

The universal overexpression of a cancer testis antigen *hiwi* is associated with cancer angiogenesis

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Abstract. The aim of this study was to determine whether the universal overexpression of *hiwi* is associated with cancer angiogenesis. The difference of mRNA expression in blood vessels derived from DLBCL and RLNH tissues was detected, and *hiwi* was overexpressed in tumor vessels of lymphoma. The *hiwi* expression in cell lines and tissues was measured by using RT-PCR and immuno-staining, as well as the Ang-2 and Tie-2 expressions in tissues. *Hiwi* was significantly expressed in cancer cell lines Hela, MCF-7, MDA-MB-231, MDA-MB-435s, A2780, 111 and 113. Compared with that of chronic cervicitis (CC), hyperplasia of mammary glands (HMG), ovarian benign lesions (OBL) and endometrium benign lesions (EBL), the expression of *hiwi*, Ang-2 and Tie-2 was increased significantly in uterine cervical cancer (UCC), breast carcinoma (BC), ovarian cancer (OC) and endometrial cancer (EC) ($P < 0.01$). Additionally, the *hiwi* expression was positively correlated with Ang-2 in UCC, BC and OC, and Tie-2 in OC. These results suggest that *hiwi* plays an important role in the oncogenesis and is a potential target for cancer angiogenesis.

Introduction

The angiogenesis plays a critical role in the progression and metastasis of cancer (1). Angiogenesis begins when a pinpoint colony of tumor cells expands to a size where the simple diffusion of nutrients is insufficient, and then new capillaries are elicited (2). Evidence was shown that tumor vessels are different from the normal ones at the molecular

level (3-5). Therefore, the understanding of the molecular pathogenesis of tumor-specific endothelial aberrations in tumor vessels could stimulate the development of new therapeutic approaches that potently and specifically target angiogenesis.

However, the isolation of tumor vessels from the mixed tissues presents technical challenges. In the present study, we sought to develop a novel technique for analyzing the global gene expression in blood vessels obtained from primary tissues. The blood vessels of diffuse large B-cell lymphoma (DLBCL) and reactive lymph node hyperplasia (RLNH) were isolated by laser capture microdissection (LCM) from tissues fixed *in situ*, and then subjected to gene expression microarray analysis (GEMA). The *hiwi* gene was found to be specifically expressed in blood vessels of lymphoma.

The *hiwi* gene, the human homolog of the *piwi* family, is located in 12q24.33 and encodes a 98.5 kDa protein (6). The *hiwi* protein contains a conserved architecture with a PAZ motif and Piwi motif (7,8), and the *hiwi* gene was originally isolated from a human testis cDNA library (6), hinting of a potential role in human spermatogenesis (9). Because of the conserved functions of *piwi* genes in stem cell maintenance and germline development, it is of interest to study the role of *hiwi* in germ cell-derived tumors (6). Although *hiwi* gene was discovered years ago, so far, little research has been done on its biological properties and mechanisms except for the spermatogenesis field. The expression of *hiwi* has been also described in hematopoietic stem cells (HSC), suggesting that *hiwi* may be a developmental regulator in the maintenance of HSCs (9). Therefore, these data support the notion that *hiwi* may be a candidate gene that plays a potential role not only in the maintenance and proliferation of germ cells and stem cells, but also in the angiogenesis of malignancy development.

To date, few studies had been performed on the role of *hiwi* in cancer. We first employed the novel strategies to analyze the expression profile of the cancer blood vessels for differential genes, and identified that the *hiwi* transcript was specifically expressed in lymphoma-derived micro-vessels. Next, we explored that *hiwi* was universally expressed in many types of cancers, and the high expression of *hiwi* was closely related to the upregulation of angiogenesis relevant factors, such as Ang-2 and Tie-2.

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Materials and methods

Patients, tissues and cell lines. The fresh tissues, pathologically diagnosed as DLBCL (n=2) and RLNH (n=2), were collected from patients undergoing surgery at the Department of Homology, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology.

The slides of paraffin-embedded tissues were obtained from the Department of Pathology at Tongji Hospital after pathological diagnosis, including hyperplasia of mammary glands (HMG) and breast carcinoma (BC) chronic cervicitis (CC) and uterine cervical cancer (UCC), endometrium benign lesion (EBL) and endometrial cancer (EC), ovarian benign lesion (OBL) and ovarian cancer (OC). The clinical and pathology data, such as age, diagnosis, disease stage and grade, were also collected at the same time.

