

## RESEARCH ARTICLE

Editorial Process: Submission:02/05/2024 Acceptance:06/10/2024

# HEPPAR1 and PIWIL2 as Panel Markers for Hepatocellular Carcinoma

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### Abstract

**Objective:** The aim of this study was to evaluate the expression profiles of PIWI-like protein- 2 (PIWIL2), and HepPar1 and their immunohistochemical (IHC) characteristics in Hepatocellular Carcinoma (HCC), and determine their correlation with clinicopathological parameters of this type of cancer to determine their diagnostic value in combination. **Methods:** Seventy-five patients with HCC were assessed for the expression of PIWIL2 in serum and tissue using real-time polymerase chain reaction (RT-PCR) and IHC was performed for PIWIL2 and HepPar1 was performed on all patients. **Results:** A statistically significantly higher level of PIWIL2 was found in HCC compared to controls ( $p \leq 0.001$ ). Both HepPar1 and PIWIL2 were detected in 84% of HCC cases, the diagnostic and prognostic factors for PIWIL2 were found to be significant in liver tumour tissue samples and non-tumorous sections  $p < 0.001$ , and the same was observed for serum samples and results of healthy serum controls ( $p < 0.001$ ) when compared to AFP. **Conclusion:** Our results affirm the hypothesis that reactivation of PIWI expression in various cancer types is crucial for cancer development, and that a possible panel maybe used for these markers HCC diagnosis.

**Keywords:** HCC- PIWIL2- HEPPAR1- RT-QPCR- Diagnosis- Prognosis

*Asian Pac J Cancer Prev*, **25** (6), 2123-2131

### Introduction

Hepatocellular Carcinoma (HCC) is a form of cancer that frequently affects individuals with chronic liver disease. It has a reputation for its aggression and high mortality rates. Hepatitis B or C infection, alcoholic liver disease, and non-alcoholic fatty liver disease are significant risk factors for HCC [1–3]. In the diagnosis, prognosis, treatment, and progression of HCC, biomarkers have played a significant role. Biomarkers for HCC may be classified as serological, genetic, or epigenetic. They can aid in the early detection, risk assessment, selection of treatment, and surveillance of HCC. Among the members of the PIWI/AGO protein family, is P-element-induced wimpy testis-like protein 2 (PIWIL2). PIWIL2 regulates stem cell self-renewal and germline development as its primary function. New evidence indicates, however, that it plays a function in carcinogenesis, including HCC [4]. According to studies, aberrant PIWIL2 expression in HCC tissues is associated with aggressive tumour characteristics and a poor prognosis [5]. PIWIL2 may promote tumour development, invasion, and metastasis by modulating diverse signalling pathways and regulating

gene expression. As such it has the potential to be utilised as a diagnostic biomarker and therapeutic target in the treatment of HCC [6–9]. HepPar1 (Hepatocellular carcinoma-associated protein 1) is an intracellular protein that is present in HCC tissues but not in normal liver cells. Because its expression levels increase with tumour growth, HepPar1 has previously shown promise as an early-stage HCC diagnostic biomarker [10–12]. In addition, HepPar1 has been linked to a poor prognosis and increased HCC recurrence rates. Subsequently, we predict that both PIWIL2 and HepPar1 could be promising biomarker candidates for HCC research. Understanding their functions and mechanisms in HCC will aid in the development of improved diagnostic tests, prognostic models, and targeted therapeutics for patients with this aggressive form of liver cancer, leading to improved patient outcomes [5, 13–15]. As HCC biomarkers, the combination of HepPar1 and PIWIL2 has received little attention. In cancer research, however, the concept of combining multiple indicators is acquiring popularity [5, 16–18]. The incorporation of multiple biomarkers into the diagnosis, prognosis, and therapy monitoring of HCC has the potential to increase precision and reliability. The

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feasibility and potential synergistic benefits of combining HepPar1 and PIWIL2 as HCC biomarkers require further investigation. Extensive studies in larger patient cohorts to examine their expression patterns, diagnostic accuracy, and clinical value would yield valuable insights into their collective efficacy as HCC biomarkers, which is the aim of this study.

