Correlation of HIWI and HILI Expression with Cancer Stem Cell Markers in Colorectal Cancer

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Abstract. Background/Aim: Cancer stem cells (CSCs) constitute a sub-population of tumor cells that possess stem cell properties, such as self-renewal and the ability of differentiation. The presence of CSCs is associated with metastatic potential, treatment resistance and poor patient prognosis. Recently, aberrant expression of P-element induced wimpy testis proteins-PIWI (HIWI and HILI) has been identified in various types of tumors. The aim of the study was to evaluate the clinical significance of the HIWI and HILI expression and its relationship with cancer stem cells markers in 72 patients with colorectal carcinoma (CRC). Materials and Methods: The expression level of HIWI and HILI and cancer stem cells markers in paired cancerous and non-cancerous tissues was measured by real-time reverse transcription-polymerase chain reaction (RT-PCR) assay. Immunohistochemistry was performed to confirm the observed changes on mRNA level and detect tissue localization of PIWI proteins. Results: Significantly higher mRNA levels of HIWI and decreased HILI mRNA were measured in colorectal cancer tissues compared to corresponding non-cancerous samples. The changes in HIWI mRNA level in cancer tissues were correlated with OCT4 expression. Positive correlations between HILI level and SOX2 were also observed in cancerous tissues. Conclusion: Our results indicate a reciprocal regulation between HIWI, HILI and some CSCs markers in colorectal cancer.

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Cancer contains a large population of genetically and epigenetically heterogeneous individual cells with different molecular and phenotypic characteristics and proliferative potential. The heterogeneity of the tumor cells within the cancer led to the concept of the existence of “cancer stem cells” (CSCs) or “tumor-initiating cells” (TICs), which present epigenetic changes and signaling pathways characteristic for stem cells, such as self-renewal capacity, fast proliferation and multilineage differentiation (1). The presence of chemotherapy-resistant cancer stem cells is considered as a primary cause for tumor recurrence (2). So far, the most widely used method to identify cancer stem cells is through their expression of particular cell surface markers, called clusters of differentiation (CD) (3). Although none of these markers identified to date are expressed exclusively by CSCs, many are also present in non-stem cells tumors and normal tissues. Therefore, characterization of the genetic and epigenetic changes that occur in cancer stem cells will provide important insights into the understanding over the processes of cancer development and metastasis. To date, some key signalling pathways have been identified, which may be aberrantly regulated in CSCs and, thus, may represent potential targets for future cancer diagnostics and therapies (4). Many studies indicate that several fundamental signaling pathways and transcription factors are responsible for the regulation of pluripotency and self-renewal of embryonic stem cells. Expression of these factors, called embryonic stem cells markers, is restricted to pluripotent stem cells and down-regulated during embryonic development (5). Based on recent studies, there might be a positive involvement of some transcription factors like OCT4, NANOG and SOX2 during tumour development and progression (6-8). However, the functional characteristics of embryonic stem cell markers’ co-expression in colorectal cancer need to be further examined.
In various cancers, besides genetic and epigenetic aberrations, changes in the expression of certain small non-coding RNAs (ncRNAs) are observed. All these changes drive the transformation of normal cells into highly malignant tumors consisting of neoplastic cells with metastatic potential and unlimited proliferation capacities (9, 10). In recent years systematically increasing interest in the biology of non-coding RNA has been observed. To date, three major classes of small regulatory RNAs have been identified: microRNAs (miRNA), short-interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs). The novel pathway plays a crucial role during the regulation of target genes and the housekeeping gene (GAPDH). All transcripts (HIWI and HILI) in colorectal cancer. The aim of these studies was to evaluate the expression profiles of the PIWI transcripts (HIWI and HILI) in correlation with cancer stem cell markers and to determine their possible prognostic significance in colorectal cancer.

**Materials and Methods**

**Clinical data collection.** Paired tissue specimens (tumor and adjacent non-cancerous samples) were obtained from 72 patients with colorectal cancer operated on at the Regional Specialist Hospital in Wroclaw, Research and Development Centre, between 2011 and 2014. The study protocol was approved by the Medical Ethics Committee of Regional Specialised Hospital, Research and Development Centre. The study was conducted in accordance with the Helsinki Declaration of 1975 and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. All samples were derived before any therapeutic procedure, including preoperative chemotherapy or radiotherapy. The fresh tissue samples were collected in RNAlater solution (Sigma, Saint Louis, MO, USA), frozen and stored at −80°C until use. All tumor and normal samples were submitted to histopathological analysis. The subjects comprised of 38 males and 34 females with ages ranging from 29 to 88 years. The clinical data of patients were reviewed according to the TNM classification for colorectal cancer (20). The patients’ clinicopathological data are listed in Table I.

