

RESEARCH ARTICLE

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Decreased Expression Levels Of *PIWIL2*, *PIWIL3* and *PIWIL4* Are Associated with Poor Prognosis and Worse Survival in Bladder Cancer Patients

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Abstract

Background: Bladder cancer (BC) remains one of the most prevalent and recurrent malignancies worldwide. Identification of early biomarkers and prognostic indicators is vital for improving diagnostic accuracy and therapeutic outcomes. PIWI-interacting RNA pathway proteins (PIWILs), known regulators of gene silencing and genome stability, have emerged as potential biomarkers in various cancers. This study evaluates the expression patterns of PIWIL1–4 in BC patients and investigates their prognostic value. **Methods:** Tumor and adjacent tissues were collected from 220 BC patients, along with urine samples from both patients and 70 healthy controls. Total RNA was extracted, followed by cDNA synthesis and real-time PCR for PIWIL1–4. Protein expression of PIWIL2 was evaluated by immunohistochemistry. Clinical data, including staging and histopathological features, were analyzed. In silico analysis (cBioPortal, miRNet) was performed to assess *PIWIL* gene variants and miRNA associations. Receiver operating characteristic (ROC) curves, logistic regression, and survival analysis were conducted for diagnostic and prognostic assessments. **Results:** Expression of all four *PIWILs* was significantly downregulated in both tissue and urine samples of BC patients compared to controls ($p < 0.001$). ROC analysis showed high sensitivity and specificity for PIWILs in distinguishing BC from controls ($AUC \geq 0.927$). Logistic regression confirmed their diagnostic and prognostic relevance ($p < 0.001$). *PIWIL2*, *PIWIL3*, and *PIWIL4* were significantly associated with tumor recurrence ($p \leq 0.01$), while all four PIWILs correlated with patient survival. Immunohistochemical analysis of *PIWIL2* supported its expression relevance. In silico findings revealed a 7% structural variant frequency for *PIWIL2* and functional associations with tumor suppressor miRNAs. **Conclusion:** PIWIL1–4, particularly PIWIL2, are significantly downregulated in BC and hold promise as non-invasive diagnostic and prognostic biomarkers. Their expression correlates with recurrence and survival, suggesting clinical utility for improving bladder cancer management. Further studies are recommended to validate these findings in larger cohorts.

Keywords: Bladder cancer (BC)- *PIWIL1*- *PIWIL2*- *PIWIL3*- *PIWIL4*- qPCR- relative expression patterns- diagnosis

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Introduction

Bladder cancer was ranked 4th leading cause of cancer in males in 2023, with 6% predominance among predicted cancers, and 4% of cancer mortalities[1–3]. Clinically, bladder cancer manifests in two primary forms: 75% account for non-muscle-invasive bladder cancer (NMIBC), while 20-25% muscle-invasive bladder cancer (MIBC), as well as 5% for metastasis. They exhibit significant variations in genetics, biology, management, and prognosis [3]. Approximately 90% are diagnosed as urothelial cell carcinoma, and the rest determined as adenocarcinoma, neuroendocrine carcinoma, or squamous cell carcinoma,

[4]. NMIBC has a 31-78% rate of advancement and a 1-45% five-year rate. While, MIBC has a 40-80% five-year rate. These rates of progression in high-grade NMIBC underline the need for effective diagnosis and surveillance [4, 5]. Currently, the major assessment for bladder cancer diagnosis and surveillance is involves urine cytology, cystoscopy, and urinary tract imaging [6, 7]. Extensive research exists regarding the clinical management of bladder cancer (BC), along with its aetiology, diagnostic tools, prognostic markers, and predictive biomarkers [3, 6, 8–12]. Diagnostic confirmation involves cystoscopy, and local and distant staging is determined from MRI and CT scans. Primary treatment includes a transurethral tumour

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excision, facilitating staging and guiding subsequent therapy. Histological prognostic markers, such as stage, grade, variable histology presence, and lympho-vascular invasion, are crucial in determining further management [13, 14]. Endoscopic resection of NMIBC is followed by intravenous chemotherapy or immunotherapy, yet recurrence rates remain substantial even with adjuvant therapy. Essential prognostic indicators, encompass various factors influencing a 5-year probability of progression, ranging from 40 to 44% in high-risk groups to >1% in low-risk groups, heavily reliant on stage and malignancy grade [3, 10]. The standard treatment for non-metastatic MIBC is neoadjuvant chemotherapy and radical cystectomy, followed by urinary diversion after surgery [8]. Surgical interferences have a mortality rate of 2%–8% and a 50% risk of complications [15–17]. Advanced disease stages, extravesical expansion, lymph node involvement, the existence of a prostatic urethra tumour, and high neutrophil-to-lymphocyte ratios are all negative postoperative prognostic indicators. Bladder cancer has five molecular subtypes with distinct genetic profiles and clinical prognoses, which are divided into basal-squamous and luminal classifications, albeit they are rarely used in clinical settings [18]. Cisplatin-based regimens remain the standard for systemic chemotherapy in metastatic bladder cancer, although response and survival rates are low, with a median survival duration of 12–14 months for individuals receiving first-line chemotherapy. Visceral metastasis growth and a Karnofsky performance level of 80% indicate poor prognostic indicators [15]. More current models include metastatic location, extent, and laboratory data such as Complete blood picture, albumin levels, and leucocyte count and haemoglobin levels. Checkpoint inhibitors provide a distinct therapeutic option for patients who are ineligible for cisplatin, with response rates ranging from 21 to 29% and median survival extending from 8 to 16 months [18]. Prognostic indicators that influence treatment success include *PD-L1* expression and gene expression patterns [19–21].

