HIWI Is Associated With Prognosis in Patients With Hepatocellular Carcinoma After Curative Resection

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BACKGROUND: PIWI protein family was found to play an important role in stem cell self-renewal. Overexpression of HIWI, the human homolog of PIWI family proteins, was found in several solid tumors, although the role of HIWI in hepatocellular carcinoma (HCC) and its prognostic value remain unclear. METHODS: HIWI expression was measured in stepwise metastatic HCC cell lines (HCCLM3, MHCC97H, MHCC97L, SMMC7721, and HepG2), the normal liver cell line (LO2), and HCC tissue samples (n = 20). Proliferation and invasion were investigated in HCC cell lines undergoing HIWI target small interfering RNA transfection. Also explored was HIWI expression in HCC tissue microarrays (n = 168) for survival analysis. RESULTS: Levels of HIWI protein and mRNA were up-regulated in highly metastatic HCC cell lines (HCCLM3, MHCC97H, and MHCC97L), whereas their proliferation and invasion significantly decreased after depletion of HIWI. Intratumoral HIWI expression was higher than that of peritumoral tissue (P < .001) and positively associated with proliferating cell nuclear antigen expression (P < .001). Positive expression of intratumoral HIWI was associated with larger tumor size (P = .047) and intrahepatic metastasis (P = .027) and was an independent risk factor for overall survival (P = .007) and recurrence-free survival (P = .036), particularly in patients with low serum α-fetoprotein and low Edmondson-Steiner grade. CONCLUSIONS: HIWI may play a key role in HCC proliferation and metastasis and can be a potential prognostic factor for HCC after curative resection, particularly with well-differentiated HCC.


KEYWORDS: HIWI, hepatocellular carcinoma, prognosis, proliferation, metastasis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent human cancers and the third most common cause of death from cancer worldwide. The 5-year recurrence rate after curative resection is 54.1% to 61.5%, with a poor overall survival (OS), although surgical resection and liver transplantation have provided valid approaches to treat HCC. The high recurrence rate and behindhand diagnosis are considered as the major causes of poor outcomes in patients with hepatectomy. Thus, exploring effective biomarkers to predict outcome and adjuvant agents to prevent postoperative recurrence and prolong the patient’s life span is important.

As component proteins of the RNA interference (RNAi) machinery, Argonaute proteins are present in all RNA-induced silencing complexes. In humans, 8 Argonaute-like proteins are divided into 2 subfamilies: AGO and PIWI. HIWI (also named PIWIL1), a human homolog of the PIWI subfamily, has been described in various species and expresses mainly in human germ cells. It is highly conserved during evolution and plays a crucial role in stem cell self-renewal, division, gametogenesis, germ cell proliferation, and RNAi in diverse organisms. Expression patterns and biologic capacities of HIWI are considered the key elements in regulation of cancer cells and a promising biomarker to detect tumors, determine prognosis, or function as a therapy target.
HIWI overexpression has been found to be related with cancer. Qiao et al. found that aberrant HIWI expression might contribute to occurrence and development of seminoma. HIWI was present in the early phase of hematopoietic cell proliferation, probably causing the arrest of cell differentiation, and was associated with the occurrence of leukemia. In addition, HIWI could be an effective prognostic factor in soft-tissue sarcoma, esophageal squamous cell carcinoma, and pancreas adenocarcinoma. However, little is known about the role and prognostic value of HIWI in HCC.

In the current study, HIWI expression was measured in stepwise metastatic HCC cell lines and HCC tissue samples with long-distance peritumoral tissue. Cell proliferation and invasion were investigated in high-metastatic HCC cell lines undergoing HIWI target small interfering RNA (siRNA) transfection. We also explored HIWI expression in HCC tissue microarrays (TMAs) to evaluate its association with clinicopathologic factors and prognosis of HCC patients. In addition, we investigated the relation between HIWI expression and proliferating cell nuclear antigen (PCNA). As a result, we found that HIWI played an important role in HCC proliferation and metastasis and was associated with prognosis of HCC patients.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Five HCC cell lines (HepG2, SMMC7721, MHCC97L, MHCC97H, and HCCLM3) and 1 normal liver cell line (L02) were used in cell experiments. HepG2 and SMMC7721 are HCC cell lines with low metastatic potential. MHCC97L, MHCC97H, and HCCLM3 are HCC cell lines with high metastatic potential. HepG2 and L02 were originally obtained from American Type Culture Collection (ATCC, Manassas, VA). HCCLM3, MHCC97H, and MHCC97L were established by the Liver Cancer Institute of Zhongshan Hospital (Shanghai, China) and SMMC7721 was established by the Second Military Medicine College (Shanghai, China). These cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Invitrogen).