Paraffin blocks of normal colorectal samples were obtained from the archives of the Department of Histology and Embryology, Wroclaw Medical University and were used for the immunohistochemistry (IHC) studies.

**Quantitative real-time RT-PCR assay.** Total RNA was extracted from tumor and non-cancerous adjacent tissues by homogenizing tissues in Trizol reagent (Sigma) and the NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. The concentration and quality of isolated RNA was measured in the NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For the reverse transcription reaction, 1μg of RNA was used. The reverse transcription reaction was performed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) according to the procedure of the manufacturer. The transcribed cDNA was used for the subsequent real time PCR using TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression Assays (Life Technologies, Carlsbad, CA, USA): Hs01041737_s1 specific for human HIWI (PIWIL1), Hs010532720_m1 for HILI (PIWIL2), Hs04260367_g1 for Oct4 (Pou5F1), Hs04260366_g1 for NANOG, Hs01053049_s1 for SOX2 and Hs03929907_g1 for GAPDH. All target genes and the housekeeping gene (GAPDH) were run simultaneously and reactions were carried-out in triplicate wells on a 96-well optical reaction plate in 20 μl reaction volume, using the StepOnePlus Real Time PCR System (Life Technologies, Carlsbad, CA, USA). For quantification, the samples were normalized against the expression of GAPDH mRNA. Relative quantification (RQ) of mRNA for the examined genes was calculated using the Pfaffl’s (2001) method (21).

**Immunohistochemistry.** IHC was performed on 4-μm formalin-fixed paraffin-embedded (FFPE) sections from human colorectal...
cancer. The slides were de-paraffinized, rehydrated and antigen retrieval was carried out by boiling the sections in EnVision™ FLEX Target Retrieval Solution high pH using a PTLink (Agilent Technologies, Dako, Glostrup, Denmark). All reactions were performed using an automated immunostainer device Autostainer Link 48 (Dako). Activity of endogenous peroxidase was blocked by 5 min exposure to EnVision™ FLEX Peroxidase-Blocking Reagent. Polyclonal goat anti-human PIWIL1 (HIWI, 1:50 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), polyclonal goat anti-human PIWIL2 (1:100 dilution; Santa Cruz Biotechnology) were used as the primary antibodies. Tested sections were incubated with primary antibodies 20 min at room temperature, followed by incubation with secondary antibody conjugated with horseradish peroxidase (EnVision™ FLEX/HRP; Agilent Technologies, Dako, Glostrup, Denmark) for 20 min. The reactions were visualized using freshly prepared substrate for horseradish peroxidase (diaminobenzidine, EnVision™ FLEX Working Solution), with incubation for 10 min. Additionally, all slides were counterstained using EnVision™ FLEX Hematoxylin for 5 min. After dehydration in graded ethanol concentrations (70%, 96%, absolute) and in xylene, all slides were coverslipped in SUB-X Mounting Medium (Agilent Technologies, Dako, Glostrup, Denmark). Primary antibodies were diluted in EnVisio™ FLEX antibody diluent.

Statistical analysis. Statistical analysis was performed with R for Windows, version 3.0 (The R Foundation for Statistical Computing, Vienna, Austria). The statistical analysis results were evaluated using the Wilcoxon test for paired data. Correlations between variables were calculated by the Spearman’s correlation coefficients. The results were considered to be statistically significant at \( p<0.05 \).
Results

The level of HIWI, HIWI and CSC-associated gene expression. Real-time PCR analysis was performed to quantify the mRNA expression levels of HIWI, HILI and CSCs markers in colorectal cancer and non-cancerous tissues. As presented in Figure 1A, significantly higher transcript levels of HIWI mRNA were measured in 51 out of 72 colorectal cancer tissues compared to adjacent non-cancerous samples. The mean expression level of HIWI transcripts in colorectal cancer tissues was 30.112 (median=2.321), whereas in non-cancerous the mean was 1.72 (median=0.321). In 13 non-cancerous tissue samples the mRNA level was undetectable, while in paired cancerous samples the HIWI expression was highly elevated. On the other hand, the expression of HILI was significantly decreased in 35 out of 72 cancer tissues compared to corresponding normal tissue samples (Figure 1B). The mean expression of HILI mRNA level was 1.48 (median=0.344) in cancer tissues and 2.48 (median=0.724) in the paired normal one. Statistical analysis using the Wilcoxon test revealed that the observed changes in the HIWI and HILI mRNA expression were statistically significant.

