**RESEARCH ARTICLE**

**Overexpression of Hiwi Promotes Growth of Human Breast Cancer Cells**

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Abstract

The Piwi subfamily comprises two argonaute (Ago) family proteins, which are defined by the presence of PAZ and Piwi domains, with well known roles in RNA silencing. Hiwi, a human Piwi subfamily member, has been shown to play essential roles in stem cell self-renewal and gametogenesis. Recently, accumulating reports have indicated that abnormal hiwi expression is associated with poorer prognosis of multiple types of human cancers, including examples in the breast. However, little is known about details of the oncogenic role of hiwi in breast cancers. In present study, we confirmed overexpression of hiwi in breast cancer specimens and breast cancer cell lines at both mRNA and protein levels. Thus both RT-qPCR and Western blot data revealed significantly higher hiwi in intratumor than peritumor specimens, overexpression being associated with tumor size, lymph node metastasis and histological grade. Hiwi overexpression was also identified in breast cancer cell lines, MDA-MB-231 and MCF-7, and gain-of-function and loss-of-function strategies were adopted to identify the role of hiwi in the MCF-7 cell growth. Results demonstrated that hiwi expression in MCF-7 cells was significantly up- or down-regulated by the two strategies. We next evaluated the influence of hiwi overexpression or knockdown on the growth of breast cancer cells. Both cell count and colony formation assays confirmed promoting roles of hiwi in MCF-7 cells, which could be inhibited by hiwi specific blockage by siRNAs. In summary, the present study confirmed overexpression of hiwi in breast cancer specimens and breast cancer cell lines, and provided evidence of promotion by hiwi cell growth. The results imply an oncogenic role of hiwi in breast cancers.

**Keywords:** Hiwi - growth - human breast cancer cells

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Introduction

Breast cancer leads the cause of death among solid tumours in women, with an increasing incidence, especially among younger woman (van Diest et al., 2004). Presently, the disease will affect 12% of all women and about 30-40% of patients will die from metastatic disease despite radical surgery (Brown et al., 2011). And several risk factors have been recognized, such as early menarche, late menopause, nulliparity, and positive family history (Purinton et al., 2014). However, there are at present no realistic options for primary prevention in patients, except hereditary breast cancer related genes, such as BRCA1 and BRCA2 (Marcus et al., 1996), PTEN (Cowden’s disease) (Eng et al., 1994), and p53 (Li Fraumeni syndrome) (Magnusson et al., 2012). Recently, multiple molecules have been recognized as pro-oncogenic factors for breast cancers by the universal screen methods basing genomics, transcriptomics, proteomics (Ocana et al., 2008), or other methods. CYP3A4 expression has been recognized as a risk factor of breast cancer in Mexican women (Floriano-Sanchez et al., 2014). ESR1 and PGR have been reported to be associated with ductal and lobular breast cancer (Medina-Jaime et al., 2014) and Expression of EMSY, a Novel BRCA2-link Protein, has been confirmed to be associated with lymph node metastasis of breast cancer (Madjd et al., 2014). Most of these factors were utterly incompetent as diagnostic or therapeutic targets, because they are basing on traditional models of tumorigenesis which suggest that malignancies develop in a series of stepwise genetic mutations culminating in the generation of cancer cells able to disseminate and metastasize (Klein et al., 2009). However, recent evidence strongly suggests that metastasis may be an early event in tumorigenesis (Husemann et al., 2008, Weng et al., 2012). The cancer stem cell (CSC) hypothesis offers new insights into tumorigenesis and metastatic progression that may lead to more effective therapies to breast cancers.

There were two models for the development of CSCs, the cancer stem cell model and the clonal evolution model. In former model, random mutations that are accumulated by normal stem cells or their early progeny (Reya et
hand, a lentivirus harboring shRNA targeting hiwi was transfected into MCF-7 cells. Then, MCF-7 cells overexpressing hiwi, with a lentivirus harboring shRNA targeting hiwi was transfected into MCF-7 cells. Then, MCF-7 cells overexpressing hiwi were used for Western Blot Analysis for hiwi expression. The Western Blot Analysis revealed that the overexpression of hiwi in MCF-7 cells leads to an increase in the growth of MCF-7 cells. It is implied that hiwi plays an important role in breast cancer cell growth and can be a potential target for anticancer therapy.

