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ORIGINAL ARTICLE

piRABC and TNFSF4 as biomarkers for diagnosis and staging of bladder cancer.

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ABSTRACT

Background: In Egypt, bladder cancer (BC) is among the most common cancers. Piwi-interacting RNA associated with bladder cancer (piRABC) expression was downregulated in bladder cancer tissue. The piRABC increase TNFSF4 levels that leads to cancer cell death. This study aimed to assess the roles of piRABC and TNFSF4 in BC detection and to evaluate their roles in cancer grading and staging.

Methods: Forty BC patients and 40 controls were enrolled. A quantitative real-time PCR was used to measure the expression of piRABC. ELISA kit was used for determining serum TNFSF4.

Results: Compared to controls, BC patients had significantly lower PiRABC expression l and TNFSF4 levels ($p<0.0001$). With an area under the ROC curve of 0.938 for PiRABC and 0.874 for the TNFSF4, they were able to detect BC. On multivariate analysis, low PiRABC and TNFSF4 levels were significant independent predictive factors of BC (AOR = 133 and 12.4, respectively). With more advanced BC stages, PiRABC expression displayed a negative trend. For BC patients' overall survival, PiRABC and TNFSF4 may be significant independent prognostic factors ($p=0.038$ and 0.026, respectively).

Conclusions: Decreased levels of piRABC expression and TNFSF4 appear to be independent predictors of BC. The low expression of piRABC had accepted prediction criteria for BC better than TNFSF4. The piRABC showed a decreasing trend with tumor grading and staging, while TNFSF4 exhibited a declining tendency throughout BC stages, despite having a small impact in BC grading. Greater expression of TNFSF4 and piRABC was linked to prolonged patient survival rates.

Keywords:

Bladder cancer; Biomarker; Diagnostic; piRABC, TNFSF4

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INTRODUCTION

The most prevalent and fatal form of urinary neoplasm is bladder cancer (BC), and its incidence has steadily risen in recent years [1]. With more than 1,300,000 individuals worldwide, BC is a frequent cancer [2].

Bladder cancer is one of the most prevalent malignancies in Egypt, where it accounts for 6.9% of cases in both sexes and 10.7% of cases in males. In terms of distribution, lower Egypt accounts for 8.8% of it, middle Egypt for 14.2%, and higher Egypt for 12.6%. Urinary schistosomiasis is the primary risk factor for bladder cancer in Egypt, and despite ongoing efforts to control it, this relationship still exists [3]. Currently, surgical resection, radiotherapy, and chemotherapy are available therapies for BC. However, in patients undergoing these treatments, BC cells' muscle invasion and medication resistance frequently lead to cancer relapse and spread [4]. Therefore, it is urgently necessary to comprehend the molecular mechanisms behind the development of BC and to create a more potent treatment strategy [5].

The piwi-interacting RNA (piRNA) class is a large group of non-coding RNA that complexes with Argonaute proteins [6]. The piRNA causes post-transcriptional and epigenetic silencing of transposable elements [7]. The ability of piRNAs to silence transposons and maintain genome integrity was first discovered to be strongly expressed in germline cells [8]. This function shielded the genome from anomalies in gametogenesis and fertility caused by transposons. Furthermore, piRNAs have been found to be broadly expressed in somatic cells [9]. They engage in a variety of regulatory functions, including controlling mRNA stability and translation, preserving stem cell potential, and interacting with other proteins. [10]. These complex regulatory mechanisms significantly broaden the range of piRNA presence and attract the interest of scientists especially in carcinogenesis [11].

In bladder cancer, piwi-interacting RNA associated with bladder cancer (piRABC) expression was downregulated. Comparing bladder cancer and healthy tissues, PiRABC showed remarkably high levels of differential expression. In vitro studies suggest that high piRABC can inhibit the induction of cell death [12]. Numerous cancers, including cervical cancer [13], gastric cancer [14, 15], breast cancer [16], multiple myeloma [17], and hepatocellular carcinoma [18], have been linked to the piRNAs. These results suggested that tumor suppressor genes, or oncogenes, both play significant roles in the development of cancer.