Levels of HIWI and HILI mRNA were compared against three genes characteristic for cancer stem cells. The expression levels of OCT4, NANOG and SOX2 in colorectal cancer tissues and adjacent non-cancerous samples were investigated. As presented in Figure 1C, the level of OCT4 transcript was significantly higher in colorectal cancer tissues (mean=1.784; median=1.243) compared to adjacent non-cancerous samples (mean=1.29; median=0.993). Statistically significant lower expressions SOX2 were measured in colorectal cancer tissues compared to adjacent non-cancerous samples (Figure 1D). The mean expression of SOX2 mRNA level was 0.717 (median=0.115) in cancer tissues and 2.695 (median=1.334) in the paired normal one. There was no significant change in the mRNA level of NANOG (Figure 1E).

Relationship of gene expression between HIWI, HILI and cancer stem cell marker levels. The gene expression results obtained by quantification of the mRNA levels were used to perform non-parametric Spearman correlations (Tables II and III).

In non-cancerous colorectal tissues, statistically significant negative correlations were observed between HIWI and HILI (\(p=0.025\)), HIWI and SOX2 (\(p=0.0004\)) and OCT4 and SOX2 expression (\(p=0.0005\)). A strong positive correlation was observed between HILI and SOX2 expression (\(p=0.0097\)).

As presented in Table III in colorectal cancer tissue, a positive correlation between HIWI expression and OCT4 (\(p=0.003\)). Negative correlations between SOX2 and NANOG markers were also described in cancer tissue samples (\(p=0.0027\)) (Table III). A moderate positive correlation was observed between OCT4 and SOX2 expression in both non-cancerous (\(p=0.0008\)) and cancerous tissues (\(p=0.0001\)) (Table II and III). High HIWI expression in colorectal cancer is associated with high level of one of the CSCs marker OCT4 and low expression of HILI and SOX2. These observations may indicate reciprocal regulation between HIWI, HILI and some CSCs markers in colorectal cancer.

Immunohistochemical analysis of the HIWI and HILI in colorectal cancer. The protein expression level of HIWI and HILI in representative FFPE sections of colorectal normal and cancer samples were analyzed using immunohistochemistry. In all cases, hematoxylin-eosin (H&E) staining was performed in order to determine tumor grade (G) and to evaluate the extent of necrosis in the tumors. Absence or weak HIWI staining was noted in non-cancerous tissue
HIWI and HILI proteins (PIWIL1, 2), members of the Argonaute family of proteins, are exclusively expressed in the testis and have been found to be critical for spermatogenesis, stem cell self-renewal and retrotransposon silencing (17). While the functions of PIWI in germline stem cells have been extensively studied, several lines of evidence have indicated that human PIWI proteins, HIWI and HILI, are aberrantly expressed in various types of cancers (18). Based mainly on immunohistochemical studies, the increased expression of HIWI has been detected in ovarian, oesophageal, pancreatic, gastric and hepatocellular carcinoma, and correlated with the histological grade of the tumour, clinical stage and poorer clinical outcomes for patients (23-27). Positive staining of HIWI in colorectal cancer tissue or matched adjacent non-cancerous tissue has been described as a poor prognostic factor for the colorectal patient (28). Our research confirms the up-regulated level of HIWI in colorectal cancer tissue compared to adjacent non-cancerous samples. In many non-cancerous tissues HIWI was undetectable, while in paired cancerous samples the HIWI expression was highly elevated. Our findings are consistent with several previous observations by other authors (28, 29). Furthermore, significant correlation between high HIWI expression and lymph node and distant metastasis occurrence were observed.

Expression of HIWI and HILI and relationship with clinical characteristics. High expression level of HIWI in cases of lymph node and distant metastasis was detected, but the difference was not statistically significant \( (p=0.73 \text{ and } p=0.08, \text{ respectively}) \) (Figure 3A and B). No relationships were found between expression levels of HIWI, HILI and CSCs markers and patient sex, age, grade of differentiation and T classification \( (p=\text{NS}, \text{ non-significant}) \).

