Overexpression of Argonaute *HIWI* Gene in Colorectal Cancer Stem Cells and Colorectal Cancerous Tissue

Soheila Moeini
Islamic Azad University
Eisa Tahmasbpour Marzouni
Pasteur Institute of Iran
Asghar Beigi Harchegani
Shahid Beheshti University of Medical Sciences School of Nursing and Midwifery
Mahdi Ghatrehsamani
Shahrekord University of Medical Science
Saeid Rahmanzadeh (s.rahmanzadeh2020@gmail.com)
Pasteur Institute of Iran

Research Article

Keywords: Cancer stem cells, colorectal cancer, HIWI expression, Real-time PCR, Western blotting, IHC.

Posted Date: January 24th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1241717/v1

Abstract

Background: Recent studies have shown that Hiwi has a crucial role in stem cell self-renewal in various organisms and it is also associated with some cancers. In the present study, *Hiwi* expression was examined in different grades of primary human colorectal cancer (CRC), colorectal cancer stem cells (CRCSCs) and HT-29 CRC cell line.

Methods and Results: CRC tissue samples were collected from 20 patients with CRC. Furthermore, HT-29 cell line, CRCSC and 13 normal colorectal tissue samples were prepared. Expression of Hiwi at mRNA and protein levels was determined by Real-time PCR, flow cytometry, Western blot and immunohistochemistry. Overexpression of *Hiwi* was detected in 40% (3.351 ± 2.94, $P<0.05$) of clinical CRC tissue specimens. We also observed a significant increase in the expression of *Hiwi* in CRCSCs (5.94 ± 0.05, $P < 0.005$). Hiwi expression in CRCSC was significantly higher compared to the colorectal cancer tissue and HT-29 cells ($p<0.01$). *Hiwi* mRNA level was significantly correlated to tumor grade ($p < 0.01$) and stage ($p < 0.01$).

Conclusions: *HiWi* can be considered as an oncogene in progressive cancer and, therefore, it can be a valuable biomarker and target in therapeutic procedures.

Introduction

Colorectal cancer (CRC) is the third most common cancer and the second most common cause of cancer-related death in both men and women [1–3]. Although multiple factors are responsible for colorectal cancer development, it is mainly resulted from genetic and epigenetic variations which convert normal glandular epithelium into invasive adenocarcinoma. At present, multiple cellular and molecular pathways have been proposed for the development of colorectal cancer [1, 4]. Identification of molecules or associated risk factors involved in colorectal cancer development is valuable for early diagnosis and also finding a better therapeutic strategy for cancer at early stages [4, 5].

Cancer stem cells (CSCs) are a rare population of cells within a tumor which serve as tumor-initiating cells or tumorigenic cells [6, 7]. CSCs have a similar expression of cell surface markers pattern like normal stem cells. Furthermore, They have self-renewing capacity and can be differentiated to various cancer cells type with common signaling pathways [6]. Unlike normal stem cells, CSCs have a tumorigenic activity that enables them to form tumors when they are transplanted into animal models [7].

Piwi like RNA-mediated gene silencing 1 (HIWI), also known as Piwi1, is a member of the highly conserved P-element induced wimpy testis (PIWI) family. are The Piwi family are important regulators of different physiological processes such as germ cell differentiation, stem cell self-renewal and differentiation, spermatogenesis and male infertility [8, 9]. Expression of Hiwi protein in spermatocytes and round spermatids during spermatogenesis indicates that this gene is critical for normal spermatogenesis.

Most of *Hiwi* mRNAs are not complementary to their target genes, which suggest that *Hiwi* family are more likely under the effect of post-transcriptional regulation rather than chromatin-associated regulation. It has been determined that Hiwi are exclusively expressed in stem cells. For example, Sharma *et al*., [10] found HIWI expression in $CD34^+$ hematopoietic stem cells (HSCs), but not in differentiated HSCs. This data indicates that HIWI may be responsible for determination or regulation of HSCs development.

Recent studies have shown that HIWI is ectopically expressed in all cancers [11–13]. *It* has been shown to be up-regulated in different cancers such as seminomas [11], gliomas [14], squamous-cell carcinomas [15], pancreatic [16], liver [17], and gastric cancers [18]. Taubert *et al*., have reported that *Hiwi* is highly expressed in primary soft-tissue sarcomas, where higher *Hiwi* mRNA levels are predictable based on fault clinical outcomes [12, 13]. However, the exact function of Hiwi in tumorigenesis is unclear. Araújo *et al*., [19] demonstrated that Hiwi up-regulation plays a crucial role in the signaling pathway of gastric cancer and promotes CSCs maintenance, tumor cell viability, migration and invasion. A previous study found overexpression of Hiwi in testicular seminoma, indicating Hiwi deregulation may contribute to the occurrence and development of testicular tumors [20]. These data mean that abnormal expression of Hiwi protein may be involved in development, progression and poorer diagnosis of various cancer forms [12, 13]. On the other hand, some studies found reverse association between Hiwi expression and cancer progression. For example, Wang *et al*., [21] revealed that overexpression of Hiwi inhibits the growth of chronic myeloid leukemia K562 cells and consequently enhance their chemosensitivity to daunomycin. These data implicate that the biological functions of Hiwi may vary between different types of tumor, necessitating its role in each cancer to be studied individually. Furthermore, Hiwi may be considered as a potential target for cancer diagnosis and therapy because most non-cancer cells would not be affected by cytotoxic effects [19]. Given the critical role of Hiwi in cancer proliferation and metastasis, we assume that altered expression of *Hiwi* may be involved in development of colorectal cancer. Therefore, this study aims to evaluate the *Hiwi* expression and its prognostic values in 20 clinical samples with non-necrotic colorectal cancer tissue and normal respective colorectal tissue. Furthermore, *Hiwi* expression levels are compared between different grades of primary human colorectal carcinoma, colorectal cancer stem cells (CRCSCs), as well as HT-29 CRC cell line.