PIWIL-like genes are members of the Argonaute gene family and are essential for stem cell self-renewal and maintenance in multicellular animals. These genes catalyse the amplification of tiny RNAs (piRNAs), which are necessary for the suppression of transposons via target destruction and epigenetic silencing. Prominently expressed in germ cells, increased expression of these molecules has also been detected in several cancers, including *PIWIL*-like 1 and *PIWIL*-like 2. *PIWIL*-like 2 suppression influences cancer hallmarks such as proliferation and apoptosis via the *STAT3/BCLXL* signalling pathway, whereas studies show the effects of *PIWIL*-like 1 silencing on gene expression and cell proliferation in glioma and breast cancer cells. *PIWIL*-like 2 also contributes to DNA damage repair and chromatin modification [22–25]. The potential of *PIWIL* proteins/piRNAs as prognostic markers and targets in bladder cancer has been indicated by the limited research performed on this subject, indicating their potential attractiveness as prognostic markers and targets for therapeutic intervention. In this study, we analysed the tumour tissue, and urine samples of 220 bladder cancer

patients for their expression pattern of *PIWIL1*, *PIWIL2*, *PIWIL3*, and *PIWIL4* and associated their expression with clinico-pathological characteristics and survival data, and we aimed to assess the influence of expression patterns diagnostic and prognostic potential.

Materials and Methods

Subjects and Methods

Sample collection and preparation

Tumourous and tumor adjacent tissue from 220 bladder cancer patients from Theodor Bilharz Research Institute were selected for this study. The criteria were for bladder cancer patients who were diagnosed prior to any therapeutic intervention, who were about to undergo tumour resection. All cases were histologically diagnosed post-resection, and samples were stored at -80°C until use. Routine processing was conducted into paraffin sections and pathologically diagnosed for tumour stage and grade, which was according to the health organization 1973 grading of the urothelial carcinoma: well-differentiated (grade1, G1), moderately differentiated (grade2, G2) or poorly differentiated (grade3, G3). Previous cancer diagnosis, metastasized cancer from other origins were excluded from the selection. For urine samples twenty ml urine samples from the 220 BC patients and 70 healthy volunteers were collected, centrifuged at 4,000 rpm for 10 minutes at 25°C, samples were processed immediately, aliquoted and stored at -20°C till use. Written informed consent was obtained from all subjects (patients and volunteers), and the study was approved by the Ethics committee with number (FWA#0000106099; Serial# PT800) at Theodor Bilharz Research Institute.

RNA Extraction and purification

Total RNA from tissue samples was isolated using magnetic beads technique by use of Abbott Kit (Abbott Molecular, USA). For RNA isolation from urine, RNeasy Mini Kit (Qiagen, Germany) was used, the samples were all extracted in duplicates, then RNA concentration and purity for urine samples were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA).

cDNA synthesis and Real time PCR

The four primer assays for *PIWIL* transcript quantification were purchased from Qiagen and were ready to use. The primers contained the four isoforms *PIWIL1/HIWI*; *PIWIL2/HILI*; *PIWIL3*, and *PIWIL4*, and all experiments were carried out according to the manufacturer's instructions. Real-time PCR amplification was carried out using QuantiTect SYBR Green PCR Kits (Qiagen, Valencia, CA). In brief, using 10 ng of total RNA, cDNA was synthesized, and *PIWIL*1–4 expression were determined using One-step RT qPCR Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. *GAPDH* was used as an internal control. The primer assays were readily acquired. The expression levels were quantified absolutely by using an amplification curve and the comparative $2^{-\Delta\Delta CT}$ method was used to determine the fold change and expression patterns of the samples.

Histopathological study

The 220 bladder samples that were formalin-fixed and paraffin-embedded came from patients who underwent radical cystectomy combined with pelvic lymphadenectomy at the urology department of Theodor Bilharz Research Institute in Giza, Egypt. All specimens were processed at the Pathology Department of Theodor Bilharz Research Institute. Clinical information, such as age, gender, symptoms, physical indicators, laboratory results, and radiological findings, was taken from medical records. Neither neoadjuvant chemotherapy nor radiation therapy were administered to any of the patients in this study. Two pathologists reevaluated sections stained with haematoxylin and eosin (H and E) independently to determine the existence, grade, and pathological TNM staging of bilharziasis, as well as UC variant and divergent differentiation. The eighth edition of the American Joint Committee on Cancer (AJCC) staging system was used to stage the tumours, and the World Health Organisation (WHO) classification system was used to classify histological variants of the urinary tract.

PIWIL2 Immunohistochemistry

Tissue sections (4 µm thick) were cut from each block and stained using the PIWIL2 antibody (Novus Biologicals, LLC., USA), NBP2-24590 PIWIL2/HILI, following the manufacturer's protocol. The sections were first incubated at 95°C for 1 hour before undergoing dewaxing, rehydration, and antigen retrieval in a DAKO autoimmunostainer PT-link using Target Retrieval Solution (Citrate buffer, Low pH 6.1, 50x, DM829). After cooling to room temperature, the sections were rinsed in distilled water for 2 minutes. Endogenous peroxidase was blocked for 5 minutes using DakoEnVision™ FLEX Peroxidase Blocking Reagent (Code SM801) and then rinsed twice with diluted phosphate-buffered saline (PBS) using DakoEnVision™ FLEX Wash Buffer (20x) (Code DM831). The sections were incubated overnight at ~4°C for 20 hours with the primary antibody, diluted 1:100 (optimal dilution). The following day, the slides were rinsed twice with PBS. After this, they were treated with a secondary antibody at room temperature for 20 minutes using DakoEnVision™ FLEX HRP (Horseradish peroxidase) (Code SM802), followed by two more PBS washes. Positive staining was visualized using diaminobenzidine (DAB) with DakoEnVision™ FLEX DAB and Chromogen (Code DM827) and Substrate Buffer (Code SM803). The sections were counterstained with ready-to-use haematoxylin using DakoEnVision™ FLEX Haematoxylin (Code SM806), then dehydrated in graded ethanol, cleared with xylene, and mounted for microscopic examination. Burkitt lymphoma tissue sections were used as both positive and negative controls (negative control by omitting the primary antibody).

Evaluation of PIWIL2 Immunostaining

PIWIL2 expression in each tumor sample was evaluated independently by two pathologists without prior knowledge of the clinical data. The staining results were analyzed based on the percentage of positive cells, the cellular location, and the staining intensity. The

histoscore (H-score) system was applied to quantify PIWIL2 expression, ranging from 0 to 300. The H-score was calculated by semi-quantitative assessment of both the staining intensity (classified as negative (0), weak (1+), moderate (2+), or strong (3+)) and the percentage of positive cells. The formula used was: $H\text{-score} = 1 \times (\% \text{ cells } 1+) + 2 \times (\% \text{ cells } 2+) + 3 \times (\% \text{ cells } 3+)$. An H-score was determined for each case, and the mean score was calculated for each group of cases.