## Materials and Methods

### *Subjects and Sampling*

The present research was conducted in Egypt at Theodor Bilharz Research Institute (TBRI). Institutional ethical approval was acquired by the TBRI research ethics committee (REC), (FWA00010609, Serial#788). The investigation was conducted in accordance with the 2013 Guidelines of the Helsinki Declaration on the Protection of Human Subjects. Seventy-five patients undergoing liver resection, tumour and nontumorous sections that are tumour-adjacent tissue were collected as well as 5 millilitres of blood samples, 50 healthy volunteers provided blood samples as well. Participants with autoimmune hepatitis, hemochromatosis, schistosomiasis, or HIV, as well as those with ischemic heart disease, were excluded in addition to those who had received immunomodulatory interferon therapy for HCV. All cases' clinical histories, pathology reports, and haematoxylin and eosin (H&E) stained slides were reviewed to confirm the diagnosis. To determine the histologic grade of HCC, the METAVIR scoring was conducted.

### *Sample Processing*

Blood samples were left to coagulate, and then they were centrifuged at 500 xg for 10 minutes, and serum was extracted, centrifuged, aliquoted, and kept at -80°C. One significant advantage of serum is its ease of collection and storage. Furthermore, serum is widely utilised in diagnostic procedures such as serological testing and biomarker discovery, making it a familiar and standardised candidate for the current study. Tumorous and non-tumorous liver slices were kept in lysis buffer at -80°C until use.

### *RNA Extraction*

Utilising the miRNeasy extraction reagent (Qiagen, Valencia, CA), total RNA was extracted. The extraction technique was carried out according to the manufacturer's instructions for both tissue and serum samples. Before extraction, approximately 70 mg of tissue was homogenised with lysis solution and 200 µl of serum was utilised. Using a NanoDrop-1000c spectrophotometer (Thermo Fisher Scientific, Cinisello Balsamo, Italy), the purity and concentration of duplicate samples were determined following the extraction.

### *qRT-PCR analysis of PIWIL2*

The PIWIL2 primer assays was purchased from Qiagen, and all experiments were conducted in accordance with the manufacturer's instructions. Real-time PCR amplification was performed with QuantiTect SYBR Green PCR Kits (Qiagen, Valencia, CA). Five microliters of RNA isolated

from serum and tissue samples were reverse transcribed utilising the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). After completing the synthesis of the first strand of cDNA, 5 µl of the material was utilised for real time PCR amplification. Each reaction was performed in triplicates. Cycle Threshold (CT) method was used to ascertain the relative expression of mRNAs in each sample.

### *Immunohistochemical staining*

The procedures were conducted in accordance with the manufacturer's instructions (Novus Biologicals, LLC., USA), NBP2-24590 PIWIL2/HILI and NBP2-45272 HepPar1 Antibody kits. Rabbit polyclonal antibody against HILI protein of human origin, at a dilution of 1:200 was used, and a mouse monoclonal antibody against HepPar1, was analysed, in four-micron thick sections of formalin-fixed, paraffin-embedded tissue blocks from all the studied cases. Deparaffinization and hydration in xylene and decreasing alcohol grades were conducted for all tissue sections. After rinsing the tissue segments in PBS with pH 6.0, citrate buffer was applied for 10 minutes in a 700-watt microwave. The peroxidase activity was inhibited by incubating the transparencies for 5 to 10 minutes in 3% hydrogen peroxide, followed by a wash buffer. The primary antibody (PIWIL2 or HepPar1) was incubated at room temperature for one hour. The antibody reaction was detected using DakoEnVision™ FLEX HRP (Horseradish peroxidase) (CAT# SM802) and diaminobenzidine (DAB) as the chromogen. Before being examined under a microscope, the sections were counterstained for 15 seconds with haematoxylin. Positive controls consisted of normal liver tissues, whereas negative controls consisted of the same tissue (normal liver) minus the primary antibody.

### *Evaluation of immunostaining for PIWIL2/HILI & HepPar1*

Two pathologists with no prior clinical knowledge independently analysed the expression of PIWIL2/HILI and HepPar1 in every segment of the tumour. Calculations were performed on the percentage of positive cells, cellular location, and intensity of staining. The Histoscore (H-score) method with a range of 0-300 was utilised to quantify PIWIL2/HILI expression. Elwy et al. [19] determined the H-score using a semi-quantitative analysis of both the intensity (classified as negative (0), weak (1+), moderate (2+), and intense (3+)) and the percentage of positive cells.  $H\text{-score} = 1 \times (\text{percentage of positive cells}) + 2 \times (\text{percentage of positive cells}) + 3 \times (\text{percentage of positive cells})$ . Each instance was assigned an H-score, and the mean score for each group was evaluated [19].