Methods And Materials

Study population and sample collection

A total number of 20 patients (aged 21–46 years old) with colorectal cancer and 13 healthy individuals who referred to Imam Khomeini Hospital (Tehran-Iran), were entered into the study between 2016 and 2018. Healthy controls were those who came to our hospital for checkup and no abnormalities were found in physical examination or laboratory results. They also did not have a history of cancer and previous medical diseases. The control subjects were matched to the patients based on age and sex. This cross-sectional study was approved by the institutional review board and ethics committee of Tehran

University of Medical Sciences (Tehran, Iran). Written informed consents were signed by all subjects at the department of cancer in Imam Khomeini Hospital. Clinical and pathological findings of the patients, including disease stage, tumor grading, metastasis status, and lymph node involvement. were also provided. Inclusion criteria for patients were: (i) patients with known colorectal cancer; (ii) complete clinical and pathologic information. Patients who met the following criteria were excluded from the study: (i) the presence of tumor in other places; (ii) previous treatments such as chemotherapy or radiotherapy; (iii) history of other chronic diseases like diabetes mellitus, and liver disease.

All colorectal cancer tissue specimens were confirmed by the surgical findings and the postoperative pathological study. All specimens were collected and transferred into RNAlater Reagent (Qiagen, USA) and kept at 4°C. The tissue samples were washed with phosphate-buffered saline (PBS) and cut into smaller pieces and stored at −80°C until use.

HT-29 cell culture

The human colorectal adenocarcinoma cell line HT-29 (ATCC® HTB-38™) was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 µg/ml streptomycin, 100 µg/ml penicillin in a humidified incubator containing 5% CO_2 at 37 ˚C.

Cell separation by CD44 positive selection

The human colorectal adenocarcinoma cell line HT-29 was passed through a MACS column [Order number: 130-095-194, Miltenyi Biotec, UK], including CD44 monoclonal antibody, thereby $CD44^+$ HT29 cells were separated from other cells, and then they were cultured. The isolation of cells was performed according to the method described by Gao *et al.* [22].

Stem cell surface marker phenotyping by flow cytometry

CD44-positive cells separated from HT-29 colorectal adenocarcinoma cells in passage 2 (10^5-10^6 cells) were used for phenotypic marker identification by flow cytometry. For fluorescent antibody cell surface staining, cells were washed with Hanks' Balanced Salts Solutions (HBSS) + 2% BSA two times and incubated with specific fluorescent-labeled monoclonal antibodies or respective isotype controls at concentrations recommended by the respective manufacturer. Cells were incubated for 20 min and then analyzed by flow cytometry. The antibodies used were: FITC Anti-CD90 / Thy1 antibody [clone F15-42-1] (ab11155), PE Anti-CD133 antibody [clone EPR20980-104] (ab252128) purchased from Abcam (Cambridge, MA, USA), APC anti-human CD34 Antibody [clone 561] (biolegend 343607) and PerCP anti-human CD45 Antibody [clone 2D1] (biolegend 368505) purchased from biolegend (San Diego, CA). Incubation was performed for 30 min. Cells were then washed twice in PBS and were analyzed using flow cytometry (Becton Dickinson, Germany and BD Biosciences Inc).

Quantitative RT-PCR

Total RNA was extracted using Qiagen RNase kit. Complimentary DNA (cDNA) was synthesized using 1 µg of RNA, according to instructions in the cDNA synthesis kit (Takara, Japan). Real-time PCR was

performed by SYBR green PCR Master Mix (Takara), on a Rotor-Gene Q, real-time thermocycler (Qiagen, USA) and Real-time RT-PCR primers for *Hiwi* and *GAPDH* were designed by Allele ID software Version 6.0 (PREMIER Biosoft, CA, USA) (Table 1). The thermal profile included 10 min at 95°C followed by 40 cycles of 15 sec at 95°C, 30 sec at 57°C, and 45 sec at 72°C. Data were normalized to *GAPDH* expression applying the comparative threshold cycle method. The PCR efficiencies for *Hiwi* and *GAPDH* were verified by generating related standard curves. The relative expression of *Hiwi* was compared based on fluorescence intensity changes of samples from endometriosis vs. compliant normal tissues. More than two-fold increase in expression was considered as over-expression, while more than two-fold decrease was considered as under-expression. The range between those two values was interpreted as no change or regular expression. All experiments were performed in triplicate.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously described by Taubert *et al*. [11, 12]. Briefly, paraffin-embedded tissue sections were deparaffinized and rehydrated. Slides were treated with 3% H_2O_2 for 10 min and 2% BSA for 30 min at room temperature before incubation overnight with 2 µg/ml polyclonal anti-PIWI (HIWI) (abcam, UK) antibody at 4° C. After 3 washes in PBS, slides were incubated with an HRP-linked anti-mouse secondary antibody for 30 min, and then washed 3 times with PBS, followed by chromogen detection with DAB for 10 min, and hematoxylin counterstaining.

Western blot analysis

Samples were collected and lysed with standard RIPA buffer. After centrifugation at 40,000 *g* at 4 ^{o}C for 45 min, the supernatant was subjected to SDS-PAGE analysis. 100 µg of protein from each clinical sample, CD44+ HT29 and HT-29 colon cancer cell line was subjected to 12% SDS–PAGE. Proteins were transferred to the nitrocellulose membrane (Millipore, USA) using Western blot technique followed by blocking using 5% bovine serum albumin (BSA, Sigma, Germany) for 60 min at room temperature along with shaking. Nitrocellulose membranes were then incubated with rabbit anti-PIWI (abcam, UK) antibody (1:1000) for 1 h followed by the incubation of membranes with HRP conjugated anti-mouse antibody at room temperature with mild shaking. Enhanced chemiluminescence (ECL) Western blotting system (GE Healthcare, USA) was used to develop the membrane on high performance chemiluminescence film (GE Healthcare) according to the company guidelines. After each step, the membrane was washed with PBS [11, 13].