Human cervical cancer cell lines Hela and Siha, human breast cancer cell lines MCF-7, MDA-MB-231 and MDA-MB-435s, human ovarian cancer cell line A2780 and human endometrial cancer cell lines 111 and 113 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and the European Collection of Cell Cultures (ECACC, Salisbury, UK), respectively. These cells were routinely cultured at 37°C in a humidified atmosphere of 5% CO₂ in RPMI-1640 or DMEM medium with 10% fetal calf serum (Gibco, USA).

LCM, total RNA isolation, RNA linear amplification and gene expression chip detection. After fixing in Zn fixation solution, the slides were subjected to immuno-staining with Dako rapid Envision™-HRP immuno-staining system (Dako Corp., Carpinteria, CA). Then the dissection of vessels were carried out through laser activation with an SL μ cut microscopic UV laser system [Molecular Machines & Industries AG (MMI) (10)].

Total RNA of the vessels was isolated by using RNAeasy® Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Because of the small amount of starting total RNA, the non-bias linear amplification was performed with MessageAmp-aRNA kit (Ambion, Austin, TX) in order to obtain sufficient amounts of labeled antisense RNA (aRNA) for microarray analysis (11,12). The quality of total RNA and aRNA was monitored with the RNA 6000 Nano LabChip Kit (Agilent Technologies, Boeblingen, Germany), and the fragmentation of cRNA was also detected quantitatively and qualitatively before hybridization. The fragmented cRNA was hybridized onto the human genome U133 plus 2.0 arrays (Affymetrix, Santa Clara, CA). The arrays with hybridized probes were scanned with the Affymetrix® GeneChip Scanner 3000 and the images were analyzed with GCOS software (Affymetrix).

Total RNA isolation and the expression of *hiwi* mRNA. Human cancer cell lines Hela, Siha, MCF-7, MDA-MB-231, MDA-MB-435s, A2780, 111 and 113 cultured in 6-well plates were harvested when they reached 80% confluence, respectively.

Total RNA was isolated from the different cell lines by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and the RT mixture was

incubated at 75°C for 5 min followed by quick-chill on ice, then at 42°C for 60 min. A fixed amount of RT products (one tenth of RT products) and 0.5 μ mol/l upper-lower primers were co-amplified at a final concentration of 1X PCR buffer, 50 μ mol/l dNTPs, 1 U Taq DNA polymerase (Ferment, USA) in a total volume of 50 μ l. The amplification profile was: denaturation at 94°C for 30 sec, primer annealing at 58°C for 40 sec, and extension at 72°C for 30 sec. *Hiwi* upper primer: 5'-CTGTGTACAGATTGCCCTACCC-3' and *hiwi* lower primer: 5'-GCAGACTTTGAGCCCATCTACCAG-3'. The housekeeping gene β -actin was used as an internal quantity control. The predicted sizes for *hiwi* and β -actin PCR products were 312 and 416 bp, respectively.

The expression of *hiwi*, *Ang-2* and *Tie-2* proteins. Cancer cells of Hela, Siha, MCF-7, MDA-MB-231, MDA-MB-435s, A2780, 111 and 113 on the glass coverslips were fixed in a 1:1 mixture of 100% ethanol and cold acetone at 4°C for 15 min and then air-dried. After incubation in 1% Triton X-100 for 15 min, the slides were incubated with 1% bovine serum albumin (BSA) at room temperature for 30 min to reduce non-specific binding. Then, the slides were incubated with a 1:500 goat polyclonal anti-*hiwi* antibody (Santa Cruz Biotechnology, CA, USA) at 37°C for 1 h. After the slides were treated with a fluorescein isothiocyanate-conjugated rabbit anti-goat IgG, they were mounted in PI solution (Sigma, USA). Finally, the morphology of cancer cells was observed with a confocal microscope system OLFV-32U2/XE-3 (Olympus, Japan).

