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Original Research Article

Integrated grade-wise profiling analysis reveals potential plasma miR-373-3p as prognostic indicator in Prostate Cancer & its target KPNA2

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ABSTRACT

Background: Plasma microRNAs (miRNAs) have recently garnered attention for their potential as stable biomarkers in the context of Prostate Cancer (PCa), demonstrating established associations with tumor grade, biochemical recurrence (BCR), and metastasis. This study seeks to assess the utility of plasma miRNAs as prognostic indicators for distinguishing between high-grade and low-grade PCa, and to explore their involvement in PCa pathogenesis.

Methodology: We conducted miRNA profiling in both plasma and tissue specimens from patients with varying PCa grades. Subsequently, the identified miRNAs were validated in a substantial independent PCa cohort. Furthermore, we identified and confirmed the gene targets of these selected miRNAs through Western blot analysis.

Results: In our plasma profiling investigation, we identified 98, 132, and 154 differentially expressed miRNAs (DEMs) in high-grade PCa vs. benign prostatic hyperplasia (BPH), low-grade PCa vs. BPH, and high-grade PCa vs. low-grade PCa, respectively. Our tissue profiling study revealed 111, 132, and 257 statistically significant DEMs for the same comparisons. Notably, miR-373-3p emerged as the sole consistently dysregulated miRNA in both plasma and tissue samples of PCa. This miRNA displayed significant overexpression in plasma and tissue samples, with fold changes of 3.584 ± 0.5638 and 8.796 ± 1.245, respectively. Furthermore, we observed a significant reduction in KPNA2 protein expression in PCa.

Conclusion: Our findings lend support to the potential of plasma miR-373-3p as a valuable biomarker for predicting and diagnosing PCa. Additionally, this miRNA may contribute to the progression of PCa by inhibiting KPNA2 expression, shedding light on its role in the disease.

1. Introduction

Prostate cancer (PCa) is the most common malignant cancer in men worldwide (20 %) and in Asia, including in countries like India (17.3 %) [1]. After lung cancer, PCa has the second highest number of newly diagnosed cases (~1.41 million), and was accountable for the 1/5th highest number of cancer-related deaths (~375 thousand) among males worldwide in the year 2020 [1]. Both the mortality rate and incidence rate of PCa are rising in India [1–3]. Due to rising and an ageing population, the number of new cases of PCa and the number of deaths caused by it are both projected to rise to 2.43 million and 740,000 respectively by the year 2040 [1].

The most common methods of monitoring and detecting PCa are serum PSA (prostate specific antigen) levels and clinical examination of the prostate gland using techniques like DRE (digital rectal examination) and evaluation of histopathology of needle PCa biopsies.Conversely, PSA specificity does have its drawbacks, as increased PSA amounts can be associated with either benign or PCa forms, and they are unable to differentiate between pathogenicity and aggressiveness of PCa [4]. In addition, the Gleason Score (GS) can be utilised for PCa tissue in order to determine the degree of disease aggression. It is helpful in the diagnosis of a patient [5,6]and gives the clinician assistance regarding the treatment that is most suitable in the given circumstance [7]. The existing diagnostic PCa biomarkers need to be re-designed or transformed to be more disease specific and precise in order to avoid overtreatment of PCa and reduce morbidity/mortality [8,9].Presently, there is a plethora of PCa biomarkers accessible for diagnosis; however, these markers have low sensitivity, specificity, and accuracy, making them inadequate

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screening clinical methods and techniques. Consequently, the optimal biomarker for PCa would be one that is non-invasive, has a high specificity, and is more reliable than PSA. This biomarker could be used by itself or in combination with PSA.

MicroRNAs (miRNAs) are small, conserved, noncoding molecules (19–22 nucleotides) that regulate post-transcriptional processes through repression, or degradation of messenger RNA (mRNA), or inhibition of translation of mRNA [10]. MicroRNA can regulate many processes and have the potential to act as either tumour suppressor genes or oncogenes, depending on the context. Significant previous studies on miRNAs have evidenced the wide range of roles that miRNAs play in biological mechanisms such as carcinogenesis, cell propagation, development, cell death, and various signalling pathways [11,12]. Study into miRNAs at the molecular level has revealed the importance of miRNA expression alterations in PCa and BPH patients, establishing miRNA as a crucial diagnostic and prognostic biomarker in PCa [13].So far, there has been little investigation into miRNAs' potential as PCa diagnostics.

To further elucidate the functional significance of miRNA in PCa tumor development, we profiled miRNAs in the plasma and tissue of low-high grade PCa patients and attempted to verify the selected miRNAs in larger PCa samples. We also find out the gene target of selected miRNAs and validate it. The goal of this preliminary research was to determine whether miRNA expression levels could be used as a diagnostic or prognostic marker for PCa.

2. Materials and methods

2.1. Study design

From the Biorepository at the Rajiv Gandhi Cancer Institute & Research Centre (RGCIRC) in New Delhi, 48 pairs of samples (plasma and tissue) were taken from PCa patients who had a prostatectomy. These specimens were tested for PSA and confirmed by histopathology (Table 1).45 paired BPH specimens were used in this study; after an expert pathologist reviewed their histopathology, they were considered appropriate for the research. In terms of PSA, all specimen of BPH were within normal ranges (<10 ng/mL) except some. The study was approval from the ethics scientific committee (Approval no.Res/SCM/31/2018/109).

The purpose of this study, which involved profiling of miRNAs in high-low grade plasma as well as tissue and further validation of selected miRNAs, was to assess miRNA expression in PCa. Further, selected miRNAs were used in a miRNA-based diagnostic approach for the early detection of PCa (Fig. 1). Histopathologists classified the specimens by stage and Gleason score, as recommended by the International Society of Urological Pathology (2005) (Table 1). In this study, we found that the ages of patients ranged from 43 to 78 years. The patients' foods and their clinicopathological features such as PSA, Gleason score, pathological stage, margins, perineural invasion etc. were analysed. In the subsequent validation analysis, we focused on the common miRNAs that demonstrated consistent expression in high-low grade plasma and tissue.

2.2. Isolation of total RNA

2.2.1. From plasma

Venous blood was collected in an EDTA 2.5 ml tube, centrifuged at 2000×*g* for 15 min to separate the plasma, and then either used for RNA extraction or frozen at −80^0 C until further processing. Total RNA was extracted and purified using a miRNeasy serum/plasma kit (QIAGEN, Germany) from 200 μL of plasma from PCa and BPH specimens, as per the manufacturer's protocol with some modifications. Using a spectrophotometer/nanodrop, we determined the concentration of the isolated RNA as well as its purity. Those samples of total RNA that had an OD 260/280 ratio of >1.8 after extraction were chosen for further experiment and were stored at −80° until use.