Patients, Specimens, and Follow-up

From March 2001 to March 2006, 168 patients who underwent curative resection for pathologically confirmed HCC were randomly enrolled. No patient received preoperative anticancer treatment. Tumor stage was determined according to the 2002 International Union Against Cancer TNM classification system. Tumor differentiation was graded by the Edmondson-Steiner grading system. Hepatitis B history was defined as history with a positive serum hepatitis B surface antigen. A tumor with complete encapsulation was defined as a tumor surrounded continuously by a fibrous capsule or a thinner pseudocapsule. Intrahepatic metastasis was defined as presence of tumor thrombi in the portal vein or satellite nodules surrounding a larger main tumor. Microvascular invasion was defined as presence of tumor cells within a vascular lumen lined by endothelium under microscopy. All patients were recruited into the current study after informed consent. This study was approved by the Research Ethics Committee of Zhongshan Hospital.

All patients were followed up until March 2008 with a median observation time of 31.6 months. As follow-up procedures, abdominal ultrasonography, serum α-fetoprotein (AFP) levels, and chest radiographs were monitored with an interval of 2 months to 6 months after the operation day. Computed tomography scanning or magnetic resonance imaging was performed when recurrence was suspected. OS was defined as the interval between surgery and death. Recurrence-free survival (RFS) was defined as the interval between surgery and recurrence. If recurrence was not diagnosed, patients were censored on the date of death or the last follow-up.

Twenty paired intratumoral and long-distance peritumoral tissue samples (at a distance > 30 mm from the tumor edge) of consecutive HCC patients undergoing curative resection were also collected. Intratumoral tissues and peritumoral tissues at 2 distances (10 mm and 30 mm) from the tumor margin were used in quantitative real-time polymerase chain reaction (qRT-PCR). All samples were collected in the operating room immediately after surgery and then were frozen in liquid nitrogen. No patient received preoperative anticancer treatment.

RNA Isolation and qRT-PCR

Total RNA was extracted from the HCC cell lines and HCC tissue samples using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. qRT-PCR was performed using a SYBR PrimeScript qRT-PCR Kit (Takara, Dalian, China) according to the manufacturer's instructions. β-actin was used as an internal control. The primers were as follows: HIWI forward primer 5'-GAAG CAGCCCTGACTTGGTCAAG-3' and reverse primer 5'-GAATCAAGGCTCAAAACCATGCTTC-3', and β-actin forward primer 5'-CCCATGAGATTCAAGA
TCATTGC-3' and reverse primer 5'-GGCCGGACTC
ATCGTACTCCTGC-3'. Relative mRNA levels were
calculated based on Ct values, corrected for β-actin
expression, according to the equation: $K = 2^{-\Delta Ct} [\Delta Ct =
Ct (HIWI) – Ct (β-actin)]$, where K is a constant. All
experiments were performed in triplicate.

**Western Blot Analysis**
Cultured HCC cells were collected and lysed with Pro-
teoJET Mammalian Cell Lysis Reagent (Fermentas,
Burlington, Canada). After centrifugation at 12,000 rpm at
4°C for 15 minutes, the supernatant was subjected to
sodium dodecyl sulfate-polyacrylamide gel electropho-
resis analysis. The resolved proteins were transferred onto
nitrocellulose membranes (Bio-Rad, Hercules, CA).
Membranes were blocked with 10% nonfat dry milk for
1 hour at room temperature and then incubated with
HIWI antibody (Abcam, Cambridge, MA; diluted 1:1000) or β-actin antibody (Abcam; diluted 1:5000) at
4°C overnight. For detection, membranes were incu-
bated with a horseradish peroxidase-conjugated second-
ary antibody (Sigma, St. Louis, MO) and the bands
were detected using the electrochemoluminescence
(ECL) detection system (Amersham, Uppsala, Sweden)
following the manufacturer’s instructions.