Discussion

HIWI and HILI proteins (PIWIL1, 2), members of the Argonaute family of proteins, are exclusively expressed in the testis and have been found to be critical for spermatogenesis, stem cell self-renewal and retrotransposon silencing (17). While the functions of PIWI in germline stem cells have been extensively studied, several lines of evidence have indicated that human PIWI proteins, HIWI and HILI, are aberrantly expressed in various types of cancers (18). Based mainly on immunohistochemical studies, the increased expression of HIWI has been detected in ovarian, oesophageal, pancreatic, gastric and hepatocellular carcinoma, and correlated with the histological grade of the tumour, clinical stage and poorer clinical outcomes for patients (23-27). Positive staining of HIWI in colorectal cancer tissue or matched adjacent non-cancerous tissue has been described as a poor prognostic factor for the colorectal patient (28). Our research confirms the up-regulated level of HIWI in colorectal cancer tissue compared to adjacent non-cancerous samples. In many non-cancerous tissues HIWI was undetectable, while in paired cancerous samples the HIWI expression was highly elevated. Our findings are consistent with several previous observations by other authors (28, 29). Furthermore, significant correlation between high HIWI expression and lymph node and distant metastasis occurrence were observed.
Recent data indicated that HILI (PIWIL2) might also play an important role in tumor development. HILI expression has been observed in various stages of breast cancer and its expression was associated with ER and Ki-67, as well as cancer progression (29). Similar observations were made for cervical cancer (30) and colon cancer (31). Enhanced expression of the HILI gene was reported by Lee et al. (32) in prostate, breast, gastrointestinal, ovarian and endometrial cancer. High levels of HILI have also been observed in testicular seminomas, but not in testicular non-seminoma tumors (32). The authors suggest that HILI acts as an oncogene by inhibiting apoptosis and promoting cell proliferation through the STAT/Bcl-XL signalling pathway (32). A significantly higher expression of HILI was observed in primary colon cancer tissue and lymph node metastasis in comparison with normal colon tissue. A high level of HILI was correlated with a lower degree of differentiation of the tumor, invasion, and poorer overall survival (32, 33). Moreover, HILI-knockdown in colon cancer cells significantly inhibited proliferation, migration and colony formation, increased apoptosis in vitro and reduced tumour growth in vivo (33). In contrast, we observed significantly decreased HILI mRNA levels in colon cancer tissues compared to corresponding normal tissue samples. Decreased levels of HILI were determined in 35 out of 72 cancer tissue samples. Moreover, a strong negative correlation between HIWI and HILI was observed in colorectal normal tissues. Similarly, Nikpour et al. (34) described the absence of HILI expression in several bladder carcinoma cell lines and bladder cancer tissues. The authors suggest that ectopic expression of HILI is not essential for the pathogenesis of human bladder carcinoma (34). We state that the lack of HILI expression in the examined colon cancer tissues does not directly indicate that HILI is not critical for cancer development but may indicate reciprocal regulation between HIWI and HILI in colon cancer. Therefore, further analysis of PIWI gene expression, at both mRNA and protein level, would provide a novel insight into the mechanisms of cancer development.

Recent studies have shown a direct connection between stem cell self-renewal and cancer development (35). However, the potential connection between cancer stem cells, e.g. in colon cancer and piRNAs-PIWI mechanism, remains unexplored. The hypothesis of the stem cell model of cancerogenesis states that PIWI acts as an oncogene whose expression is re-activated in cancer cells and, therefore, up-regulated levels of PIWI proteins contribute to the development of cancer (36). There exist numerous data showing changes in the expression of embryonic stem cell (ESC)-associated proteins in many types of cancers (37). OCT4, a transcription factor of the POU protein family, expressed in both embryonic and adult stem cells, has been suggested to be associated with the pluripotency, proliferative potential and self-renewal of embryonic stem cells and germ cells (38). NANOG, a downstream target of OCT4, which contributes to cell fate determination of the pluripotent inner cell mass during embryonic development, is also specifically expressed in human embryonic pluripotent stem cells (39). Patients with co-expression of OCT4 and NANOG have been shown to have significantly worse overall survival and poor prognosis in several malignancies, including oral, glioma, gastric and hepatocellular cancer (40-43). Over-expression of NANOG was strongly correlated with poor prognosis, lymph node metastasis and Dukes classification of colorectal cancer (43). SOX2, along with OCT4 and NANOG, plays a crucial role in colorectal cancer.
role in the maintenance of embryonic stem cell pluripotency. A number of studies have demonstrated that SOX2 is involved in promoting tumorigenesis, proliferation and dedifferentiation of human lung squamous cell carcinoma and breast cancer (44, 45). Changes in the SOX2 expression have also been observed in pancreatic intraepithelial neoplasia, gastric and prostate cancers. These changes were correlated with the invasion and high metastatic capacity of the tumor (46). In spite of the above-mentioned data, in our study, changes in the NANOG expression correlated with the level of OCT4, both in cancer and normal tissue samples were observed. Therefore, we state that these genes cannot be exclusive markers of colorectal cancer cells. However, a positive correlation was observed between HIWI and OCT4 mRNA level, as well as HILI and SOX2 in cancer tissue samples. Although changes in the expression of HILI and SOX2 are not consistent with some literature data, this may indicate a different regulation of these genes during the development of colorectal cancer, characterized simultaneously by high expression of the HIWI gene. Aberrant expression of HIWI and HILI showed a strong prognostic value, suggesting the PIWI gene as a stem cell-associated gene, which may have an impact on the metastatic potential determination. However, the experiments presented in this paper point to the importance of the interaction of different cancer stem cell markers and PIWI genes on cancer development.

Conflicts of Interest

The Authors declare that they have no conflict of interest.

References


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