P- element Induced Wimpy Testis (PIWI), a Novel Powerful Candidate Diagnostic Marker for Hepatocellular Carcinoma

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Abstract

P-Element-induced wimpy testis (PIWI) proteins are engaged in epigenetic regulation of gene expression in germline cells when they are in combination with PIWI-interacting RNA (piRNA). Different types of tumour cells have been found to exhibit abnormal expression of piRNA and PIWI proteins. HCC is majorly diagnosed in later stages, which is associated with lower survival rates. There is an urgent need for improved, more specific and less invasive diagnostic methods that can detect earlier stages of the disease. Purpose: The aim of this study was to assess the diagnostic potential by determining the expression profiles of PIWI-like protein-1, -2, -3, & -4 (PIWIL1, PIWIL2, PIWIL3, & PIWIL4) in hepatocellular carcinoma, and determine its association with clinicopathological features. Methods: Samples from patients comprising serum and frozen fragments of paired tissue specimens (tumour and adjacent non-malignant liver tissue) were employed for molecular analyses. Real-time polymerase chain reaction was performed on 50 tissue and serum samples from HCC patients and 25 serum samples from healthy controls. Results: The PIWIL1, PIWIL2, PIWIL3, & PIWIL4 levels were significantly (<0.001) higher in both HCC tissue and serum samples than in the controls. Additionally, the diagnostic performance was assessed by ROC curves, the PIWIL1-4 levels in serum showed better potential and sensitivity to screen HCC patients than their levels in tissues. Moreover, some PIWIL elements were found here to be significantly correlated with the HCC profile. Conclusion: PIWI expression is considered as a powerful non-invasive diagnostic tool for HCC. Our findings also support the theory that PIWIL expression is critical for cancer progression.

Introduction

Liver cancer continues to be a global health problem, with global rates increasing (Villanueva, 2019; Rumgay et al., 2022). By 2025, the disease is predicted to affect over 1 million people (Llovet et al., 2021). Hepatocellular Carcinoma (HCC) is the most common type of primary liver cancer, accounting for around 90% of cases. In Egypt, HCC is seen as a problematic health problem, with the number of patients more than doubling over the last decade (Rashed et al., 2020). The most important risk factors for the development and progression of HCC include viral infection Hepatitis B virus (HBV) and hepatitis C virus (HCV). Patients with viral clearance for HCV and HBV who have cirrhosis are thought to be at a high risk of developing HCC (Llovet et al., 2021). Along with mutational changes ascribed to tobacco and aristolochic acid (AA) as probable pathogenetic cofactors in HCC, non-alcoholic steatohepatitis (NASH) is a growing aetiology of HCC (Schulze et al., 2015).

HCC is typically diagnosed using non-invasive criteria. Screening and sensitive imaging techniques such as ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI) have advanced dramatically in recent years. These diagnostic tools demonstrate increased detection of HCC at an earlier stage, hence improving patient outcomes (Grandhi et al., 2016). There is a widely accepted protocol for diagnosing chronic liver disorders (CLD), which involves evaluating liver function using a series of serum-level enzyme assays and a significant tumour marker, α-fetoprotein (AFP). Serum biomarkers have long been used to screen for and diagnose HCC, and AFP is one of the most thoroughly studied biomarkers in

solid tumours, inflammatory diseases, and premalignant lesions (Dimitroulis et al., 2017). As a result, AFP is the most often used tumour marker for HCC in clinical practise. The sensitivity and specificity of AFP in the early phases of hepatocarcinogenesis vary according to numerous investigations. However, AFP has exhibited suboptimal results in terms of therapeutic surveillance and early identification (Wang et al., 2021). As a result, much effort has been made to develop a reliable molecular marker for HCC in its early stages (Craig et al., 2020). There is a need for more accurate serum biomarkers with increased sensitivity and specificity that complement AFP and improve clinical outcomes for patients.

Recent studies have shown that other proteins could be used as markers for their diagnostic and prognostic values, including P-element-induced wimpy testis (PIWI) proteins (Jiang et al., 2021; Hanusek et al., 2022). Some of these biomarkers have been proposed as potential, accurate, simple and non-invasive biomarkers for early detection of HCC. In humans, PIWI protein family consists of four proteins: PIWIL1/ HIWI, PIWIL2/ HILI, PIWIL3, and PIWIL4/HIWI2 (Yu et al., 2019). PIWI family is a class of genes protected during evolution that play a key role in stem cell regeneration, division, gametogenesis, germ cell proliferation and RNA silencing (Lingel & Sattler, 2005; Kuramochi-Miyagawa et al., 2001). The expression of PIWI proteins has been linked to cancer with characteristics that include maintaining proliferative signalling, evading growth suppressors, activating invasion and metastasis, mediating genomic instability and mutation, and boosting cell growth, to mention a few (Quinn et al., 2015). The abnormal expression of PIWIs in cancer were first discovered in 2011(Esteller, 2011) and the molecular mechanisms underlying PIWI's oncogenic actions are still being studied. Moreover, PIWIs have been found to regulate the appearance of cancer hallmarks (Schulze et al., 2015).

Studies have also shown that PIWI proteins are involved in proliferation, apoptosis, metastasis, and invasion of cancer cells and act as potential diagnostic factors and biomarkers of cancer prognosis (Han et al., 2017). Overexpression of PIWIL1/HIWI gene is seen in various cancers, including seminoma cell hyperplasia (Han et al., 2017), oesophageal squamous cell carcinoma, gastric cancer (Liu, 2006) and pancreatic adenocarcinoma (Grochola et al., 2008). This study aimed to determine the efficacy of the 4 PIWI mRNA in humans (PIWIL1/HIWI, PIWIL2/ HILI, PIWIL3 and PIWIL4/HIWI2) as diagnostic biomarkers for HCC patients in both tissue and serum, and to correlate their expression with clinicopathological parameters.