Table 1
Benign prostatic hyperplasia (BPH) and prostate cancer (PCa) patients' (plasma & tissue) clinical-pathological and other characteristics (BPH) used in the study.

Variables	Study of miRNA profiling			Study of miRNA testing/validation	
	PCa Plasma + Tissue		BPH Plasma + Tissue	PCa plasma + Tissue	BPH Plasma + Tissue
	High grade	Low grade			
Total, n	3 + 3	3 + 3	3 + 3	42 + 42	42 + 42
Age, mean + SD	67.66 ± 4.49		56 ± 3.55	65.52 + 8.10	63.8 + 5.9
Food Habit					
Vegetarian	1	2	0	23	20
Non-Vegetarian	2	1	3	19	22
Smokers	1	1	2	12	22
Non-Smokers	2	2	1	30	20
Alcoholic	1	1	0	15	13
Non-Alcoholic	2	2	3	27	29
BMI					
Normal	1	2	3	12	10
Over-weight	2	1	0	30	32
PSA (n)					
≤10 (ng/ml)	1	1	3	12	34
>10 (ng/ml)	2	2	0	30	8
PSA, mean ± SD	60.86 ± 59.29		3.32 ± 0.30	27.94 ± 31.57	7.671 + 4.64
Gleason Score (n)					
6 (3 + 3)	0	1	–	9	–
7 (3 + 4)	0	1	–	8	–
7 (4 + 3)	0	1	–	9	–
8 (4 + 4)	1	0	–	6	–
9 + 10	2	0	–	10	–
Pathological stage (n)					
≤2	0	3	–	21	–
≥3	3	0	–	21	–
Unknown	0	0	–	0	–

2.3. From tissue

Total RNA was extracted using a miRNeasy Mini kit (QIAGEN, Germany) from fresh frozen tissue from PCa patients and BPH samples, as per the manufacturer's instructions. Spectrophotometer/Nanodrop measured isolated RNA concentration and purity. Only RNA samples with an OD 260/280 ratio >1.8 were selected for the further experiment. The size and quality of the RNA was evaluated by electrophoresis on an agarose gel with 1X MOPS-formaldehyde.

2.4. MicroRNA RT-qPCR profiling

The RNA was reverse transcribed into cDNA with a miRNA-specific QuantiMir RT kit (RA420A-1) (system biosciences, USA) in order to conduct a Sybr green-based miRNA profiling. Following is a brief summary of the procedure: cDNA first strand was reverse transcribed from 10 μL of eluted plasma and tissue RNA according to manufacturer's protocol. Quantitative RT- PCR (qPCR) assays were performed on a LightCycler® 96 Instrument (Roche, Switzerland) using diluted reverse-transcribed cDNA (5x for plasma, 10x for tissue) containing 476 mature microRNAs (Human miRnome microRNA profiler panel, System Biosciences, USA) from miRBase (www.mirbase.org/). Muñoz X. et al., 2015 [14], had already described the specifics and attributes of the amplification curves.

Cq values < 40 were considered for miRNA expression in a minimum of two of three replicates. To determine the relative quantification (RQ) of the miRNA expression, the relative quantitative of $2^{-\Delta\Delta Cq}$ method was utilised [15].

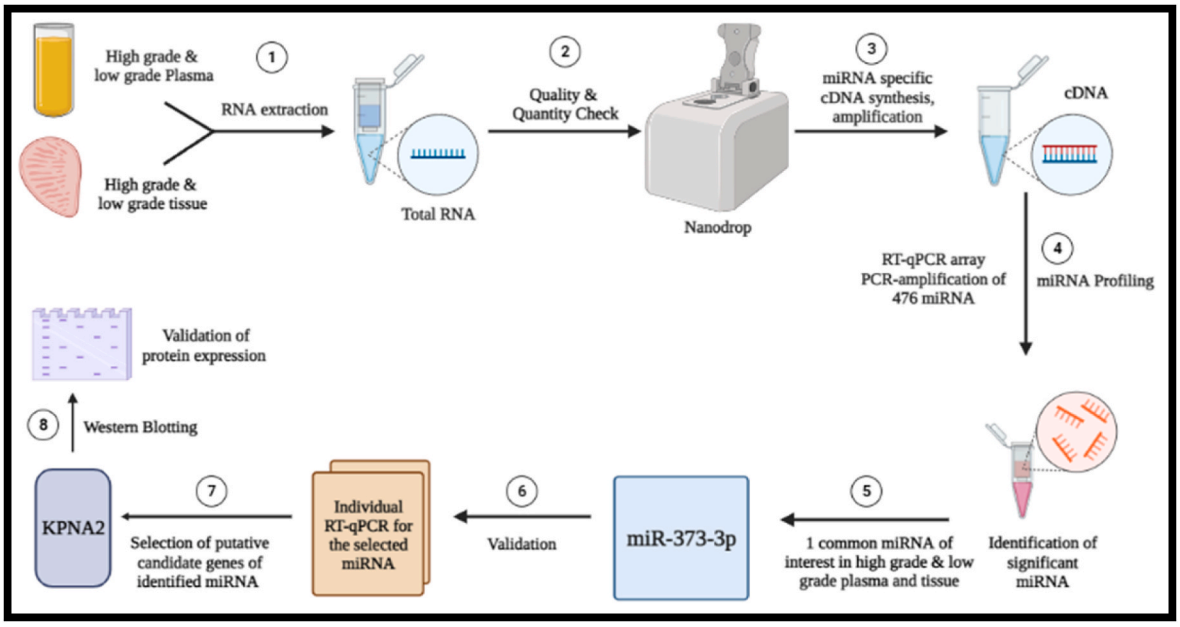

Fig. 1. The study's layout is represented as a diagram in the flowchart. Information regarding the total number of miRNAs and individual's miRNA evaluated at each investigation stage is displayed.