### *Statistical analysis*

Statistical analysis was conducted using SPSS 2016 (Statistical Package for the Social Sciences), A sample size calculation was done to determine the appropriate number of patients, which was based on previous research projects involving HCC diagnosis, and the power test was also carried out to confirm the number of participants.

The continuous variables were described as mean

standard deviation (SD) or median and interquartile range (IQR) according to their distribution, which was evaluated using a normality test. For categorical variables, we used frequencies and percentages. A  $p$  value of 0.05 was used to define statistical significance. The Mann-Whitney U test was utilised to compare means. The  $X^2$  test or Fisher's exact test was employed to determine the distribution of categorical variables between groups.

## Results

Our cohort was well-balanced in terms of sex, and the mean age of patients was 57 years. In terms of association, a significance was detected for the expression of PIWIL2 and the age of patients  $p < 0.05$ . A total of 75 patients, were assessed for immunohistochemistry. The tissue samples were categorized as HCV (for nontumorous tissues), HCC (tumorous tissues). For HCC patients, and their pathological diagnosis, in terms of tumour grade, 10(20%) were diagnosed Low Grade 44%, and 56% were diagnosed as High Grade of carcinoma. The nontumorous samples of the HCC patients had the following characteristics, the hepatitis activity index (HAI) was found as (20%) for 0, (32%) for 1, (28%) at 2, and (20%) at 3. In terms of Fibrosis, (20%) were 0, (4%) were 1, (24%) were 2, (16%) were 3, and (36%) were 4. Steatosis was found to have a median and IQR of 50(10 – 77.5) (Table 1). Findings were assessed via METAVIR scoring, which defines stages according to fibrosis level, with end stage being cirrhosis, and grade according to activity level i.e., inflammatory or immune reaction A0-A3 meaning no reaction to severe inflammation. HCC grading additionally has patterns in hepatology, such as acinar, insular, sarcomatous (spindle), inflammatory etc... The expression values were analysed via Kruskal Wallis Test for association with fibrosis of tissue, and the results showed a significant association between PIWIL2 expression with liver fibrosis  $p < 0.001$  (Table 1) for HCC patients. Moreover, an association was found for tumour grades and expression of PIWIL2  $p < 0.001$  (Table 1), and tumour patterns  $p < 0.05$ .

### *PIWIL2 mRNA expression and Diagnostic performance for HCC*

Figure 1 A, showed that mRNA levels for PIWIL2 were upregulated both in HCC serum samples compared to controls and also an upregulation was observed in tumour tissue samples compared to nontumorous, while Figure 1 B, showed the Receiver Operating Characteristic (ROC) curve of PIWIL2 in the studied samples. Figure 1 C revealed an increased concentration of PIWIL2 in HCC serum samples compared to normal serum samples of the healthy volunteers. ROC curve for AFP measured by ELISA in HCC serum samples was illustrated in Figure 1 D.

Table 2, showed that the diagnostic performance of serum PIWIL2 expression was with sensitivity of 100% and specificity of 100%, with an area under the curve (AUC) of 1 ( $p < 0.001$ , 95% Confidence Interval, 95% C.I = 1.0 – 1.0), a sensitivity of 96%, specificity of 60%, an AUC of 0.8 ( $p < 0.001$ , 95% C.I = 0.72 – 0.88) was found for tumour tissue PIWIL2 expression samples. AFP was

measured using ELISA, the diagnostic performance had sensitivity of 84%, specificity of 64% with an AUC of 0.828 ( $p < 0.001$ , 95% C.I = 0.746 - 0.91).

### *Immunohistochemistry*

For PIWIL2 and HepPar1, liver tissue sections in cases of hepatitis C (HCV) showed an increase in the level of immunohistochemical expression of both PIWIL2 and Hep-Par1 with increasing hepatitis activity index and stage of fibrosis. There was also an increase in their expression in the high grades of HCC compared to low grade tumours (Table 3, Figures 2&3). Sixty three of 70 (84%) were positive cases in HCC for both PIWIL2 and HepPar1 expression, representative cases with diffuse and focal positive staining for both markers are shown in Figure 2 for PIWIL2 and Figure 3 for HepPar1. Most cases were positive for both of the studied markers as shown in Table 3, and were both found to be significant  $p < 0.001$ ,  $p < 0.05$  for PIWIL2 and HepPar1 respectively. For distinguishing positive cells, HepPar1 intensity was also found to be significant  $p < 0.01$ , however PIWIL2 intensity score was not significant. Table 4 indicates the diagnostic accuracy for PIWIL2 and HepPar1 through the percentage of positive counts, the highest accuracy was found for the usage of both markers at 89.4%, in addition to specificity and positive predictive value (PPV), and specificity scoring at 100% when both markers were found.