In Silico variant detection for PIWIL genes

Bladder cancer database used for detection of possible mutation variants analysis for PIWIL1, PIWIL2, PIWIL3, PIWIL4 from the publicly available database cBioPortal for Cancer Genomics and the cancer genome atlas project (<https://www.cbioportal.org>) to predict most prevalent variants implicated in BC, additionally miRNA net was used to predict miRNA associations with bladder cancer.

Statistical analysis

The appropriate patient sample size was determined through a calculation based on previous research focused on diagnosis in BC [26]. Continuous variables were expressed as mean ± standard deviation (SD), with their distribution assessed using a normality test. Categorical variables were analyzed using frequencies and percentages. Statistical significance was considered for p-values less than 0.05. Data analysis was performed with Microsoft Excel 2016 and IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, N.Y., USA). The student's t-test was used to compare the means of normally distributed variables between groups, while the χ^2 test was used to evaluate the distribution of categorical variables. Receiver operating characteristic (ROC) curves were used to assess the diagnostic performance of the markers, with the area under the curve (AUC) calculated. Logistic regression analysis was conducted to determine diagnostic and prognostic accuracy, along with the risk of bladder cancer. Survival analysis was performed using Kaplan-Meier curves and the Log Rank (Mantel-Cox) test.

Results*Patient and control characteristics*

The study enrolled a total of 220 participants diagnosed with bladder cancer (BC), with an average age of 59.1 ± 8.7 years. Of these, 195 were male (88.6%) and 25 were female (11.4%). Additionally, a control group comprised 70 healthy individuals without a history of bladder disease, including 49 males (70%) and 21 females (30%), with an average age of 54.5 ± 9.6 years. Among the BC patients, 39 had low-grade BC with a mean age of 58.9 ± 6.9 years, and 72 had high-grade BC with a mean age of 60.2 ± 8.3 years. Smoking history was gathered from medical records, classifying those who had smoked less than an average of one cigarette per day for fewer than 12 months in their lifetime as non-smokers. This group accounted for 55 patients (25%), while the remaining 165 (75%) were categorized as smokers. Additionally, 175 cases (79.5%) tested positive for bilharziasis, while 45 cases (20.5%) tested negative (Table 1). Demographic analysis revealed

a significant difference between BC patients and controls regarding age ($P < 0.001$), and there was also a statistically significant difference in sex distribution ($P < 0.001$), with a higher prevalence of males among BC patients.

Expression of the studied PIWILs in samples of BC patients

Using GAPDH as a housekeeping gene, we observed a significant downregulation of all four PIWIL mRNAs in both urine and tissue samples from bladder cancer (BC) patients compared to controls. Statistical analysis via Student's t-test demonstrated that the expression levels of PIWIL-1, PIWIL-2, PIWIL-3, and PIWIL-4 in the urine of BC patients were markedly lower than those in controls, with p-values of < 0.001 , 0.004, < 0.001 , and < 0.001 , respectively (Table 2, Figure 1a). This trend of decreased expression was similarly observed in tumor tissue samples from BC patients when compared to adjacent non-tumor tissues, which served as a control group. The statistical analysis confirmed significant differences in the expression of PIWIL-1, PIWIL-2, PIWIL-3, and PIWIL-4 between tumor and non-tumor tissues, with all p-values being < 0.001 (Table 2, Figure 1b).

Diagnostic performances of the studied PIWILs regarding urine and tumour tissue samples

ROC curves were employed to assess the diagnostic performance of PIWIL-1, PIWIL-2, PIWIL-3, and PIWIL-4 in urine samples from BC patients versus controls, as well as in tumour tissue samples from BC patients compared to non-tumour tissues. These analyses aimed to evaluate the specificity and sensitivity for predicting bladder cancer (BC) and to determine the discriminatory capacity between patients and healthy individuals.

For distinguishing BC patients from controls using urine samples, PIWIL-1 had a cut-off value of > 0.5 , with a sensitivity of 100% and specificity of 96%. The area under the curve (AUC) was 0.964, with a 95% confidence interval (C.I) of 0.939–0.988 and a p-value of

0.001. PIWIL-2, with a cut-off value of > 0.5 , achieved 100% sensitivity and 93% specificity, with an AUC of 0.927, a 95% C.I of 0.893–0.962, and a p-value of 0.001. For PIWIL-3, the cut-off value was > 0.45 , yielding a sensitivity of 100% and specificity of 94%. The AUC was 1.0, with a 95% C.I of 1.0–1.0, and a p-value of 0.001. Similarly, PIWIL-4 had a cut-off value of > 0.5 , with 100% sensitivity and 96% specificity. The AUC was 0.964, with a 95% C.I of 0.939–0.988, and a p-value of 0.001 (Table 3, Figure 2a).

In tissue samples, PIWIL-1, PIWIL-2, PIWIL-3, and PIWIL-4 all showed cut-off values of > 0.5 , with both sensitivity and specificity reaching 100%. The AUC for each was 1.0, with a 95% C.I of 1.0–1.0, and a p-value of < 0.001 (Table 3, Figure 2b).

Univariate logistic regression analysis for prognostic values

Logistic regression analysis was conducted on the four PIWILs to assess their efficacy as predictors and/or prognostic markers for bladder cancer (BC). The findings revealed a significant statistical association with BC.

In urine samples, the four PIWILs showed strong potential as prognostic biomarkers for BC patients compared to controls, with the logistic regression analysis confirming statistical significance. Specifically, a one-unit increase in PIWIL-1 was associated with an odds ratio (OR) increase of 0.068 (95% C.I = 0.016–0.284, $P < 0.001$). For PIWIL-2, each unit increase in its levels resulted in an odds increase by a factor of 0.296 (95% C.I = 0.124–0.709, $P = 0.006$). In the case of PIWIL-3, the odds increased by a factor of 0.051 (95% C.I = 0.013–0.254, $P < 0.001$). Finally, PIWIL-4 demonstrated statistical significance in urine samples with an OR of 0.063 (95% C.I = 0.015–0.262, $P < 0.001$) (Table 4).