2.5. Validation of miRNA by RT-qPCR analysis

For the purpose of validation, we chose miRNAs that were consistently expressed in both plasma and tissue samples. The validation method was the same as outlined previously for profiling. There were a total of 42 PCa and BPH involved in the verification phase for both plasma and tissue samples. Reverse transcription of 200 ng of RNA in 10 µl was performed using a miRNA specific cDNA QuantiMir RT kit (system biosciences, USA) to generate the first strand of cDNA. In a total volume of 10 µl, a PCR reaction was performed using KAPA SYBR® FAST [KAPA BIOSYTEMS (Sigma-Aldrich), Germany] qPCR master mix and the reverse-transcribed cDNA from plasma and tissue at a 5x:10x dilution. On a LightCycler® 96 instrument, the assays [Human miRnome microRNA qPCR primer (SBI)] were carried out with three replicates of each reaction. The expression levels of targeted microRNAs were analysed and normalized to the average levels of expression for endogenous microRNAs such as miR-16 and U1 snRNA. We utilised the $2^{-\Delta\Delta Cq}$ method for the relative expression analysis.

2.6. Determining target genes of miRNAs by in silico method

The target genes along with associated pathways of miRNA were predicted via online targets databases such as miRTarBase [16], miRDB (http://mirdb.org/), miRTarBase (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php), TargetScanHuman (https://www.targetscan.org/vert_80/), and miRTargetLink 2.0 (https://ccb-compute.cs.uni-saarland.de/mirtargetlink2) for acquiring common gene targets(s) of the key miRNA(s). The miRTarbase's gene targets are tested utilizing reporter assays, western blots, RT-qPCR, microarrays, NGS, pSILAC, and CLIP-Seq.

2.7. Western blot analysis

Whole protein lysate was isolated from PCa, BPH tissue samples treated with different conditions using RIPA lysis buffer (50 mMTris-HCl with pH 7.4, 150 mMNaCl, 1 % NP-40, 0.1 % sodium dodecylsulfate). The protein concentrations were measured using the Bradford method. We used SDS-PAGE (Sodium dodecylsulfate-polyacrylamide gel electrophoresis) to separate protein samples ranging from 30 to 50 µg. After the protein had been separated, PVDF (Polyvinylidenedifluoride) (Millipore) membrane was utilised for the protein transfer.After blocking for 1 h at room temperature, we incubated the membrane with a 1:250 dilution of rabbit polyclonal anti-human KPNA2 antibody (immunotag, cat no. ITT06510) and B-actin (abcam, cat no. ab8227) overnightat 4^0C. The proteins were incubated with a secondary antibody for 1 h at room temperature. According to the instructions provided by the manufacturer, immunoreactive bands were observed using the ECL Western Blotting reagent (Abbkine) in the ChemiDoc System (Biorad). These bands were consequently quantified by densitometry utilizing imageJ software.

2.8. Genetic alteration and survival analysis of KPNA2

The cBioportal for Cancer Genomics checked for alterations in KPNA2 gene [17]. The cBioPortal for Cancer Genomics generated KPNA2 gene oncoplot. The KPNA2 gene expression was analysed for their role in patient survival using the web-based programme UALCAN, which is based on the TCGA database [18]. Expression levels in PCa patients were divided into high, medium, and low categories using transcripts per million (TPM) enrichment evaluation. The prognostic significance of KPNA2 was assessed in combination with Gleason Score using the Kaplan-Meier (KM) survival method ($p < 0.05$).

2.9. Statistical analysis

Unpaired two-tailed Student's t-test was used to examine the relative expression of significance miRNA differences in the miRNA expression analysis. Receiver operating characteristic (ROC) curve analysis used RQ values to grade the aggressiveness of prostate cancer samples [19]. Accuracy was calculated using the area under the curve (AUC) method [19]. The method used to determine the threshold value was based on the use of Youden's index (sensitivity + specificity-1) [20]. The insignificant values were minimized as much as possible. The tool GraphPad Prism 8.0.2 was used to analyse the statistical data, and a p value $p \leq 0.05$was considered to be statistically significant.

3. Results

3.1. Characterization of studied population

In total, the study incorporated the samples of 48 PCa cases and 45 BPH cases in the study, with each case represented by a pair (plasma + tissue). The clinicopathological and many other properties of PCa and BPH are described in Table 1. The median age of PCa patients was 65.95 (range, 43–78), and 29 of the PCa cases had Gleason scores of 6 or 7, indicating a low to intermediate Gleason scores, while only 19 had Gleason scores of 8 or 10, indicating a high Gleason score (8,9,10). The PCa category consisted of 26 vegetarians, 22 non-vegetarian, 14 smokers, and 34 non-smokers, and 17 alcoholics, 31 non-alcoholic. In addition, the BPH category included of 20 vegetarians, 25 non-vegetarian, 24 smokers, 21 non-smokers, 13 alcoholics, and 32 non-alcoholics. There were 15 patients with a normal body mass index (BMI) and 33 patients with an excessively high BMI among PCa catogory. Furthermore, 13 BPH patients had a healthy body mass index, while 32 were overweight. The 24 patients were classified as having stages 1 and 2, while other 24 patients were classified as having stages 3 and 4.

3.2. Differentially expressed circulating MicroRNA expression profile

To identify miRNA expressional modification in plasma that are linked to PCa, the first stage of the study involved a high throughput assessment of the expression level of human miRNAs in a selected group of patients (High grade and low grade).

As a first step, profiling of miRNAs in plasma samples was performed using the Human miRnome microRNA profiler, which contains 476 mature miRNAs. Differentially expressed miRNAs (DEMs) were identified via using the log10 ($2^{-\Delta Ct}$) fold change method by comparing miRNA levels between the groups in PCa and BPH samples. The High grade PCa and BPH groups had a total of 98 DEMs that were statistically significant ($P < 0.05$), 54 of which were miRNAs that were overexpressed and 44 that were miRNAs that were downexpressed (Supplementary file). A low grade PCa and BPH groups had a total of 132 DEMs that were statistically significant ($P < 0.05$), 76 of which were miRNAs that were overexpressed and 56 that were miRNAs that were downexpressed (Supplementary file). The High grade PCa vs low grade PCa groups had a total of 154 DEMs that were statistically significant ($P < 0.05$), 106 of which were miRNAs that were overexpressed and 44 that were miRNAs that were downexpressed (Supplementary file). We found 32 common DEMs across all the groups i.e.High grade PCa plasma vs BPH, Low grade PCa plasma vs BPH, High grade PCa plasma vs Low grade PCa plasma (Table 2).