## Discussion

PIWI proteins formulate along with a piRNA dependent or independent complex to regulate gene expression at the epigenetic post-transcriptional stage. PIWI proteins were previously discovered as essential factors for germline development, stem cell self-renewal, and gametogenesis in germline cells [13, 20]. Distinct PIWI types, as well as distinct piRNAs, have been found to be expressed abnormally at the mRNA and protein levels in tumours [14, 20–22]. PIWIL2 expression has been found to be elevated in a several cancers, including breast, cervical, gastric, ovarian, prostate, and colorectal [14, 23, 24]. For colon and bladder cancer, there was a statistically significant rise in PIWIL2 [25–27]. PIWIL4 expression was shown to be higher in renal cancer than in other members of the PIWIL protein family. Furthermore, statistically significant down-regulation of PIWIL2 and PIWIL4 in breast tumour tissues was described [15]. It has been postulated that reactivation of diverse PIWI proteins is critical for cancer growth and progression; thus, PIWI proteins are very likely to be essential markers for the advancement of various cancer types. However, the precise process driving the alterations in PIWI protein expression remains unknown. The detection of piRNAs in blood or cancer tissues could be a reliable tool for detecting circulating or cancer stem cells [28, 29]. Martinez et al. [14] described the somatic and malignant expression patterns of a variety of piRNAs, and they found that numerous piRNAs were found to be overexpressed in tumour tissues and were linked to cancer malignancy and clinical characteristics [14]. Although piRNA-PIWI complexes are detected in somatic

Table 1. Demographics and Clinico-Pathological Characteristics for HCC Patients and Their Association with *PIWIL2* Expression

Clinico-pathological characteristics	Total number of patients N=75 (%)	Association with expression PIWIL2	Significance P<0.05
Age (Mean±SD)	57.2±8.1	-0.289	0.042*
Sex			
Female	33 (44.0)	0.047	0.741
Male	42 (56.0)	0.072	0.35
ALT			
Up to 40 U/L	61.4±15.5	-0.158	0.274
AST			
Up to 40 U/L	65.8±16.6	-0.118	0.414
Alb			
Up to 5.5 g/dL	2.3±1.0	0.076	0.601
Bilirubin			
0.3-1.2mg/dL	2.9±1.1	0.047	0.745
AFP			
Up to 11	75.0 (40.0- 150.0)	-0.027	0.86
No. of masses	1.1±0.2	-0.102	0.481
Tumour size	2.25 (0.75- 4.25)	-0.091	0.556
Grade of Carcinoma			
Low Grade	33 (44%)	6.5 (4.6- 10.9)	0.001**
High Grade	42 (56%)	9.9 (5.9- 39.9)	
Pattern			
Acinar	45 (60%)	6.4 (3.8- 25.8)	0.03*
Solid	25 (34%)	6.1 (4.1- ) 6.7	
Acinar/Solid	5 (6%)	10.9 (9.9- 10.9)	
Steatosis	0.02 (0.02- 0.04)	-0.028	0.848
Stage (Fibrosis)			
0	15(20%)	7.0 (4.5- 14.3)	0.001*
1	3(4%)	30.0 (30.0- 30.0)	
2	18(24%)	14.5 (9.0- 50.0)	
3	12(16%)	4.0 (2.3- 5.8)	
4 Cirrhosis	27 (36%)	6.0 (3.0- 11.8)	
HAI			
0	15 (20%)	14.1 (6.6- 27.5)	
A1	24 (32%)	6.2 (3.9- 9.8)	0.1
A2	21(28%)	4.6 (3.7- 9.9)	
A3	15(20%)	6.8 (3.8- 39.9)	
Hepatomegaly	62 (82%)	6.1 (4.1- 14.1)	0.5
Negative	13 (18%)	7.5 (4.2- 25.8)	
Positive	45 (60%)	6.6 (3.9- 17.0)	0.6
Ascites			
Negative	30 (40%)	5.2(4.3- 22.7)	
Positive	32 (42%)	6.4(4.3- 19.9)	0.3
Splenomegaly			
Negative			
Positive	43 (58%)	6.6(3.5- 19.9)	
Oedema Lower Limbs		6.1(4.1- 8.7)	0.7
Negative	56 (74%)		
Positive	19 (26%)	6.8(4.1- 25.8)	

Age, Alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (Alb), Bilirubin and No. of masses are represented as Mean and SD. But Alpha fetoprotein (AFP), Tumour size, and Steatosis (Fatty degeneration of hepatocytes (% of cells)) are represented as Median and Interquartile Range IQR (25% -75%). While Sex, Grade, Pattern, Stage, HAI (Hepatitis Activity Index (grade of hepatitis), Hepatomegaly, Ascites, Splenomegaly, and oedema Lower Limbs are represented as Frequency and percent.