Association analysis between the expression of the studied PIWILs and the clinicopathological factors among BC patients

No significant associations were detected between

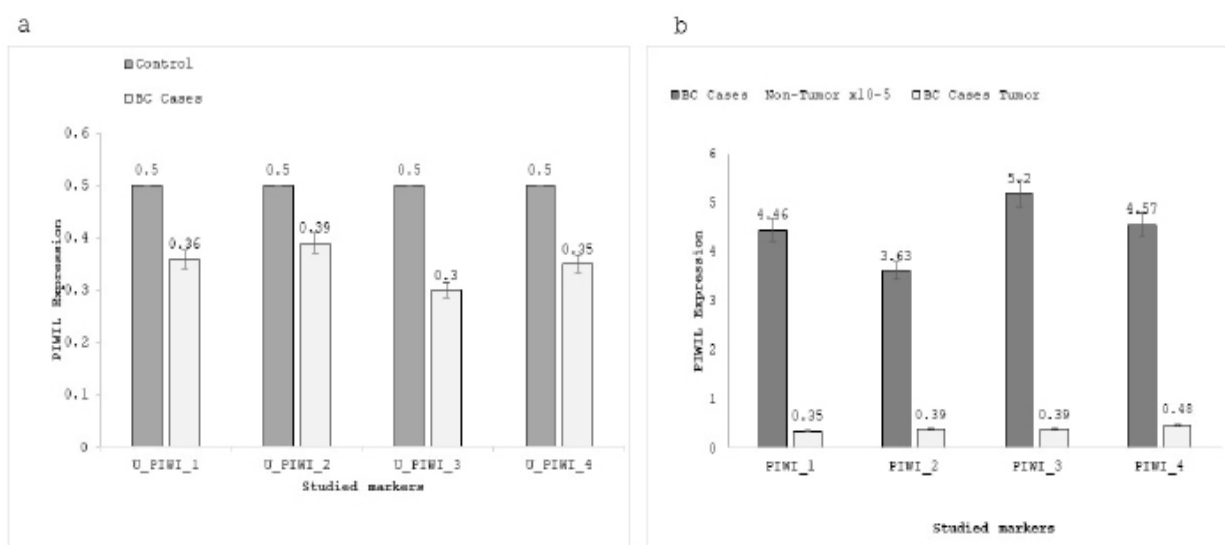


Figure 1. a) PIWIL expression in urine samples; b) in Tissue samples of BC cases.

Table 1. Clinico-Pathological Features for Patients

		Control N=70	BC Cases N=220	P. value
Age		54.5±9.6	59.1±8.7	<0.001**
Sex (%)	Female	21 (30.0)	25 (11.4)	<0.001**
	Male	49 (70)	195 (88.6)	
Smoking (%)	No	0 (0.0)	55 (25.0)	
	Yes	0 (0.0)	165 (75.0)	
Bilharziasis (%)	No	0 (0.0)	45 (20.5)	
	Yes	0 (0.0)	175 (79.5)	
Clinical Ex. (%)	T1	0 (0.0)	50 (22.7)	
	T2	0 (0.0)	100 (45.5)	
	T3	0 (0.0)	70 (31.8)	
Cytology (%)	Negative	0 (0.0)	70 (31.8)	
	Positive	0 (0.0)	150 (68.2)	
Histopathological type of the Tumour (%)	ScC	0 (0.0)	40 (18.2)	
	Tcc	0 (0.0)	180 (81.8)	
Clinical staging (%)	T1	0 (0.0)	25 (11.4)	
	T2	0 (0.0)	50 (22.7)	
	T3	0 (0.0)	125 (56.8)	
	Ta	0 (0.0)	20 (9.1)	
Serum Creatinine (%)	Normal	0 (0.0)	180 (81.8)	
	High	0 (0.0)	40 (18.2)	
Lymph Node (%)	Negative	0 (0.0)	110 (50.0)	
	Positive	0 (0.0)	110 (50.0)	
Tumour Grade (%)	GI	0 (0.0)	20 (9.1)	
	GII	0 (0.0)	60 (27.3)	
	GIII	0 (0.0)	140 (63.6)	
PIWI2 IRS		10.21±1.74	3.97±1.92	P <0.01*
Recurrence (%)	No		130 (59.1)	
	Yes		90 (40.9)	
5 Y Survival (%)	Live		180 (81.8)	
	Die		40 (18.2)	

Age and PIWI2 immunoreactive score (IRS) are represented as Mean±SD; the data were analysed by t test. While sex, smoking, bilharziasis, clinical Ex., cytology, histopathological type of the tumour, clinical staging, serum creatinine, lymph node, tumour grade, recurrence and survival are represented as frequency and percent; the data were analysed by X² test.