3.3. Differentially expressed MicroRNA profile in tissue

Profiling of miRNAs in tissue samples was performed using the Human miRnome microRNA profiler. According to the tissue profiling study, there were 111 statistically significant ($P < 0.05$) DEMs between high grade PCa vs BPH, 56 of which were overexpressed miRNAs and 55 that were downexpressed miRNAs (Supplementary file). There were 132 statistically significant ($P < 0.05$) DEMs between low grade PCa vs BPH, 81 of which were overexpressed miRNAs and 51 that were downexpressed miRNAs (Supplementary file). Furthermore, there were 257 statistically significant ($P < 0.05$) DEMs between high grade PCa vs low grade PCa, 142 of which were overexpressed miRNAs and 115 that were downexpressed miRNAs (Supplementary file). We found 59 common DEMs across all the groups i.e. High grade PCa tissue vs BPH, Low grade PCa tissue vs BPH, High grade PCa tissue vs Low grade PCa tissue (Table 3).

Table 2
The common significant overexpressed and downexpressed miRNAs in different groups of plasma samples.

Common miRNAs between High grade PCa plasma vs BPH, Low grade PCa plasma vs BPH, High grade PCa plasma vs Low grade PCa plasma				
S. No.	**miRNAs**	High grade vs BPH	Low grade vs BPH	High grade vs low grade
		Fold change	**Fold change**	**Fold change**
1.	hsa-miR-154	0.771105413	1.022192327	0.754364313
2.	hsa-miR-205	1.551144762	0.789129147	1.965641197
3.	hsa-miR-208b	1.245449622	0.741404786	1.679851069
4.	hsa-miR-219-5p	0.889870106	0.553504391	1.607701981
5.	hsa-miR-302d	0.827405623	0.624886755	1.32408891
6.	hsa-miR-320a	1.979313313	1.425696274	1.38831345
7.	hsa-miR-339-5p	3.138336392	2.614738494	1.200248667
8.	hsa-miR-370	0.524252352	1.24977351	0.419477887
9.	hsa-miR-371-5p	1.383510251	1.375541818	1.005792941
10.	hsa-miR-373-3p	3.034937206	1.185092771	2.560927954
11.	hsa-miR-423-5p	1.258466372	1.972465409	0.638016954
12.	hsa-miR-433	1.273089047	1.103178822	1.154018752
13.	hsa-miR-450a	3.020945171	1.468320253	2.05741572
14.	hsa-miR-483-3p	0.770215111	0.401461441	0.641712949
15.	hsa-miR-502-3p	0.751754411	0.340721919	2.206357644
16.	hsa-miR-502-5p	1.273089047	0.92444966	1.377131825
17.	hsa-miR-504	3.625885414	1.252664439	2.894538475
18.	hsa-miR-505	0.560583039	0.385107556	1.455653183
19.	hsa-miR-508-3p	3.185824218	1.946433874	1.636749268
20.	hsa-miR-512-5p	3.426336076	0.936272247	3.659551039
21.	hsa-miR-513a-5p	1.817136337	1.769489662	1.026926789
22.	hsa-miR-514	1.375541818	2.006943497	0.685391402
23.	hsa-miR-518a-5p	2.605692186	1.55293775	1.67791155
24.	hsa-miR-518f	1.012788784	0.256731697	3.944930818
25.	hsa-miR-525-5p	1.355038022	2.383915887	0.568408487
26.	hsa-miR-532-5p	0.379367151	0.744838732	0.509327905
27.	hsa-miR-548a-3p	0.544939006	1.536875181	0.354575969
28.	hsa-miR-548c-3p	0.435275282	0.572031566	0.760928779
29.	hsa-miR-548d-3p	0.589814556	0.467595624	1.261377409
30.	hsa-miR-576-5p	0.554784736	0.868541486	0.638754446
31.	hsa-miR-616	1.761331747	1.900878955	0.926588062
32.	hsa-miR-617	1.21981864	0.878633452	1.38831345

3.4. Validation

After analysing the miRNA profiling outcomes, we tested and verified a slected miRNAs as possible markers of PCa in a large patient population. We selected miRNAs that were statistically significant across all groups i. e. high-low grade plasma as well as tissue tumor and had a Cq value of less than 40 for validation. Only one of the eligible miRNAs, miR-373-3p, was found to be commonly dysregulated in both plasma and tissue samples, and its expression was the same in across all the groups (Fig. 2).

Table 3
The common significant overexpressed and downexpressed miRNAs in different groups of tissue samples.