It has been demonstrated that the co-stimulatory checkpoint protein tumour necrosis factor receptor superfamily member 4 (TNFSF4) increases T lymphocytes' anti-neoplastic function [19]. Numerous immunological and non-immune cell types express TNFSF4. Researchers discovered a potential connection with TNFSF4 and proposed that piRABC might increase TNFSF4 levels, thereby promoting bladder cancer cell death [20]. Because of the above evidence, this study aimed to assess the roles of piRABC and TNFSF4 in the detection of BC and to evaluate their roles in cancer grading and staging.

METHODS

Study design:

With the use of Epi Info program 6 (Atlanta, Georgia, USA), the sample size for this case-control study was calculated. A research power of 95%, a confidence level of 95%, and a case-to-control ratio of 1:1 were the parameters used in the calculation. In a recent investigation on serum TNFSF4, the mean and standard deviation values of patients and controls were taken as factors for calculation [21]. There were 80 subjects in total, with 40 in each group.

Subjects:

Between December 2022 and August 2023, 40 BC patients were selected from the Urology Department. As a control group, 40 healthy people were chosen. By examining the histopathology of a cystoscopy bladder biopsy, patients with newly diagnosed BC are identified. Patients who had other cancer sources and BC patients who had begun therapy were excluded. Every BC patient underwent a thorough clinical examination. BC patients were graded and staged according to the following references [22, 23]. Table 1 lists the clinicopathological features of the study subject.

Outcome:

Overall survival was the study's primary outcome. From the date of diagnosis through the date of death, or the censor date, the survival period was determined. A 6-month follow-up period included monthly assessments of survival.

Sampling:

In plain vacutainers that were manufactured by Becton Dickinson, which is located in Franklin Lakes, NJ, USA, two mL of whole blood were collected from all participants. The blood was left undisturbed until it formed a clot before the serum was separated and kept at a temperature of -20 °C.

TNFSF4 assessment:

The measurement of serum TNFSF4 was carried out as per the manufacturer's guidelines using a sandwich ELISA kit from LS BIO, USA. The catalogue number is LS-F12800. The coefficients of variability for the intra- and inter-assays were 6.4% and 10.3%, respectively.

piRABC expression:

To quantify the expression of piRABC in the serum, a quantitative real-time polymerase chain reaction was employed. The steps in this process are extraction of the messenger RNA (mRNA), reverse transcription (RT) to produce cDNA, and real-time PCR using DNA fluorescent dye for selective amplification.

By utilization of the miRNeasy Serum Plasma kit (Catalogue no. 217184; Qiagen, Hilden, Germany), serum total RNA was isolated. The steps were followed in line with the manufacturer's instructions. The RNA was evaluated using gel electrophoresis and spectrophotometer (Nano Drop 1000, Wilmington, DE, USA).

The miScript RT II kit from QIAGEN GmbH in Hilden, Germany utilizes 1 µg of extracted RNA, 1 µL of miScript reverse transcriptase, and 4 µL of miScript HiFlex Buffer to transform all RNA types into complementary DNA (cDNA). The thermal profile was 60 minutes at a temperature of 37°C, followed by 5 minutes at 95°C. The reverse transcription (RT) process was carried out using a thermocycler from Perkin Elmer in Singapore. The cDNA was then stored at -80°C until PCR runs.

We utilized the StepOneTM System (Applied Biosystems, USA) and the miScript SYBR Green PCR Kit (Qiagen, Germany). The 2XQuantiTect SYBR Green PCR Master Mix (10 µL), universal reverse PCR primer for small RNA (1.5 µL), forward PCR primer (1 µL), cDNA (4 µL), and distilled water (3.5 µL) were combined to create the PCR mixture (20 µL).

The forward primer for piRABC had the following sequence: 5'-TTCAAGCCAGGAAAGCACTG-3' and the forward primer for RNU6 had the following sequence: 5'-CTCGCTTCGGCAGCACATATACT-3' (Sangon Biotech, Shanghai, China). PiRABC level normalization was done using RNU6.

The PCR process was carried out using the following parameters: an initial step of heating the reaction to 95 °C for 15 minutes, followed by 40 cycles each of three stages: heating to 94 °C, cooling to 60 °C, and heating to 70 °C. Times of these stages were 15 seconds, 30 seconds, and 30 seconds, respectively. RNU6 served as an internal control and was used to normalize the expression level of piRABC. The $2-\Delta\Delta CT$approach was used to assess the extent of piRABC expression change in patients compared to controls.