Materials & Methods

Patients

The present investigation was conducted at Theodor Bilharz Research Institute (TBRI), Egypt. Hundreds of patients are regularly admitted to TBRI and to the National Hepatology and Tropical Medicine Research Institute (NHTMRI), for evaluation of any form of chronic liver disease HCV or HCC or otherwise related, patients were diagnosed with HCC by multi-slice triphasic CT and increased alpha feto protein levels were selected. Institutional Approval was acquired from the Research Institute office for IRB (NHTMRI-IRB) (Serial:2-2019), and Theodor Bilharz Research Institute (TBRI-IRB). The research was conducted

according to the declaration of Helsinki for human subject research guidelines (2013). Prior to enrolment, all patients and controls signed an informed consent form. Participants' personal, clinical, and laboratory data were collected in strict confidence. Samples were collected from 50 HCC patients, and 25 healthy controls, the study was conducted between January and December 2019. For tissue samples 50 patients undergoing liver resection were sampled for tumour and tumour-adjacent samples, the surgery for liver resection was conducted within the department of surgery NHTMRI Hospital, Egypt. Individuals suffering from other liver diseases (e.g., Auto immune hepatitis, Hemochromatosis, Schistosoma), or diseases such as HIV, and ischemic heart diseases were excluded. Patients with HCV who were taking immunomodulatory interferon therapy were also excluded from the research.

Sample collection

Blood was drawn from 50 patients and 25 control individuals, then it was allowed to clot. samples were centrifuged at 500 xg for 10 minutes. Serum was then aliquoted after centrifugation at -80°C. Liver tissues were collected from 50 patients (tumorous and non-tumorous) who underwent liver resection to remove the tumour (non-tumorous sections of the liver from those patients served as controls). Liver sections were stored in lysis buffer at -80°C until use.

Biochemical Parameters

Laboratory tests including alanine aminotransferase (ALT), aspartate aminotransferase (AST), Bilirubin, albumin (ALB) and alpha-fetoprotein (AFP) were performed for all subjects as routine tests upon admission.

RNA extraction

The miRNeasy extraction kit was used to extract total RNA (Qiagen, Valencia, CA). The extraction procedure was conducted according to the manufacturer's instructions for both tissue and serum samples. Roughly 70 mg of tissue, homogenized with lysis solution prior to extraction, and 200 µl serum were used. Samples were extracted in duplicates, then the quality and concentration of the samples were measured using a NanoDrop-1000c spectrophotometer (Thermo Fisher Scientific, Cinisello Balsamo, Italy).

Quantitative Real-Time Reverse-Transcription (qRT-PCR)

For quantification of PIWI transcripts, the four primer assays were readymade and acquired from Qiagen. The primers included the four isoforms PIWIL1/HIWI, PIWIL2/HILI, PIWIL3, PIWIL4 and all assays were performed according to the manufacturer's instructors. The real time PCR amplification was performed by QuantiTect SYBR Green PCR Kits (Qiagen, Valencia, CA). Briefly, 5 µl of the RNA extracted from the serum and tissue samples were reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Upon the completion of 1st strand cDNA synthesis, 5µl of the sample was used for the real time PCR amplification step. All reactions were run in duplicates. Finally, the ΔΔCT method was used for the relative quantification of mRNAs in all samples (Wong & Medrano, 2005).

Statistical analysis:

To determine the appropriate number of patients, a sample size calculation was conducted, and adapted from other research projects involving HCC diagnosis (Xie et al., 2013). The continuous variables were described as mean ± standard deviation (SD) or median and interquartile range (IQR) according to their distribution, which was determined using a normality test. The frequencies and percentage were used for categorical variables. A *p value* of < 0.05 was considered statistically significant. To compare the means Mann-Whitney U was used. Chi^2 test or Fisher's exact test was used to determine the distribution of categorical variables between groups. The diagnostic performance of the studied markers was assessed by receiver operating characteristic (ROC) curves. The area under the ROC (AUC) was calculated as an accuracy index for prognostic performance of selected tests. Logistic regression was conducted to determine the risk level, for HCC patients. Pearson Correlation was done to correlate between the studied parameters with the studied biomarkers.

Results

Patient characteristics

A total of 50 HCC participants were recruited in this study, the patients included 28 males (56%) and 22 females (44%), with a mean age of 57.2±8.1 and 25 healthy individuals with no history of liver disease or alcohol consumption were included as controls. Individual demographic and clinical data of the studied groups are shown in (Table 1).

Recruitment was carried out post admission and diagnosis, biochemical lab tests included alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (Alb), Bilirubin, creatinine and alpha-fetoprotein (AFP) for all subjects as routine tests. The results were represented as mean±SD for these tests, and had values of 61.4±15.5, 65.8±16.6, 2.3±1.0, 2.9±1.1, 0.9±0.4 respectively, AFP however was 75.0 (40.0- 150.0).

By computed tomography, the number of tumour masses was detected with mean of 1.1±0.2, while tumour size and steatosis were found at 2.25 (0.75- 4.25) and 0.02 (0.02- 0.04) respectively.

Regarding pathological diagnosis, 29(38.7%) were diagnosed GI, 11 (14.7%) were with GII and 10 (13.3%) were GIII, 30 (60.0%) were with acinar pattern tumour, 17 (34.0%) with solid tumour and only 3 patients (6.0%) were with acinar/solid tumour.

Tumour staging results indicated that only 8 patients (16%) were fibrotic with the predominant number for the cirrhotic patients 42 (84%). Histopathological examination was performed using METAVIR scoring system to determine the hepatitis activity index (HAI), 15 (30.0%) patients were rated as AI, 35 (70.0%) were A2 while none of the patients were A3.

Abdominal ultrasounds detected hepatomegaly in 9 (18%) patients, ascites in 20 (40%) patients and splenomegaly in 13 (26%) patients and finally 29 (58.0%) out of the 50 HCC patients were detected with

oedema lower limbs (Table 1).