Statistical analysis

Data were analyzed using the SPSS software. Pearson's correlation assessed correlation between HIWI expression levels in various cancer stages and grades. Comparison of the gene expression between all groups was evaluated by ANOVA, Tukey test analysis. P value <0.05 was considered to be statistically significant.

Results

Cancer stem cell isolation and identification

Colorectal cancer stem cells ($CD44^+$) were successfully isolated from HT-29 colorectal adenocarcinoma cells line after positive immune-selection to deplete CD44 cells (Figure 1). As shown in figure 1, the morphological appearance of colorectal cancer stem cells ($CD44^+$) is different from HT-29 cells line. Colorectal cancer stem cells ($CD44^+$) appeared as cuboidal-shaped cells with scant cytoplasm and granules around the nuclei (Figure. 1B). In compared to the HT-29 cell line (Figure. 1D) derived from the adenocarcinoma patients, they have a same proliferation capacity. Flow cytometry analysis revealed that colorectal cancer stem cells were strongly positive for surface markers CD44, CD133, CD90, but they were negative for CD45 and CD34.

Real time PCR analysis of Hiwi Gene

The relative expression of *Hiwi* in CD44 positive colorectal cancer cells, HT-29 cell line, and normal colorectal cells was analyzed by Real time PCR. As shown in Figure 2, the relative expression of *Hiwi* gene was higher in colorectal adenocarcinoma samples and CD44 positive colorectal cancer stem cells compared to normal cells. This result indicates that *Hiwi* gene is down-regulated in normal colon samples.

Immunohistochemistry (IHC) staining

Hematoxylin & Eosin staining of normal tissues (Figure 3A) and colorectal cancer (Figure 3B) immunohistochemically for HIWI was localized in the colon of cancerous patients (Figure 3C). IHC analysis showed increased expression of HiWi protein in colorectal tissues compared to normal colon tissues.

Western blot analysis

Western blot analysis was performed to confirm the expression of HIWI protein in colorectal tissue extract. Expression of HIWIprotein was observed under western blot analysis in tissue extract (Figure 4).

Hiwi mRNA expression in different disease stages and grades

The expression levels of *Hiwi* mRNA were normalized with respective mRNA levels of GAPDH as a housekeeping gene. Mean expression of *Hiwi* mRNA in colorectal tumor tissue was significantly higher than healthy colon sample ($p<0.001$). There were statistically significant differences in *Hiwi* expression in patient's group according to the disease stage and grade (Figure 5). Ten patients (50%) were in stage I/II, and the others (50%) were in stage III and IV (Table 2). The mean expression of *Hiwi* mRNA levels in patients with disease stage I/II was significantly higher than that in patients with disease stage III/IV ($p<0.001$; Fig. 5). The relative expressions of *Hiwi* gene in patients with early and advanced stages of colorectal cancer were 3.92 and 1.58, respectively (Figure 5).

There was a significant difference in the mean expression of *Hiwi* mRNA levels between patients with different disease grades ($p<0.01$). Ten patients (50%) were in grade I, 5 patients (25%) in grade II and 5 patients (25%) in grade III (Table 2). The mean expression of *Hiwi* mRNA levels in grade I was significantly higher than that in patients with grade II and III ($p<0.01$; Fig. 5).

Discussion

Due to the high incidence of colorectal cancer and its adverse effects on social life, it is essential to identify and target molecules or factors that are involved in cancer development and progression. Cancer cells have similar proliferation and differentiation patterns to stem cells. Therefore, it is important to study the genes/proteins that are responsible cancer stem cells differentiation to various cancer types. Recent studies have proposed that *HIWI* plays a crucial role in cancer development and progression. Therefore, it can be considered as a valuable target for cancer treatment. In the current study, we considered the expression pattern of Hiwi at both protein and mRNA levels in CD44 positive colorectal cancer stem cells, HT-29 cell line, healthy and cancerous colorectal tissues via real-time quantitative PCR, western blot assay, and immunohistochemical staining methods. We found overexpression of *Hiwi* in 40% of clinical colorectal cancer specimens. Similarly, Raeisossadati *et al*., [23] reported overexpression of *Hiwi* gene in 34.8% of clinical colorectal cancer specimens. In the study by Zeng *et al*., [24], they found overexpression of HIWI protein in 25.6% of patients with colorectal cancer. These findings are comparable with results of our study. We also found that relative expression of Hiwi at mRNA and protein levels was significantly higher in patients with colorectal cancer than healthy individuals. Interestingly, this expression pattern in CCSC was even higher than in cancerous colorectal tissues and colorectal cancer cells line. More importantly, the Hiwi expression profile was significantly correlated to the disease stages and grades. Patients with diseases stage I/II and grade I showed significantly higher *Hiwi* expression level compared to those with disease stage III/IV and grades II and III. These data indicate that Hiwi may be involved in colorectal cancer development and progression and, therefore, it can be considered as a potential prognostic biomarker for patients with colorectal cancer, especially at early stages.