Statistical analysis:

To test the data, by Shapiro-Wilk test the study's data was found to deviate from normality. As a statistical test for categorical data, the chi-square test was applied. To find out if there is a difference between groups, the Mann-Whitney U test or Kruskal-Wallis test were used. When a Kruskal-Wallis test yields statistically significant findings, Dunn's test is used to ascertain precisely which groups differ. An odds ratio (OR) was calculated from a univariate analysis to analyze the connection between an exposure and a result. When more than one variable is examined, the adjusted odds ratio—an OR that has been adjusted to consider additional predictor variables in a model—can be determined. A graphical curve called a receiver operating

characteristic curve (ROC) is used to show a marker's capacity for diagnosis. A Kaplan-Meier survival plot was created to do a survival study. If we wish to determine whether the survival distributions of two groups differ, we apply the log-rank test. If the p-value is less than 0.05, an actual effect is present. The SPSS Inc., Chicago, IL, USA software package, SPSS 20, was utilized to conduct several kinds of data analysis and interpretation.

RESULTS

The features of BC patients and healthy controls are shown in Table 1. Age, sex, smoking, bilharziasis history, and family history of cancer had non-significant difference between cases and controls. The BC cases were categorized as G1 (13 cases, 32.5%), G2 (20 cases, 50%), or G3 (7 cases, 17.5%). Stages 1 (25%) and 4 (25%) were the two most prevalent stages among BC patients. When compared to controls, patients with BC had significantly lower serum PiRABC expression levels and TNFSF4 levels ($p<0.0001$).

Figure 1 illustrates the PiRABC and TNFSF4 ROC curves for the diagnosis of BC. With an area under the ROC curve of 0.938 for the PiRABC and 0.874 for the TNFSF4, respectively, they were able to detect BC. The diagnostic performance criteria of the markers were assessed. The Youden index of PiRABC and TNFSF4 was 0.83 and 0.65, respectively. In terms of detecting BC, the PiRABC, when the cutoff level is set to be less than a 0.88-fold change, demonstrated excellent performance. It achieved a sensitivity of 87.5%, a specificity of 95%. Furthermore, it had a positive predictive value (PPV) of 94.6% and a negative predictive value (NPV) of 88.4%. TNFSF4 at a cutoff level <146.5 pg/mL yielded sensitivity, specificity, PPV, and NPV of 82.5%, 85%, 84.6%, and 82.9%, respectively.

The logistic regression analysis was performed to detect any potential predictors of BC (Table 2). PiRABC and TNFSF4 were univariate predictors for BC. On multivariate analysis, low PiRABC was a significant independent predictor of BC. PiRABC had an adjusted odds ratio of 133 (95% confidence interval: 10-1672) ($p<0.001$). Also, the presence of a family history of carcinoma and low levels of TNFSF4 were significant independent predictive factors of BC (AOR = 82 and 12.4, respectively).

The relationships between BC characteristics and marker levels have been investigated. The difference in PiRABC levels between tumor grades was significant ($p = 0.001$). The median piwi expression levels in G1 were 0.75-fold change, G2 were 0.5-fold change, and G3 were 0.32-fold change. Figure 2A shows that the serum piwi was significantly lower in G2 than in G1 ($p= 0.009$) and in G3 than in G2 ($p= 0.013$). Regarding TNFSF4, there is no significant difference among different BC grades ($p= 0.09$) (Figure 2B).

Between various BC stages, there was a statistically significant variation in PiRABC expression ($p<0.0001$). The median PiRABC expression varied by stage 0a, stage 1, stage 2, stage 3, and stage 4, with changes of 0.97, 0.75, 0.56, 0.38, and 0.18 folds, respectively. With more advanced BC stages, PiRABC expression displayed a trend towards considerable decline (Figure 3A). Also, TNFSF4 shows a negative trend with more advanced BC stages, but stage 1 and stage 2 showed a non-significant difference between them (Figure 3B).