Table 1: Demographics and Clinico-pathological characteristics for HCC patients

Clinico-pathological characteristics	Total number of patients N=50 (%)
Age (Mean±SD)	57.2±8.1
Sex	22 (44.0)
Female	
Male	28 (56.0)
ALT	61.4±15.5
AST	65.8±16.6
Alb	2.3±1.0
Bilirubin	2.9±1.1
AFP	75.0 (40.0- 150.0)
S-creatinine	0.9±0.4
No. of masses	1.1±0.2
Tumour size	2.25 (0.75- 4.25)
Tumour Grade	29(38.7)
I	
II	11 (14.7)
III	10 (13.3)
Pattern	30 (60.0)
Acinar	
Solid	17 (34.0)
Acinar/Solid	3 (6.0)
Steatosis	0.02 (0.02- 0.04)
Stage	8 (16.0)
Fibrosis	
Cirrhosis	42 (84.0)
HAI	15 (30.0)
A1	
A2	35 (70.0)

A3	0 (0.0)
Hepatomegaly	41 (82.0)
Negative	
Positive	9 (18.0)
Ascites	30 (60.0)
Negative	
Positive	20 (40.0)
Splenomegaly	21 (42.0)
Negative	
Positive	13 (26.0)
Oedema Lower Limbs	37 (74.0)
Negative	
Positive	29 (58.0)

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (Alb) and alpha-fetoprotein (AFP). No. of masses are represented as Mean and SD. But Alpha feto-protein, 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.

Biochemical Assessments

The biochemical parameters ALT, AST, Bilirubin, ALB and AFP were performed for all subjects as routine tests, their results can be found in the supplementary table (Table 13). Correlation between these results and the expression of PIWIL mRNA, Pearson Correlation was conducted, and no significant difference was found (Table 13).

Expression of PIWIL's in Serum

To assess the significance of expression for PIWIL1, PIWIL2, PIWIL3 and PIWIL4 RNA in the serum of HCC patients, results showed high significance between their levels in patients and healthy controls (p<0.001) (Figure 1 a, b, c and d). This indicates that there was overexpression for all assessed PIWIL mRNAs in the serum samples of patients, when compared to the healthy controls.

Expression of PIWIL's in Tissue

To assess the significance of expression for PIWIL1, PIWIL2, PIWIL3 and PIWIL4 mRNA in the tissue of HCC patients and the corresponding adjacent non-tumour tissue. Results showed significance for patients' tissue expression (p<0.01) (Figure 2 a, b, c and d). It indicated that there was overexpression for all assessed PIWIL mRNAs in the tissue samples of patients, when compared to the tumour adjacent tissue samples.

Logistic regression analysis of PIWIL's

To assess the relative risk of HCC presented from PIWIL mRNA, logistic regression analysis model was performed (Table 2). It revealed that PIWIL1-4 is significantly associated with increased risk for HCC in serum (p<0.001) of HCC patients, and in tissue samples of patients (p<0.01).

Table 2: Logistic Regression Results for both tissue and serum samples of PIWIL mRNA in HCC patients

Serum groups	**HCC Risk determination (Logistic Regression)**	
	OR (95% C. I)	***p value***
PIWIL1	3.87(1.23 – 8.36)	<0.001**
PIWIL2	4.21(1.04 – 7.23)	<0.001**
PIWIL3	3.26(1.14 – 8.21)	<0.001**
PIWIL4	2.99(1.57 – 9.23)	<0.001**
Tissue groups	***OR (95% C. I)***	***p value***
PIWIL1	2.35(0.17 – 5.32)	0.01*
PIWIL2	2.21(0.15 – 4.24)	0.01*
PIWIL3	2.46(0.23 – 6.27)	0.01*
PIWIL4	1.65(0.19 – 3.14)	0.01*

The studied genes were represented as Median and Interquartile Range IQR (25% -75%), the Risk of HCC was conducted by logistic regression. * p<0.05, ** p<0.001. Fold change values were multiplied by 10^5 for approximation. OR: Odds ratio, CI: Confidence interval.

Diagnostic performance:

Receiver Operating characteristic (ROC) analysis is essential for evaluating the performance of diagnostic tests, and the accuracy of a statistical model. In our investigation, ROC Curve was conducted to assess the diagnostic performance of PIWIL1, PIWIL2, PIWIL3 and PIWIL4 in both serum, and tissue samples of HCC patients, to evaluate their specificity and sensitivity in HCC prediction and to evaluate their discriminatory properties for the patients and healthy individuals. Analysis of the diagnostic value of the

4 PIWI-RNAs showed promising results, indicating that they are associated with convenient disease characteristics and could differentiate between serum of HCC patients with those of controls. For discrimination, it was found that the 4 PIWIL mRNAs were with sensitivity of 100% and specificity of 100% with an area under curve (AUC) of 1.0 ($p < 0.001$, 95% C.I: 1.0 – 1.0).

Regarding the diagnostic performance of the 4 PIWILs RNA in HCC tissue, the analysis revealed a highly statistical significance with PIWIL1, it was with sensitivity of 80.0% and specificity of 72.0% with an area under curve (AUC) of 0.80 ($P < 0.001$, 95% C.I: 0.71 – 0.89). For PIWIL2, it was with sensitivity of 84.0% and specificity of 64.0% with AUC of 0.80 ($p < 0.001$, 95% C.I: 0.71 – 0.89). While for PIWIL3, it was with sensitivity of 68.0% and specificity of 84.0% with AUC of 0.79 ($p < 0.001$, 95% C.I: 0.70 – 0.88) and finally a statistical significance was observed regarding PIWIL4, with sensitivity of 64.0% and specificity of 68.0% with AUC of 0.66 ($p < 0.01$, 95% C.I: 0.56 – 0.77), the above results showed that the 4 studied PIWIs RNA can be used to differentiate between tumour and non-tumour tissue samples (Table 3) (Figure 3a, b). A combined ROC curve was prepared to show an inclusive diagnostic performance appraisal, serum had an overall sensitivity and specificity of 100%, and an AUC of 1.0. Tissue samples exhibited sensitivity of 70.7%, specificity of 68%, and AUC of 0.733. Both of which were significant p<*0.001*.

Table 3: ROC curve analysis for diagnostic performance for each of the studied PIWIL mRNAs in serum and tissue samples.

