

Effect of RNA interference-related HiWi gene expression on the proliferation and apoptosis of lung cancer stem cells

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Received November 14, 2011; Accepted March 22, 2012

DOI: 10.3892/ol.2012.677

Abstract. The aim of this study was to investigate the effect of HiWi gene silencing on lung cancer tumor stem cell proliferation and apoptosis using gene transfection and RNA interference. Moreover, we examined the feasibility of using the HiWi gene as a molecular target for the inhibition of lung cancer tumor stem cells (TSCs). shRNA eukaryotic expression vectors, pGenesil-2-HiWi1, pGenesil-2-HiWi2263 and pGenesil-2-control, targeting the HiWi gene were constructed. PBS served as the control group. The expression vector of the target HiWi gene shRNA was transfected into lung cancer TSCs with PEI as the medium. The conditions of lung cancer TSC proliferation and apoptosis in each group were examined using an MTT assay, fluorescence-activated cell sorting and Annexin V staining. The results showed that 24 h after transfection, the proliferation inhibition rates in the pGenesil-2-HiWi2263 (81.62%) and pGenesil-2-HiWi1 (73.16%) groups were higher as compared to the proliferation inhibition rate in the pGenesil-2-control group (8.54%). The apoptotic ratios in the pGenesil-2-HiWi1 and pGenesil-2-HiWi2263 groups were 26.16 ± 1.21 and $28.06 \pm 1.78\%$, respectively, were higher as compared to those in the pGenesil-2-control group $2.86 \pm 0.09\%$ ($P < 0.01$). Our results suggest that HiWi gene silencing decreases proliferation and promotes apoptosis of lung cancer TSCs. Therefore, the HiWi gene could be used as a molecular target for the inhibition of the growth of lung cancer TSCs, which has potential value for the treatment of lung cancer.

Introduction

Advances have been made with regards to the diagnosis and treatment (surgery and concurrent chemoradiotherapy) of lung

cancer. However, treatment results have not been effective. In the last 5 years, the survival rate for patients with lung cancer has been lower than 15% (1). Therefore, finding a new and effective lung cancer treatment strategy is crucial. Following 20 years of research, tumor gene therapy has become a feasible alternative for the treatment of lung cancer, besides surgery and concurrent chemoradiotherapy.

Tumor proliferation mainly depends on the tumor stem cells (TSCs) within the tumor. The self-renewal, infinite proliferation and potential for differentiation of the TSCs is of vital importance for the occurrence, development and metastasis of tumors. Recent studies have demonstrated that the HiWi gene is involved in the self-renewal of TSCs. The HiWi gene is an important factor that affects cell differentiation and proliferation. Its overexpression can lead to the excessive proliferation of stem cells, and cause a tumor. However, the expression and effect of the HiWi gene in lung cancer TSCs remains uncertain. Therefore, the aim of this study was to investigate the effect of HiWi gene silencing on lung cancer tumor stem cell proliferation and apoptosis as well as whether the HiWi gene serves as a molecular target for the inhibition of lung TSCs.

Materials and methods

Lung cancer TSCs. The lung adenocarcinoma cell strain, SPC-A1 cell line was purchased from the Cell Bank of Shanghai Institute of Life Sciences, China. The cell line was cultivated in serum-free medium until spheres formed. Flow cytometry was applied to these spheroid cells using an Aldefluor kit (Bath, UK) to separate the SSCloALDEbr cells and obtain the TSCs, as described in a previous study (2).

Short hairpin RNA (shRNA) eukaryotic expression vector pGenesil-2 plasmid. The shRNA eukaryotic expression vector pGenesil-2 plasmid was purchased from Jingsai Shengggong Biological Co., Ltd., China.

Construction of shRNA eukaryotic expression vector targeting the HiWi gene. As described in a previous study (3), shRNA sequences were selected using software from Ambion Co. (Grand Island, NY, USA). The two shRNA sequences and a negative control shRNA sequence were designed according to the

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Key words: lung cancer tumor stem cells, HiWi gene, RNA interference, gene silencing

HiWi gene mRNA sequence in GenBank. The sequences were then transferred into the pGenesil-2 vector. shRNA eukaryotic expression vectors, pGenesil-2-HiWi, pGenesil-2-HiWi2263 and pGenesil-2-control, targeting the HiWi gene were constructed. These vectors were identified using enzyme restriction cuts and sequencing analysis. In brief, the culture liquid was obtained, plasmid DNA as a small volume of culture was removed using a pipette small volume kit (McMurray, PA, USA) according to the manufacturer's instructions, and the extracted plasmid was cut using an *SalI* enzyme. The plasmid DNA was eluted in deionized (1 μ l, 10X buffer H 1 μ l, *SalI* 1 μ l) and incubated in 37°C water for 3 h. Reactant (5 μ l) was pipetted to perform 1% agarose gel electrophoresis. The fully-constructed recombinant plasmid was sent to Shanghai Yingjun Biotechnology Co., Ltd. for the sequencing test.