The median follow-up duration for the mortality data was 4.75 months; however, it ranged from 0.5 to 6 months. Eleven patients passed away throughout the follow-up period. One patient died from postoperative complications, three from metastatic cancer, and seven from problems from chemotherapy or radiotherapy. We calculated the survival rate by using a graph called the Kaplan-Meier curve. Patients with BC had an overall survival rate of 72.5%. For BC patients' overall survival, PiRABC and TNFSF4 may be significant independent prognostic factors ($p =0.038$ and 0.026, respectively) (Figure 4).

Table 1: Demographic and clinical characteristics of the subjects

Parameter	**Control (No. = 40)**	**Bladder cancer (No. = 40)**	**p**
Age (years)	63 [47-73]	65 [45-81]	0.27
Sex: Male/ Female	34/6 (85/15)	31/9 (77.5/22.5)	0.39
Smoking	22 (55)	28(70)	0.16
Family history of carcinoma	1 (2.5)	4 (10)	0.17
History of bilharziasis	0 (0)	3 (3.5)	0.08
Tumor grade (G)**:**			
G1	—	13 (32.5)	
G2	—	20 (50)	
G3	—	7 (17.5)	
TNM Staging:			
Stage 0a	—	5 (12.5)	
Stage 1	—	10 (25)	
Stage 2	—	8 (20)	
Stage 3	—	7 (17.5)	
Stage 4	—	10 (25)	
PiRABC (Fold change)	1 [0.77-1.1]	0.52 [0.1-1.05]	<0.0001*
TNFSF4 (pg/mL)	150 [135-163]	129.5 [86-169]	<0.0001*

No.: number of subjects. Data are presented as No. (%) or median [range]
** Significant*

Table 2: Logistic regression analysis of bladder cancer prediction

Parameters	**Univariate**		**Multivariate**	
	OR (95%CI)	**p**	**AOR (95%CI)**	**p**
Age	1.02 (0.96-1.1)	0.41	0.91 (0.8-1.03)	0.12
Sex	1.65 (0.53-5.1)	0.39	4.2 (0.5-33)	0.16
Smoking	1.9 (0.8-4.7)	0.17	1.8 (0.3-12.8)	0.56
Family history of carcinoma	4.3 (0.5-40.6)	0.19	82 (1.5-445)	0.030*
History of bilharziasis	1.7 (0.5-3)	0.91	1.06 (0-2.4)	0.99
PiRABC	183 (21-1570)	<0.001*	133 (10-1672)	<0.001*
TNFSF4	44 (9.2-214)	<0.001*	12.4 (1.2-129)	0.036*