The tissue sections of CC, UCC, HMG, BC, OBL, OC, EBL and EC were deparaffinized and hydrated in serial dilutions of alcohol, and the sections were microwaved in 40 mM Borate buffer (pH 8.2) supplemented with 1 mM EDTA and 1 mM NaCl for 20 min for antigen retrieval (13,14). Slides were treated with 3% H₂O₂ for 10 min to quench endogenous peroxidase activity, then, immersed in 1% BSA at room temperature for 30 min. The tissues were incubated with a goat polyclonal anti-*hiwi* antibody (1:600 dilution), a rat anti-*Ang-2* polyclonal antibody and a rat anti-*Tie-2* polyclonal antibody (Santa Cruz Biotechnology, 1:150 dilutions) overnight at 4°C, respectively. After rinsing, the slides were further incubated with a secondary antibody for an additional 30 min. The chromagen detection was developed with 3'-diaminobenzidine (Jinqiao, Beijing, China) for 5 min, and the slides were counterstained with Meyer's hematoxylin, dehydrated, and mounted with Canada balsam for examination.

Evaluation of results of immunohistochemical staining. The Sinicrope *et al* scoring method was applied to evaluate both the staining intensity and the proportion of the stained cells (15). The staining intensity was sub-classified as follows: 1, weak; 2, moderate; or 3, strong. The percentage of the total number of positive cells was quantified, and then assigned into one of the following five categories: 0, <5%; 1, 5-25%; 2, 26-50%; 3, 51-75%; and 4, >75%. Next, the positive percentage and the staining intensity were multiplied to generate the immunoreactivity score (IS) for each specimen. Each lesion was separately examined and scored by two pathologists. The cases with discrepant scores were discussed to obtain a consensus.

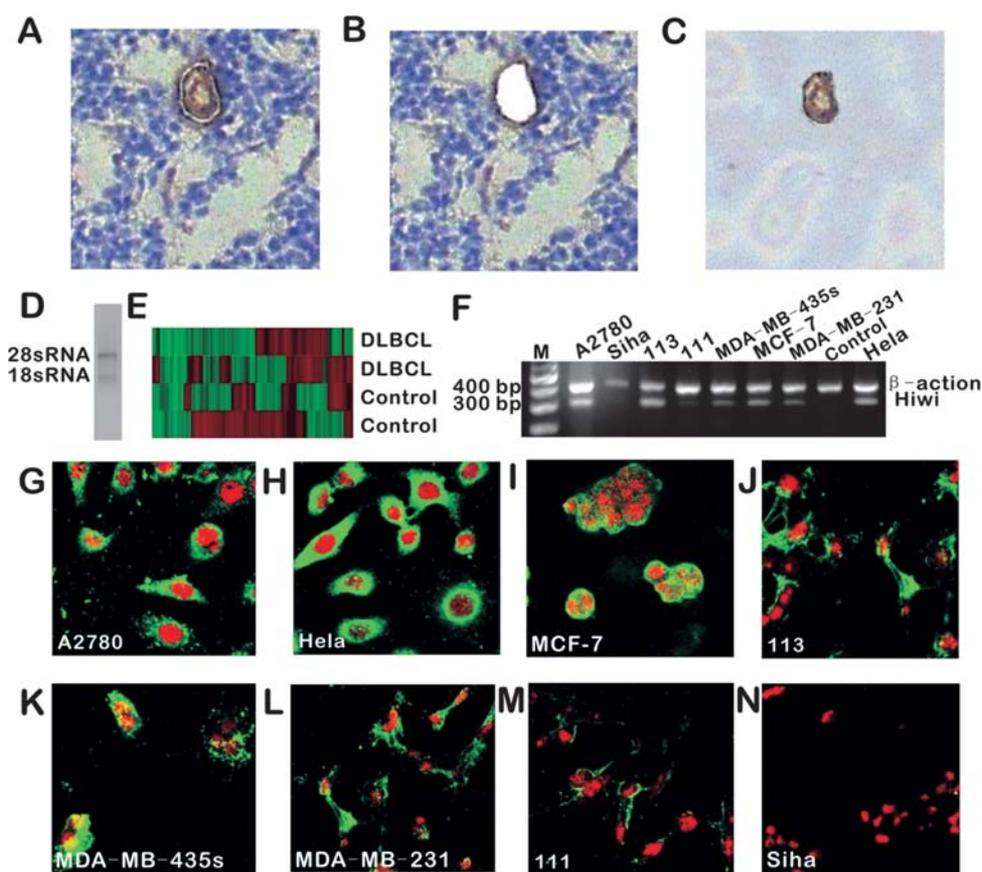


Figure 1. (A-C) The labeled vessels in DLBCL and RLNH tissues were captured efficiently by using LCM. (D) The ratios of 28S to 18S rRNA bands of vessels were 2:1. (E) GEMA. (F) The *hiwi* mRNA expression in cancer cell lines by using RT-PCR. (G-N) The *hiwi* protein expression in cancer cell lines by using immuno-fluorescence assay.

