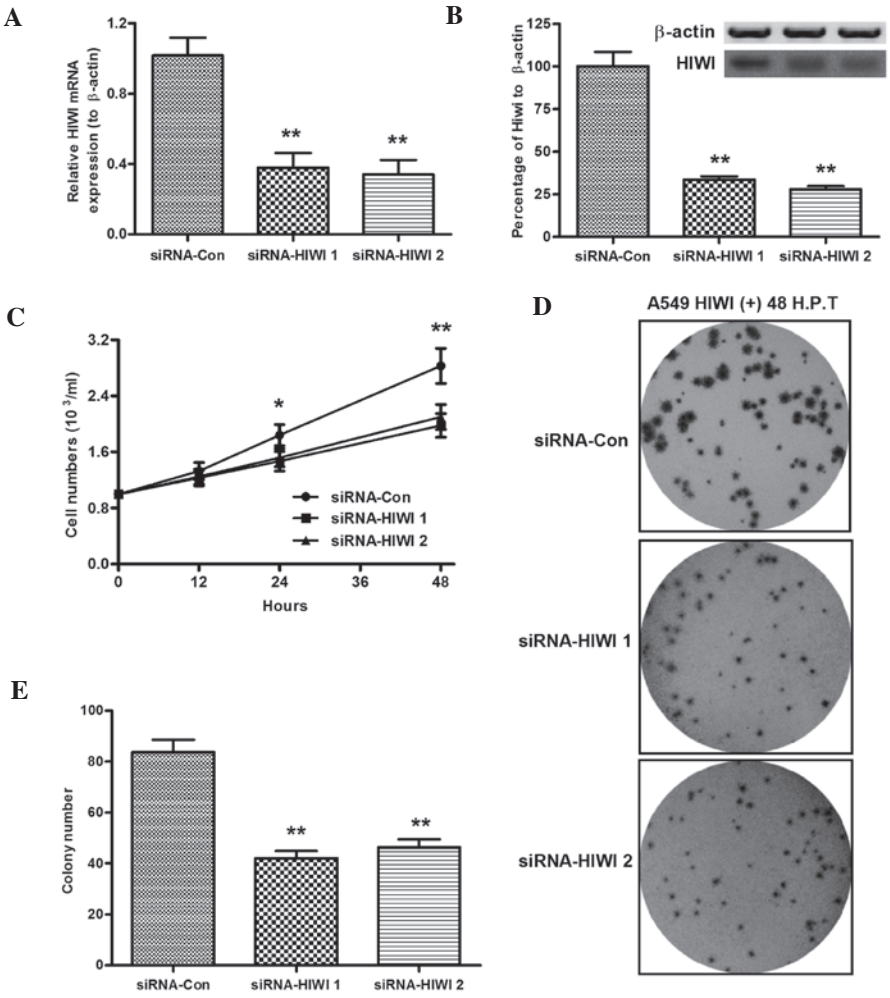

Figure 4. Knockdown of *hiwi* by siRNA transfection inhibited the proliferation of A549 HIWI (+) cells. (A) mRNA and (B) protein expression levels of HIWI were significantly downregulated in the A549 HIWI (+) cells at 24 h following transfection with siRNA-HIWI 1 and siRNA-HIWI 2, as compared with the cells transfected with siRNA-Con. (C and D) The proliferation of A549 HIWI (+) cells transfected with siRNA-HIWI 1 or siRNA-HIWI 2 was significantly reduced, as compared with the cells transfected with siRNA-Con, as demonstrated using the (C) cell counting kit-8 assay and (D) the colony formation assay. (E) The graph indicates the colony number of cells transfected with siRNA-Con, siRNA-HIWI 1 or siRNA-HIWI 2. Experiments were performed independently in triplicate. $^{*}P<0.05$ and $^{**}P<0.01$ vs. the siRNA-Con group. siRNA, small interfering RNA.

Discussion

HIWI is a member of Argonaute (Ago) family, which exert RNase activity similar to RNase H endonuclease (26). Previous studies have reported that the *piwi* gene family, including *hiwi*, may be associated with a novel class of small RNAs, called 'piwi-interacting RNAs' (piRNAs). piRNAS have previously been associated with mammalian spermatogenesis (11,13,27-29), and are a type of regulatory small non-coding RNA (ncRNA) (30-32). In order to perform their functions, small ncRNAs are incorporated into Ago complexes to form highly specific small-RNA-binding modules in RNA-silencing pathways (33,34). HIWI has previously been shown to be highly expressed in numerous types of human cancers, and HIWI overexpression has been associated with a poor prognosis (11,5-7). In addition, HIWI gene silencing was shown to decrease the proliferation and promote the apoptosis of lung cancer tumor stem cells (8). Furthermore, it was proposed that the *hiwi* gene may be a key regulator in the maintenance of CSC populations in lung cancers (9,20). However, it is currently unclear how this ribonucleoprotein-like molecule may promote tumorigenesis.

Yang *et al* demonstrated that the inhibition of HIWI expression was able to significantly reduce tumor growth in a xenograft mouse model (35). Furthermore, immunohistochemical analyses suggested that downregulation of HIWI was associated with a reduced number of aldehyde dehydrogenase (ALDH)-1 positive cells in xenograft tumor samples (36). Since ALDH-1 is a marker of lung cancer stem cells (37), it has been hypothesized that the use of small hairpin RNA-mediated *hiwi* gene silencing may decrease the number of lung cancer stem cells and suppress tumor growth in nude mice. Therefore, HIWI may have a crucial role in the regulation of lung cancer stem cell growth.

The present study detected HIWI overexpression in NSCLC tissue samples at the mRNA and protein level; significantly higher HIWI mRNA and protein expression levels were detected in the intratumor specimens, as compared with the peritumor specimens. Gain-of-function and loss-of-function strategies were adopted in order to investigate the effects of *hiwi* overexpression on A549 NSCLC cell proliferation. Notably, the mRNA and protein expression levels of HIWI were significantly higher in the A549 HIWI (+) cells, and this

was stable following serial passages. The CCK-8 and colony formation assays suggested that overexpression of HIWI promoted A549 cell proliferation, and this was confirmed by RNAi, in which the knock-out of HIWI by siRNAs resulted in attenuation of the HIWI-associated promotion of A549 cell proliferation.

In conclusion, the present study demonstrated that HIWI was overexpressed in NSCLC specimens, and identified a positive correlation between the expression levels of HIWI and the proliferation of NSCLC A549 cells, using gain-of-function and loss-of-function strategies. Therefore, the results of the present study suggested that HIWI may have an oncogenic role in the tumorigenesis of NSCLC; however, the mechanisms underlying the promotion of NSCLC cell proliferation by HIWI requires further investigation.

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