Table 2. Diagnostic Performance for the Studied Genes in the Studied Samples

	Sample type	Cut-off	Sensitivity	Specificity	AUC	S. E	95% C. I		P. value
							Lower Bound	Upper Bound	
PIWIL-2	Serum	>0.55	100	100	1	0	1	1	<0.001**
	Tissue	>3.15	96	60	0.8	0.043	0.72	0.88	<0.001**
ELISA/ AFP	Serum	>0.97	84	64	0.828	0.07	0.746	0.91	<0.001**

Sn, Sensitivity; Sp, Specificity; AUC, Area under curve and C.I: 95% Confidence Interval; \* p value <0.05 is significant; \*\* p value <0.001 is highly significant.

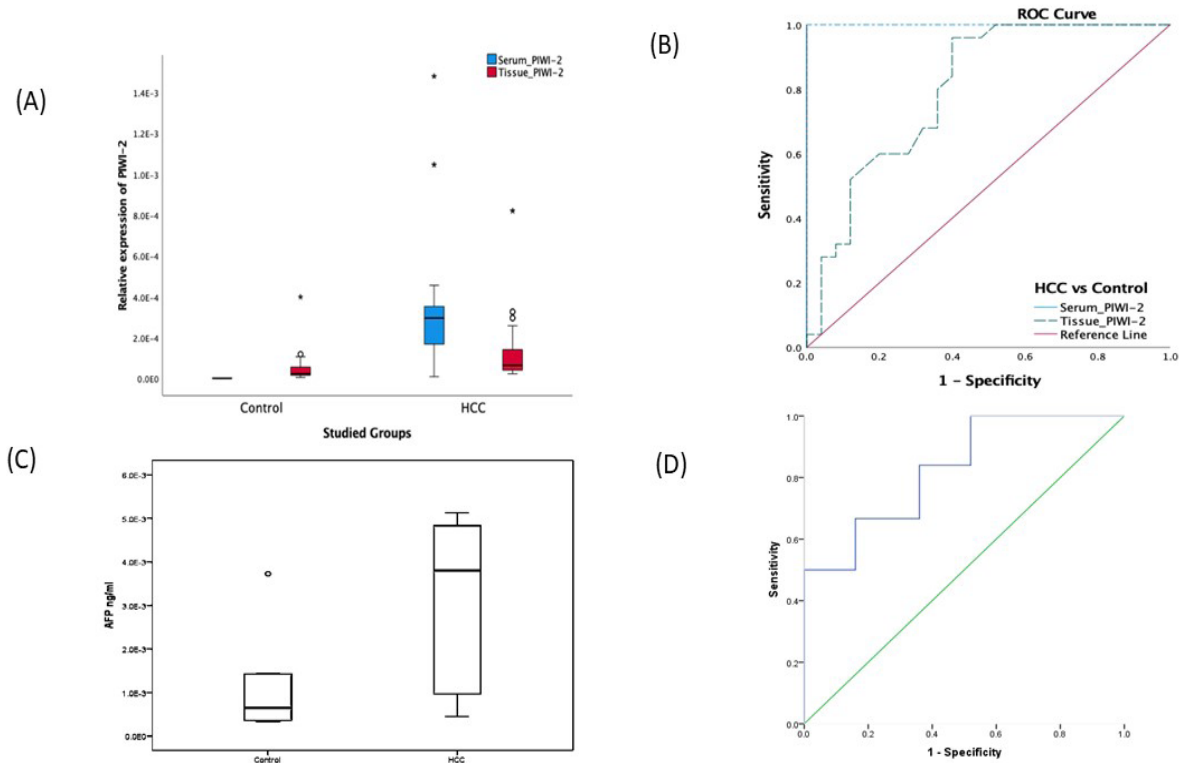


Figure 1. (A) *PIWIL2* gene expression in the studied samples for serum and tissue; (B) ROC curve of *PIWIL2* in the studied samples (C) Expression of AFP via ELISA for HCC and control serum samples. (D) ROC curve for AFP in HCC serum samples.

