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

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Abstract

Background: Recent studies have demonstrated that *Hiwi* has a crucial role in stem cell self-renewal in various organisms, and it is associated with some cancers.

Objectives: 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: The CRC tissue samples were collected from 20 patients with CRC. Furthermore, the HT-29 cell line, CRCSC, and 13 normal colorectal tissue samples were prepared. The expression of Hiwi at mRNA and protein levels was determined by real-time polymerase chain reaction (Real-time PCR), flow cytometry, Western blot, and immunohistochemistry.

Results: The 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 was significantly higher in CRCSC compared to that in 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).

Conclusion: As evidenced by the obtained results**,** *Hiwi* can be considered an oncogene in progressive cancer and, therefore, can be a valuable biomarker and target in therapeutic procedures.

Keywords: Cancer stem cells, Colorectal cancer, *Hiwi* expression, IHC, Real-time PCR, Western blotting

1. Background

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 mainly results 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). The identification of molecules or associated risk factors involved in colorectal cancer development is valuable for early diagnosis and 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 that serve as tumor-initiating cells or tumorigenic cells (6, 7). The CSCs have a similar expression of cell surface markers pattern like normal stem cells. Furthermore, they have a self-renewing capacity and can be differentiated into various cancer cell 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. The *Piwi* families are important regulators of different physiological processes, such as germ cell differentiation, stem cell self-renewal and differentiation, spermatogenesis, and male infertility (8, 9). The expression of HIWI protein in spermatocytes and round spermatids during spermatogenesis indicates that this gene is critical for normal spermatogenesis.

Most *Hiwi*mRNAs 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 instance, 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 the determination or regulation of HSCs development.

Recent studies have pointed out that *Hiwi* is

ectopically expressed in all cancers (11-13). It has been shown to be upregulated 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). Nonetheless, 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 the overexpression of *Hiwi* in testicular seminoma, indicating that *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 the development, progression, and poorer diagnosis of various cancer forms (12,13). On the other hand, some studies found a 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 enhances 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 a potential target for cancer diagnosis and therapy since 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 the development of colorectal cancer.

2. Objectives

In light of the aforementioned issues, the present study aimed 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 were compared between different grades of primary human colorectal carcinoma, colorectal cancer stem cells (CRCSCs), and HT-29 CRC cell line.

3. Methods

3.1.Study population and sample collection

A total number of 20 patients (aged 21-46 years old) with colorectal cancer and 13 healthy individuals who were 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 checkups, and no abnormalities were found in physical examination or laboratory results after considering their biopsies. Moreover, they did not have a history of cancer or previous medical diseases. The control subjects and patients were matched in terms of age and gender. This cross-sectional study was approved by the institutional review board and Ethics Committee of Tehran University of Medical Sciences (Tehran, Iran). Written informed consent forms 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.

The inclusion criteria for patients were: (i) patients with known colorectal cancer and (ii) complete clinical and pathologic information. Patients who met the following criteria were excluded from the study: (i) the presence of tumors in other places, (ii) previous treatments such as chemotherapy or radiotherapy, (iii) a history of other chronic diseases, such as 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.

3.2. 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, and 100 µg/ml penicillin in a humidified incubator containing 5% CO_2 at 37°C.

3.3.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).

3.4. Stem cell surface marker phenotyping by flow cytometry

The 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).

3.5. Quantitative reverse transcription polymerase chain reaction

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 colorectal cancer biopsies vs. compliant normal tissues. More than a two-fold increase in expression was considered over-expression, while a more than two-fold decrease was regarded as under-expression. The range between those two values was interpreted as no change or regular expression. All experiments were performed in triplicate.

Table 1. Primer sequence for *Hiwi*

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

3.6. Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously described by Taubert et al. (11, 12). In brief, 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 three washes in PBS, slides were incubated with an HRP-linked anti-mouse secondary antibody for 30 min and then washed three times with PBS, followed by chromogen detection with DAB for 10 min and hematoxylin counterstaining.