	Groups	Cut-off	Sensitivity	Specificity	AUC	95% C. I		*p* value
						Lower Bound	Upper Bound	
Serum	PIWIL1	<1.6	100.0	100.0	1.0	1.0	1.0	<0.001**
	PIWIL2	<0.56	100.0	100.0	1.0	1.0	1.0	<0.001**
	PIWIL3	<8.87	100.0	100.0	1.0	1.0	1.0	<0.001**
	PIWIL4	<1.02	100.0	100.0	1.0	1.0	1.0	<0.001**
Tissue	PIWIL1	<4.76	80.0	72.0	0.80	0.71	0.89	<0.001**
	PIWIL2	<3.69	84.0	64.0	0.80	0.71	0.89	<0.001**
	PIWIL3	<4.5	68.0	84.0	0.79	0.70	0.88	<0.001**
	PIWIL4	<0.94	64.0	68.0	0.66	0.56	0.77	<0.01*

Sn: Sensitivity, Sp: Specificity, AUC Area under curve and C.I: 95% Confidence Interval. * $p<0.05$, ** $p<0.001$.

Table 4: ROC curve analysis for diagnostic performance for the studied PIWIs RNA in serum and tissue samples overall.

Groups	Sensitivity	Specificity	AUC	95% C. I		*p value*
				Lower Bound	Upper Bound	
Serum	100.0	100.0	1.0	1.0	1.0	<0.001**
Tissue	70.7	68.0	0.733	0.683	0.783	<0.001**

Sn: Sensitivity, Sp: Specificity, AUC Area under curve and C.I: 95% Confidence Interval. * p<0.05, ** p<0.001.

Analysis of the association between the tumour grade and gene expression of the studied PIWIs RNA:

Regarding serum samples, no significant association was observed between the expression and the tumour grade of the patients. As for tissue samples, a significant association was observed with *p<0.05* for PIWIL1 and patients diagnosed grade II versus (Vs) grade I, PIWIL4, had an association between the expression in tissue and patients diagnosed with grade III Vs grade I. *p<0.05* (**Table 5**).

Table 5: The association between the tumour grade and expression of the studied PIWIs RNA in serum and tissue samples.

	Groups	Tumour grade			*p value*		
		I N=29	II N=11	III N=10	II Vs I	III Vs I	III Vs II
Serum	PIWIL1	71.0(18.2-100.3)	60.1(18.2-120.2)	100.3(49.3-128.2)	0.9	0.1	0.2
	PIWIL2	29.6(24.8-41.6)	29.6(16.8-37.5)	30.1(22.9- 39.5)	0.6	0.9	0.7
	PIWIL3	55.3(29.0-122.5)	55.3(36.0-170.0)	189.7(32.5-236.3)	0.9	0.3	0.4
	PIWIL4	32.7(17.0-97.8)	22.8(11.8-39.7)	39.7(15.7-182.9)	0.5	0.6	0.2
Tissue	PIWIL1	6.2(4.4- 13.4)	28.0(5.9- 36.5)	10.1(5.8- 30.0)	0.05*	0.08	0.8
	PIWIL2	6.8(3.5- 25.8)	6.4(4.6- 9.9)	6.2(4.6- 8.3)	0.9	0.9	0.9
	PIWIL3	6.0(3.5- 9.0)	9.0(3.0- 20.0)	7.0(2.8- 20.0)	0.5	0.5	0.9
	PIWIL4	1.7(0.8- 2.9)	1.0(0.6- 7.8)	0.7(0.4- 2.6)	0.5	0.04*	0.2

The studied genes are represented as Median and Interquartile Range IQR (25% -75%), the data were analysed by Mann-Whitney *U* test. * p<<0.05, ** p<<0.001.

Analysis of the association between the tumour pattern and expression of the studied PIWILs RNA:

In terms of the association between tumour pattern and PIWILs expression, no significant difference was detected in serum, while, in the tissue, PIWIL2 was significantly associated with patients *p<0.05*, with acinar/solid Vs solid with 10.9(9.9- 10.9) (**Table 6**).

Table 6: The association between the tumour pattern and expression the studied PIWIs RNA in serum and tissue samples.

	Groups	Tumour Pattern			*p value*		
		Acinar N=30	Solid N=17	Acinar/Solid N=3	Solid Vs Acinar	Acinar/Solid Vs Acinar	Acinar/Solid Vs Solid
Serum	PIWIL1	65.6(18.2-90.4)	71.5(18.2-152.2)	80.4(3.1-80.4)	0.3	0.6	1.0
	PIWIL2	30.1(24.9-39.5)	29.6(8.9-35.3)	37.5(0.9-37.5)	0.3	0.7	0.5
	PIWIL3	55.3(37.5-179.8)	55.3(22.0-189.7)	75.0(22.0-75.0)	0.5	0.7	0.6
	PIWIL4	34.9(17.0-84.9)	22.8(11.8-55.9)	32.7(11.8-32.7)	0.4	1.0	0.7
Tissue	PIWIL1	7.9(5.1-13.5)	7.9(4.1-28.8)	31.3(6.2-31.3)	0.7	0.1	0.3
	PIWIL2	6.4(3.8-25.8)	6.1(4.1-) 6.7	10.9(9.9-10.9)	0.5	0.2	0.03*
	PIWIL3	6.0(3.0-11.8)	9.0(3.0-20.0)	20.0(7.0-20.0)	0.9	0.1	0.2
	PIWIL4	1.7(0.6-2.2)	1.0(0.5-5.9)	6.8(1.2- 6.8)	0.8	0.2	0.2

The studied genes are represented as Median and Interquartile Range IQR (25% -75%), the data were analysed by Mann-Whitney *U* test. * *p*<<0.05, ** *p*<<0.001.

Analysis of the association between the tumour stage and expression of the studied PIWIs RNA:

Our findings showed only PIWIL1 expression in serum was significantly associated with cirrhotic patients with 71.5(18.2- 128.2) and *p<0.05* and no association was observed between PIWILs tissue expression and tumour stage neither in fibrotic nor cirrhotic patients (**Table 7**).