To support our findings, a growing number of studies reported overexpression of *Hiwi* in different types of cancers [12, 13]. For example, Zeng *et al*., [24] found that patients with colorectal cancer and positive Hiwi expression had significantly lower survival rate compared to patients with negative Hiwi expression. Litwin *et al*., [25] found increased expression of *Hiwi* mRNA in colorectal cancer tissues compared to non-cancerous samples, which is in accordance with the findings of our study. Some studies indicated that up-regulation of *Hiwi* is associated with clinical features of cancerous patients [12, 13, 26]. Similarly, we found a significant relationship between colorectal cancer disease stages and degrees with *Hiwi* expression pattern. Our data also revealed that *Hiwi* is up-regulated in colorectal cancer cells and its function is altered in aberrant cancer state. Therefore, Hiwi can be considered as a direct tumorigenic or oncogenic factor. To support this hypothesis, Raeisossadati *et al*., [23] considered Hiwi as a tumorigenic factor in colorectal cancer. They showed that increased expression of *Hiwi* in colorectal cancer cells was significantly associated with the depth of tumor invasion, the stage of tumorigenesis progression and lymph node metastasis of tumor cells [23]. Wang *et al*., [27] reported overexpression of *Hiwi* in both

clinical breast cancer specimens and breast cancer cell's lines. Additionally, they found that increased expression of *Hiwi* was significantly associated with the growth of human breast cancer cells, tumor size, lymph node metastasis and histological grade [27]. A previous study reported increased expression of *Hiwi* in the cytoplasm of esophageal cancer cells which was associated with histological grade, disease stage and clinical outcome [28]. We also found increased expression of HIWI protein in undifferentiated human colorectal sarcomas; however, the exact molecular mechanism for this up-regulation in sarcomas is unclear. In a previous study, Liu *et al*., [29] found overexpression of *Hiwi* in human gastric cancer which was associated with the proliferation of cancer cells. A recent study has revealed that Hiwi promotes proliferation of colorectal cancer cells through upregulation of global DNA methylation [30].

According to the previous accomplished data and findings of our study, Hiwi can be considered as an oncogene for colorectal cancer development and progression. Therefore, it can be targeted for colorectal cancer therapy, especially at early stages.

Conclusion

In summary, our results showed that Hiwi expression at both mRNA and protein levels is significantly increased in clinical colorectal cancer tissues compared to normal specimens. Furthermore, the expression of Hiwi in CCSC was significantly higher than in colorectal cancer tissues than normal specimens. We also found a close relationship between *Hiwi* expression and colorectal cancer stage and degree. These results suggest that Hiwi may be involved in the development and progression of colorectal cancer and can be considered as a prognosis factor for the early diagnosis of this cancer. Besides, the oncogenic role of *Hiwi* indicates that it may be a potential target for cancer therapy. Further studies are merit to discover the underlying mechanisms by which Hiwi promotes colorectal cancer development.

Declarations

Acknowledgements

The authors are deeply grateful of colleagues in Enzyme Technology Lab., Genetics & Metabolism Research Group, Pasteur Institute of Iran, Tehran, Iran. Also, we kindly thank the colleagues who helped us to collect specimens, especially Imam Khomeini Hospital.

Conflict of interests

The authors declare that they have no conflict interests.

Compliance with Ethical Standards

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical approval: This study was approved by the institutional review board and ethics committee of Tehran University of Medical Sciences (Tehran, Iran). Written informed consents were signed by all subjects at the department of cancer in Imam Khomeini Hospital. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional committee and with the 1964 Helsinki declaration.

References

1. Ozawa, T., et al., Log odds of positive lymph nodes as a prognostic indicator in stage IV colorectal cancer patients undergoing curative resection. Journal of surgical oncology, 2015. **111**(4): p. 465-471.

2. Dolatkhah, R., et al., Colorectal Cancer in Iran: Molecular Epidemiology and Screening Strategies. Journal of Cancer Epidemiology, 2015. **2015**.

3. Hassan, C., et al., Efficacy and safety of endoscopic resection of large colorectal polyps: a systematic review and meta-analysis. Gut, 2015: p. gutjnl-2014-308481.

4. Sun, D.-w., et al., Prognostic significance of MMP-7 expression in colorectal cancer: A meta-analysis. Cancer epidemiology, 2015. **39**(2): p. 135-142.

5. Vatandoost, N., et al., Early detection of colorectal cancer: from conventional methods to novel biomarkers. Journal of cancer research and clinical oncology, 2015: p. 1-11.

6. Dawood, S., L. Austin, and M. Cristofanilli, Cancer Stem Cells: Implications for Cancer Therapy. Oncology (Williston Park, NY), 2014. **28**(12).

7. Kozovska, Z., V. Gabrisova, and L. Kucerova, Colon cancer: Cancer stem cells markers, drug resistance and treatment. Biomedicine & Pharmacotherapy, 2014. **68**(8): p. 911-916.

8. Cox, D.N., et al., A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev, 1998. **12**(23): p. 3715-27.

9. Seto, A.G., R.E. Kingston, and N.C. Lau, The coming of age for Piwi proteins. Mol Cell, 2007. **26**(5): p. 603-9.

10. Sharma, A.K., et al., Human CD34(+) stem cells express the hiwi gene, a human homologue of the Drosophila gene piwi. Blood, 2001. **97**(2): p. 426-34.

11. Qiao, D., et al., Molecular characterization of hiwi, a human member of the piwi gene family whose overexpression is correlated to seminomas. Oncogene, 2002. **21**(25): p. 3988-3999.

12. Taubert, H., et al., Expression of the stem cell self-renewal gene Hiwi and risk of tumour-related death in patients with soft-tissue sarcoma. Oncogene, 2007. **26**(7): p. 1098-1100.

13. Taubert, H., et al., Stem cell-associated genes are extremely poor prognostic factors for soft-tissue sarcoma patients. Oncogene, 2007. **26**(50): p. 7170-7174.