3.7. Western blot analysis

Tissue samples were collected and lysed with standard RIPA buffer. After centrifugation at 40,000 *g* at 4°C for 45 min, the supernatant was subjected to SDS-PAGE analysis. Thereafter, 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 the 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 one hour, 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).

3.8. Statistical analysis

Data were analyzed using the SPSS software. Pearson's correlation assessed the correlation between *Hiwi* expression levels in various cancer stages and grades. The comparison of the gene expression between all groups was evaluated by ANOVA and Tukey test analysis. A p-value less than 0.05 was considered to be statistically significant.

4. Results

4.1. 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 illustrated In Figure 1, the morphological appearance of colorectal cancer stem cells ($CD44^+$) is different from the HT-29 cell line. Colorectal cancer stem cells ($CD44^+$) appeared as cuboidal-shaped cells with scant cytoplasm and granules around the nuclei (Figure. 1B). In comparison with 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, and CD90; nonetheless, they were negative

for CD45 and CD34.

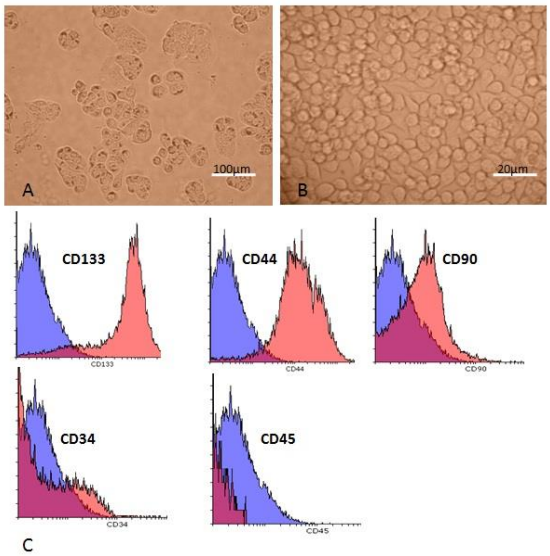

Figure 1. (A) Morphology of HEC-1B cell line, (B) invert microscopy image from CD44+ cancer stem cells, (C) positive expression of CD44, CD90, CD133, and negative for CD34 and CD45 in CD44 sorted cancer stem cell. The blue color line indicates the negative area of the diagram and red color line shows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

4.2. Real-time polymerase chain reaction 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 displayed 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.

4.3. Immunohistochemistry 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). The IHC analysis showed an increased expression of HIWI protein in colorectal tissues compared to normal colon tissues.

4.4. Western blot analysis

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

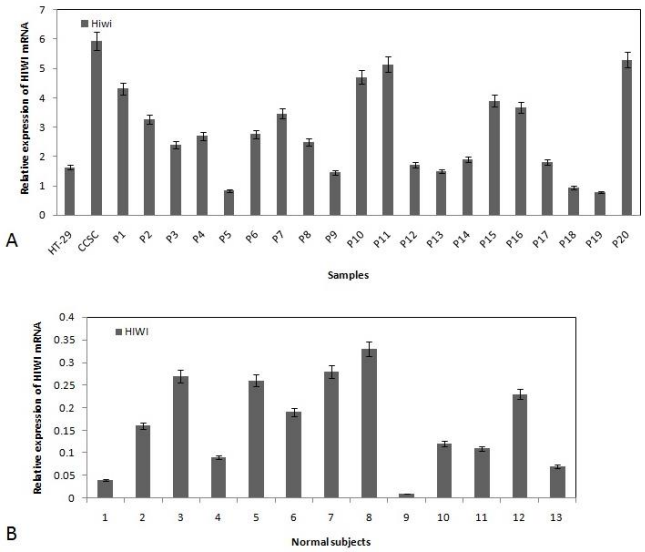

Figure 2. Real-time PCR results for comparing relative gene expression levels in colorectal cancer, HT-29 cell line, colorectal cancer stem cell, and normal colon samples. A: Bar graphs show relative expression of *Hiwi* mRNA in colon cancer (n = 20), $CD44^{+}$ CSC, and HT-29 cell line, B: normal colon (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 $P < 0.05$