**siRNA-Mediated Silencing of HIWI**
The target siRNA sequences used in our study were:
5'-GCCGUUCAUAAGACUAATT-3'; antisense: 5'-UUAGUCUUGAUAAGCCGCTT-3' (HIWI-
1510), sense: 5'-GUGGGCCUUAAUCAGAUUTT-3'; antisense: 5'-AUACUGAUAAAGGGCCACTT-3' (HI-
WI-970), and sense: 5'-UUCUCGGAACGUGCAGC
UTT-3'; antisense: 5'-ACGUGACAGGUUCCGGAGA
ATT-3' (control). RNA duplexes were synthesized by
BioTNT (Shanghai, China), HCCLM3, MHCC97H, and
MHCC97L cells were used in siRNA analysis, and trans-
fection of siRNA was performed using Lipofectamine 2000
(Invitrogen) according to the manufacturer’s instructions.

**Cell Proliferation and Invasion**
Cells were seeded onto a 96-well plate (3000 per 100 μl/
well), incubated for 24 hours, and then treated with
HIWI siRNA for 6 hours. After replacing the medium
with 100 μl DMEM containing 10% FBS, 10 μl of CCK-
8 solution (Dojindo, Kyushu, Japan) was added at various
time points (24 hours, 48 hours, 72 hours, 96 hours, 120
hours, and 144 hours), and plates were incubated for a
further 1 hour. The absorbance at 450 nm was measured
to determine the number of viable cells in each well.

Cell invasion analysis was performed using a Trans-
well (Corning, NY). A total of 5000 cells per 200 μl/well
of HIWI siRNA-transfected cells in serum-free DMEM
were added into the well of an 8-μm pore membrane
chamber coated with Matrigel (BD Biosciences, Bedford,
MA). The bottom chamber contained 600 μl of 10% FBS
in DMEM as a chemoattractant. After 48 hours, migrated
cells on the bottom surface were fixed with 4% paraformal-
dehyde and counted after staining with Giemsa (Baso,
Zhuhai, China). Any cells that had not penetrated the
filters were removed by scrubbing with cotton swabs. Cells
that migrated to the bottom surface of the filter were con-
sidered to have invaded through the matrix and were
counted under a microscope. All experiments were per-
fomed in triplicate.

**Immunohistochemistry (IHC)**
In total, 336 pairs of cores of representative formalin-fixed,
paraffin-embedded tumor tissue and liver tissue adjacent to
the tumor from recruited patients mentioned above were
constructed into TMA. Two duplicate cylinders from 2 dif-
ferent areas, a total of 4 punches from each patient, were
obtained. The IHC protocols were described in our previous
studies.23 Primary antibodies were rabbit anti-human anti-
obodies combined with HIWI (Abcam; diluted 1:100) and
PCNA (Abcam; diluted 1:500). Components of the EnVi-
vision-plus detection system (EnVision+/HRP/Mo; Dako,
Carpinteria, CA) were used. Reaction products were visu-
alyzed by incubation with 3, 3'-diaminobenzidine. Negative
controls were treated identically but with the primary anti-
obodies omitted. The intensity of positive staining was mea-
sured using a computerized image system composed of a
Leica charge-coupled device camera DFC420 connected to
a Leica DM IRE2 microscope (Leica, Cambridge, UK).
Under high-power magnification (×200), photographs of 4
representative fields were captured by the Leica QWin Plus
v3 software; identical settings were used for all photographs.