Statistical analysis. SPSS 13.0 statistical software was used to perform statistical analysis. The Fisher's exact test, Pearson's χ^2 test, ANOVA SNK test and Nemenyi-Wilcoxon-Wilcox test were used to assess the differences. Spearman's rank correlation was used to determine whether there was a positive or negative correlation. The level of $P < 0.05$ was considered to be statistically significant.

Results

Hiwi is overexpressed in the lymphoma-derived microvessels.

The labeled vessels were readily identified under microscopy and harvested by using LCM technique. As seen in Fig. 1A-C, the microvessels in DLBCL and RLNH tissues were labeled with brown staining to improve the capture efficiency of LCM. The ratios of A_{260} to A_{280} values of the RNA prepared in vessels are shown in the range of 1.8-2.0. The integrity of total RNA was determined and based on the ratio of 28S to 18S rRNA bands, which were 2:1 (Fig. 1D). Therefore, the intact mRNA in vessels was successfully isolated from the fresh samples. Hybridization results revealed that 80 genes were upregulated and 72 genes were downregulated in DLBCL. Of these candidates, *hiwi* continually demonstrated to be positive in DLBCL tissues, but negative in RLNH tissues (Fig. 1E). Therefore, the *hiwi* gene was chosen for further analysis.

The hiwi gene is expressed in many types of cancer cell lines.

The special mRNA expression of *hiwi* was positively detected in 7 cancer cell lines except for the cervical cell line Siha. The levels of *hiwi* mRNA in A2780, HeLa, MCF-7 and 113 cell lines were high, while the expressions in 111, MDA-MB-231 and MDA-MB-435s cell lines were weaker (Fig. 1F). The PCR products were confirmed by sequencing. Similarly, as shown in Fig. 1G-L, it was clear that *hiwi* protein was also detectable in the cytoplasm of 7 cell lines, especially in A2780, HeLa and MCF-7 cell lines.

The expression levels of hiwi, Ang-2 and Tie-2 proteins are detected in the different tissues. The expressions of *hiwi* and angiogenesis relevant factors Ang-2 and Tie-2 in various carcinomas and non-carcinomas tissues were analyzed by using immunohistochemistry.

In case of *hiwi*, although the expression was also found in cytoplasm of the matched non-carcinoma, the levels were rather low. However, the aberrant expression of *hiwi* was frequently observed in carcinoma. Compared with those of CC, HNC, OBL and EBL, the levels of *hiwi* were strikingly higher in UCC, BC, OC and EC with statistical significance, respectively (Figs. 2 and 3) ($P < 0.01$). The *hiwi* levels in the early stage cancer were obviously increased in comparison to those in the matched benign lesions ($P < 0.05$). While there was no difference in the *hiwi* levels between the early-stage