**Materials and Methods**

**Breast cancer specimens, Cell lines and culture**

47 human breast cancer specimens and 47 peritumor specimens (as control; at a distance ≥10mm from the tumor edge) were resected from breast cancer patients, with complete clinico-pathologic data, who were registered in the China-Japan Union Hospital, Jilin University, from April 2007 to Jun 2012, with informed consent and agreement. Clinico-pathological data of the patients were recorded prospectively, including the age at diagnosis, menstruation status, tumor size, axillary lymph node metastasis and histological grade. All fresh specimens were resected before being exposed to radiotherapy and chemotherapy, were immediately frozen in liquid nitrogen and stored at -80°C post resection. And 10 peritumor specimens of breast cancers were used as control for hiwi expression in breast cancer cell lines. Present study has been approved by the medical ethics committee of China-Japan Union Hospital, Jilin University. Breast cancer cell lines, MDA-MB-231, MCF-7 and MCF-12A were purchased from Shanghai Bioleaf biotech co.Ltd (Shanghai, China) and were cultured at 37°C in a humidified atmosphere of 5% CO2 in Dulbec-co’s modified Eagle’s medium (DMEM) or RPMI640 medium (for MCF-12A) (both from Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; GIBCO, Rockville, MD, USA).

**RNA extraction and quantitative real-time polymerase chain reaction**

Total RNA from the intra-tumor, peritumor specimens, or the breast cancer cell lines was extracted using the RNaseasy plus mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions, and added with Rnase inhibitor (TaKaRa Bio Inc., Tokyo, Japan). Quantitative real-time RT-PCR (RT-qPCR) was performed using a SYBR PrimeScript RT-qPCR Kit (TaKaRa Bio Inc., Tokyo, Japan) according to the kit manual, with β-actin as an internal control. The primers for hiwi and β-actin were synthesized by Shanghai Sangon company (Shanghai, China) according to the reported sequences (Zhao et al., 2012). The RT-qPCR was performed with a Lightcyler 480 II (Roche, Mannheim, Germany). All data were normalized to β-actin and expressed as the fold change over control and calculated with the ∆∆Ct method (Livak et al., 2001).

**Western Blot Analysis for hiwi expression**

Breast cancer specimens for western blot analysis were homogenized before protein isolation. The homogenized breast specimens or cultured breast cancer cells were collected and lysed with a lysis reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manual, and were supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). Protein samples were
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Manipulation of hiwi level in MCF-7 cells
To generate a hiwi overexpressed MCF-7 cell line, the wild hiwi coding sequence was amplified and cloned into the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA, USA). And the Hiwi-2A-eGFP-pcDNA3.1 (+) or control pcDNA3.1(+) vectors was transfected into MCF-7 cells by lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The hiwi and eGFP positive cell clones were selected in the presence of 1.5mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA), and maintained in medium containing G418 at 1 mg/mL. The 50 nM hiwi specific siRNAs, siRNA-Hiwi 1, siRNA-Hiwi 2 or siRNA-Con (Sangon, Shanghai, China) was transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) into the MCF-7 Hiwi (+) cells to abrogate the Hiwi expression.

Statistical analysis
Statistical analyses were performed using SPSS16.0 software (IBM SPSS, Armonk, NY, USA). All data were expressed as mean±standard error of the mean (SEM). The expression of hiwi in mRNA or protein level, the cell number or the colony number two groups were analyzed by Student’s t test. A number or the colony number two groups were analyzed by paired-samples t-test. And the significant high hiwi mRNA level was also separated by 10% SDS-PAGE gel, and were transferred to a PVDF membrane. The hiwi and β-actin were detected, via successively using anti-hiwi or anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a peroxidase-conjugated secondary antibody (Sigma, St. Louis, MO, USA) and the electrochemoluminescence (ECL) detection system (Amersham, Uppsala, Sweden) following the manufacturer’s instructions.

Cell count assay and Colony formation assay
Cell count assay was performed as previously described (Li et al., 2013). Briefly, cells plated in 12-well plates and were incubated at 37°C for various periods of time and then trypsinized. The number of viable cells was counted in a hemocytometer with the use of trypan blue staining. For the colony formation assay, 500 cells in each group were seeded into a 12-well plate. After 6 days, cells were stained by 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) and the electrochemoluminescence (ECL) detection system (Amersham, Uppsala, Sweden) was used to quantify the colonies.