OR: Odds ratio; CI: Confidence interval; AOR: Adjusted OR.
**: Significant*

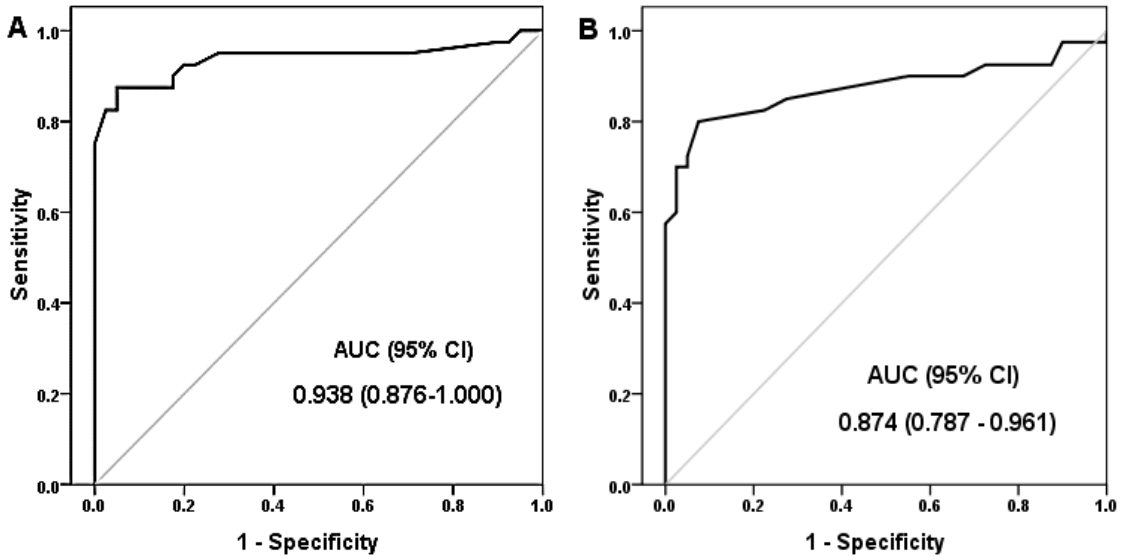


Figure 1: ROC curves of (A) PiRABC and (B) TNFSF4 in cancer bladder prediction

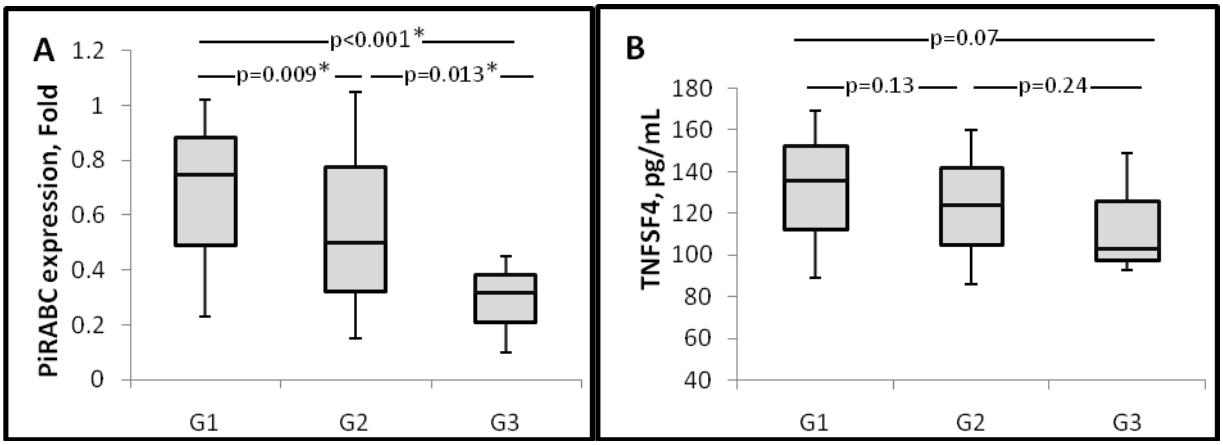
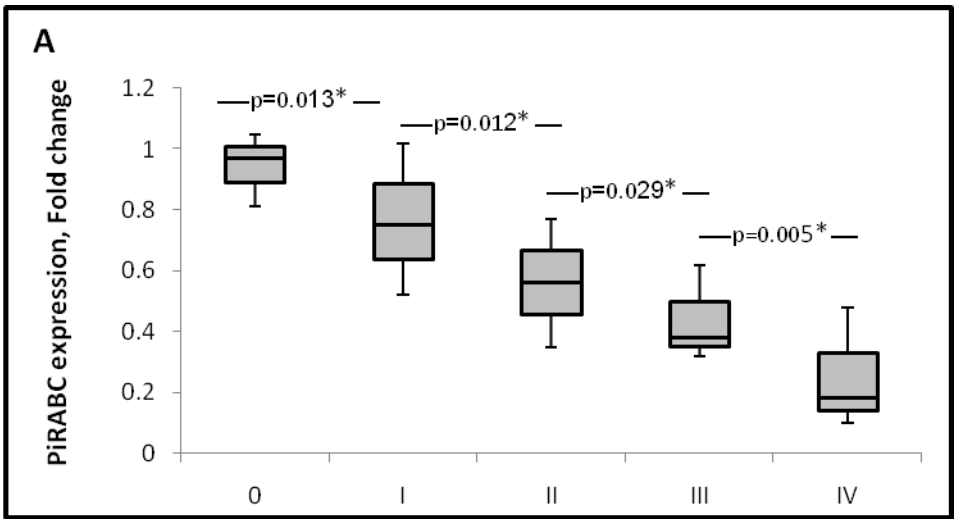


Figure 2: Levels of (A) PiRABC and (B) TNFSF4 in BC patients with different grades.