14. Sun, G., et al., Clinical significance of Hiwi gene expression in gliomas. Brain research, 2011. **1373**: p. 183-188.

15. He, W., et al., Expression of HIWI in human esophageal squamous cell carcinoma is significantly associated with poorer prognosis. BMC cancer, 2009. **9**(1): p. 426.

16. Grochola, L., et al., The stem cell-associated Hiwi gene in human adenocarcinoma of the pancreas: expression and risk of tumour-related death. British journal of cancer, 2008. **99**(7): p. 1083-1088.

17. Jiang, J., et al., Expression of HIWI in human hepatocellular carcinoma. Cell biochemistry and biophysics, 2011. **61**(1): p. 53-58.

18. Liu, X., et al., Expression of hiwi gene in human gastric cancer was associated with proliferation of cancer cells. International journal of cancer, 2006. **118**(8): p. 1922-1929.

19. Araújo, T., et al., Piwi like RNA-mediated gene silencing 1 gene as a possible major player in gastric cancer. World J Gastroenterol, 2018. **24**(47): p. 5338-5350.

20. Qiao, D., et al., Molecular characterization of hiwi, a human member of the piwi gene family whose overexpression is correlated to seminomas. Oncogene, 2002. **21**(25): p. 3988-99.

21. Wang, Y., et al., Overexpression of Hiwi Inhibits the Growth and Migration of Chronic Myeloid Leukemia Cells. Cell Biochem Biophys, 2015. **73**(1): p. 117-24.

22. Gao, Y., et al., Isolation and characterization of proliferative, migratory and multidrug-resistant endometrial carcinoma-initiating cells from human type II endometrial carcinoma cell lines. Oncology reports, 2012. **28**(2): p. 527-532.

23. Raeisossadati R, et al., Aberrant expression of DPPA2 and HIWI genes in colorectal cancer and their impacts on poor prognosis. Tumour Biol, 2014. **35**: p. 5299-305.

24. Zeng Y, et al., HIWI expression profile in cancer cells and its prognostic value for patients with colorectal cancer. Chin Med J 2011. **124**: p. 2144-9.

25. Litwin M, et al., Correlation of HIWI and HILI Expression with Cancer Stem Cell Markers in Colorectal Cancer. Anticancer Res, 2015. **35**: p. 3317-24.

26. Strimpakos, A., et al., The impact of carcinoembryonic antigen flare in patients with advanced colorectal cancer receiving first-line chemotherapy. Annals of oncology, 2010. **21**(5): p. 1013-1019.

27. Wang DW, et al., Overexpression of hiwi promotes growth of human breast cancer cells. Asian Pac J Cancer Prev, 2014. **15**: p. 7553-8.

28. He W, et al., Expression of HIWI in human esophageal squamous cell carcinoma is significantly associated with poorer prognosis. BMC Cancer, 2009. **9**: p. 426.

29. Liu X, et al., Expression of hiwi gene in human gastric cancer was associated with proliferation of cancer cells. Int J Cancer, 2006. **118**: p. 1922-9.

30. Yang L, et al., Hiwi Promotes the Proliferation of Colorectal Cancer Cells via Upregulating Global DNA Methylation. Disease Markers, 2015. **383056**: p. 1-10.

Tables

Table 1: Primer sequence for *HIWI*

Sequence (5'->3')	Product length	Tm
Hiwi		
Forward primer: GGTGATTTGGCCTGGCTTCA	301bp	60
Reverse primer: GTAGTATTCTAAGAAGCTGACCCC		
Gapdh		
Forward primer: CCTTCATTGACCTCAACTACATG	115 bp	59
Reverse primer: GGGATTTCCATTGATGACAAGC		

Table 2 : Histological and clinical data

	Total (n)	High *Hiwi*(n)	Medium *HiWi*(n)	Low *HiWi* (n)
	20	8	9	3
Men/women	10/10	3/5	6/3	2/1
Tumor grade				
I	10	8	2	-----
II	5	-----	5	-----
III	5	-----	2	3
Tumor stage				
I/II	9	8	1	-----
III/IV	11	-----	8	3

Tumor grade was determined according to van Unnik (1995) and tumor stage was ascertained according to UICC classification (Wittekind and Wagner, 1997).