Plasmid transfection. The plasmid was immediately transfected using PEI (Sigma Co., St. Louis, MO, USA). SSCloALDEbr cells (1×10^4 and 1×10^5 per well) were inoculated onto 96- and 24-well plates, respectively, one day prior to transfection. When the cell density reached 70-80%, the cells were transfected. A total of 0.2 μ g plasmid, 10 μ l 1X PBS and 0.8 μ g PEI were added to the 96-pore plate, while a total of 2 μ g plasmid, 100 μ l 1X PBS and 8 μ g PEI were added to the 24-pore plate. The cells were mixed and left to rest for 15 min. The supernatant was discarded and 10 and 100 μ l PEI/DNA and 90 and 900 μ l complete medium were added to each pore of the 96- and 24-pore plates, respectively. The plates were cultivated until analysis.

Experimental group. The experimental groups comprised pGenesil-2-HiWi1, pGenesil-2-HiWi2263, pGenesil-2-control and PBS (no cells, only nutrient solution), which was used as the blank for zeroing. There were four wells in each group at each time, and 1×10^4 cells was added to each well.

Proliferation of lung cancer TSCs using the MTT assay. The nutrient solution was removed 24, 48 and 72 h following transfection. A total of 10 μ l MTT (5 mg/ml) was added to the pores, and placed into a CO₂ culture box for 4 h. The nutrient solution was removed, and 100 μ l DMSO was added into each pore. The solution was agitated to inhibit crystallization. In order to test the light absorbance, the absorbance of each pore was reduced in enzyme immunoassay instrument at a wavelength of 490 nm. The cell proliferation inhibition rate was calculated using the formula: Cell proliferation inhibition ratio (%) = (absorbance in the control wells - absorbance in the test wells) / (absorbance in the control wells - absorbance in the blank wells) \times 100. Statistical analyses were conducted simultaneously.

Detection of lung cancer TSCs and apoptosis with Annexin V staining. Between 48 and 72 h after transfection in each group, the cells were digested into mono-cell suspension by pancreatin (0.125%), 4°C, 1000 rpm \times 5 min, PBS (0.01 M, pH 7.2). Cell suspension occurred at 4°C, 1000 rpm \times 5 min. The supernatant was discarded and 500 μ l suspension liquid was added to the centrifugal tube bottom for resuspension. The solution was removed and 5 μ l Annexin V-FITC (ShangHai Biotechnology Co., Ltd., China) labeled solution was added. The solution was fully blended, and flow cytometry was initiated 5 min later.

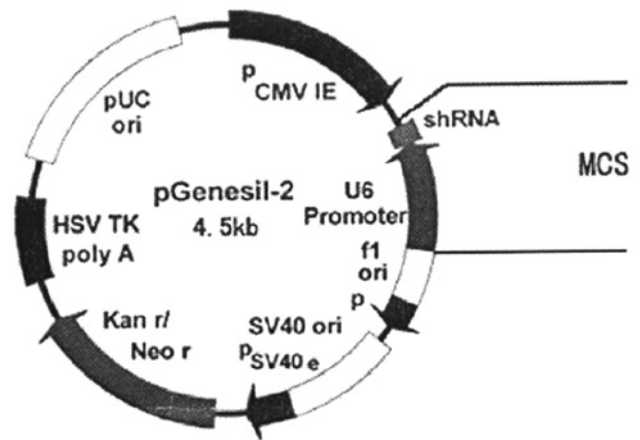


Figure 1. Map of recombinant plasmid. shRNA, short hairpin RNA.

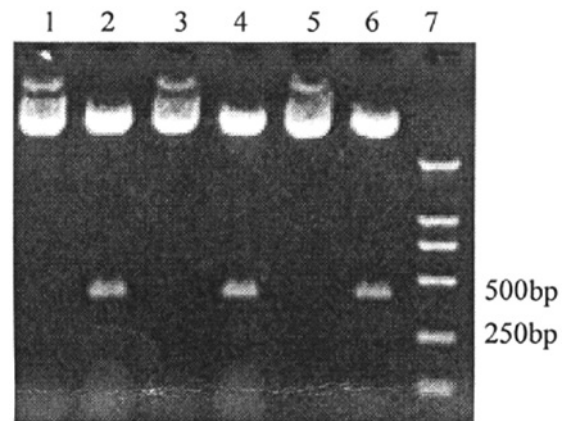


Figure 2. Confirmation of the successful construction of the recombinant plasmid by *SalI* restrictive enzyme digestion analysis. Lane 1, pGenesil-2-HiWi1; lane 2, pGenesil-2-HiWi1 + *SalI*; lane 3, pGenesil-2-HiWi2263; lane 4, pGenesil-2-HiWi2263 + *SalI*; lane 5, pGenesil-2 negative; lane 6, pGenesil-2 negative + *SalI*; lane 7, DL2000 marker.