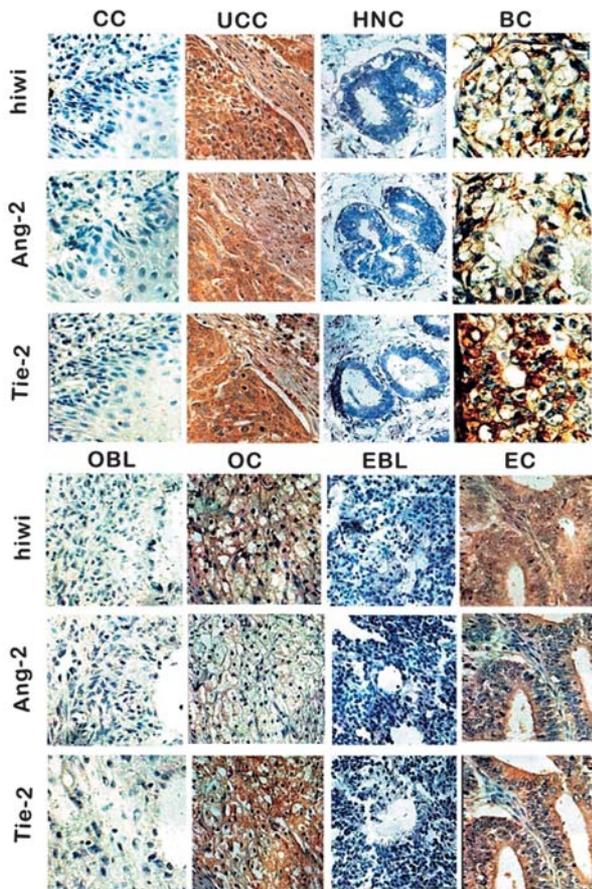


Figure 2. The *hiwi* protein expression in different tissues by using immunohistochemistry. CC, chronic cervicitis; UCC, uterine cervical cancer; HMG, mammary glands; BC, breast carcinoma; OBL, ovarian benign lesion; OC, ovarian cancer; EBL, endometrial benign lesion; EC, endometrial cancer.

and late-stage cancers except for UCC ($P \geq 0.05$) (Table I). We further analyzed the frequency of high levels with the IS higher than 3 in neoplastic versus non-neoplastic tissues. The high cytoplasmic expression of *hiwi* was rare in the matched non-carcinoma lesions, but the rates were much higher in UCC, BC, OC and EC ($P < 0.01$) (Table II).

Similar to *hiwi*, both Ang-2 and Tie-2 exhibited cytoplasmic expression pattern. The expressions of Ang-2 and Tie-2 were generally low in overall matched benign lesions, however, the Ang-2 and Tie-2 levels were higher in various carcinomas ($P < 0.01$) (Figs. 2 and 3). On the other hand, compared with those of the matched benign non-cancer lesions, Ang-2 and Tie-2 levels were obviously upregulated in the early stage cancers ($P < 0.05$), but there was no difference between the early-stage and the late-stage cancers ($P \geq 0.05$). The rates of Ang-2 and Tie-2 with the IS higher than 3 were very low in the matched benign lesions, and much higher in several carcinoma tissues except for OC (Table II).

The *hiwi* expression is correlated with angiogenesis-relevant factors Ang-2 and Tie-2. The expression of *hiwi* was positively correlated with the Ang-2 expression in UCC ($\rho = 0.7696$, $P = 0.0013$), BC ($\rho = 0.9087$, $P = 0.0018$) and OC ($\rho = 0.7225$, $P = 0.0429$). Moreover, a positive correlation with cancer angiogenesis assessed by Tie-2 was observed for *hiwi* in OC ($\rho = 0.7554$, $P = 0.0302$) (Table III).

Discussion

So far, only a very limited number of studies support a role of a cancer testis antigen *hiwi* in cancer (9,16). Furthermore, the correlation between *hiwi* and angiogenesis in cancers was