Results
Overexpression of hiwi in breast cancer specimens and breast cancer cell lines, and is associated with the cancer malignance.
To identify a possible oncogenic role of hiwi in breast cancers, we determined the hiwi expression in breast cancer cell lines, revealing by western blot analysis. And the significant high hiwi level was also significantly higher than in the peritumor specimens (p<0.01, paired-samples t-test).

Table 1. Association of Hiwi Expression with Clinicopathological Variants in Breast Cancers

| Clinicopathological Variants | n  | Relative Hiwi levela | p-value |
|-----------------------------|----|----------------------|---------|
| Age (yr)                    |    |                      |         |
| ≤50                         | 22 | 1.986±0.244          | 0.2536  |
| >50                         | 25 | 2.185±0.225          |         |
| Menopause                   |    |                      |         |
| Premenopausal               | 23 | 2.165±0.241          | 0.3042  |
| Postmenopausal              | 24 | 0.022±0.228          |         |
| Tumor size (cm)             |    |                      |         |
| T1                          | 0  | /                    | 0.0360  |
| T2                          | 13 | 1.825±0.203          |         |
| T3                          | 19 | 2.131±0.219          |         |
| T4                          | 15 | 2.275±0.315          |         |
| Axillary lymph node metastasis |    |                      |         |
| Negative                    | 21 | 1.881±0.352          | 0.0143  |
| Positive Histological grade |    |                      |         |
| I/II                        | 19 | 1.732±0.294          | 0.0093  |
| III                         | 28 | 2.336±0.210          |         |

*: The Relative Hiwi level was normalized to β-actin, and relative to the hiwi expression to each peritumor tissue (which was designated as “1”).

Figure 1. Overexpression of Hiwi Protein in Breast Cancer Specimens and Cell Lines. A) Relative mRNA level of Hiwi in the intratumor (n=47) or peritumor tissues (n=47) of breast cancers, revealing by quantitative real-time RT-PCR; B) Relative mRNA level of Hiwi in the breast cancer cell lines; C) Overexpression of Hiwi in protein level in the intratumor (n=15) or peritumor tissues (n=15) of breast cancers, revealing by western blot analysis. D) Overexpression of Hiwi in protein level in breast cancer cell lines, revealing by western blot analysis. Statistical significance was considered with a p<0.05 or less, * p<0.05, ** p<0.01, ns, no significance.
Da-Wei Wang et al. found in breast cancer cell lines, MDA-MB-231, MCF-7, but not in MCF-12A, compared to the primary breast cells (Figure 1B, \( p < 0.01 \) for MDA-MB-231 or MCF-7, \( p > 0.05 \) for MCF-12A, paired-samples t-test). And Figure 1D also indicated that there was a significant high level of hiwi expression in protein level in both MDA-MB-231 and MCF-7 cells (\( p < 0.05 \), unpaired-samples t-test), but not in MCF-12A cells (\( p > 0.05 \), unpaired-samples t-test). Taken together, we confirmed the overexpression of hiwi in breast cancer specimens and breast cancer cell lines.

Construction of MCF-7 stable cell line overexpressing hiwi

To identify the oncogenic role of hiwi in breast cancer cells, we generate an hiwi and eGFP co-expressing MCF-7 cell line. Hiwi and eGFP cDNA were amplified and then over-lapped with a 2A peptide coding sequence between them, and then were cloned into the eukaryotic expression vector, pcDNA3.1 (+). As shown in Figure 2A, the co-expressing method with 2A peptide guarantees both molecules to be transcribed into one mRNA molecule, but be translated into two separate proteins (Szymczak et al., 2004. Szymczak-Workman et al., 2012). MCF-7 cells were transfected with the recombinant hiwi-2A-EgfpcDNA3.1 (+) plasmid and selected under the G418 pressure. Post serial passages with 1.5 mg/mL G418, MCF-7 Hiwi (+) cells were isolated and propagated, with a eGFP positive (Figure 2B). Then the overexpression of hiwi were determined. A stabilized Hiwi overexpression in mRNA level in MCF-7 Hiwi (+) cells were determined by the RT-qPCR method with the hiwi specific premers. A significantly higher level of hiwi mRNA was observed in the MCF-7 Hiwi (+) cells with various passages (t-test, \( p < 0.01 \) respectively, compared to the control cells, MCF-7 cells) (Figure 2C). And the hiwi overexpression in protein