Statistical analysis. The statistical analysis was performed using statistical software SPSS 13.0. $P < 0.05$ was considered to indicate a statistically significant difference. The Chi square test and Fisher's exact test were used to compare the counted data between the groups.

Results

Identification of enzyme-cut recombinant plasmid. Within the structure of the recombinant plasmid, the short hairpin RNA (shRNA) sequence is the designed RNA interference target sequence, and the multiple cloning site (MCS) is the clone starting point (Fig. 1). The results of electrophoresis showed that the enzyme cut the recombinant plasmid at the position point of *SalI* in the gene segment. The restriction enzyme *SalI* was used to cut 400 bp-long segments within the recombinant plasmid. Segments (400 bp) were cut within the pGenesil-2HiWi1, pGenesil-2HiWi2263 and blank plasmid (Fig. 2). Therefore, the designed shRNA interference segments were inserted successfully into the pGenesil-2 plasmid vector. The results from the sequencing analysis are shown in Fig. 3. There

Table I. Recombinant plasmid cell proliferation inhibitory rates following 24, 48 and 72 h of transfection.

Group	Cell proliferation inhibitory rate (%)		
	24 h	48 h	72 h
A (pGenesil-2-HiWi1)	73.16 ^a	56.96 ^a	44.26 ^a
B (pGenesil-2-HiWi2263)	81.62 ^a	62.42 ^a	47.19 ^a
C (pGenesil-2-control)	8.54	12.38	10.37
A vs. C	$\chi^2=34563.451$, P<0.01	$\chi^2=17548.646$, P<0.01	$\chi^2=11569.834$, P<0.01
B vs. C	$\chi^2=43143.228$, P<0.01	$\chi^2=21390.388$, P<0.01	$\chi^2=13228.323$, P<0.01

^aThe difference between the data in this group and in the pGenesil-2-control group was statistically significant (P<0.01).

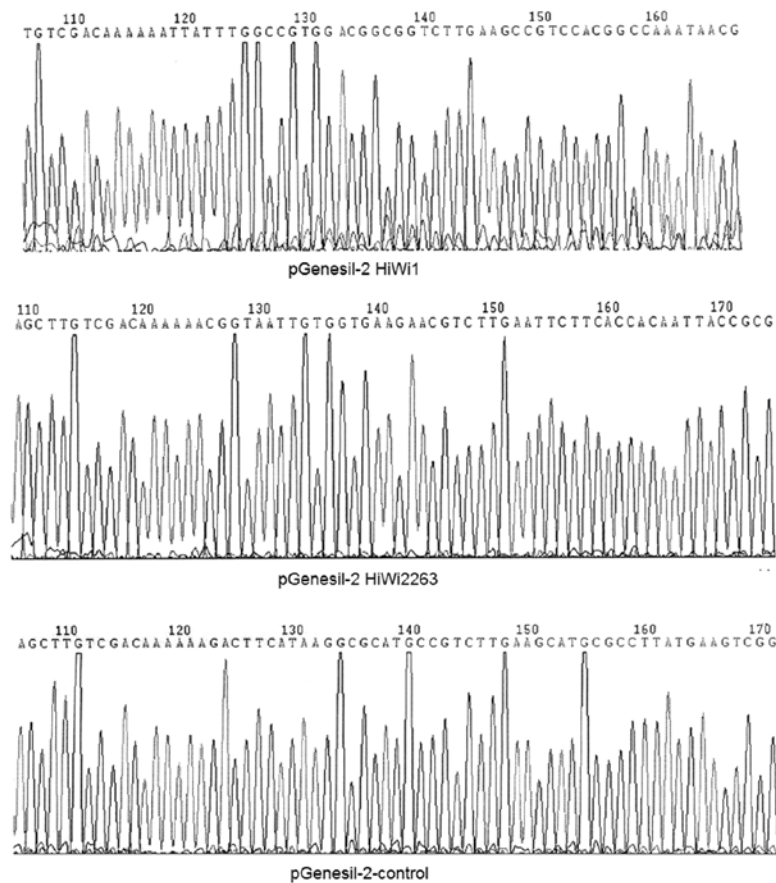


Figure 3. Confirmation of the exactness of the bases, which were inserted into the plasmid by sequencing. Three oligonucleotides had no mutation.

was a C to G base mutation in the HiWi inserted sequences. According to the principle of RNA interference, the bases in the siRNA sequence play vital roles and the mutated base is not located in the siRNA sequence; therefore this recombinant plasmid could still be used. This enabled other sequences to be inserted correctly.