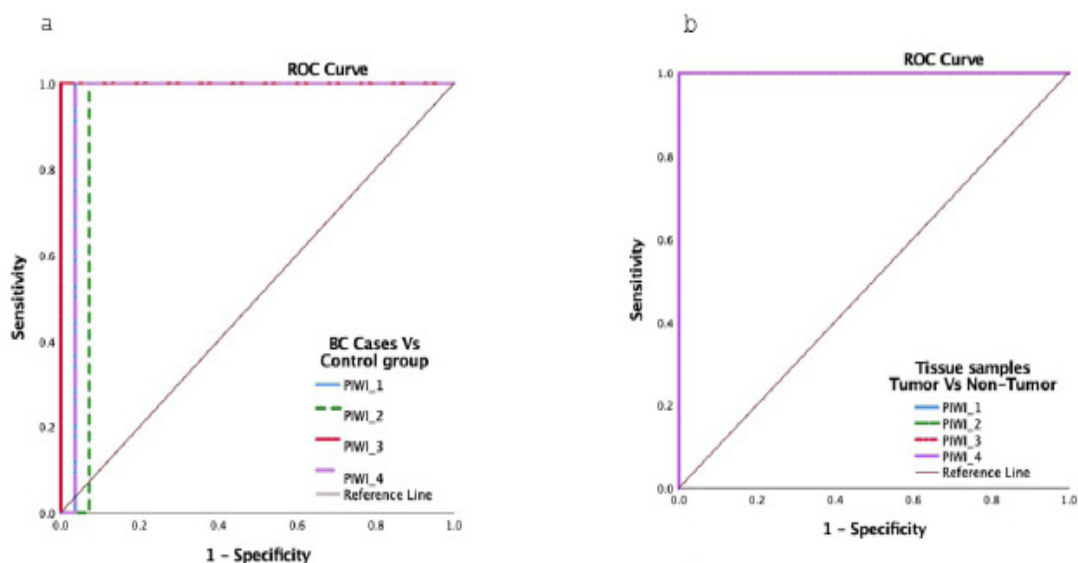


Figure 2. ROC Curve of PIWIL a) in urine samples and b) in Tissue samples

Table 2. *PIWIL* Expressions in the Studied Groups

	Urine samples		p. value	BC Cases		
	Control N=70	BC Cases N=220		Non-Tumour x10 ⁻⁵	Tumour	P. value
<i>PIWIL-1</i>	0.5±0.01	0.36±0.25	<0.001**	4.46±1.83	0.35±0.27	<0.001**
<i>PIWIL-2</i>	0.5±0.01	0.39±0.31	0.004**	3.63±1.04	0.39±0.3	<0.001**
<i>PIWIL-3</i>	0.5±0.01	0.30±0.12	<0.001**	5.2±1.6	0.39±0.31	<0.001**
<i>PIWIL-4</i>	0.5±0.01	0.35±0.26	<0.001**	4.57±1.2	0.48±0.39	<0.001**

PIWIL-1-4 expression is represented as Mean±SD; the data were analysed by t test. * P value <0.05 is significant, ** P value <0.001 is highly significant.

Table 3. The Diagnostic Performance of *PIWIL* in Distinguishing BC Patients from Controls Using ROC Curve Analysis

Sample type		Cut-off	Sn. %	Sp.%	AUC	S. E	95% C. I		P. Value
							Lower	Upper	
Urine samples	<i>PIWIL-1</i>	>0.5	100	96	0.964	0.013	0.939	0.988	<0.001**
BC patients and control group.	<i>PIWIL-2</i>	>0.5	100	93	0.927	0.018	0.893	0.962	<0.001**
	<i>PIWIL-3</i>	>0.45	100	94	1	0	1	1	<0.001**
	<i>PIWIL-4</i>	>0.5	100	96	0.964	0.013	0.939	0.988	<0.001**
	<i>PIWIL-1</i>	>0.5	100	100	1	0	1	1	<0.001**
Tumour and non-tumour tissue in BC patients.	<i>PIWIL-2</i>	>0.5	100	100	1	0	1	1	<0.001**
	<i>PIWIL-3</i>	>0.5	100	100	1	0	1	1	<0.001**
	<i>PIWIL-4</i>	>0.5	100	100	1	0	1	1	<0.001**
	<i>PIWIL-1</i>	>0.5	100	100	1	0	1	1	<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.

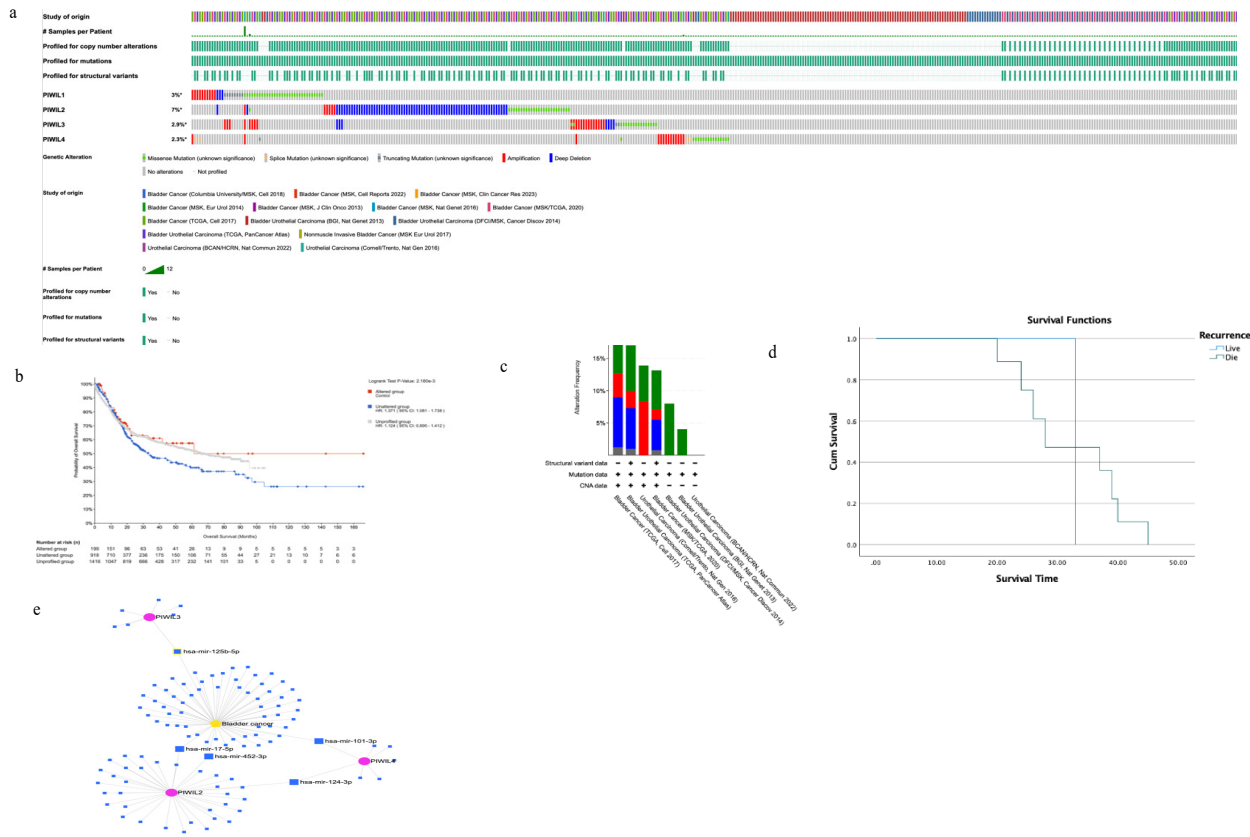


Figure 3. (a) Frequency of mutated *PIWIL* genes and their predicted variants' implication for BC through TCGA; via BioPortal for Cancer Genomics (<https://www.cbioportal.org>). (b) In silico kaplanmeier analysis for predicted cases (c) Reported cases with mutated *PIWIL* genes in bladder cancer (d) kaplanmeier analysis for analyzed cases in the study e) miRNet prediction of *PIWIL* genes and BC

Table 4. Prognostic Performance of *PIWIL* in the Studied Groups.