Common miRNAs between High grade PCa tissue vs BPH, Low grade PCa tissue vs BPH, High grade PCa tissue vs Low grade PCa tissue				
S. No.	**miRNAs**	High grade vs BPH	Low grade vs BPH	High grade vs low grade
		Fold change	**Fold change**	**Fold change**
1.	hsa-miR-107	0.850667161	0.716149817	1.187834082
2.	hsa-miR-128	2.982798801	1.290860708	2.310705394
3.	hsa-miR-130b	0.941696017	1.435612775	0.655954052
4.	hsa-miR-134	0.871556844	0.700601832	1.244011653
5.	hsa-miR-142-5p	0.711203009	1.51221856	1.396355868
6.	hsa-miR-146b-3p	2.639015822	1.442262066	1.829775519
7.	hsa-miR-147	1.225468443	0.533416121	2.29739671
8.	hsa-miR-147b	1.988480848	0.936272247	2.123827608
9.	hsa-miR-15a	1.112136086	1.174190644	0.947151207
10.	hsa-miR-15b	1.159363791	1.757266904	0.659753955
11.	hsa-miR-181b	1.792119284	1.467472363	1.221228644
12.	hsa-miR-187	0.804780172	0.785491009	1.024556823
13.	hsa-miR-18a	0.721964598	0.686183655	1.052144848
14.	hsa-miR-190b	0.567752215	0.467055748	1.215598388
15.	hsa-miR-193b	1.114708637	1.404444876	0.793700526
16.	hsa-miR-200a	0.704660378	0.893991694	0.788218036
17.	hsa-miR-208a	0.261823531	0.380244688	0.688565912
18.	hsa-miR-215	1.130269389	1.526259209	0.740548776
19.	hsa-miR-216b	1.450617005	1.008119503	1.43893358
20.	hsa-miR-302e	4.495038066	1.517468603	2.962195105
21.	hsa-miR-31	0.640972041	0.502315837	1.276033908
22.	hsa-miR-326	0.60990932	0.520029967	1.172834949
23.	hsa-miR-329	0.879649076	0.933032992	0.942784536
24.	hsa-miR-330-3p	1.804584459	2.032609864	0.887816443
25.	hsa-miR-331-3p	0.860551437	0.880665874	0.977159968
26.	hsa-miR-339-3p	1.198862886	1.486239551	0.806641759
27.	hsa-miR-34a	1.404444876	0.822640117	1.707240927
28.	hsa-miR-365	0.489710149	0.454809197	1.076737568
29.	hsa-miR-367	0.820741609	0.373280832	2.198724227
30.	hsa-miR-369-5p	0.366021424	0.599431443	0.610614322
31.	hsa-miR-371-3p	1.574615953	0.693355267	2.271008858
32.	hsa-miR-373-3p	1.464085696	1.445598252	2.025577568
33.	hsa-miR-412	0.638754446	1.115997143	0.57236208
34.	hsa-miR-423-3p	0.769325838	1.401203665	0.549046407
35.	hsa-miR-432	1.600289959	1.469168633	1.089248656
36.	hsa-miR-493	1.538651675	1.501772904	1.024556823
37.	hsa-miR-496	1.687631592	0.729510172	2.313376368
38.	hsa-miR-503	2.260538779	2.039666575	1.108288387
39.	hsa-miR-509-3p	0.848703971	0.440332937	1.927414237
40.	hsa-miR-513c	2.292094724	2.459445922	0.931955732
41.	hsa-miR-516b	0.744838732	1.311908104	0.567752215
42.	hsa-miR-517a	2.234574276	0.891928519	2.505328877
43.	hsa-miR-518d-3p	3.123867654	3.282966435	0.951538103
44.	hsa-miR-520e	2.411615655	1.549353844	1.556529946
45.	hsa-miR-524-5p	1.386710534	1.494849249	0.927659117
46.	hsa-miR-525-3p	0.892959511	0.211686328	4.218314518
47.	hsa-miR-527	0.742261785	0.544624328	1.362887677
48.	hsa-miR-548k	0.797376688	0.556068043	1.433955248
49.	hsa-miR-548l	2.080119868	1.611420856	1.290860708
50.	hsa-miR-548p	0.363073448	0.587773953	0.617709319
51.	hsa-miR-548s	0.360982299	0.666649339	0.541487523
52.	hsa-miR-548t	1.84889932	1.995384353	0.926588062
53.	hsa-miR-566	1.156688184	1.08422687	1.066832243
54.	hsa-miR-567	2.173469725	2.3000523	0.944965349
55.	hsa-miR-586	3.045473744	2.138599997	1.424050196
56.	hsa-miR-592	1.144724161	1.264295179	0.905424761
57.	hsa-miR-605	2.924790589	2.867910496	1.019833287
58.	hsa-miR-615-3p	0.756983277	0.576343173	1.313424556
59.	hsa-miR-96	2.639015822	1.268684494	2.080119868

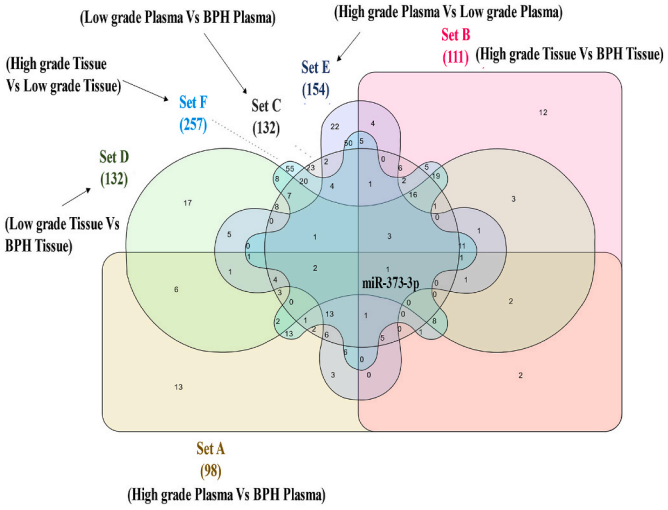

Fig. 2. Venn diagram illustrating a significant DEM across the different groups of the patient's plasma and tissue in PCa.

Therefore, since the association between this miRNA namely, miR-373-3p and PCa is the least established, we decided to concentrate on validating miR-373-3p separately in plasma and tissue specimens. The levels of miR-373-3p were found to be significantly overexpressed in plasma and tissue samples after validation in larger population, with fold changes of 3.584 ± 0.5638 (p value < 0.0001) and 8.796 ± 1.245 (p value < 0.0001), respectively (Fig. 3A & B).

3.5. Diagnostic significance of miRNAs

The ROC curve analysis demonstrated the sensitivity and specificity of the this most remarkable miRNAs for PCa detection: miR-373-3p. Statistical evaluations between PCa and BPH patient's cohorts were carried out for this study.ROC analysis of the potential of miR-373-3p as a biomarker in plasma and tissue samples showed an AUC of 0.8594 (95 % CI: 0.7838–0.9350) and 0.9461 (95 % CI: 0.9021–0.9902), as shown

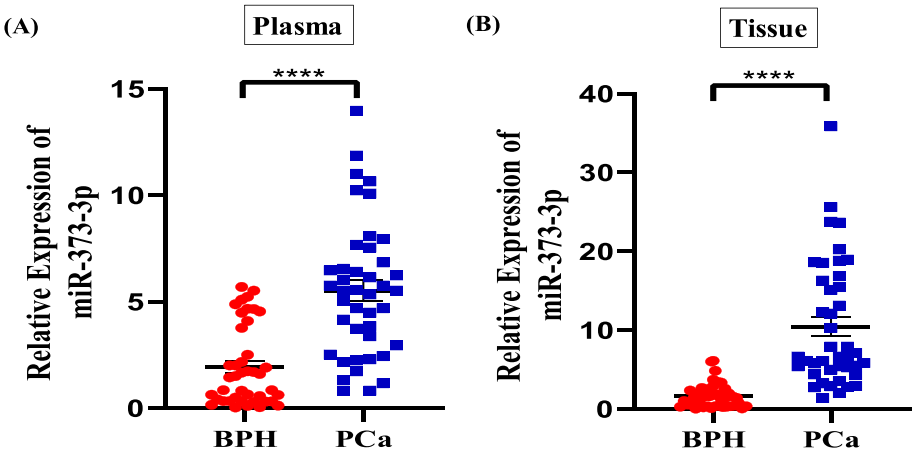

Fig. 3. Plasma miR-373-3p normalized relative expression (left) and tissue miR-373-3p normalized relative expression (right) in PCa vs BPH samples (n = 42).

in Fig. 4A and B, respectively. The PSA test has a high rate of both false-positive and false-negative results, despite the fact that it is used very frequently. Therefore, combining the use of plasma miRNA and PSA may be useful for enhancing diagnostic accuracy in PCa.