Proliferation of lung cancer TSCs using the MTT assay. Our results showed that 24 h after transfection, the lung cancer TSC inhibition rate for the pGenesil-2-HiWi2263, pGenesil-2-HiWi1 and pGenesil-2-control groups were 81.62,

73.16 and 8.54%, respectively. After 48 h, the rates of the first two groups, 62.42 and 56.96%, respectively, decreased as compared to the increase in percentage of the pGenesil-2-control groups, 12.38%. Similarly, after 72 h, the rates of the first two groups decreased to 47.19 and 44.26%, respectively, as compared to the control groups, 10.37%. This finding demonstrates that the lung cancer TSC proliferation inhibition rate was increased (P<0.01), with the inhibition rate following 24 h being the highest. This observation revealed that RNA-related interference of the HiWi gene is effective in inhibiting the growth of lung cancer TSCs (Table I).

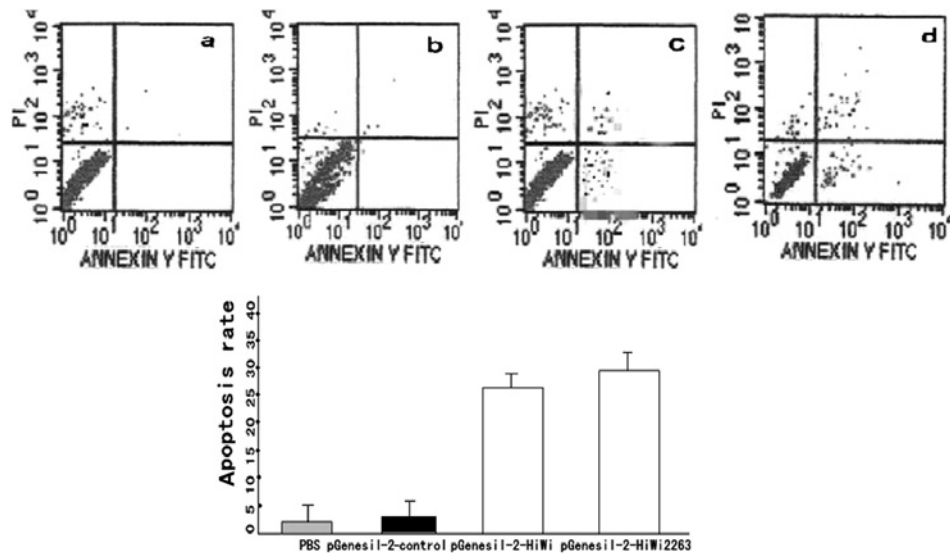


Figure 4. The apoptotic rates of (A) PBS, (B) pGenesil-2-control, (C) pGenesil-2-HiWi1 and (D) pGenesil-2-HiWi2263 groups.

Annexin V staining on lung cancer TSCs induced apoptosis. Results from the Annexin V staining on lung cancer TSC apoptosis demonstrate that: the apoptotic rates of pGenesil-2-HiWi1 and pGenesil-2-HiWi2263 were 26.16 ± 1.21 and $28.06 \pm 1.78\%$, respectively. No statistically significant difference was observed ($P > 0.05\%$). The apoptotic rates of the pGenesil-2-control were found to be $(2.25 \pm 0.058)\%$ and $(2.86 \pm 0.09)\%$. Again, there was no statistical significant difference ($P > 0.05$). However, the rates in the pGenesil-2-HiWi1 and pGenesil-2-HiWi2263 groups were higher compared to those in the PBS group and the empty plasmid group ($P < 0.01$) (Fig. 4).

Discussion

RNA interference technology is a common method used to control gene expression. Post-transcriptional gene silencing occurs following the induction of double-stranded RNA, which causes the breaking and degrading of messenger RNA, which is mediated by double-strand RNA. During tumor gene therapy, RNA is capable of specifically inhibiting the overexpression of cancer, cancer-related and mutator genes, confining them to a state of dormancy or silencing them. Therefore, RNA may be used as a new tumor treatment strategy.