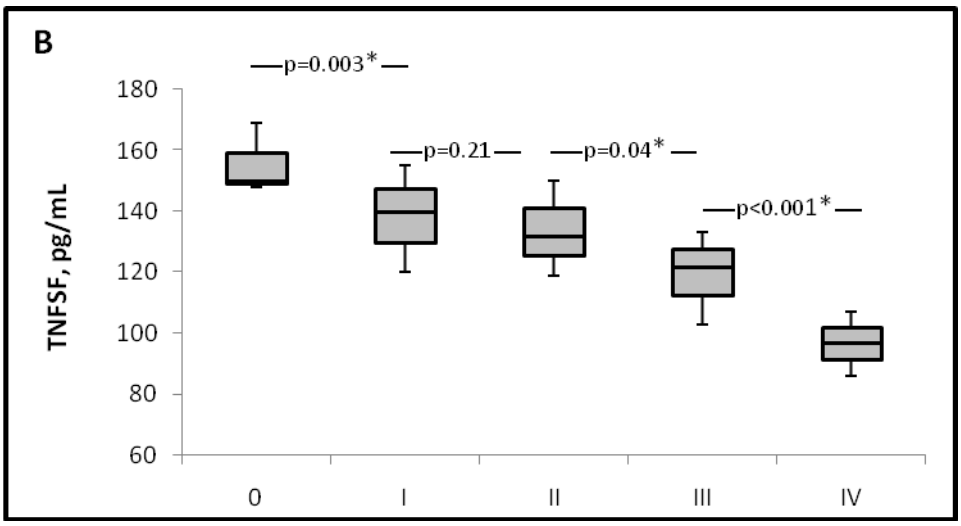


Figure 3: Levels of (A) PiRABC and (B) TNFSF4 in BC patients with different stages.

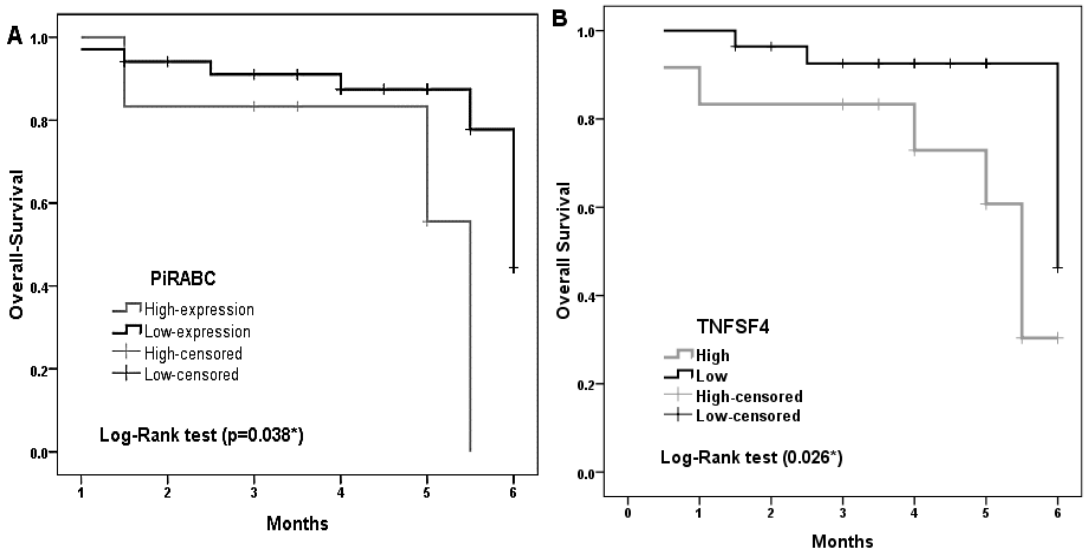


Figure 4: Kaplan-Meier curves of (A) PiRABC and (B) TNFSF4

DISCUSSION

Approximately Over 70% of the human genome is actively transcribed, yet only 2% of its codes for proteins. Instead, noncoding RNAs (ncRNAs) are most transcripts [24]. P-Element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs) are examples of short noncoding RNAs [25].

The piRNAs are a class of short, non-coding RNAs. There are around 30,000 kinds of piRNA in the human genome. Understanding the aberrant expression of piRNAs in different types of cancer is crucial. Thus, they can be utilized to differentiate them and offer prospective prognostic and diagnostic indicators throughout the course of cancer development [26].