	Urine samples			Tissue samples		
	OR	95% C. I	P. value	OR	95% C. I	P. value
<i>PIWIL-1</i>	0.068	0.016-0.284	<0.001**	0.043	0.013-0.102	<0.001**
<i>PIWIL-2</i>	0.296	0.124-0.709	0.006**	0.035	0.016-0.110	<0.001**
<i>PIWIL-3</i>	0.051	0.013-0.254	<0.001**	0.041	0.014-0.131	<0.001**
<i>PIWIL-4</i>	0.063	0.015-0.262	<0.001**	0.039	0.015-0.123	<0.001**

OR, Odd Ratio; C.I, Confidence Interval; P value calculated depend on logistic regression analysis. * P value<0.05, **P=<0.001

the expression levels of the studied *PIWIL*s and most clinicopathological factors in bladder cancer (BC) patients, including smoking history, bilharziasis, clinical examination findings, urine cytology results, histopathological tumor types, clinical staging, lymph node involvement, and tumor grade. However, a highly significant association was observed between tumor recurrence and the expression of *PIWIL-2* and *PIWIL-3*, with mean $\pm$ SD values of 0.33 ± 0.23 ($P = 0.001$) and 0.28 ± 0.12 ($P = 0.001$), respectively. Additionally, for *PIWIL-4*, a significant association was found between its expression and tumor recurrence, with a mean $\pm$ SD of

0.31 ± 0.12 ($P = 0.01$).

Regarding patient survival, significant correlations were identified with the expression levels of *PIWIL-1*, *PIWIL-2*, *PIWIL-3*, and *PIWIL-4*, showing mean $\pm$ SD values of 0.12 ± 0.02 ($P = 0.02$), 0.36 ± 0.27 ($P = 0.01$), 0.12 ± 0.02 ($P = 0.01$), and 0.12 ± 0.02 ($P = 0.001$), respectively (Table 5). These findings suggest that while *PIWIL* expression may not correlate with many clinical factors, it could be indicative of tumor recurrence and patient survival in BC cases.

Table 5. Associations between *PIWIL* Repressions with Demographic and Clinical Data

Characteristic	Type	<i>PIWIL-1</i> Mean $\pm$ SD	P. value	<i>PIWIL-2</i> Mean $\pm$ SD	P. value	<i>PIWIL-3</i> Mean $\pm$ SD	P. value	<i>PIWIL-4</i> Mean $\pm$ SD	P. value
Sex	Female	0.35 $\pm$ 0.26	0.9	0.33 $\pm$ 0.25	0.3	0.29 $\pm$ 0.14	0.7	0.34 $\pm$ 0.11	0.9
	Male	0.36 $\pm$ 0.25		0.40 $\pm$ 0.32		0.30 $\pm$ 0.12		0.35 $\pm$ 0.27	
Smoking	No	0.36 $\pm$ 0.24	0.9	0.34 $\pm$ 0.24	0.1	0.29 $\pm$ 0.13	0.4	0.36 $\pm$ 0.26	0.6
	Yes	0.36 $\pm$ 0.25		0.41 $\pm$ 0.33		0.30 $\pm$ 0.12		0.34 $\pm$ 0.26	
Bilharziasis	No	0.38 $\pm$ 0.27	0.6	0.37 $\pm$ 0.31	0.7	0.28 $\pm$ 0.13	0.2	0.33 $\pm$ 0.22	0.6
	Yes	0.35 $\pm$ 0.24		0.40 $\pm$ 0.32		0.31 $\pm$ 0.12		0.35 $\pm$ 0.26	
Clinical Ex.	T1	0.42 $\pm$ 0.34	0.1	0.41 $\pm$ 0.32	0.9	0.28 $\pm$ 0.13	0.4	0.36 $\pm$ 0.32	0.8
	T2	0.34 $\pm$ 0.20		0.39 $\pm$ 0.31		0.30 $\pm$ 0.12		0.34 $\pm$ 0.27	
	T3	0.33 $\pm$ 0.23		0.39 $\pm$ 0.31		0.31 $\pm$ 0.12		0.35 $\pm$ 0.18	
Cytology	-ve	0.33 $\pm$ 0.18	0.2	0.37 $\pm$ 0.29	0.6	0.32 $\pm$ 0.12	0.1	0.34 $\pm$ 0.28	0.9
	+ve	0.37 $\pm$ 0.28		0.40 $\pm$ 0.33		0.29 $\pm$ 0.13		0.35 $\pm$ 0.25	
Histopathological type of the Tumour	Sc	0.40 $\pm$ 0.34	0.3	0.41 $\pm$ 0.36	0.7	0.28 $\pm$ 0.12	0.2	0.32 $\pm$ 0.13	0.5
	Tcc	0.35 $\pm$ 0.23		0.39 $\pm$ 0.30		0.30 $\pm$ 0.12		0.35 $\pm$ 0.28	
Clinical staging	T1	0.32 $\pm$ 0.12	0.07	0.36 $\pm$ 0.26	0.8	0.34 $\pm$ 0.21	0.4	0.50 $\pm$ 0.45	0.8
	T2	0.42 $\pm$ 0.34		0.39 $\pm$ 0.33		0.38 $\pm$ 0.28		0.44 $\pm$ 0.44	
	T3	0.31 $\pm$ 0.13		0.29 $\pm$ 0.12		0.31 $\pm$ 0.12		0.27 $\pm$ 0.13	
	Ta	0.36 $\pm$ 0.37		0.34 $\pm$ 0.27		0.35 $\pm$ 0.24		0.30 $\pm$ 0.12	
Serum Creatinine	Normal	0.36 $\pm$ 0.26	0.9	0.39 $\pm$ 0.32	0.7	0.30 $\pm$ 0.13	0.9	0.34 $\pm$ 0.23	0.6
	High	0.35 $\pm$ 0.21		0.41 $\pm$ 0.31		0.30 $\pm$ 0.12		0.37 $\pm$ 0.35	
Lymph Node	-ve	0.36 $\pm$ 0.27	0.7	0.39 $\pm$ 0.32	0.9	0.29 $\pm$ 0.13	0.4	0.35 $\pm$ 0.26	0.9
	+ve	0.35 $\pm$ 0.22		0.39 $\pm$ 0.31		0.31 $\pm$ 0.12		0.35 $\pm$ 0.26	
Tumour Grade	GI	0.43 $\pm$ 0.39	0.4	0.39 $\pm$ 0.38	0.9	0.29 $\pm$ 0.14	0.8	0.31 $\pm$ 0.12	0.8
	GII	0.35 $\pm$ 0.24		0.38 $\pm$ 0.30		0.30 $\pm$ 0.13		0.35 $\pm$ 0.29	
	GIII	0.35 $\pm$ 0.23		0.40 $\pm$ 0.31		0.30 $\pm$ 0.12		0.35 $\pm$ 0.26	
Recurrence	No	0.38 $\pm$ 0.27	0.1	0.48 $\pm$ 0.39	0.001**	0.33 $\pm$ 0.12	0.001**	0.40 $\pm$ 0.37	0.01*
	Yes	0.34 $\pm$ 0.23		0.33 $\pm$ 0.23		0.28 $\pm$ 0.12		0.31 $\pm$ 0.12	
5 Y Survival	Alive	0.36 $\pm$ 0.27	0.02*	0.45 $\pm$ 0.07	0.01*	0.30 $\pm$ 0.12	0.01*	0.34 $\pm$ 0.28	0.001**
	Died	0.12 $\pm$ 0.02		0.36 $\pm$ 0.27		0.12 $\pm$ 0.02		0.12 $\pm$ 0.02	