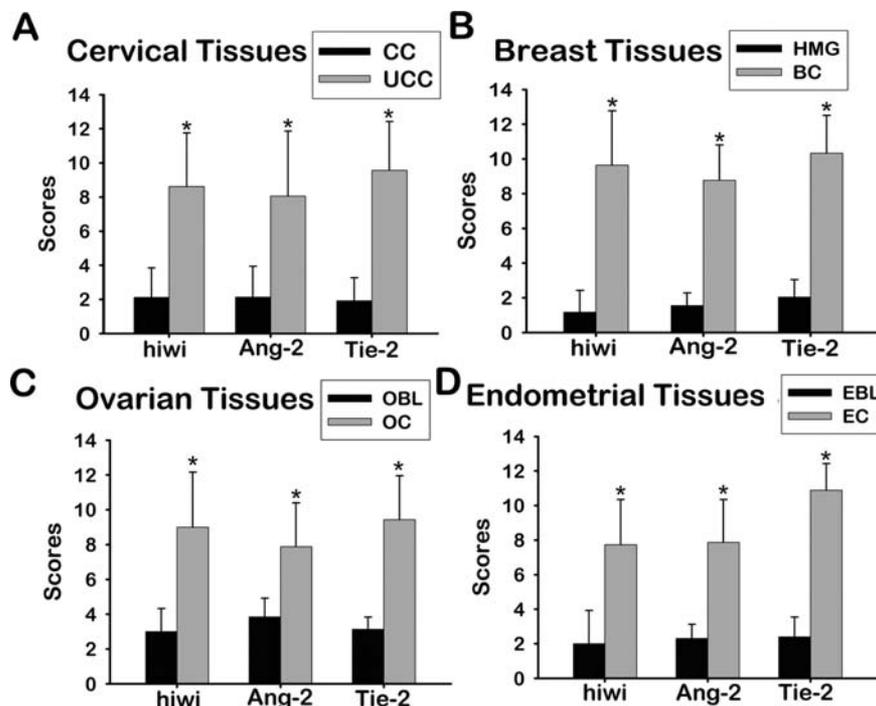


Figure 3. The means of immunoreactivity scores (IS) of *hiwi*, Ang-2 and Tie-2 in different tissues. (A) Cervical tissues, (B) breast tissues, (C) ovarian tissues and (D) endometrial tissues.



The expressive difference of *hiwi*, Ang-2 and Tie-2 in the different non-cancer and cancer tissues.

Subjects	Factor	N vs. E			E vs. L		Chi-value	P-value
		Chi-value	P-value		Chi-value	P-value		
Cervix	hiwi	9.1096	0.0105		8.9802	0.0112	46.0768	<0.0001 ^a
	Ang-2			L=E>N			F=16.47	<0.0001 ^b
	Tie-2	10.3961	0.0055		0.9536	0.6208	22.6434	<0.0001 ^a
Breast	hiwi	8.7398	0.0127		0.4395	0.8027	19.0100	<0.0001 ^a
	Ang-2			L=E>N			F=49.75	<0.0001 ^b
	Tie-2	5.4881	0.0443		0.0702	0.9655	13.3845	0.0012 ^a
Ovary	hiwi			L=E>N			F=10.65	0.0007 ^b
	Ang-2			L=E>N			F=4.44	0.0305 ^b
	Tie-2			L=E>N			F=15.82	0.0003 ^b
Endometrium	hiwi			L=E>N			F=13.01	0.0004 ^b
	Ang-2			L=E>N			F=12.29	0.0020 ^b
	Tie-2	6.7517	0.0342		0.0088	0.9956	9.2130	0.0100 ^a

N, non-cancer; E, early-stage cancer; L, late-stage cancer. ^aAVONO SNK test; ^bNemenyi-Wilcoxon-Wilcox test ($\alpha=0.05$).

Table II. The expression levers of *hiwi*, Ang-2 and Tie-2 in the different tissues.

Subjects (n)	Factor	Non-cancer		Cancer		Chi-value	P-value
		Low (%)	High (%)	Low (%)	High (%)		
Cervix (Nnc=25) (Nc=40)	hiwi	18 (82.0)	7 (18.0)	3 (7.5)	37 (92.5)	29.2658	<0.0001 ^a
	Ang-2	21 (84.0)	4 (16.0)	8 (20.0)	32 (80.0)	25.5019	<0.0001 ^a
	Tie-2	19 (76.0)	6 (24.0)	4 (10.0)	36 (90.0)	29.3106	<0.0001 ^a
Breast (Nnc=13) (Nc=14)	hiwi	11 (84.6)	2 (15.4)	0 (0.0)	14 (100.0)	19.9904	<0.0001 ^a
	Ang-2	8 (61.5)	5 (38.5)	1 (7.2)	13 (92.8)		0.0038 ^b
	Tie-2	10 (76.9)	3 (23.1)	3 (21.4)	11 (78.6)		0.0039 ^b
Ovary (Nnc=7) (Nc=12)	hiwi	5 (71.4)	2 (28.6)	1 (8.3)	11 (91.7)		0.0093 ^b
	Ang-2	5 (71.4)	2 (28.6)	3 (25.0)	9 (75.0)		0.0739 ^b
	Tie-2	4 (57.1)	3 (42.9)	2 (16.7)	10 (83.3)		0.1287 ^b
Endometrium (Nnc=8) (Nc=10)	hiwi	5 (62.5)	3 (37.5)	1 (10.0)	9 (90.0)		0.0302 ^b
	Ang-2	5 (62.5)	3 (37.5)	1 (10.0)	9 (90.0)		0.0302 ^b
	Tie-2	6 (75.0)	2 (25.0)	1 (10.0)	9 (90.0)		0.0088 ^b