IHC staining was assessed by 3 experienced investi-
gators, who performed the evaluation independently.
Conflicting scores were resolved at a discussion micro-
scope. All of these investigators were isolated from the
related clinical information. For HIWI staining, we deter-
mined the percentage of cells with a positive score for
staining in the cytoplasm, membrane, or nucleus in the
whole biopsy cylinder. Specifically, the scoring procedure
was as follows: the staining intensity was scored (0, nega-
tive; 1, weak; 2, moderate; 3, high), and the percentage of
positive cells was scored (0, 0% positive cells; 1, 1%-10% positive cells; 2, 11%-50% positive cells; 3, >50% positive cells). The final score of each sample was obtained by multiplying the scores for the staining intensity and percentage of cells. Samples were classified as negative when the final scores were 0 to 3 and positive when 4 to 9. For PCNA, a positive result was recorded when >50% of the cells exhibited strong staining.24

**Statistical Analysis**

Values are expressed as the mean ± standard error of the mean. Unpaired t test or Pearson correlation test was used.
to compare quantitative variables. Pearson chi-square test or Fisher exact test was applied to compare qualitative variables. The patients’ survival curve was plotted using the Kaplan-Meier method, and the log-rank test was used to determine the significant difference among groups. The Cox regression model was used to perform multivariate analysis. Analysis was performed using SPSS 16.0 for Windows (SPSS Inc, Chicago, IL). \( P < .05 \) was considered statistically significant.

**RESULTS**

**HIWI Expression in HCC Cell Lines and HCC Tissues**

HIWI expression in HepG2, SMMC7721, MHCC97L, MHCC97H, and HCCLM3 cells was significantly higher than in L02 cells (\( P < .05 \)). Furthermore, HIWI expression increased in parallel with the metastatic potential of HCC cell lines: the HIWI mRNA and protein levels in MHCC97L, MHCC97H, and HCCLM3 cells were significantly higher than those in SMMC7721 and HepG2 cells (\( P < .05 \)), whereas no significant difference was found among high metastatic cells as well as among low metastatic potential cells (Figs. 1A and 1B).

In HCC tissue samples, HIWI expression was significantly higher in intratumoral tissues than in peritumoral tissues regardless of the distance from the tumor margin (\( P < .001 \)), but no significant difference was found between the peritumoral tissues at 10 mm and 30 mm (\( P = .094 \); Fig. 1C).

**Inhibition of HIWI Reduced Proliferation and Invasion of HCC Cell Lines**

To investigate the functions of HIWI in HCC cell lines, we depleted HIWI in HCCLM3, MHCC97H, and MHCC97L cells by siRNA transfection, which was confirmed by qRT-PCR and Western blotting. HIWI-970 and HIWI-1510 demonstrated significant suppression effects compared with control siRNA sequences (\( P < .001 \)) and HIWI expression was significantly lower after HIWI-1510 transfection than after HIWI-970
transfection ($P < .001$; Fig. 2A). In all cell lines, down-regulation of HIWI caused significant suppression of cell proliferation at 72 hours after transfection ($P < .001$; Fig. 2B). Decreased HIWI expression was also accompanied by a decrease in invasion of HCC cells as measured in the Matrigel Transwell assay ($P < .001$; Fig. 2C).

**IHC Findings**

HIWI stained the cytoplasm, membrane, and nucleus of HCC cells and exhibited various staining patterns with respect to staining intensity and percentage of positive cells (Figs. 3A, 3B, and 3C). All the stromal cells were negative staining. Most cells showed diffuse or focal staining patterns with intermediate or strong staining intensity. We observed 26.19% (44 of 168) of HCC patients with a positive score for HIWI. Most of the peritumoral tissues showed negative or low HIWI expression; only 2.98% (5 of 168) of cases showed positive HIWI expression. A significant difference in HIWI expression was found between intratumoral tissues and peritumoral tissues ($P < .001$; Figs. 3D, 3E, and 3F). Patients with positive expression of intratumoral HIWI had a high incidence of large tumor size ($P = .047$), more intrahepatic metastasis ($P = 0.027$), and more lymph node metastases ($P < .001$).