Table 7: The association between the tumour stage and expression of the studied PIWIs RNA

	Groups	Tumour Stage		*p value*
		Fibrosis	**Cirrhosis**	
		N=8	**N=42**	
Serum	**PIWIL1**	18.2(6.7- 68.3)	71.5(18.2- 128.2)	0.02*
	PIWIL2	29.6(24.8- 32.2)	30.1(16.8 - 39.5)	0.5
	PIWIL3	52.1(40.7- 55.3)	55.3(32.5- 209.3)	0.4
	PIWIL4	22.8(17.0- 63.3)	32.7(11.8- 84.9)	0.8
Tissue	**PIWIL1**	7.9(5.3- 12.9)	8.0(4.9- 28.0)	0.8
	PIWIL2	8.3(3.5- 25.8)	6.2(4.2- 14.1)	0.8
	PIWIL3	6.0 (3.3 - 9.0)	8.0(3.0- 20.0)	0.7
	PIWIL4	1.9(0.5- 3.5)	1.4(0.6- 3.1)	0.9

The studied genes are represented as Median and Interquartile Range IQR (25% -75%), the data were analysed by Mann-Whitney *U* test. ***p<0.05, **p<0.001.***

Other parameters were investigated including associations between HAI, hepatomegaly, ascites, splenomegaly, oedema lower limbs, however no significant difference was detected (Supplementary Data: **Tables 8-12** respectively). The correlation of the biochemical parameters AST, ALT, Alb, Bilirubin, AFP, S-creatinine, in addition to the number of masses, tumour size, and steatosis were analysed versus gene expression results and no significant difference was observed (Supplementary Data: **Table 13**).

Discussion

HCC is estimated to be the result of a history of chronic liver disease (CLD) in 70%-90% of patients, with major causes being attributed to HCV or HBV infections (Llovet et al., 2021). In Egypt, HCC is perceived as a particular health challenge, with an approximate two-fold rise in the number of cases, over the course of a decade (Rashed et al., 2020). Currently, AFP is the only widely employed serological marker for screening for tumours, diagnosing HCC. However, serum AFP has a specificity of 76%- 94%, and a sensitivity of 39%- 65% for HCC, thus, hindering its clinical use (Muscari & Maulat, 2020). Ample efforts were placed into detecting higher performing biomarkers, AFP-L3, GP73, DCP, and GPC3, but to date, clinical performance was not outshone from AFP. Thus, a biomarker with potential for the diagnosis and detection of HCC at earlier stages is of imperative need.

In terms of the demographic data for the patients, the mean age was 57.2±8.1, 44% were females and 56% were males, which is typical for the risk factors associated with HCC. We found no association between the parameters, and expression of PIWIL. This was confirmed for HAI, hepatomegaly, ascites, splenomegaly, oedema lower limbs, number of masses. The aforementioned findings were in alignment

with Taubert, et al., 2015, who observed similar outcomes for no associate with clinicopathological features for colon cancer patients (Taubert et al., 2015), but was observed by Zhang, et al., and Li, et al., in breast and colon cancer respectively (Zhang et al., 2013, Li et al., 2012). For tumour grade, PIWIL1 & PIWIL4 had significant difference with tissue expression, which was confirmed in a study by Litwin, et al., for PIWIL1, & PIWIL2 in breast cancer (Litwin et al., 2018).

Recently, several reports have indicated that aberrant expression of PIWI at the mRNA and protein levels occurs in various types of tumours (Suzuki et al., 2012, Yang & Li, 2014). PIWIL1/HIWI was previously linked with several types of cancers, with a pattern of overexpression, moreover, it was correlated with tumour grading and staging (Dong et al., 2021, Krishnan et al., 2016). Our findings confirmed the apparent role of PIWIL mRNAs for HCC, with a pattern observed of overexpression for all PIWIL isoforms. Previously, PIWIL2/HILI was identified as overly expressed in colorectal, prostate, breast, cervical, gastric and bladder cancer, all of which were determined to be overly expressed for both PIWIL1& PIWIL2 (Yang & Li, 2014). PIWIL3 & PIWIL4 was observed in renal cell carcinoma. The involvement of PIWI proteins was linked with multiple hallmarks of cancer including invasion, apoptosis evasion, metastasis and cell proliferation, as such they possess prospective diagnostic factors and biomarkers for cancer prognosis (Han et al., 2017, Liu et al., 2019). Significant increased level of PIWIL1 was reported for colon, bladder, and hepatocellular carcinoma. Expression of the four members of the PIWI proteins was viewed as distinct in tumour tissue when compared with the adjacent non-tumorous tissue (Cai et al., 2022, Yu et al., 2019). PIWIL3 and PIWIL1 were assessed for expression levels by (Feng et al., 2020) in colorectal cancer, and had non-significant expression statistically. Among all PIWIL genes, those assessed for expression and correlated with overall survival and recurrence-free survival were PIWIL3 & PIWIL4, in invasive urothelial bladder cancer (Litwin et al., 2018). A limitation of a study was not assessing the expression of PIWIL1 and PIWIL2 at the mRNA level, and conversely depending on Immunohistochemistry (Erber et al., 2020), but stated that they studied the diagnostic and prognostic potential, and had reports of higher expression levels (Erber et al., 2020).

Research on the expression of PIWIL proteins has yet to be fully established. In the current study, expression of all PIWIL was assessed using real-time PCR method in HCC patients. The levels of expression of PIWIL1, PIWIL2, PIWIL3, PIWIL4 in tumour and nontumorous adjacent tissue was carried out in addition to serum samples from HCC patients and healthy controls, the expression was compared with clinical parameters. When compared to adjacent non-cancerous tissues, we found a significantly elevated expression for PIWIL1, PIWIL2, PIWIL3, &, PIWIL4 in HCC samples (p <0.001). These findings are consistent with prior findings in colorectal cancer, in which PIWIL1 mRNA levels in non-cancerous tissue were low or undetectable, but were dramatically raised in associated malignant tissue (Zeng et al., 2011). Additionally, they are confirmed in another study, where PIWIL1/HIWI was reported to have marked expression levels in HCC tissue, for patients who had undergone curative resection (Zhao et al., 2012). In breast cancer, PIWIL1 and PIWIL3 gene expressions were reported to be upregulated, whereas PIWIL2 and PIWIL4 were downregulated compared with normal breast tissue.