The most frequent cancer of the urinary tract is bladder cancer, and according to Siegel et al. [27], In men, it ranks as seventh most common type of cancer. Immune system attacks can target any type of cancer cell, including cancer stem cells, which are heterogeneous subtype cells. To learn more about the underlying mechanisms, connective functional components and lymphocyte functions were examined[28].

TNFSF4 belongs to the family of ligands known as tumor necrosis factor. In interactions between T cells and antigen-presenting cells, TNFSF4 promotes the adhesion of activated T cells to target cells. According to Zhang et al. [29], TNFSF4 has the ability to attach to TNFRSF4 and work together to promote T cell proliferation and cytokine production. TNFSF4 overexpression was shown to be connected with more active lymphocytes; these findings

suggested that TNFSF4 may be able to partially operate by specifically neutralizing stem cells and reawakening the immune response [28]. Chalbatani et al. [30] demonstrated a potential connection between piRABC and TNFSF4, suggesting that piRABC could induce apoptosis in bladder cancer cells by upregulating TNFSF4.

In our study, when compared to controls, patients with BC had significantly lower serum PiRABC expression levels. In terms of detecting BC, PiRABC ROC curves for the diagnosis of BC showed an area under the ROC curve of 0.938. The diagnostic performance criteria were assessed. The Youden index of PiRABC was 0.83. In terms of detecting BC, the PiRABC at a cutoff level < 0.88-fold change provided 87.5% sensitivity, 95% specificity, 94.6% PPV, and 88.4% NPV. The logistic regression analysis was performed to detect any potential predictors of BC. PiRABC was a univariate predictor for BC. On multivariate analysis, low PiRABC was a significant independent predictor of BC. The relationships between BC characteristics and PiRABC levels have been investigated. The difference in PiRABC levels between tumor grades was significant. Between various BC stages, there was a statistically significant variation in PiRABC expression. With more advanced BC stages, PiRABC expression displayed a trend towards considerable decline. For BC patients' overall survival, PiRABC may be a significant independent prognostic factor.

Silencing transposable elements (TEs) are the primary function of piRNAs, which can relocate via a process called cutting and reintegration and may potentially lead to instability in the genome. Thus, transposon silencing is essential to preserving the integrity of the genome. TE activity is increased when piRNAs and PIWI proteins are absent, indicating their critical roles in this pathway [31]. Research on lung cancer has demonstrated that this piRNA is downregulated, which accelerates the rate at which cancer cells divide [32]. Comparably, glioma malignant cells exhibit a decrease in this piRNA expression, which promotes the spread of cancer [33]. Similar to this, a prior study found that overexpression of piRABC has been shown to inhibit colony formation, promote cell death, and inhibit bladder cancer cell growth. PiRABC is also important in the development of bladder cancer [31]. Taubert et al.[34] performed a study that assessed the expression of Piwi-like 2 (PIWIL2) in tumor samples from patients with BC treated with chemotherapy. The findings indicated that a poor prognosis for BC patients is linked to low PIWIL2 expression.

However, a different investigation carried out by the same group showed that patients with muscle-invasive bladder cancer who expressed high levels of both PIWIL2 and PIWIL1 experienced poorer outcomes in terms of both disease-specific survival and recurrence-free survival [35]. The prior study included patients receiving chemotherapy, whereas only 28% of patients in the second trial got chemotherapy, according to the authors, who suggested that this discrepancy could be caused by this [31].

Another study that assessed the expression of piRNAs in BC found 91 downregulated and 106 upregulated piRNAs. The highest downregulated piR_DQ594040 (piRABC) was identified. In a cell line used to study bladder cancer, its overexpression reduced colony formation and cell proliferation while promoting apoptosis, suggesting a role for piRABC in the development of bladder cancer [12].