3.6. The putative candidate genes of the identified miRNAs

To learn how specific miRNAs play a role in disease development, we need to find the linked candidate genes. Because of the strong association between these two molecular regulators and numerous diseases, including cancer, many investigations have concentrated on the interplay between miRNA regulation and mRNA expression [21]. Information about the genes that may be targeted by miRNAs was gathered using the bioinformatics tool. From the TargetScan, miRDB, MiRTarBase, and miRTargetLink databases, we retrieved a total of 543, 899, 1589 and 529 gene targets corresponding to miR-373-3p. As can be seen in the Venn diagram depicted in Fig. 5, there are five gene targets that are shared by all four groups. These five genes are HOOK3, SUZ12, FEM1C, NR2C2 and KPNA2**.** Manual curation was done for these five gene targets. Based on their association in PCa and this miR-373-3p, two gene targets were shortlisted, out of which only KPNA2 has shown a matching result between expected and experimental.

We used TargetScan database to further confirm target of miR-373-3p. This miR-373-3p had binding on the seed location of 101 at 3' UTR region of KPNA2 (Fig. 6). The miR-373-3p sequence showing full complementarity to KPNA2 sequence (Fig. 6).

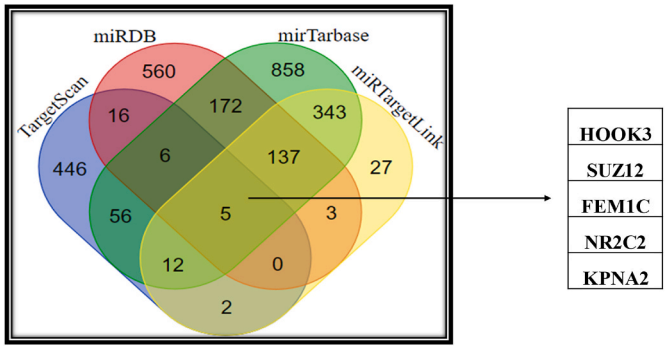

Fig. 5. Venn diagram showing the putative common candidate genes of the identified miRNAs through Insilco analysis.

3.7. Expression of KPNA2 via western blotting

KPNA2 (Karyopherin Subunit Alpha 2) expression was analysed in PCa tumour tissues and BPH tissue using immunoblotting to determine its significance in PCa carcinogenesis. The expression of KPNA2 was examined in forty-two tissue samples from the PCa, and BPH groups. The

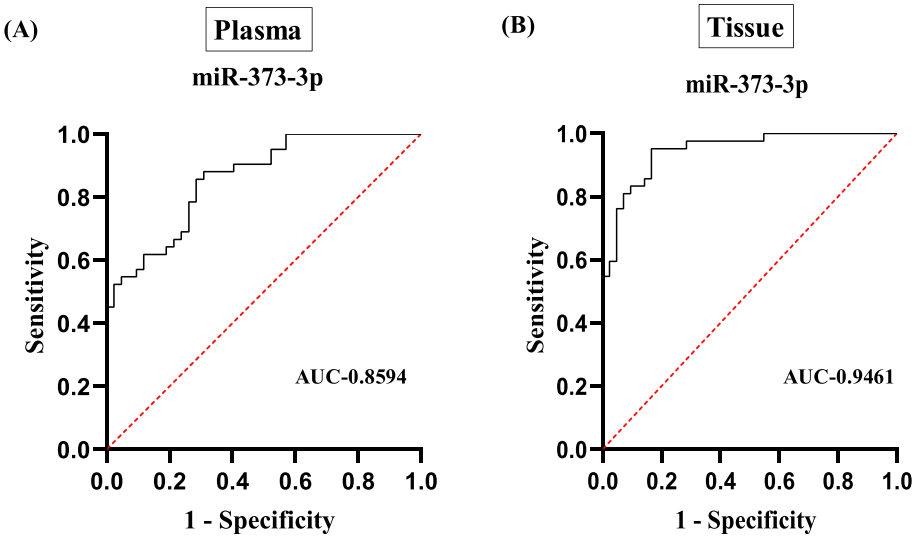

Fig. 4. The ROC curve analysis of miR-373-3p in plasma **(left)** and ROC curve analysis of miR-373-3p in tissue **(right)** in PCa vs BPH samples.

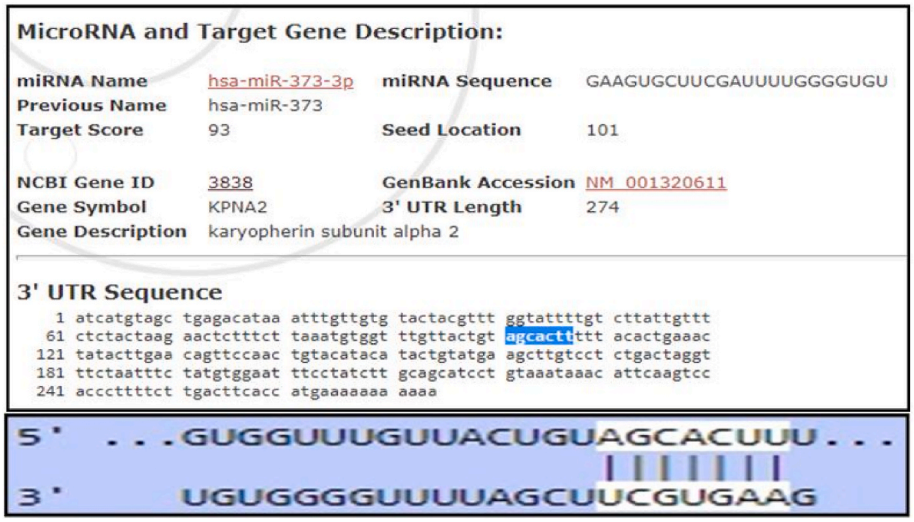

Fig. 6. The miR-373-3p and KPNA2 target gene description: upper graph showing miR-373-3p sequence and KPNA2 3′ UTR sequence and lower graph showing sequence similarity between miR-373-3p and targeted KPNA2.