In lung cancer gene therapy with RNAi, the choice of targeted gene silencing is crucial. Lung cancer is caused by a series of complex signaling pathways, involving a number of cell factors. Thus, selection of a cell target is critical when cancer occurs as the treatment target is crucial. In 1997, Lin and Spradling identified the PiWi gene (4) in the reproductive stem cells of fruit flies. This gene is involved in the process of stem cell division and is correlated with stem cell self-renewal, gamete formation, RNA interference and translation regulation. Overexpression of PiWi has been shown to lead to cell differentiation disorders and ultimately cancer generation (5). Lee *et al* (6) identified PiWi12 expression in tumors including, renal cell carcinoma, prostate, endometrial and breast cancer, and gastroenteric and ovarian tumor. Gao (7) demonstrated that the PiWi12 gene was important for TSC development in

the generation of tumor cells. The PiWi gene is homologous to the human HiWi gene, which is located in the long arm of chromosome 12. It was first cloned from the cDNA library of testes. Sharma *et al* (8) identified that HiWi was highly expressed in human prostate, brain, liver and other tissues, but was highly expressed in fetal kidney and adult testes. The HiWi gene also had a regulatory role in the division of hematopoietic stem cells. Grochola *et al* (9) found that an extremely high or extremely low expression of HiWi gene was correlated with male survival in patients with pancreatic cancer. In their study, Liu *et al* (10) found that expression of the HiWi gene in different stomach cancer cell lines correlated with the degree of malignancy. The study revealed that upon inhibition of the expression of the HiWi gene, the growth of stomach cancer cells was inhibited by RNA interference. Therefore, these authors considered that the HiWi gene could be used as a marker of tumor proliferation and as an evaluation index of cancer prognosis (11). Huang *et al* (12) revealed that tumor growth and proliferation in the body depended on cancer stem cells. Moreover, the ability of self-renewal, differentiation and proliferation of TSCs was correlated with the HiWi gene. Overexpression of the HiWi gene is the significant cause of malignancy and tumor. Therefore, it is essential to study the involvement of the HiWi gene in TSCs.

The purpose of RNA interference technology is to create siRNA segments. These are short strands of double-stranded RNA, which are able to degrade homologous complementary sequences of mRNA, known as the RNA interference pathway. Some of the most common methods of siRNA preparation include chemical synthesis, *in vitro* transcription, degradation of long sequences of RNA and PCR expression and production of siRNA in the cells. siRNA may be generated by encoding its carrier, which is capable of self-renewal as well as the inhibition of the expression of target genes in the cells over a long period of time. Thus, this method is convenient and cost-effective. In this study, the shRNA eukaryotic expression vector of targeting Hiwi gene was constructed using this method. Complying with the design requirements of shRNA

carriers, this carrier employed U6 to initiate shRNA transcription and pol III to terminate transcription. The *salI* site is located within the PGenesil-2 carrier and so the same enzyme site is designed in the shRNA fragments. If the fragment is inserted correctly, then it can be cut into 400-bp sections by the *salI* enzymes. From the results from the *salI* restriction enzyme digestion analysis, it can be observed that the plasmid enzyme-cut sections are consistent with the expected sections, and there are no sequence base mutations.

Results from the MTT assay and flow cytometry revealed that the growth of the lung cancer TSCs was inhibited, and the proliferation rate was increased following HiWi gene silencing. Immediately following transfection, the cell growth density was expected to reach 70-90%, however, 48 and 72 h following transfection the 96-pore plate was saturated with cells. Therefore, the difference between the long-time inhibition effect of the RNA interference group and the control group could not be identified. Nevertheless, it was observed that following gene silencing the apoptotic rates in the lung cancer TSCs were higher than those in the control group. Thus, results of this study have demonstrated that the HiWi gene is involved in the differentiation and proliferation of lung cancer TSCs. These results are also significant for the potential to inhibit lung cancer TSC growth and target apoptosis.

This study suggests that following silencing of the HiWi gene, the pathway regulating lung cancer TSC growth and inhibition may have been activated. Therefore, we hypothesize that abnormal function of the HiWi gene may be the key to the growth, inhibition and apoptosis of lung cancer TSCs. The HiWi gene may be crucial in the proliferation and differentiation of lung cancer TSCs. Overexpression of the HiWi gene may be the cause of viscous proliferation of lung cancer TSCs and ultimately lung cancer. We consider that the HiWi gene has the potential to become a molecular target for the inhibition of lung cancer TSCs. Silencing the HiWi gene may have potential for the treatment of lung cancer, thereby setting the basis for treatment surgeries.

Acknowledgements

This study was supported by the Heilongjiang Youth Science Foundation (Fund Code: QC2009C95).

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