Nnc, number of non-cancer; Nc, number of cancer. ^aPearson's χ^2 tests; ^bFisher's exact test.

almost unknown. Although the exact function and biological effects of *hiwi* remain unknown, the evidence that the over-expression of *hiwi* might be a key element in stem cell division and caused seminomatous malignancy development suggested that *hiwi* may be involved in carcinogenesis (6,9,16). To our knowledge, this was the first observation that *hiwi* was universally upregulated in many types of cancers and associated with tumor vessels and angiogenic factors Ang-2 and Tie-2.

The most comprehensive of our hybridization outcome raised the hypothesis that *hiwi* enriched in tumor vessels might be associated with carcinogenesis and tumor angiogenesis. To validate the microarray data, the *hiwi* expression was generally measured in several cancer cell lines and tissues, including cervical, breast, ovarian and endometrial cancer.

In brief, the mRNA and protein of *hiwi* were found in 7 cancer cell lines, especially in A2780, HeLa and MCF-7. Moreover, the *hiwi* protein expression was obviously increased in UCC, BC, OC and EC tissues ($P<0.05$) and the rates of high expression were also significantly improved in cancers ($P<0.01$). Additionally, the *hiwi* levels in the early-stage cancers were dramatically increased in comparison to those in the matched benign lesions ($P<0.05$), while there was no difference in the *hiwi* levels between the early-stage and late-stage cancers except for UCC ($P\geq 0.05$). The present study has clearly demonstrated that the universal overexpression of *hiwi* was closely correlated with a malignant state. The expression of *hiwi* was not correlated with the clinical stages except for UCC.

Table III. The correlation of *hiwi*, Ang-2 and Tie-2 in the different cancer tissues.

Subjects	Factor	<i>hiwi</i>	Ang-2	Tie-2
UCC	<i>hiwi</i>	1.0000		
		0.0000		
	Ang-2	0.7696	1.0000	
		0.0013	0.0000	
	Tie-2	0.3239	0.40268	1.0000
		0.2586	0.1534	0.0000
BC	<i>hiwi</i>	1.0000		
		0.0000		
	Ang-2	0.9087	1.0000	
		0.0018	0.0000	
	Tie-2	0.6620	0.6325	1.0000
		0.0737	0.0924	0.0000
OC	<i>hiwi</i>	1.0000		
		0.0000		
	Ang-2	0.7225	1.0000	
		0.0429	0.0000	
	Tie-2	0.7554	0.5564	1.0000
		0.0302	0.1521	0.0000
EC	<i>hiwi</i>	1.0000		
		0.0000		
	Ang-2	0.3078	1.0000	
		0.6144	0.0000	
	Tie-2	-0.7404	-0.8660	1.0000
		0.1524	0.0577	0.0000

Correlation significant at the 0.05 level (2-tailed). Spearman correlation coefficients.

The literature regarding the biological properties of *hiwi* in cancer angiogenesis is rare. Ang-2, believed to antagonize the stabilizing action of Ang-1, binds with the endothelial cell tyrosine kinase receptor Tie-2 (17). It has been revealed that the low Ang-1/Ang-2 gene expression ratio and the high Tie-2 gene expression are significantly associated with the high MVD (18). With the presence of *VEGF* in cancer tissues, Ang-2 seems to be the strengthening factor for the initiation of angiogenesis and for vascular sprouting (19,20). The current findings indicated that *hiwi* was overexpressed in the tumor vessels. They also revealed that *hiwi* was significantly, positively correlated with not only Ang-2 levels in UCC, BC and OC, but also Tie-2 levels in OC ($P < 0.05$). Therefore, *hiwi* may be a potential target involved in tumor angiogenesis. The implications of these findings need to be clarified, and should be examined in a future study with a larger number of patients and more homogeneous characteristics.

In summary, these results suggest that *hiwi* may play an important role in oncogenesis and is a potential angiogenesis target for cancer biotherapy. Further investigation is necessary to lead to development of a new molecular target therapy for epithelial cancers based on the present results.

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