KPNA2 protein expression was found to be significantly down expressed in PCa patients compared to BPH patients ($p = 0.003$). The KPNA2 expression was shown to be differentially expressed amongst BPH patients and PCa patients, with mean relative expression levels of -0.6210 ± 0.15. The B-actin was used as a control to determine the relative expression of KPNA2. The findings of the KPNA expression levels are shown as the Mean ± SEM along with the p value (Fig. 7A & B).

3.8. Genetic alteration and survival analysis of KPNA2

A total of 54 (~41 %) of 133 PCa patients had evidence of genetic change in the KPNA2 gene, as determined by data assessment using cBioPortal for Cancer Genomics. The most common genetic change was deletion (Fig. 8A). KPNA2 expression analysis and survival evaluation of PCa patients were conducted using TCGA-based UALCAN cancer transcriptome data. The Gleason Score (GS) approach is used to categorise PCa tumour tissue, gleason scores of 6, 3 + 4, 4 + 3, 8, 4 + 5, 5 + 4, and 10 are associated to Gleason Grading Groups 1, 2, 3, 4, and 5, correspondingly [22]. Overall survival in patients with PCa was found to be significantly linked with KPNA2 gene. Overall survival was shown to be considerably lower in the low/medium expression group compared to the high expression group for the KPNA2 gene (Fig. 8B).

4. Discussion

To effectively treat PCa, it is crucial to identify the disease at its earliest stage. Since its introduction thirty years ago, the PSA test has been a highly accurate method for detecting PCa, leading to a decline in the disease mortality rate. Also, there has been some debate about PSA testing findings that showed PCa was overdiagnosed and overtreated [4]. A pathologist double-checked all biopsies in this study to reduce the possibility of false positive. Increased use of miRNAs in early cancer diagnosis, targeted treatment, and precise prognosis has brought the miRNAs linked with PCa to the spotlight [23–25]. The most interesting finding of this study was the possibility that plasma miRNAs can serve as circulating biomarkers for PCa because they carry remarkable signatures of the carcinoma. Significant amounts of circulating miRNAs have been detected in a number of biological fluids, indicating their utility as diagnostic markers for a range of physiopathological diseases, including PCa [26,27]. Because of their ability to detect pathological changes early and with high accuracy, their low sensitivity to background noise, and their lack of invasiveness, circulating miRNAs serve as an excellent biomarker [28]. Many studies have been done to look into this, but so far, only a small fraction of them have shown any kind of consistency in their results.

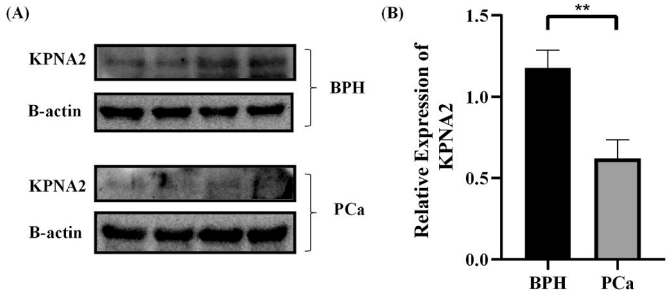

Fig. 7. The Immunoblotting of KPNA2 expression (A) The KPNA2 protein expression pattern by western blotting in BPH and PCa tissues, loading control is represented by B-actin (B) Densitometric quantification of KPNA2, B-actin was used to normalize KPNA2 expression.

The recently discovered miR-373-3p belongs to the miRNAs-371-372-373 family, which is found on chromosome 19q13.42. The profiling and validation phases of this study revealed that miR-373-3p expression was significantly overexpressed in plasma from PCa patients than in BPH patients. Concurrently, miR-373-3p expression was also overexpressed in tissue samples from PCa patients compared to BPH patients. In order to demonstrate the significance of diagnostic characteristic evaluation, we used ROC analysis, and our results showed that plasma and tissue miR-373-3p expression could reliably differentiate PCa patients from BPH patients with high sensitivity and specificity. Therefore, miR-373-3p may be utilised as a diagnostic biomarker in PCa. In line with our findings, overexpression of miR-373-3p was reported in disorders including choriocarcinoma, cervical cancer [29], pancreatic ductal adenocarcinoma (PDAC) [30], tongue squamous cell carcinoma (TSCC) [31], non-small cell lung cancer (NSCLC), clear cell renal cell carcinoma (ccRCC) [32]. A previous study reported that overexpression of serum miR-373-3p was associated with early tumour progression in PDAC [30]. Moreover, miR-373-3p regulates proliferation through the amyloid precursor protein (APP) in lung adenocarcinoma patients [33]. Another finding suggested that SNHG16 stimulated cell proliferation and migration by activating the miR-373-3p/TGF R2/SMAD axis in PCa patients [34]. Qiu et al. revealed that the inhibition of miR-373-3p by TR4 may serve as a suppressor function, causing changes in TGFR2/p-Smad3 expression and thereby promoting PCa metastasis [35]. Interestingly, some of the research into miR-373-3p suggest it