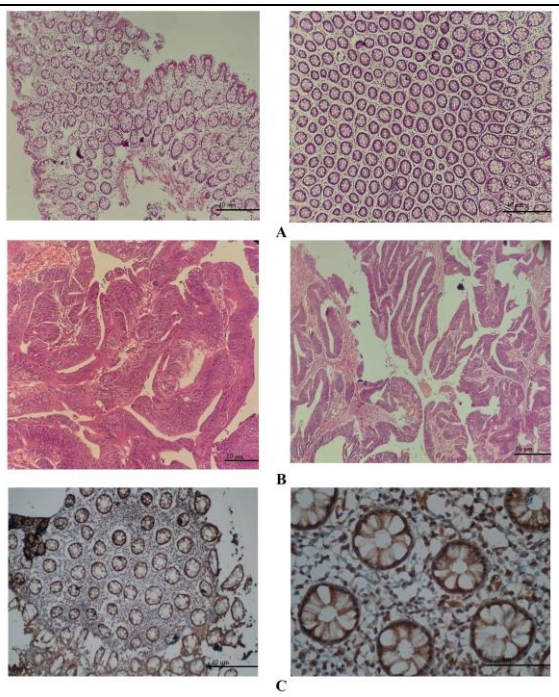

Figure 3. Sections of the normal colon (A) and colorectal cancer tissue (B) were stained with H&E
The presence of HIWI protein was detected by immunohistochemistry (C) in colorectal cancer tissue. Magnification: ×40

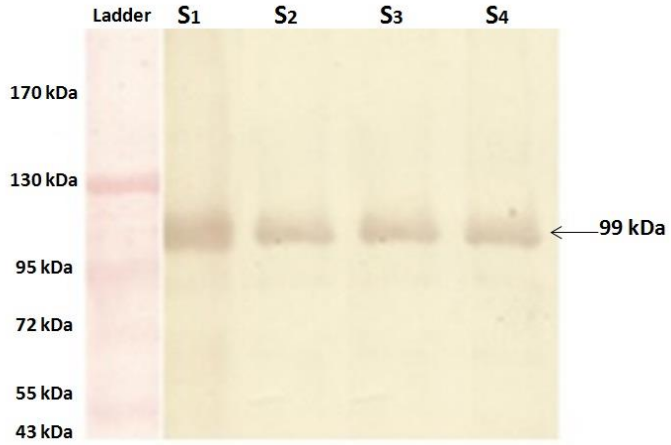

Figure 4. Overexpression of HIWI protein in colorectal cancer was detected by Western blotting. GAPDH was used as a loading control. S1 and S2, cancer samples (n = 2); S3, CD44 positive CSCs; S4, HT-29 colorectal cancer cell line

4.5. 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. The mean expression of *Hiwi* mRNA in colorectal tumor tissue was significantly higher than that in the healthy colon sample (P<0.001). There were statistically significant differences in *Hiwi* expression in the patient's group according to the disease stage and grade (Figure 5). A number of 10 (50%) patients were in stage I/II, and the others (50%) were in stages 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; Figure 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). It is noteworthy that 10 (50%) patients were in grade I, 5 patients (25%) were in grade II, and 5 patients (25%) were in grade III (Table 2). The mean expression of *Hiwi* mRNA levels in

grade I was significantly higher than that in patients with grades II and III (P<0.01; Figure 5).