Table 3. Immunohistochemical Expression of *PIWIL2* & *HepPar1* in Relation to Pathological Findings

Groups	<i>PIWIL2</i>				<i>Hep-Par1</i>		
	Positive Cases		Positive cells	Intensity	H-Score	Positive cells	Intensity
	Number	%	Mean % ± S. D.	Mean Score ± S. D.	Mean % ± S. D.	Mean Score ± S. D.	Mean Score ± S. D.
Benign	12	16%	22.41±19.67	1.81±0.34	1.71±0.88	46.30±24.08	1.81±0.77
Hepatitis Activity							
Low activity	6		19.11±16.81	1.79±0.16		20.15±12.47	1.64±0.97
High activity	6		26.21±21.67	1.94±0.40		19.84±10.62	2.13±0.64
Fibrosis Stage							
F1&2	3		14.15±12.33	1.94±0.56		39.25±14.81	1.94±0.56
F3	3		28.11±19.71	1.61±0.27		42.66±32.01	1.70±0.89
Cirrhosis	6		49.21±23.84	1.84±0.36		51.18±27.12	2.29±0.88
Malignant	63	84%	71.35± 32.08	2.09±1.07	6.28±2.39	89.41±10.71	2.08±0.57
Low grade	49		59.25±38.84	1.13 ±0.62		76.90±8.35	1.53±0.78
High grade	14		92.04±20.01	2.86 ±1.56		93.22±12.12	2.78±0.44
P value	P<0.01*		P<0.001**	P=0.08234	P<0.01*	P<0.01*	P<0.01*

Values are represented as mean and S.D. standard deviation. The qualitative parameters are represented as frequency and percent; the data were analysed by X<sup>2</sup> test. \*P<0.05, \*\*P<0.001.

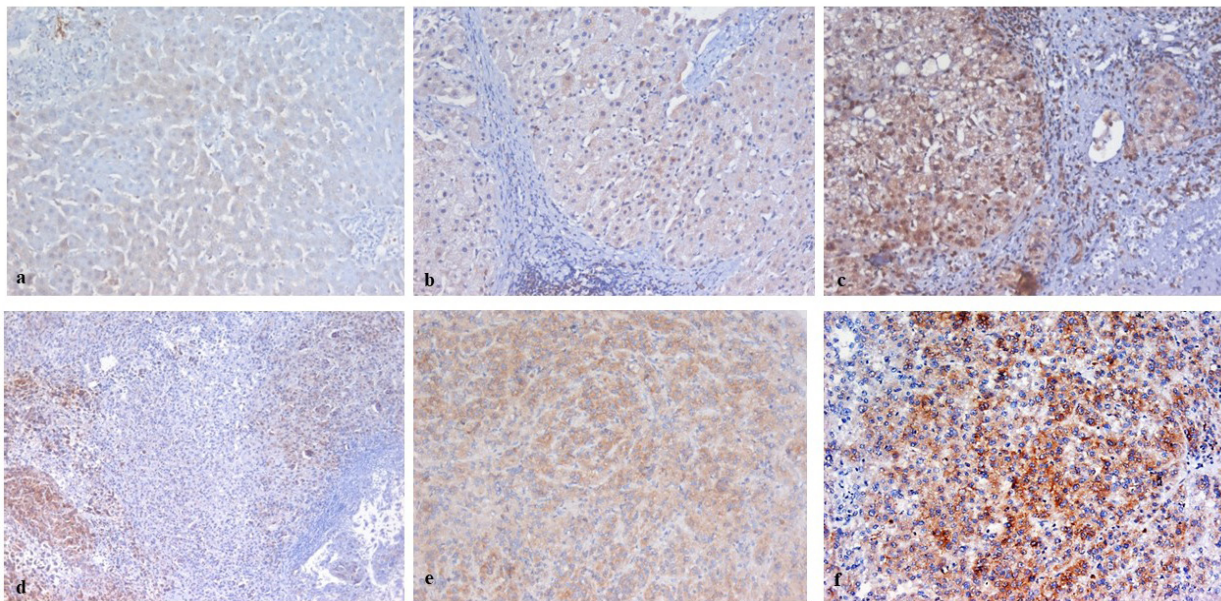


Figure 2. Sections in Liver of HCV Hepatitis (A-C) and HCC samples (D-F); showing (a) mild expression of PIWI2 in case of low activity(A1) and mild fibrosis(F1), (b) moderate expression of PIWI2 in case of moderate activity(A2) and moderate fibrosis(F2), (c) high expression of PIWI2 in case of moderate activity(A2) and cirrhosis (F4) (d) mild expression of PIWI2 in case of low grade HCC (X100), (e) diffuse positive staining of PIWI2 (X200), (f) strong diffuse positive staining indicating high expression of PIWI2 in case of high grade HCC (IHC for PIWI2,DAB, X200).