**Table 1. Relation Between Intratumor HIWI and PCNA Expression and Clinicopathologic Features**

<table>
<thead>
<tr>
<th>Clinicopathologic Features</th>
<th>HIWI Expression</th>
<th>PCNA Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative No.</td>
<td>Positive No.</td>
</tr>
<tr>
<td>Age, y$^{a,b}$</td>
<td>50.94</td>
<td>48.05</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>115</td>
<td>40</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>HBV History</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>85</td>
<td>31</td>
</tr>
<tr>
<td>No</td>
<td>39</td>
<td>13</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>97</td>
<td>32</td>
</tr>
<tr>
<td>–</td>
<td>27</td>
<td>12</td>
</tr>
<tr>
<td>Tumor size, cm$^a$</td>
<td>8.41</td>
<td>11.26</td>
</tr>
<tr>
<td>Intrahepatic metastasis$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>52</td>
<td>27</td>
</tr>
<tr>
<td>–</td>
<td>72</td>
<td>17</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>–</td>
<td>124</td>
<td>40</td>
</tr>
<tr>
<td>Microvascular invasion$^d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>52</td>
<td>23</td>
</tr>
<tr>
<td>–</td>
<td>72</td>
<td>21</td>
</tr>
<tr>
<td>$\alpha$-fetoprotein$^{a,b}$$</td>
<td>4465</td>
<td>8989</td>
</tr>
<tr>
<td>Tumor encapsulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>51</td>
<td>15</td>
</tr>
<tr>
<td>None</td>
<td>73</td>
<td>29</td>
</tr>
<tr>
<td>Edmondson-Steiner grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>97</td>
<td>35</td>
</tr>
<tr>
<td>III-IV</td>
<td>27</td>
<td>9</td>
</tr>
<tr>
<td>TNM stage$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>33</td>
<td>8</td>
</tr>
<tr>
<td>II</td>
<td>43</td>
<td>11</td>
</tr>
<tr>
<td>IIIA</td>
<td>43</td>
<td>22</td>
</tr>
</tbody>
</table>

$^{a}$Student $t$ test for quantitative variables.

$^{b}$Several patients’ data ($n < 6$) were not obtained.

$^{c}$Intrahepatic metastasis was defined as the presence of tumor thrombi in the portal vein or the presence of satellite nodules surrounding a larger main tumor.

$^{d}$Microvascular invasion was defined as the presence of tumor within a vascular lumen lined by endothelium under microscopy.

HBV, hepatitis B virus.

* indicates a significant difference in HIWI expression between intratumoral and peritumoral tissues ($P < .001$; Figs. 3D, 3E, and 3F). Patients with positive expression of intratumoral HIWI had a high incidence of large tumor size ($P = .047$), more intrahepatic metastasis ($P = 0.027$), and more lymph node metastases ($P < .001$; Figs. 3D, 3E, and 3F).
metastasis ($P = .004$). PCNA staining occurred mainly in the nucleus in tumor or peritumoral liver tissue. In intratumoral tissue, the positive rate of PCNA expression was 97.73% (43 of 44) in tissue with positive HIWI expression, whereas it was 66.13% (82 of 124) in tissue with negative HIWI expression. PCNA expression was positively correlated with HIWI ($P < .001$; Figs. 3G, 3H, and 3I; Table 1). In addition, serum AFP in well-differentiated tissue was significantly lower than that in poorly differentiated tissue ($612.99 \pm 172.29$ ng/ml vs $8793.08 \pm 1839.37$ ng/ml; $P < .001$).

**Relation Between HIWI Expression and Patients’ Survival**

The 1-, 3-, and 5-year OS rates of patients with positive HIWI expression were 61%, 34%, and 34%, respectively,
which were much lower than those of patients with negative HIWI expression (77%, 54%, and 49%, respectively; \(P = .021\)). Furthermore, the 1-, 3-, and 5-year RFS rates of patients with positive HIWI expression (54%, 40%, and 40%, respectively) were significantly lower than those of patients with negative HIWI expression (69%, 59%, and 58%, respectively; \(P = .037\); Figs. 4A and 4B). Larger tumor size, advanced microvascular invasion, and more intrahepatic metastasis were also risk factors for both OS and RFS in univariate analysis. Older age and hepatitis B virus history were only associated with OS, whereas advanced TNM stage and higher serum AFP were only associated with RFS. Factors showing significance by univariate analysis were adopted in multivariate Cox proportional hazards analyses. Positive intratumoral HIWI expression, larger tumor size, and advanced microvascular invasion were independent risk factors for both OS and RFS, in addition to older age being an independent risk factor only for OS. Peritumoral HIWI expression was associated with neither OS nor RFS (Table 2).