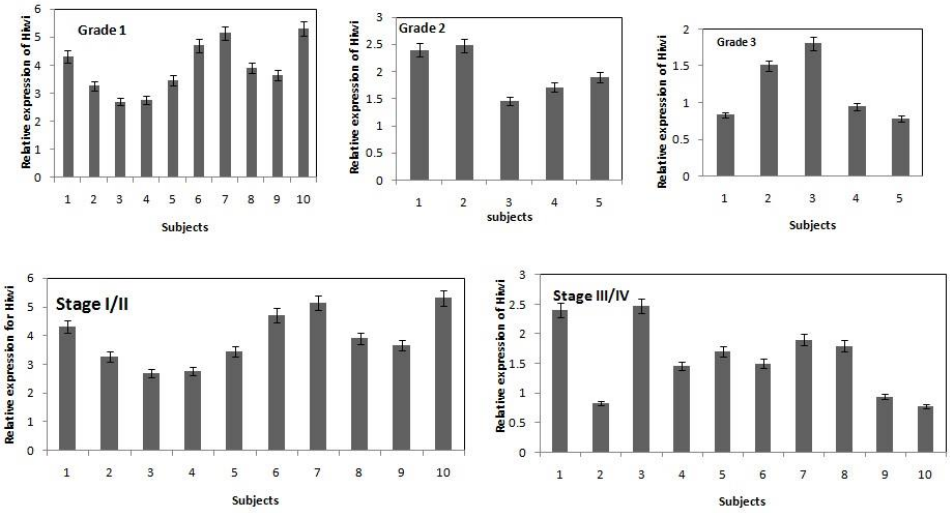

Figure 5. Ratios of *Hiwi* mRNA expression in colorectal cancer correlated best with the corresponding ratios normalized for GAPDH (R=0.674; P<0.001). Ratios of *Hiwi* mRNA expression were not associated with gender (men vs. women, P=1.0), tumor grade (P=0.0667), or stage (P=0.0514)

Table 2. Histological and clinical data

	Total (n)	High *Hiwi*(n)	Medium *Hiwi*(n)	Low *Hiwi* (n)
20	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)

5. Discussion

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. The overexpression of Hiwi was detected in clinical CRC tissue specimens. We also observed a significant increase in the expression of *Hiwi* in CRCSCs. *Hiwi* expression in CRCSC was significantly higher compared to the colorectal cancer tissue and HT-29 cells. Hiwi mRNA level was significantly correlated to tumor grade and stage.

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 for cancer stem cell differentiation into various cancer types. Recent studies have proposed that *Hiwi* plays a crucial role in cancer development and progression. Therefore, it can be considered a valuable target for cancer treatment. We found overexpression of *Hiwi* in 40% of clinical colorectal cancer specimens. In a similar vein, Raeisossadati et al. (23) reported overexpression of *Hiwi* gene in 34.8% of clinical colorectal cancer specimens.

In their study, Zeng et al. (24) found overexpression of HIWI protein in 25.6% of patients with colorectal cancer. These findings are comparable with the results of our study. We also found that the relative expression of *Hiwi* at mRNA and protein levels was significantly higher in patients with colorectal cancer than in healthy individuals. 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 levels 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 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 rates compared to patients with negative *Hiwi* expression. Litwin et al. (25) found an 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 the up-regulation of *Hiwi* is associated with the clinical features of cancerous patients (12,13,26). In a similar vein, we found that colorectal cancer disease stages and degrees are significantly correlated with *Hiwi* expression patterns.

Our data also revealed that *Hiwi* is upregulated in colorectal cancer cells, and its function is altered in an aberrant cancer state. Therefore, *Hiwi* can be considered 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 an 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 lines. In addition, they found that an 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 an increased expression of *Hiwi* in the cytoplasm of esophageal cancer cells, which was associated with histological grade, disease stage, and clinical outcome (15). We also found an 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. (18) 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 the proliferation of colorectal cancer cells through the upregulation of global DNA methylation (28).

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

6. Conclusion

In summary, the results of this study pointed out 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 in colorectal cancer tissues than in normal specimens. It was also detected that colorectal cancer stage and degree were correlated with *Hiwi* expression. These results suggest that *Hiwi* may be involved in the development and progression of colorectal cancer and can be considered a prognosis factor for the early diagnosis of this cancer. Furthermore, the oncogenic role of *Hiwi* indicates that it may be a potential target for cancer therapy. Future studies are merit to discover the underlying mechanisms by which *Hiwi* promotes colorectal cancer development.

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Footnotes

Conflicts of Interest: The authors declare that they have no conflict of interest.
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