Table 4. Diagnostic Accuracy for Detection of Hepatocellular Carcinoma Using One or Two Markers

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
One marker					
PIWIL2	96.3	89.4	95.5	88.5	88.4
HepPar1	73.7	86.3	82.2	64.3	77.8
Two markers					
Both (2) positive	70.6	100	100	77.6	89.4
At least 1 positive	84.3	82.4	89.6	86.4	74.2

PIWIL2 Piwi-like 2, HepPar1 hepatocyte paraffin 1, NPV negative predictive value, PPV positive predictive value.

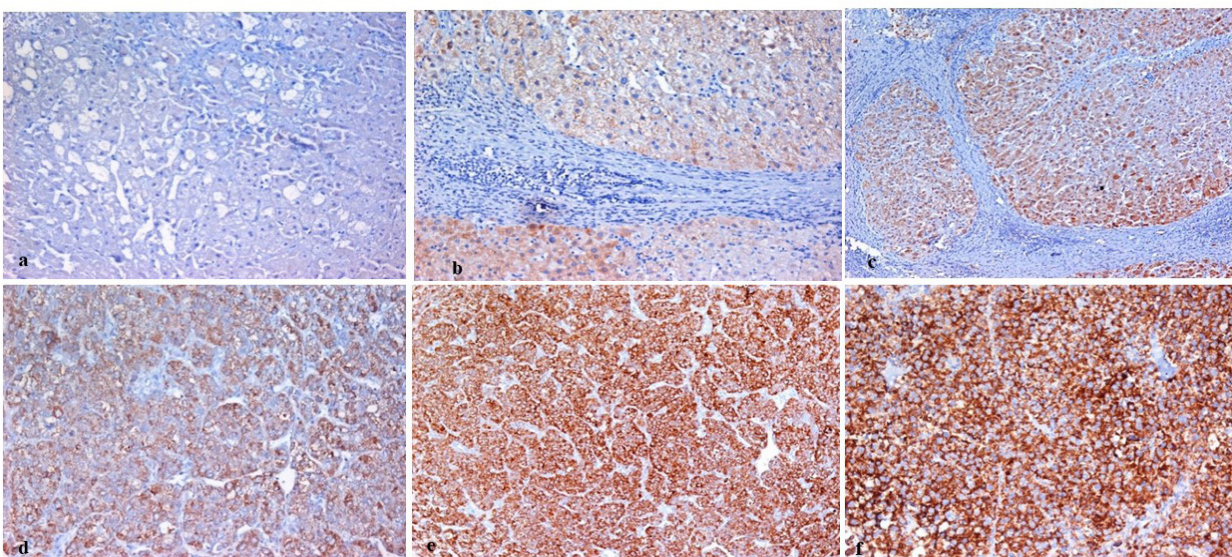


Figure 3. Sections in Liver of HCV Hepatitis (A-C) and HCC tissue (D-F); showing: (a) negative expression of HepPar-1 in case of low activity(A1) and mild fibrosis(F1), (b) moderate expression of HepPar1 in case of moderate activity(A2) and moderate fibrosis(F2) & (c) high expression of HepPar1 in case of moderate activity(A2) and cirrhosis(F4) (IHC for HepPar1, DAB, X200). (d): mild expression of HepPar1 in case of low-grade HCC, (e) higher expression of HepPar1 in case of moderately differentiated HCC, (f) highest expression of HepPar1 in case of high-grade HCC (IHC for HepPar1, DAB, X200).