PIWIL expressions are represented as Mean $\pm$ SD; the data were analysed by t test. * P value <0.05 is significant, ** P value <0.001 is highly significant.

Table 6. Kaplan-Meier Analysis of Recurrence Regarding Survival Time

		Mean time/ month	Std. Error	Log Rank (Mantel-Cox)	P. value
Recurrence	Living	33	0	0.324	0.569
	Deceased	32.028	1.39		
	Overall	32.125	1.25		

P. value depending on the Kaplan-Meier test. * P value <0.05 is significant, ** P value <0.001 is highly significant.

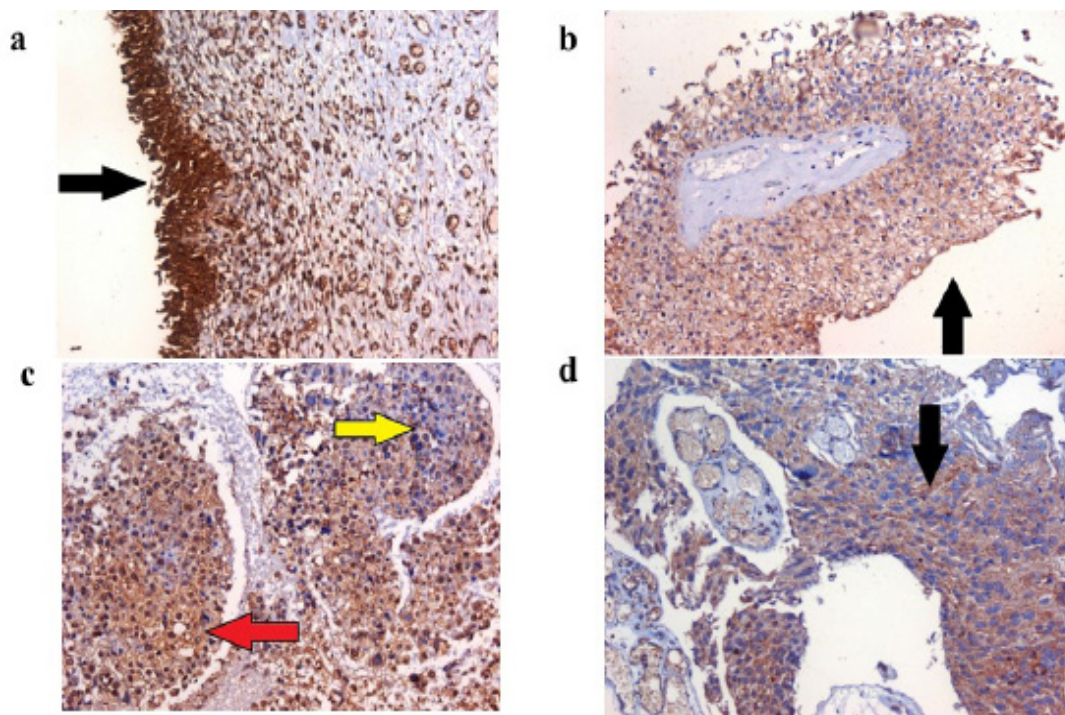


Figure 4. a) section in a case of chronic cystitis showing high expression of *PIWIL2* in the lining urothelium (black arrow), b) section in papillary transitional cell carcinoma showing lower expression of *PIWIL2* in urothelial cells (black arrow) compared to non-malignant urothelium; c) section in case of invasive high grade UC showing focal positive (red arrow) and focal negative (yellow arrow) expression of *PIWIL2* in malignant cells.; d) sections in invasive poorly differentiated squamous cell carcinoma showing low expression of *PIWIL2* in malignant cells (black arrow) (Haematoxylin and eosin stain, X200).

Survival Analysis

The Kaplan-Meier (KM) method is a widely recognized approach for analyzing “time-to-event” data, making it especially useful in survival analysis. This technique helps researchers evaluate various factors, including patients or participants who may have been lost to follow-up, dropped out of the study, developed the disease of interest, or survived it. In the current study, the Log Rank (Mantel-Cox) test was employed for survival analysis, which indicated no significant association between bladder cancer (BC) patients regarding tumor recurrence and survival time. The mean survival times for living and deceased patients were 33 months and 32.028 months, respectively, with a log rank value of 0.324 and a p-value of 0.569.