It is noteworthy to state that our research detected significant association between PIWIL1 & PIWIL4 expression with increasing tumour grade (p<0.05). Moreover, PIWIL1 in serum detected an association between tumour stage and its expression. PIWIL2 expression in tissue was also found to be associated with tumour pattern. These findings are similar to those of previous studies, which found elevated expression throughout the initial phases of cancer, implying the significant role of PIWIL-RNAs in cancer development (Cai et al., 2022, Dong et al., 2021, Liu et al., 2019, Fu et al., 2015).

In terms of diagnostic performance, the provision of non-invasive approaches for the diagnosis of HCC is of utter significance. Our findings showed that serum had an overall sensitivity and specificity of 100%, and an AUC of 1.0, meaning that they could be a powerful diagnostic marker for HCC. Tissue samples exhibited sensitivity of 70%, specificity of 68%, and AUC of 0.733. Both of which were significant *p<0.001*. Although PIWIL3 and PIWIL4 were prognostic, PIWIL1 and PIWIL2 did not show significant impacts on survival (Krishnan, et al., 2016). Research on the usefulness of PIWIL and PiRNAs was determined with higher sensitivity e.g., 83.3% sensitivity and 89.3% specificity for colorectal cancer (CRC), in addition to varying percentages, all of which were higher than tissue is CRC patients (Mai et al., 2020, Qu et al., 2019, Sabbah et al., 2021, Gu et al., 2020). For prognostic performance, and the determination of HCC risk logistic regression was done with odd ratios indicating statistical significance (p<0.001) in serum, and (p<0.05) in tissue, these results are in agreement with Mai, et al., with odd-ratio (OR) values for CRC cases diagnosed 1-3 years at 7.23, 2.80, 2.45, and 1.24, respectively (Mai et al., 2020) and similar findings were also reported (Weng et al., 2018). Tosun et al**.,** evaluated the predictive value of the serum expression level of PIWIL2 in prostate cancer, and was for similar outputs (Tosun et al., 2019). The reactivation of PIWI expression in cancer clearly suggests that these proteins are involved in the growth and differentiation of tumours (Rizzo et al., 2016).

In conclusion, our findings provide a novel perspective on the roles of PIWIL at the mRNA level in cancer development, and their promise of being powerful diagnostic candidates for HCC diagnosis. Differential protein expression in various cancer types could indicate reciprocal regulation between different piRNAs and PIWI genes. The epigenetic regulation of the observed variations in PIWIL1, PIWIL2, PIWIL3, PIWIL4 at the transcriptional and protein levels is an important problem that needs to be investigated further. Abnormal mRNA expression has substantial diagnostic value, and should be evaluated further for prognostic potential, and its effects on survival for HCC.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, practical work, data collection and analysis were performed by Gehan Hammad, Samah Mamdouh and Gehan Safwat. Samples were provided by Mohamed Ismail. The first draft of the manuscript was written by Gehan Hammad, and reviewed and edited by Rania Hassan, and Dina Seoudi. All authors read and approved the final manuscript. Statistical analysis was done by a Biostatistician.

Data Availability

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

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Research Institute office for IRB (NHTMRI-IRB) (Serial:2-2019).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