In addition, stratified analyses according to serum AFP levels and Edmondson-Steiner grade were also conducted to confirm the prognostic value of HIWI. HIWI was a risk factor associated with OS and RFS in the AFP \(\leq 300\) ng/ml subgroup (\(P = .002\) and \(P < .001\), respectively; Figs. 4C and 4D), Edmondson-Steiner I-II subgroup (\(P = .038\) and \(P = .022\), respectively; Figs. 4E and 4F), and also the combination of the 2 factors (\(P = .012\) and \(P = .007\), respectively; Figs. 4G and 4H). However, in the AFP \(> 300\) ng/ml (\(P = .463\) and \(P = .987\), respectively) and Edmondson-Steiner III-IV subgroups (\(P = .331\) and \(P = .912\), respectively), HIWI was not associated with prognosis of HCC patients.

**DISCUSSION**

Recent studies have suggested that cancer stem cells play a crucial role in hepatocarcinogenesis.\(^{25}\) As a key element in the basic mechanism governing stem cell division, HIWI has also been confirmed to participate in carcinogenesis, tumor angiogenesis, and proliferation\(^{12,15,26}\) in several tumors. However, in HCC, only Jiang et al.\(^{27}\) found that positive HIWI expression in HCC tissue was correlated with HCC local and/or remote lymph node metastasis and remote metastasis. In our study, we also found that all cases with lymph node metastasis occurred in patients with positive HIWI expression, although sample size bias could not be completely excluded. Moreover, we found that both mRNA and protein levels of HIWI expression were up-regulated in 3 high metastatic potential HCC cell lines that were isolated from the same parent cell MHCC97.\(^{28}\) Interestingly, HIWI expression was not different among these HCC cell lines with different metastatic potential. It is possible that as a conservative protein, HIWI may just present different expression in tumor cells from different individuals with different metastatic capabilities. Its underlying mechanism remains unclear and needs to be further investigated. Nevertheless, the role of HIWI expression in invasive capability of HCC cell lines has been verified in our study. Cell invasion could be significantly inhibited by depletion of HIWI. In addition, our data showed that intratumoral

### Table 2. Univariate and Multivariate Analyses of Factors Associated With Survival and Recurrence

<table>
<thead>
<tr>
<th>Factor</th>
<th>OS Univariate</th>
<th>RFS Univariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(P)</td>
<td>(P)</td>
</tr>
<tr>
<td></td>
<td>Hazard Ratio</td>
<td>Hazard Ratio</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age, y: &gt;51 vs (\leq 51) years</td>
<td>.017</td>
<td>.100</td>
</tr>
<tr>
<td>Sex: female vs male</td>
<td>.685</td>
<td>NA</td>
</tr>
<tr>
<td>Hepatitis B virus history: yes vs no</td>
<td>.039</td>
<td>.176</td>
</tr>
<tr>
<td>Liver cirrhosis: yes vs no</td>
<td>.206</td>
<td>.168</td>
</tr>
<tr>
<td>Tumor size: &gt;5 cm vs (\leq 5) cm</td>
<td>&lt; .001</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Intrahepatic metastasis: yes vs no</td>
<td>.005</td>
<td>.002</td>
</tr>
<tr>
<td>Microvascular invasion: yes vs no</td>
<td>.007</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>(\alpha)-fetoprotein: &gt;300 ng/ml vs (\leq 300) ng/ml</td>
<td>.222</td>
<td>.004</td>
</tr>
<tr>
<td>Tumor encapsulation: complete vs none</td>
<td>.684</td>
<td>.646</td>
</tr>
<tr>
<td>Edmondson-Steiner grade: III-IV vs I-II</td>
<td>.819</td>
<td>.060</td>
</tr>
<tr>
<td>TNM stage: IIIA vs II vs I</td>
<td>.051</td>
<td>NA</td>
</tr>
<tr>
<td>Intratumoral HIWI: negative vs positive</td>
<td>.021</td>
<td>.007</td>
</tr>
<tr>
<td>Peritumoral HIWI: negative vs positive</td>
<td>.988</td>
<td>.825</td>
</tr>
</tbody>
</table>