Figures

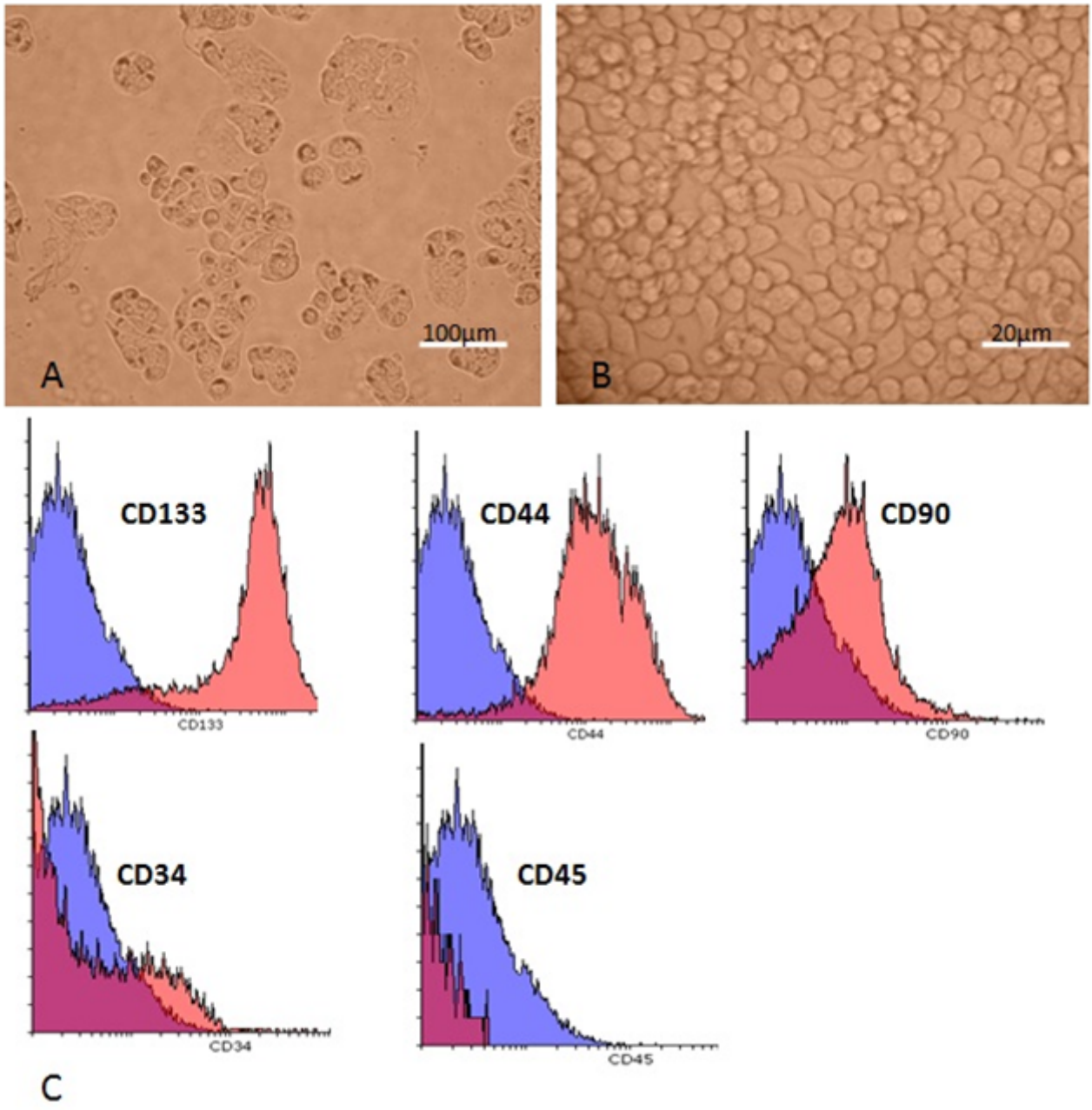

Figure 1

(A) morphology of HEC-1B cell line, (B) invert microscopy image from CD44+ cancer stem cells, (C) positive expression of CD44, CD90, CD133 and negative for CD34 and CD45 in CD44 sorted cancer stem cell. Blue color line indicates the negative area of diagram and red color line shows positive area. Scale bar: 100µm and 20µm.