cells, their functions are still unknown. The expression of PIWIL2 was assessed using IHC and real-time PCR methods in the current investigation, which was conducted on a well-characterized group of patients with HCC. We detected significantly elevated expression of PIWIL2 in HCC serum and tumour tissue samples and high diffused significant expression in tissue (Figure 1A and Figure 2). Real-time PCR was used to determine if gene expression directly impacts the protein levels of PIWIL2 (Table 2). Interestingly, in the great majority of cases, PIWIL2 expression was considerably raised in associated malignant samples compared to the controls in both serum and tissue samples. It is also worth noting that our study found an association between PIWIL2 expression and advancing cancer grades, tumour patterns, patient age, and tumour staging (Table 1). These findings are similar to previous investigations that were published by Liu et al., which showed elevated expression of PIWIL2 during the start phases of breast cancer, and imply that PIWIL2 plays a significant role in breast cancer development [30]. PIWIL2 was found to be ubiquitously and distinctively expressed in different stages of breast cancer, and its expression pattern was linked to oestrogen receptor (ER) expression and the proliferative marker Antigen Kiel 67(Ki67) [31]. Histological changes caused by HCC are varied in different patients that cause enormous problems in diagnosis. Therefore, accurate detection methods are necessary to conclusively enable the diagnosis of HCC at earlier stages. HepPar1 is one of the key factors in the urea metabolism cycle and can be highly sensitive and specific in the detection of hepatocytes [32]. The main objective of this study was to evaluate the expression pattern of PIWIL2 and HepPar1 in patients with HCC, to evaluate their combination for HCC diagnosis. The staining pattern of hepatocytes by PIWIL2 was mainly diffuse cytoplasmic and patchy nuclear reactivity for HCC liver samples. Patients with HCC had significant higher levels of HepPar1 and PIWIL2 than adjacent nontumour tissue. Also, PIWIL2 was more specific than HepPar1 and could be a suitable biomarker to increase the specificity and sensitivity when used collectively. The results of HepPar1 functions in cancer diagnosis, in terms of staining and expression patterns, are similar to those of other investigations [12, 33, 34]. Alternatively, a diagnostic response to both proteins were found and we postulate based on current evidence that the combination of HepPar1 and PIWIL2 improved the accuracy, which can be helpful in disease monitoring, since patients were diagnosed with a 100% specificity, when using a combined model of PIWIL2 and HepPar1. One of the complex issues in the detection of liver malignancies is diagnosing hepatic failures which are susceptible to advanced stages of liver disease, the association of expression for PIWIL2 was found to be significant with tumour staging, grading and pattern (Table 1) making it an interesting finding for our study. In this study, IHC results showed appropriate findings or performance for HepPar1 and PIWIL2, and our findings give fresh perspectives on the molecular control of PIWIL in cancer development [12, 23]. The observed discrepancies in protein expression may imply that different piRNAs and PIWI genes are

regulated in different cancer types. The reactivation of PIWI expression in cancer clearly shows that these proteins are involved in tumour growth and differentiation processes. The epigenetic regulation of the observed variations in PIWIL2 at the transcriptional and protein levels is a significant problem that requires additional exploration. According to current evidence, PIWI-piRNA complexes contribute to cancer development by causing aberrant DNA methylation, which results in genomic silencing and promotes cancer cell stemness [23, 35]. The current study had several limitations. Despite the results obtained in this research, until larger studies of HepPar1 and PIWIL2 are evaluated, the probability of any changes in antibody-antigen reactions must be reviewed. In the current study, and other studies [32, 36, 37] the cytoplasmic staining pattern of HEPPAR1 has been considered only. However, in some normal and/or HCC liver tissue, PIWIL2 is expressed both in the nucleus and in the cytoplasm but the significance in the cell nucleus is still unknown [36]. To conclude, abnormal mRNA expression and protein levels of PIWIL2 protein have a substantial predictive significance in HCC and can contribute to disease progression.

## Author Contribution Statement

All authors contributed to equally to the research project. S.M. Conception and writing of manuscript, GH, practical work, and manuscript preparation, TA, Practical for IHC, data analysis, MM, study design, editing and reviewing the manuscript, AA samples, clinical data, and approval acquisition, and review of the manuscript. All authors have read and agreed to the published version of the manuscript.

## Acknowledgements

The authors thank Prof Ayman Diab, Prof Gehan Safwat and Prof. Mohamed Shemis, for their suggestions and critical comments throughout the progress of this project.

### *Ethical approval and Consent to participate*

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Research ethics committee at Theodor Bilharz research institute (TBRI-REC); (FWA00010609, Serial#788). Informed consent was acquired from all participants.

### *Availability of data and materials*

The datasets generated during and/or analysed during the current study are available from the corresponding author or S.M. on reasonable request.

### *Conflict of interest*

The authors declare no conflict of interest and that this manuscript is not published or submitted elsewhere and the authors have no relevant financial or non-financial interests to disclose.

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