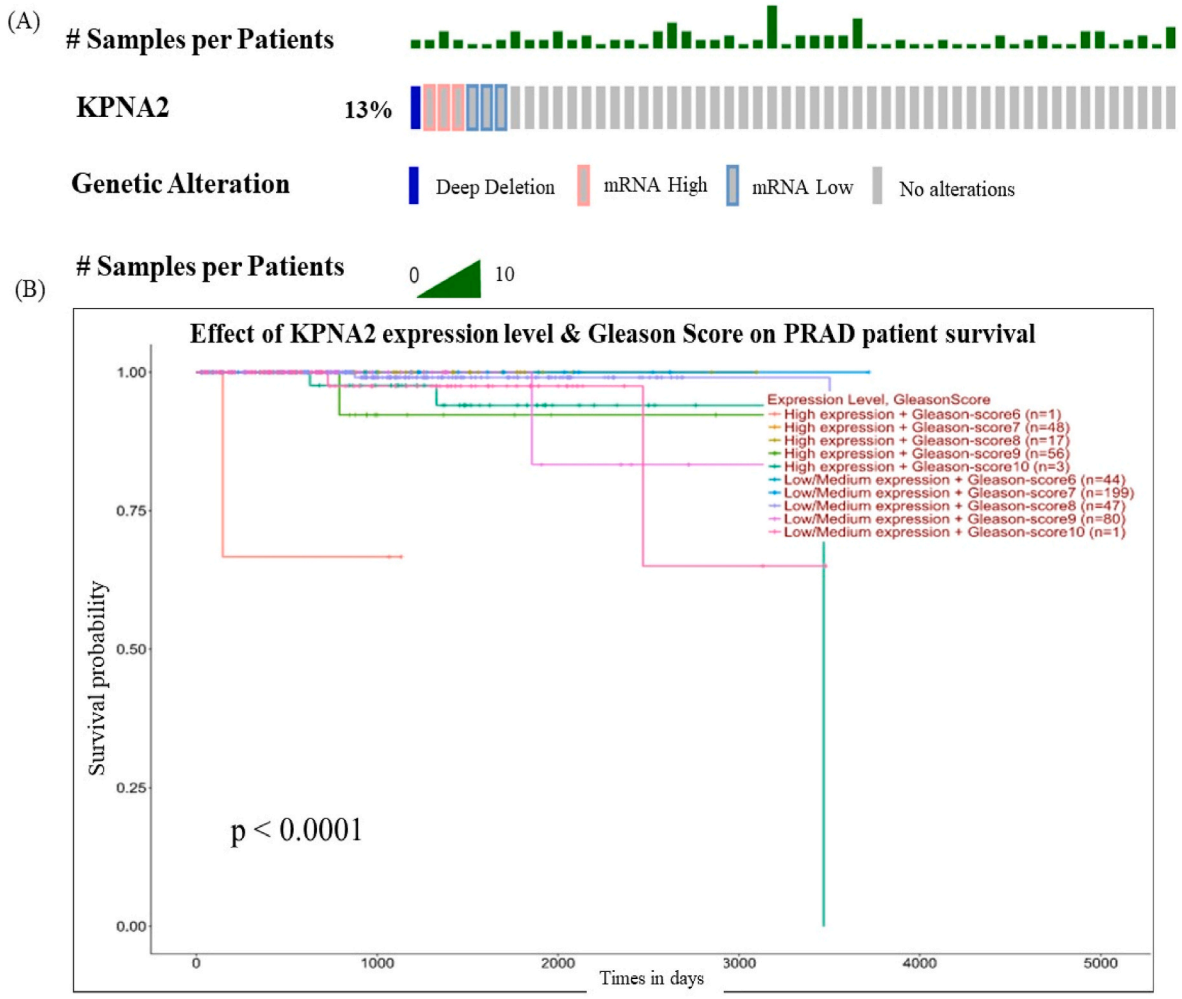

Fig. 8. Genetic change (A) The oncoplot of KPNA2 and (B) survival analyses of KPNA2 gene associated with PCa using TCGA data using the Kaplan-Meier approach.

hasan oncogenic ability in testicular germ-cell cancers [36] and breast cancer [37]. Some studies suggest that miR-373-3p has been shown to act as a tumour suppressor in other types of cancer, including breast cancer [38] and pancreatic cancer [39]. Therefore, these findings suggest that miR-373-3p has a dual function as both a promoter and a suppressor in different cancer types, which plays a role in cell growth, invasion, and metastasis.

Our target prediction analysis by miRTarbase revealed that miR-373-3p interacts with many genes associated with the progression of cancer. The miR-373-3p targeted the genes named RAD23B, RECK, VEGFA, LATS2, CSDC2, CD44, and TNFAIP1 verified by strong validation methods such as reporter assay, Western blot, and qPCR. The genes including KIF23, GBAS, C2orf18, PHC2, CD24, NUPL1,MYBL1, KLHL12, HERPUD1, LUC7L2, GPSM2, CENPF, CFL2, TUSC2, HOOK3, SUZ12, FEM1C, NR2C2, and KPNA2 targeted by miR-373-3p were verified by less strong validation methods such as microarray, NGS, pSILAC, clip-seq.

KPNA2 (also known as importinα-1 or RAG cohort 1) is a 58 kDa protein that facilitates the transport of cargo proteins into the nucleus. By binding cargoproteins that have a classical nuclear localization signal, it facilitates their import into the nucleus and performs an adaptor function [40]. KPNA2 is part of the karyopherin α group. Numerous genes encoding Karyopherin-α are found in mammals; these genes fall into three distinct subtypes based on their expression patterns in various tissues [41,42]. An earlier study demonstrated that KPNA2 is predominantly expressed in undifferentiated embryonic stem cells [43]. Multiple cellular processes, such as transformation, transcriptional regulation, immunological reaction, virulent infection, cell restoration, and tumorigenesis, have been linked to KPNA2.In addition, numerous types of cancer have shown aberrant KPNA2 expression, which may play a role in tumor development [44]. In some cases, KPNA2 has been shown to be a tumour suppressor [45], however, it has also been reported to play a oncogenicrole [46]. Using Western blot assay, we were able to find out the expression pattern of KPNA2 in PCa and confirm it as a potential downstream target of miR-373-3p. The decreased expression of KPNA2 has been linked to non-small cell lung cancer [45], head and neck squamous cell carcinoma (HNSCC) [47]. Various cancers, including endometrial [48], ovarian [49], prostate [50], gastric [51], and bladder cancers [52], has revealed that KPNA2 is an independent prognostic marker of poor survival. Data suggests that the KPNA2/NBS1/Snail axis was accountable for inducing nuclear β-catenin deposition and Wnt/-catenin signalling stimulation in gastric cancer [51]. A correlation was found between KPNA2 expression and serum AFP level, vascular invasion, BCLC stage, and early relapse in hepatocellular carcinoma [53]. Additionally, KPNA2 is a reliable indicator of chemo-resistance in both breast cancer and PCa [54,55].

5. Conclusion

Our finding suggested that low level of KPNA2 expression is linked to poor outcomes in PCa. Lower KPNA2 expression may reduce the nuclear availability of the repair complex MRN, resulting in the collapse of the DNA damage signalling pathway as well as decreased stimulation of the apoptotic cell death pathway. Failure to activate cell death pathways

may allow the tumour to continue growing. Based on our findings, KPNA2 could play a crucial role in facilitating the propagation and metastatic potential of PCa. Moreover, we found that miR-373-3p could help PCa spread by suppressing KPNA2 expression, which in turn would help the cancer progression. The results of our study add to the evidence supporting a regulatory role for KPNA2/miR-373-3p in PCa. These results could lead to new aspects of designing better treatment approaches for PCa patients. More study on a large population is required before significant miRNAs can be established as biomarkers.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (Approval no. Res/SCM/31/2018/109).

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

CRediT authorship contribution statement

Mohd Mabood Khan: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vineeta Sharma:** Writing – review & editing. **Mohammad Serajuddin:** Writing – review & editing. **Annet Kirabo:** Writing – review & editing.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2024.04.004.

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