Figure 2. Construction of MCF-7 Stable Cell Line Overexpressing Hiwi. A) Schematic diagram of Hiwi and eGFP co-expression with a 2A peptide sequence, all of which were cloned into pcDNA3.1 vector; B) The GFP-positive MCF-7 cells overexpressed Hiwi. C) RT-qPCR analysis of the over-expressed Hiwi mRNA in MCF-7 Hiwi (+) cell line post serial passages, compared to \( \beta \)-actin. D and E) Western blot indicated Hiwi overexpression in protein level in serially propagated MCF-7 Hiwi (+) stable cell line, normalized to \( \beta \)-actin. Statistical significance was showed as **\( p < 0.01 \). Data represent the mean±SEM of three independent experiments

Figure 3. Overexpression of Hiwi Promotes the Growth of Human Breast Cancer Cells, MCF-7. A) Significantly high growth of MCF-7 Hiwi (+) cells than the MCF-7 cells after an inoculation of 103 /mL cells. B) Significantly high growth of MCF-7 Hiwi (+) cells than the MCF-7 cells after an inoculation of 104 /mL cells. C) Difference in cell growth by colony formation assay between the MCF-7 Hiwi (+) cells and MCF-7 cells. The experiments were performed separately in triplicate. Statistical significance was showed as * \( p < 0.05 \), ** \( p < 0.01 \)

Figure 4. RNA Interference Blocks Hiwi Expression and Inhibits the Growth of MCF-7 Hiwi (+) cells. A) Significant suppression by siRNA of the hiwi mRNA in MCF-7 Hiwi (+) cells 24 h post the transfection of hiwi specific siRNAs, compared to the siRNA control, revealing by quantitative real-time RT-PCR. B) Hiwi expression in protein level in MCF-7 Hiwi (+) cells 48 h post transfection of hiwi specific siRNAs or siRNA control, revealing by western blot analysis. \( \beta \)-actin was used as an internal control. C) Significant less growth of MCF-7 Hiwi (+) cells in siRNA-Hiwi 1 or shRNA-Hiwi 2 group than in control siRNA group. The growth curve of both cells were evaluated by cell count assay. D and E) Cell growth by colony formation assay in MCF-7 Hiwi (+) cells with or without Hiwi knockdown by siRNAs. The experiments were performed separately in triplicate. Statistical significance was showed as * \( p < 0.05 \), ** \( p < 0.01 \)
Hiwi overexpression promotes the growth of breast cancer cells, MCF-7

We then evaluated the influence of hiwi overexpression on the growth of breast cancer cells. The growth of breast cancer cell in vitro was assessed by cell count assay and colony formation assay. Firstly, the cell count assay was performed for cell counting every other day for three times post cell inoculation. As shown in Figure 3A, when inoculated with 10^4/mL, MCF-7 Hiwi (+) cells grew more efficiently than MCF-7 cells, particularly at 4 or 6 day post inoculation, with a significant difference (5.64±0.70×10^3 /mL for MCF-7 Hiwi (+) cells and 3.72±0.54×10^3/mL for MCF-7 cells at 4 day post inoculation, and 6.43±0.81×10^3 /mL for MCF-7 Hiwi (+) cells and 4.45±0.62×10^3/mL for MCF-7 cells. p<0.054, p<0.05, respectively). The growth difference was reconfirmed with an inoculated with 10^4/mL, the growth of MCF-7 Hiwi (+) cells was also significantly more efficient than MCF-7 cells (Figure 3B; p<0.01, respectively). Furthermore, the colony formation for both MCF-7 Hiwi (+) and MCF-7 cells was evaluated. As shown in Figure 3C, there was more colonies formed by MCF-7 Hiwi (+) cells than MCF-7 cells, post an inoculation of same cell number (p<0.01). Thus, we confirmed the promotory role of hiwi to the growth of breast cancer cells by both kinds of assays.