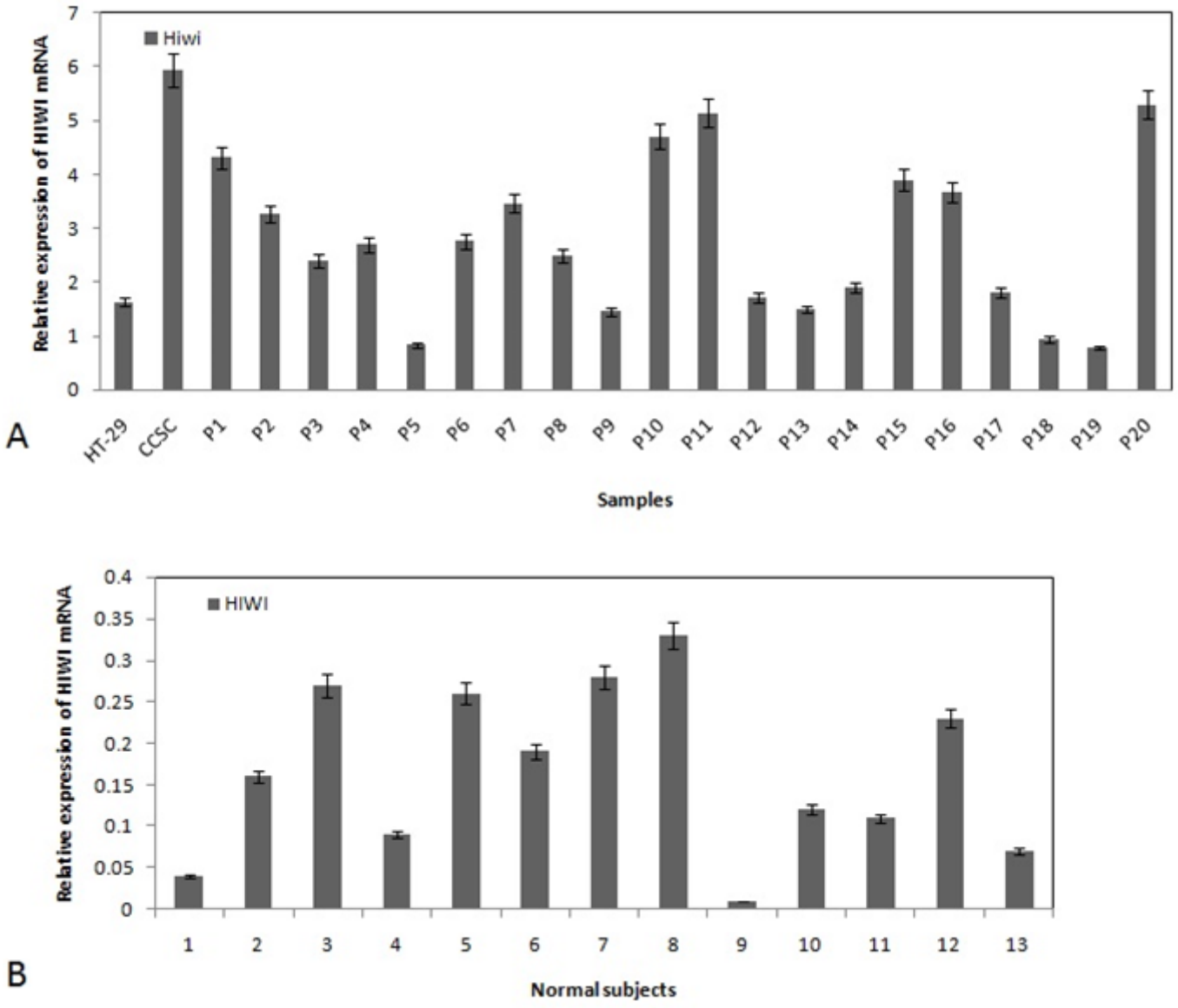

Figure 2

Real time PCR results for comparing relative gene expression levels in colorectal cancer, HT-29 cell line, colorectal cancer stem cell and normal colon samples. A: Bar graphs show relative expression of *HIWI* mRNA in colon cancer (n = 20), $CD44^{+}$ CSC and HT-29 cell line, B: normal colon (n=13) determined by real-time PCR. Values on the y-axis represent an arbitrary unit derived from the mean expression value for *HIWI* with values. Results are presented as mean ± SEM of mRNA expression $P < 0.05$.

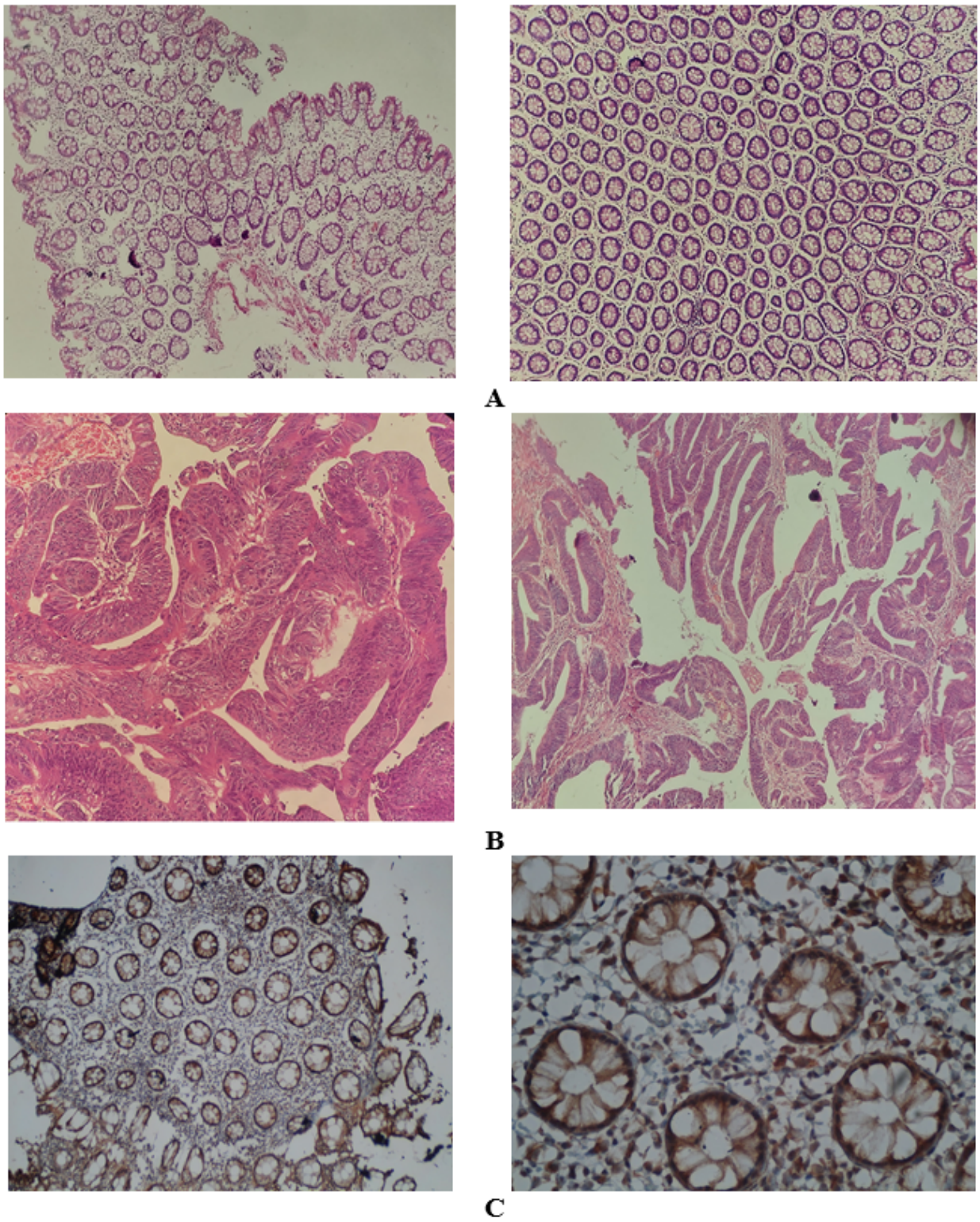

Figure 3

Sections of normal colon (A) and colorectal cancer tissue (B) were stained with H&E. The presence of HIWI protein was detected by immunohistochemistry (C) in colorectal cancer tissue. Magnification: ×40.