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Figures

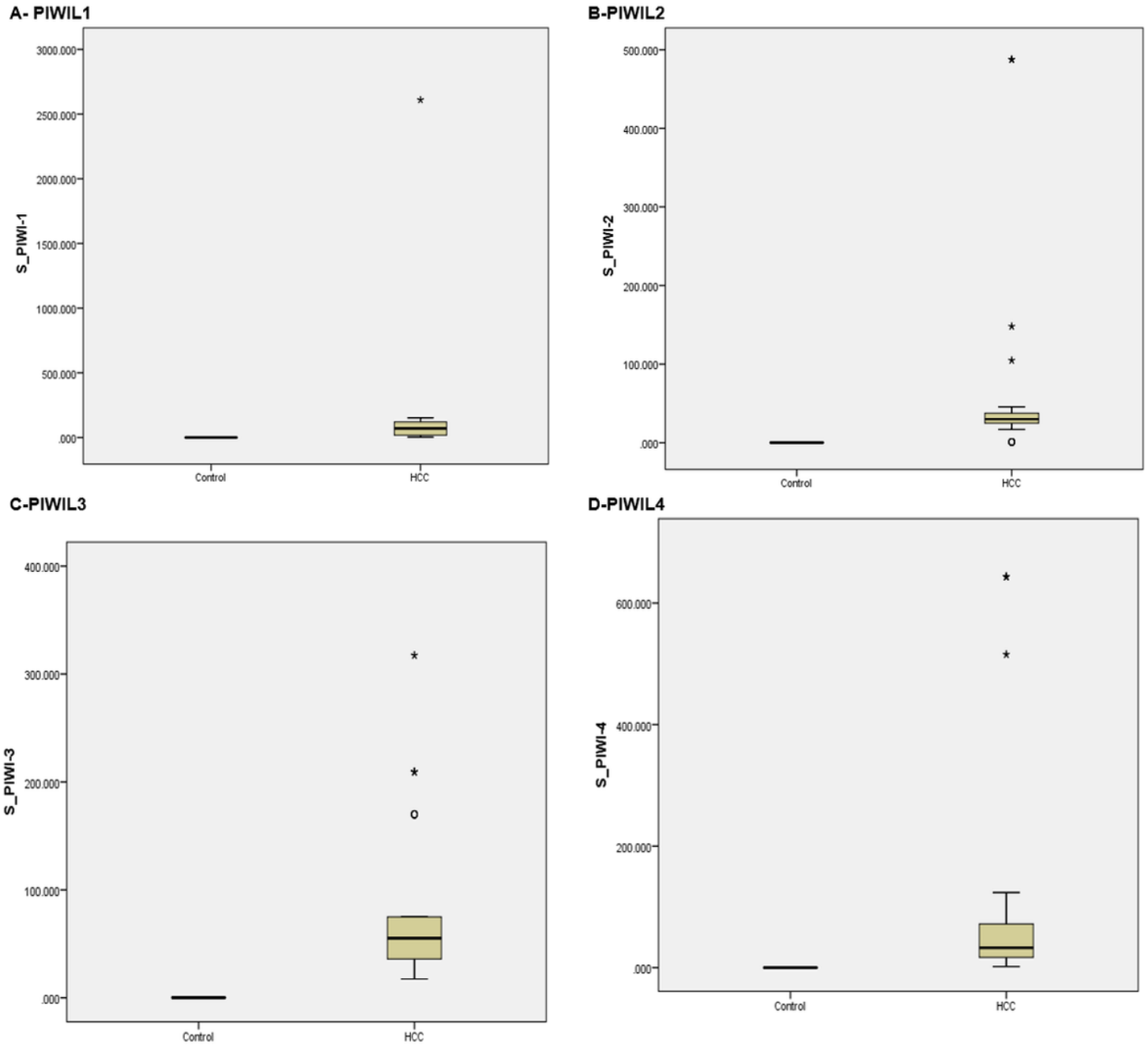

Figure 1

Relative expression of A) PIWIL1, B) PIWIL2, C) PIWIL3 and D) PIWIL4 in serum of HCC patients and controls.

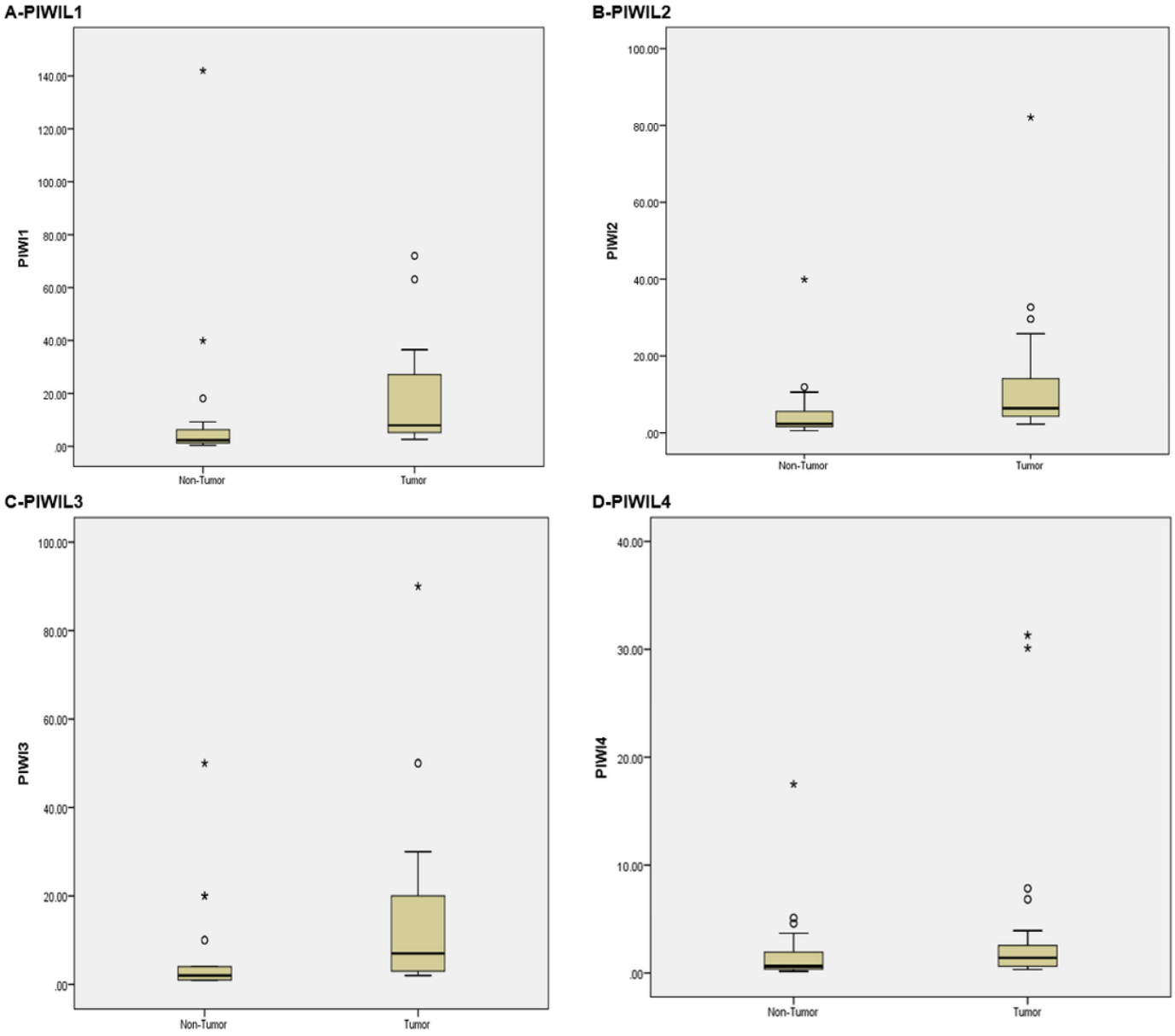

Figure 2

Relative expression of A) PIWIL1, B) PIWIL2, C) PIWIL3 and D) PIWIL4 in HCC tumour and adjacent non-tumour tissues.