In Silico Analysis for *PIWIL* genes

Figure 3 illustrates the role of *PIWIL* genes in the progression of bladder cancer (BC), with *PIWIL2* emerging as the most significant, showing a 7% detection rate of structural variants associated with BC. Panel 3b provides a predicted survival analysis for BC in relation

to these *PIWIL* genes. Furthermore, miRNet predictions were utilized to explore the association between BC and the *PIWIL* genes (Figure 3e), revealing direct links between *PIWIL2*, *PIWIL3*, and *PIWIL4* with *miR-124-3p*, *miR-125-5p*, *miR-452*, *miR-17-5p*, and *miR-101-3p*. These microRNAs are recognized as potential tumor suppressors involved in various processes, including proliferation, apoptosis, and metastasis. Additionally, the frequency of *PIWIL* mutations was analyzed through TCGA and cBioPortal (Figure 3c), indicating that the frequency of reported mutations surpassed 15%. The alterations identified included structural changes, copy number variations, and mutations.

Immunohistochemistry

In bladder cancer (BC) cases, the immunohistochemical analysis indicated a decrease in *PIWIL2* expression levels in bladder tissue sections, yielding an immunoreactive score (IRS) of 3.97 ± 1.92 (Figure 4). This reduction was statistically significant compared to control cases, which had an IRS of 10.21 ± 1.74 ($P < 0.01$) (Table 1).

Discussion

The incidence of bladder cancer is increasing in developing countries [27]. Timely and accurate diagnosis plays a critical role in the effective treatment of cancer [15]. Recently, more and more teams are interested in the use of molecular-genetic methods in the early diagnosis of a bladder tumour [8]. The identification of molecular markers in urine, tissue, or blood presents promising opportunities to enhance our understanding of the biological mechanisms underlying cancer and their effects on both the microenvironment and macroenvironment. This approach can facilitate targeted therapies, improve prognostic predictions, enable early disease detection, and assist in risk stratification for patients. Understanding and identifying the molecular pathways and biomarkers involved in the development of bladder cancer is essential, as it paves the way for personalized treatment plans in clinical practice.

In the current study, expression of all PIWIL mRNAs were assessed using real-time PCR in 220 BC patients. The levels of expression of *PIWIL1*, *PIWIL2*, *PIWIL3*, *PIWIL4* in tumour and nontumorous adjacent tissue was carried out in addition to urine samples from BC patients and healthy controls, the expression was associated with clinicopathological features and survival data. In terms of expression, all PIWIL mRNAs were found to be significantly downregulated in both urine and tissue samples (Table 2, Figure 1). ROC curve analysis additionally indicated significant performance in terms of diagnostic capability, as well as prognostic potential through odd ratios (Table 3, Figure 2, and Table 4). In terms of clinicopathological features, PIWIL repression was found to be significantly associated to 5-Year survival, as well as a significant relationship involving the expression patterns of *PIWIL2*, *PIWIL3*, and *PIWIL4* and tumour recurrence (Table 5).

The *PIWIL* genes are known to produce multiple transcripts, some of which may be driven by putative intragenic promoters rather than the canonical promoter, a characteristic linked to cancer development [28]. Research has demonstrated that the expression of PIWIL genes, particularly *PIWIL2*, *PIWIL3*, and *PIWIL4*, directly influences the progression of bladder cancer (BC). This influence is mediated through interactions with specific microRNAs (miRNAs), including miR-17-5p, miR-452-3p, miR-105-3p, and miR-125b-3p, as well as an interaction between *PIWIL2* and *PIWIL4* via miR-124-3p (Figure 3e). Numerous studies have shown that dysregulation of miRNA expression is associated with poor prognosis and survival outcomes across various cancers, such as breast, prostate, and bladder cancers [29–35]. Our findings reveal a novel direct interplay between *PIWIL2*, *PIWIL4*, and miR-124-3p. This pathway underscores several target miRNAs that could potentially serve as therapeutic targets, as highlighted in this study. Thus, the aberrant expression of *PIWIL2* and *PIWIL4* may facilitate the inactivation of tumour suppressor mechanisms (Figure 3e). Furthermore, the implicated miRNAs present attractive opportunities for therapeutic intervention, enabling the development of combination

therapies that target either specific antibodies against *PIWIL2* or the PIWI/miRNA RISC complex [36], this is particularly evident when observing Table 5, which indicated a predicted significant co-occurrence between *PIWIL2* and *PIWIL4*, with bladder cancer. In Figure 3a and b, the highest frequency of alterations was observed for *PIWIL2* for BC, and as such this marker was used for IHC analysis (Figure 4). *PIWIL2* was found to be significantly repressed in BC tissues, and IRS score (Table 1) was found to be significantly correlated to BC tissues like findings indicated by [37, 38]. These findings are consistent with earlier research demonstrating that Piwi-like 2 protein is linked to histone acetylation. This supports the notion that bladder cancer (BC) patients who test positive for Piwi-like 2 expressions may derive significant benefits from therapies involving DNA methyltransferase inhibitors or histone deacetylase inhibitors. Furthermore, given the observed expression patterns, microRNA interventions may also offer therapeutic potential for these patients [37, 39–43].

The current study's patients had a mean age of 59.1 ± 8.7 , with 11.4% females and 88.6% males, which is consistent with the risk factors linked with BC (Table 1). The table includes all typical parameters. We observed a substantial association between age and tissue expression for PIWIL mRNA, while other clinical variables were not statistically significant. The findings were in accordance with Taubert, et al. [43], and Al-Janab et al. [38], who observed similar outcomes for no association with clinicopathological features apart from age for colorectal cancer and renal cell carcinoma, respectively [38, 40, 43, 44]. In conclusion, prior research has shown that *PIWIL2* may inhibit apoptosis, potentially influencing patient prognosis and responses to therapy. However, further exploration of its specific role is necessary. Additionally, *PIWIL2*, *PIWIL3*, and *PIWIL4* have been found to correlate significantly with clinicopathological factors and overall survival. This indicates that these PIWIL proteins hold promise as prognostic biomarkers for bladder cancer patients, offering insights that could enhance personalized treatment strategies.

Author Contribution Statement

All authors contributed equally in this study.

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Declarations

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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); (FWA#0000106099;

Serial# PT800). 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 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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