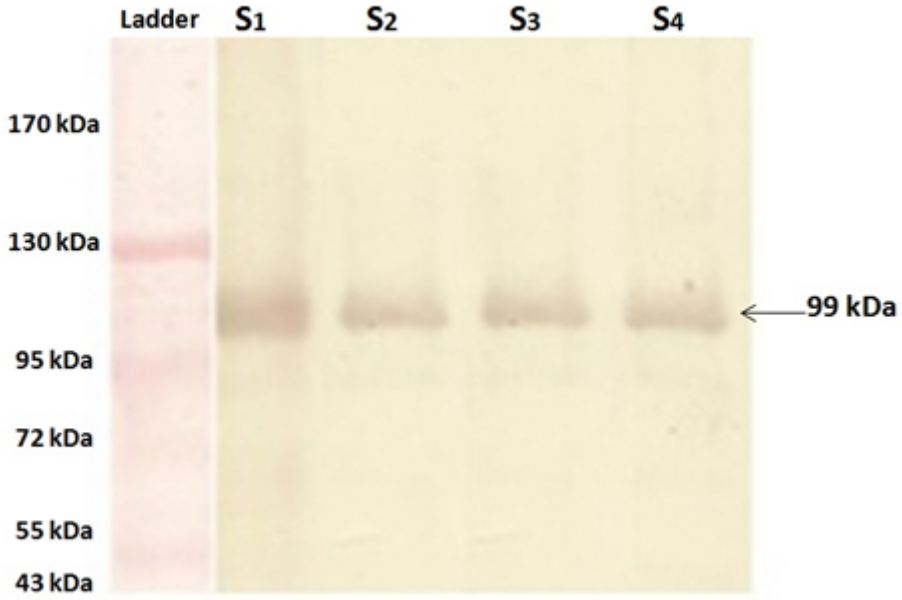

Figure 4

Overexpression of HIWI protein in colorectal cancer was detected by Western blotting. GAPDH was used as a loading control. S1 and S2, cancer samples (n = 2); S3, CD44 positive CSCs; S4, HT-29 colorectal cancer cell line.

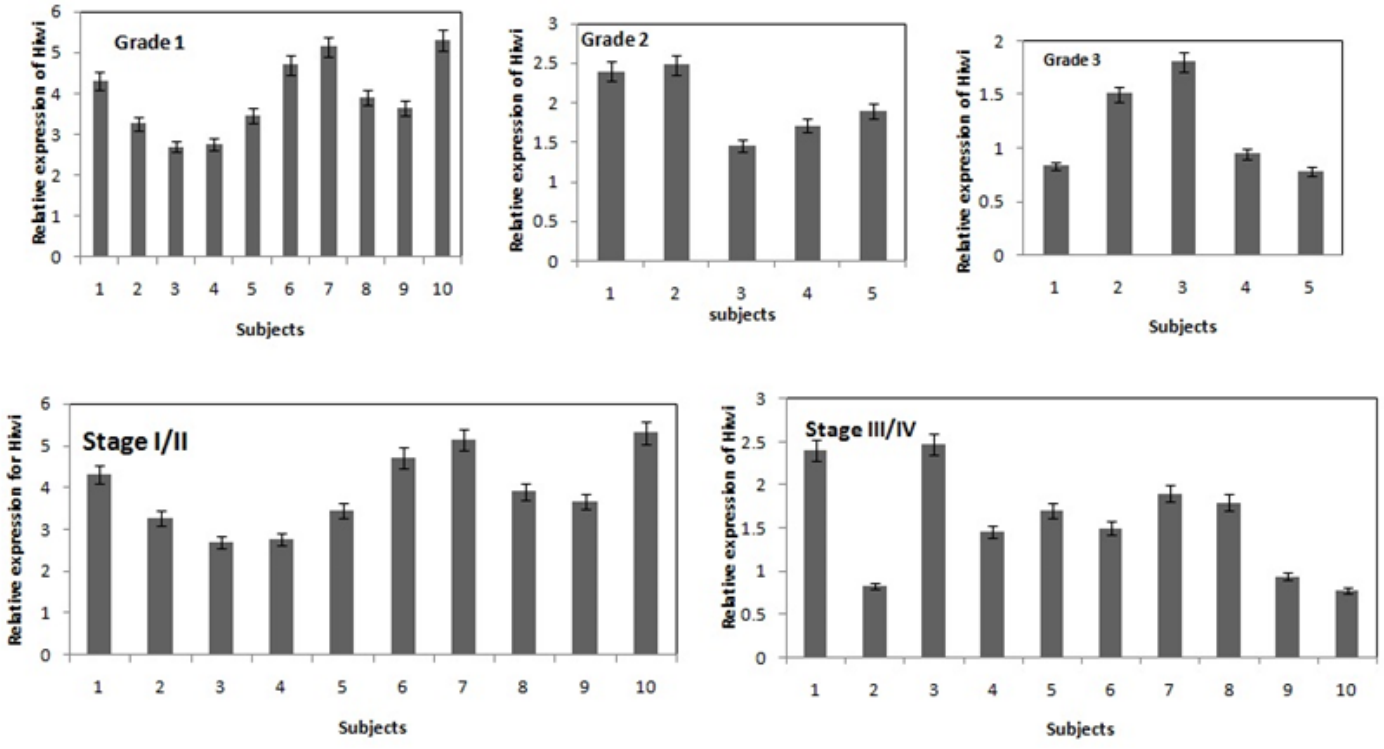

Figure 5

Ratios of Hiwi mRNA expression in colorectal cancer, correlated best with the corresponding ratios normalized for GAPDH ($R = 0.674$; $P < 0.001$). Ratios of *Hiwi* mRNA expression were not associated with gender (men vs. women, $P = 1.0$), tumor grade ($P = 0.0667$) or stage ($P = 0.0514$).