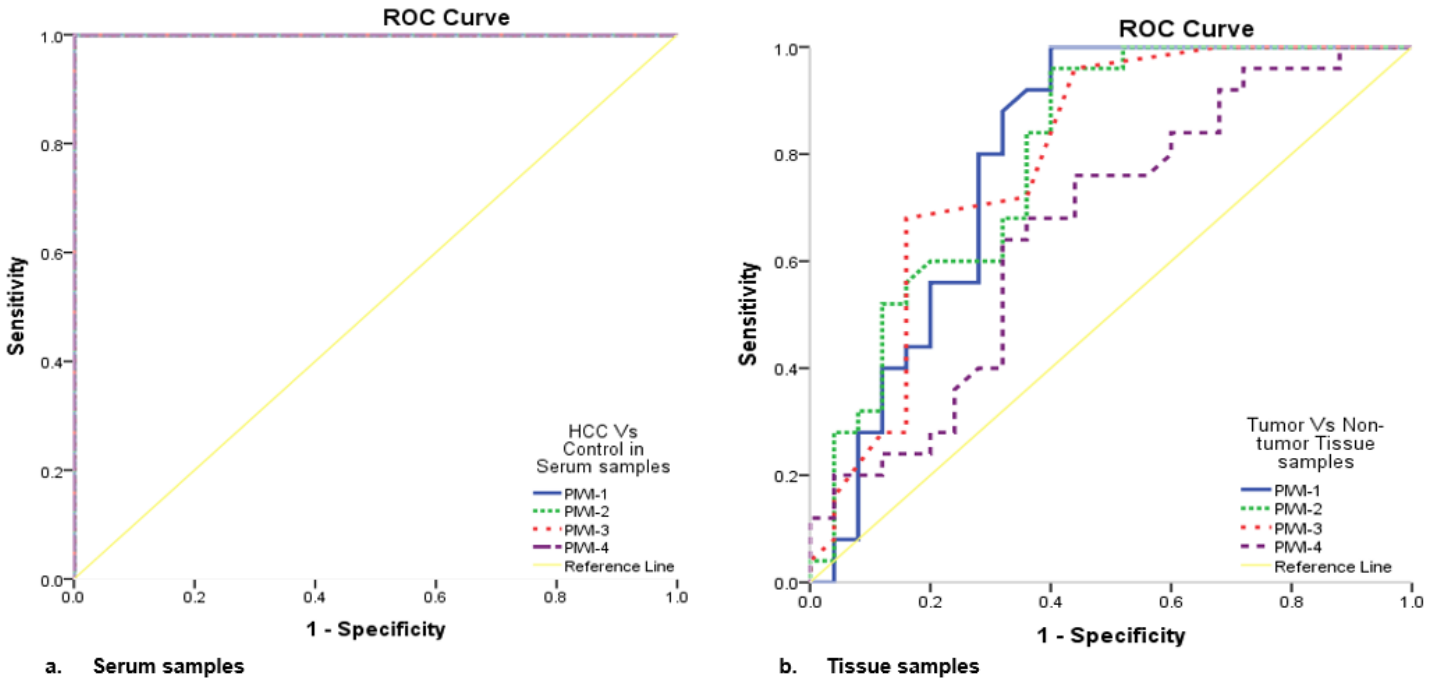

Figure 3

ROC curves of the studied PIWIs RNA in (A) serum and (B) tissue samples. Results showcase the diagnostic performance of all PIWIL mRNAs in terms of specificity.

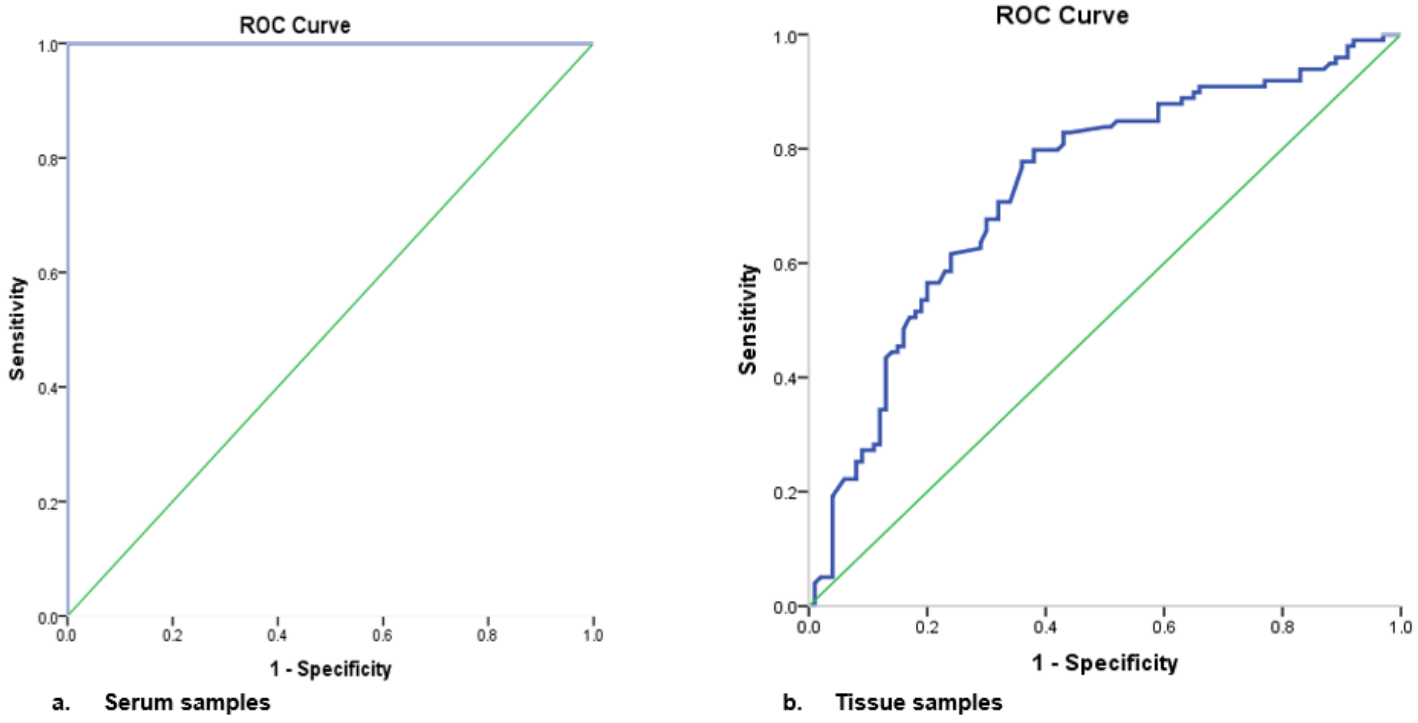

Figure 4

Combined ROC curves of the studied PIWIL mRNAs in (A) serum and (B) tissue samples. The graphs show a combined roc curve to show diagnostic performance.

Supplementary Files

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