RNAi-mediated hiwi knockdown blocks the promotion of overexpressed hiwi to the MCF-7 cell growth

To further confirm the promotion by hiwi to the growth of breast cancer cells, we knockdown the hiwi with hiwi specific siRNAs in the MCF-7 Hiwi (+) cells, and then to determine the influence of hiwi knockdown on the breast cancer cell growth. Then MCF-7 Hiwi (+) cells were transfected with the 30 nM siRNA-Hiwi 1, siRNA-Hiwi 2, or siRNA-Con according to the product manual. Figure 4A and 4B indicated that both siRNAs significantly blocked the hiwi expression in both mRNA and protein levels, compared to the siRNA-Con (p<0.01 respectively, unpaired t test). We then evaluated the influence of hiwi knockdown on the growth of breast cancer cells. by cell count assay and colony formation assay. Firstly, the cell count assay was performed for cell counting every other day for three times post the inoculation of 10^4/mL MCF-7 Hiwi (+) cells. As shown in Figure 4C, both siRNAs significantly inhibited the MCF-7 Hiwi (+) cell growth at 4 or 6 day post inoculation, compared to the siRNA-Con (either p<0.05, unpaired t test). As shown in Figure 4D and 4E, the numbers of MCF-7 Hiwi (+) formed colonies in both siRNA-Hiwi 1 and siRNA-Hiwi 2 groups were significantly less than in the control siRNA group (either p<0.05, unpaired t test). Thus, we reconfirmed the promotory role of hiwi to the growth of breast cancer cells by the loss-of-function strategy.

Discussion

As a member of Argonaute (Ago) family, the hiwi protein also contains a conserved architecture with a PAZ motif and Piwi motif (Cerutti et al., 2000, Yan et al., 2003). And the Piwi motif has a structural homology to RNome H endonuclease (Parker et al., 2004). Proteins with Piwi domains have been described as components of ribonucleoprotein complexes that act in the microRNA/ RNA interference pathway of gene silencing (Fetzer et al., 2002, Lingel et al., 2005). Recently, it was shown that the Piwi gene family are associated with a new class of small RNAs socalled ‘piwi-interacting RNAs’ (piRNAs) in mammalian spermatogenesis (Hall et al., 2005, Houwing et al., 2007, Hutvagner et al., 2008, Peters et al., 2007), which is one of gene expressing regulatory small non-coding RNAs (ncRNAs). Small ncRNAs are naturally conserved regulators of gene expression described in almost all eukaryotic species including humans (Elbashir et al., 2001, Meister et al., 2004, Sana et al., 2012). To perform their effector functions, small ncRNAs must be incorporated into Ago complexes and form highly specialized small-RNA-binding modules in RNA-silencing pathways. However, it is not clear how the ribonucleoprotein-like molecule promotes the tumorigenesis.

More recently, professor Yang and his colleagues have confirmed that the hiwi expression inhibition could significantly reduce the tumor growth in a xenograft mouse model. Further immunohistochemistry analyses confirmed that the hiwi blockage also induced a significant suppression in ALDH-1 positive cells in xenograft tumor samples (Liang et al., 2013). Since ALDH-1 is a confirmative marker of the lung cancer stem cells (Liang et al., 2012), it implied that delivery of shRNA-mediated Hiwi gene silencing resulted in the decreased number of lung cancer stem cells and suppressed tumor growth in nude mice. Thus, hiwi may play a crucial role in the regulation of lung cancer stem cell growth.

In present study, we confirmed the overexpression of hiwi in breast cancer specimens and breast cancer cell lines in both mRNA and protein levels. Both RT-qPCR and western blot analysis revealed a significant high hiwi level in intratumor specimens than in the peritumor specimens. And the hiwi overexpression was significantly associated with the tumor size, lymph node metastasis and histological grade. Moreover, the hiwi overexpression was also identified in breast cancer cell lines, MDA-MB-231, MCF-7, but not in MCF-12A. Then, both the gain-of-function and loss-of-function strategies were adopted to identify the role of hiwi in the MCF-7 cell growth. Results demonstrated that the hiwi expression in MCF-7 cells was significantly up- or down- regulated by the two strategies. We next evaluated the influence of hiwi overexpression or knockdown on the growth of breast cancer cells. Both cell count assay and the colony formation assay confirmed the promotory role of hiwi in MCF-7 cells, and the promotion could be inhibited by the hiwi specific blockade by siRNAs. Taken together, our study implied an oncogenic role of hiwi in breast cancer. However, the mechanism of the oncogenic regulation by hiwi needs to be further recognized.

In summary, present study confirmed the overexpression of hiwi in breast cancer specimens and breast cancer cell lines, identified the promotion of hiwi to the growth

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of breast cancer cells, using both the gain-of-function and loss-of-function approaches: Overexpressed hiwi promoted MCF-7 cell growth, while hiwi knockdown by RNAi method inhibited the promotion by hiwi. It implied the oncogenic role of hiwi in breast cancers.

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