A prior study noted that the acquisition of epigenetic factors and the spread of the disease might be altered by overexpression of piRNAs. The role of piRNAs in carcinogenesis and tumor growth is becoming increasingly evident. The proliferation, invasiveness, and malignant transformation of cancer cells are attributed to dysregulation of piRNAs. These phenotypes could be reversed by altering the expression of piRNAs [36]. They discovered that a pertinent piRNA that is downregulated in bladder cancer is piRABC. In the study conducted by Chu et al. [37], they discovered that the expression levels of piRABC differed significantly between bladder cancer and normal tissues. The difference observed was quite remarkable. In contrast, it was discovered that multiple myeloma cell lines and patients had elevated levels of piR-823. In contrast to

gastric cancer, where piR-823 was down-regulated, piR-823 was positively linked with clinical stage [26]. Additionally, research conducted in vitro on human BC cell lines revealed that piRABC overexpression may prevent the growth, death, and colony formation of cells [30]. Furthermore, numerous investigations have shown that PIWI proteins function as putative biomarkers for cancer [26].

In our study, the presence of a family history of carcinoma and low levels of TNFSF4 were significant independent predictive factors for BC. The TNFSF4 ROC curve for the diagnosis of BC was assessed, and the area under the ROC curve of 0.874 was able to detect BC. TNFSF4 at a cutoff level < 146.5 pg/mL yielded sensitivity, specificity, PPV, and NPV of 82.5%, 85%, 84.6%, and 82.9%, respectively. The logistic regression analysis was performed to detect any potential predictors of BC. TNFSF4 was a univariate predictor for BC. The relationships between BC characteristics and TNFSF4 levels have been investigated, and there is no significant difference among different BC grades. On the other hand, TNFSF4 shows a negative trend with more advanced BC stages. Patients with BC had an overall survival rate of 72.5%. For BC patients' overall survival, TNFSF4 may be a significant independent prognostic factor. Chu et al.[37] found that control subjects had higher levels of TNFSF4 protein expression than bladder cancer subjects, which is consistent with our findings. Furthermore, TNFSF4 was discovered to be strongly expressed in all types of breast cancers, and abnormal overexpression of this protein was linked to decreased disease-free and overall survival [28]. Significantly, it was also discovered that TNFSF4 is intimately associated with T cell profiles and many immunological effector molecules involved in antitumor immunity. Furthermore, it demonstrated increased importance in forecasting both overall survival and disease-free survival[28].

A potential correlation between piRABC and TNFSF4 was reported, suggesting that piRABC could stimulate TNFSF4 upregulation to induce apoptosis in BC cells [20]. In three bladder cancer tissues investigated using piRNA microarray assays to look at global piRNA expression, 106 piRNAs were elevated and 91 were reduced. The most highly elevated piRNA among them was piRABC, which inhibited colony formation and cell proliferation while stimulating apoptosis in bladder cancer cells via TNFSF4. The most downregulated piRNA was piRNA DQ594040 (piRABC), and overexpression of DQ594040 increased the production of TNFSF4, which in turn promoted cell death by inhibiting proliferation and colony formation [33].

Previous research using luciferase reporter gene assays showed that piRABC might raise TNFSF4's luciferase activity, with similar results. Additionally, TNFSF4 protein expression was found to be upregulated in controls as compared to bladder cancer subjects, according to Western blotting studies and ELISA assays[12].Furthermore, by targeting TNFSF4, a binding partner of OX40, it was concluded that piR-60152 prevented bladder cancer via triggering apoptosis in the cells [37].The TNFSF4 protein can be upregulated to prevent BC cell invasion and proliferation when piRNAs are overexpressed. PiRNAs play a collective role in the invasion, metastasis, and apoptosis of cancer cells. It may be utilized as a predictive and diagnostic biomarker as cancer develops [26].

This study was limited in that the participants were not chosen in a randomized manner, and the follow-up period lasted just six months. It is important to approach the interpretation of these findings with caution. To ascertain the function of piRABC and TNFSF4 in BC carcinogenesis, more research is required. Their involvement in the diagnosis, staging, grading, response to therapy, and prognosis of BC need to be confirmed by larger-scale research. The established significance of piRABC and TNFSF4 in BC will aid in the development of novel preventative and therapeutic approaches.

CONCLUSIONS

Decreased levels of piRABC expression and TNFSF4 appear to be independent predictors of BC. The low expression of piRABC had accepted prediction criteria for BC better than TNFSF4. The piRABC showed a decreasing

trend with tumor grading and staging, while TNFSF4 exhibited a declining tendency throughout BC stages, despite having a small impact in BC grading. Greater expression of TNFSF4 and piRABC was linked to prolonged patient survival rates.

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