CI, confidence interval; NA, not adopted; NS, not significant; OS, overall survival; RFS, recurrence-free survival.
HIWI expression was higher compared with that of peritumoral tissue and demonstrated that the tissue at a longer distance from the tumor margin may express lower HIWI despite the lack of a significant difference between the peritumoral tissues at different distances. Liu et al.\textsuperscript{12} have confirmed that accumulation of HIWI was consistent with precancerous development of gastric cancer. Thus, we speculate that HIWI plays a pivotal role in the processes of liver cell malignant transformation. Furthermore, association of HIWI expression with metastatic potential of HCC cell lines and their invasive capability indicated that HIWI expression was related to the malignant phenotype of HCC cell as well.

A previous study has shown that HIWI may be correlated with gastric cancer cell proliferation.\textsuperscript{12} High proliferative activity of tumor cells is known to be associated with tumor progression.\textsuperscript{24} In our study, expression of PCNA, a nuclear protein that is synthesized in the G1-S phase of the cell cycle, was shown to be positively correlated with HIWI in HCC tissue. We also observed that depletion of HIWI could induce significant inhibition of the proliferation status of HCC cell lines. Furthermore, expression of HIWI in HCC tissue was positively correlated with tumor size, which was different from Jiang’s study. This discrepancy may be due to the difference in sample size between the 2 studies. Nevertheless, our result indicates that HIWI may participate in HCC cell proliferation. Further prospective studies with a larger sample size are needed to confirm this finding.

The prognostic value of HIWI has been validated in several other malignancies. We also tried to investigate the prognostic value of HIWI in 168 HCC patients undergoing curative resection. HIWI was confirmed to be an independent risk factor for OS and RFS. Moreover, we stratified patients by serum AFP level and Edmondson-Steiner differentiation grade. AFP is the most widely used tumor marker in the diagnosis of HCC and monitoring postoperative recurrence and metastasis in the patients with positive serum AFP.\textsuperscript{29} However, no ideal prognostic biomarker exists for the 30% to 40% of HCC patients with low or negative serum AFP.\textsuperscript{30,31} The outcomes of patients with low serum AFP and well-differentiated HCC are difficult to predict using conventional clinical indexes.\textsuperscript{30,32} Interestingly, HIWI demonstrated a better association with OS and RFS under these conditions regardless of low serum AFP and well-differentiated status existing respectively or simultaneously but not conversely. A previous study indicating that HIWI was a negative developmental regulator underlies the unique biologic properties associated with hematopoietic stem and progenitor cells.\textsuperscript{15} HIWI appearing in the immature cell to regulate its development is normal; however, HIWI will vanish along with cell differentiation. Thus, HIWI in well-differentiated cells is abnormal.\textsuperscript{15} In addition, the consistency of serum AFP and HCC cell differentiation grade was found in our study. Therefore, we propose that low serum AFP may be associated with better tumor differentiation status and speculate that mal-expression of HIWI also plays an important role in well-differentiated HCC. The cell subpopulation with overexpression of HIWI will obtain more naive cell behavior, implying a more malignant phenotype (self-renewal and aggressive capability). Therefore, HIWI may be an effective prognostic factor complemented with serum AFP and cell differentiation grade.

In summary, our data have demonstrated that HIWI may be a useful prognostic factor for HCC patients after curative resection, particularly in conditions of low serum AFP and low Edmondson-Steiner grade. In addition, HIWI probably plays a crucial role in carcinogenesis, proliferation, and metastasis of HCC. However, the precise internal mechanism responsible for HIWI in these aspects of biologic functions needs further evaluation.

FUNDING SUPPORT
This study was jointly supported by National Natural Science Funds of China (30973499); National S&T Major Project (2008ZX10002-021); and Dawn Program of Shanghai Education Commission (07SG04).

CONFLICT OF INTEREST DISCLOSURES
The authors made no disclosures.

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