Prostate International 11 (2023) 150–158

Contents lists available at ScienceDirect

Prostate International

journal homepage: https://www.journals.elsevier.com/prostate-international

Research Article

Screening and validation of novel serum panel of microRNA in stratification of prostate cancer

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ARTICLE INFO

Article history:
Received 27 March 2023
Received in revised form
11 June 2023
Accepted 15 June 2023
Available online 21 June 2023

Keywords:
Benign prostatic hyperplasia
Biomarker
Diagnosis
MicroRNA
Non-invasive
Prostate cancer

ABSTRACT

Background: Owing to the heterogeneous nature of prostate cancer (PCa) and errors in the characterization of the disease, researchers have been trying to unveil molecular biomarkers like microRNA (miRNA) as diagnostic markers. The purpose of our study is to demonstrate the precision of a panel of miRNAs as biomarkers with diagnostic potential for risk stratification.

Materials and methods: The present study demonstrates the comparative expression profiles of miRNA-141,-1290,-100, and -335 in both tissue and serum, including Benign Prostate Hyperplasia (BPH) and PCa, with healthy volunteers. Firstly, we demonstrate the expression of all miRNAs in the discovery cohort, including metastasis and benign tissue, and later validate their non-invasive diagnostic potential in BPH and PCa with healthy volunteers. MiRNA was isolated from tissue and serum to be quantified by RT-PCR and analyzed for biomarker potential by receiver operating characteristic (ROC) curve analysis, followed by targetome analysis of each miRNA.

Results: Among the non-invasive miRNA assessed, it was seen that miRNA 141 ($P = 0.0003$) and miRNA 1290 ($P < 0.0001$) are oncogenic with significantly higher expression, while miRNA 100 ($P = 0.0002$) and miRNA 335 are tumor suppressor, in PCa as compared to controls. While for BPH, miRNA 141 ($P = 0.003$) and miRNA 335 ($P = 0.0002$) were found to be significantly oncogenic and tumor suppressors, respectively. The analysis of the ROC curve of panel miRNAs (miRNA-141,-1290, and -100) portrayed a significant area under the curve with greater sensitivity and specificity. Moreover, in-silico prediction of their respective targetomes represents their extensive involvement in PCa progression and various other cascades that aid in PCa networks.

Conclusions: To the best of our knowledge, we are going to report for the first time this panel of miRNA that can be used to accurately and efficiently diagnose BPH and PCa patients from healthy males.

1. Introduction

The abnormal, uncontrolled increase in cells in the prostate gland is known as prostate cancer (PCa). It accounts for the second-most common malignancy in men and the fifth-largest cause of cancer-related death.[1] According to the ICMR, the incidence of PCa in India has reached up to 7.5 percent in the male population, commencing at a young age of 40 years and more. PCa diagnosis and suspicion have changed since the discovery of serum-based prostate-specific antigen (PSA).[2]Furthermore, patients with a PSA level of less than 2 ng/ml and a suspected DRE have a positive predictive value of 5–30%.[3,4] Non-categorized treatment of PCa concerning the incidence and development of cancer was a major restraint behind earlier diagnostic approaches such as serum PSA level, DRE, and trans-rectal ultrasound-guided biopsy. Therefore, molecular markers involved in major pathways triggering PCa progression have yet to be explored for better understanding due to the molecular heterogeneity of PCa and the time course of recurrence.

One of the most prominently researched molecular markers for the past decade has been microRNAs, due to their extremely survival-resistant mechanism in harsh conditions and protection from degradation. They are short strands up to 22 bases in length

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https://doi.org/10.1016/j.prnil.2023.06.002
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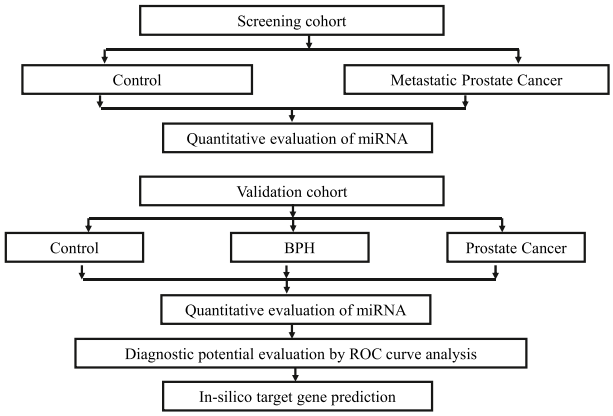

Fig. 1. Study design.

Table 1
Primers for RT-PCR

SNo.	MicroRNA	Accession ID	Primer
1	141-3p	MIMAT0000432	TAACACTGTCTGGTAAAGATGG
2	1290	MIMAT0005880	TGGATTTTTGGATCAGGGA
3	100-5p	MIMAT0000098	AACCCGTAGATCCGAACTTGTG
4	335-5p	MIMAT0000765	TCAAGAGCAATAACGAAAAATGT

that play major roles in the various crucial processes like cell cycle, apoptosis, cell differentiation, proliferation, etc., and indirectly or directly target many genes that form part of complex networks responsible for cancer progression and development.

Researchers' past-a-decade has been spent exploiting the ambiguous nature of miRNA for better understanding and diagnosis of PCa. Numerous studies have been carried out to identify dysregulated potential miRNAs in clinical samples, solid and liquid biopsies, by comparing expression profiles with healthy controls.[5] One of the recently studied miRNAs is miRNA 141, which is reported to be overexpressed in metastatic PCa. It is reported to be released into the circulation in epithelial-origin cancers and can differentiate metastatic PCa from healthy controls. Moreover, miRNA 100 has been reported to be significantly downregulated in PCa tissues in various studies. Keeping this in mind, our present study is designed to explore the roleplay of miRNA 141, miRNA 100, miRNA 1290, and miRNA 335 as non-invasive circulating miRNAs as potential biomarkers to identify and characterize patients from healthy individuals with BPH and PCa.

2. Material and methods

2.1. Patient and sample collection

The patients were recruited from the Department of Urology, King George's Medical University, Lucknow. The tissue and blood samples were collected with prior informed, voluntary, and written consent. In this study, a screening cohort and a validation cohort are considered (Fig. 1).

The screening cohort contains control ($n = 32$) and metastatic PCa (mPCa) ($n = 28$) along with details of age, PSA level, Gleason score, pathological (TNM staging) grade, and prostate volume. The mPCa subjects were chosen according to their increasing PSA level and undergoing the TRUS procedure, while the control was chosen with suspected PCa but with no evidence of histological confirmation of disease. The tissue samples collected were stored in RNAlater by cutting them in 0.5 cm and properly dipping them in solution.

Another validation cohort is an independent cohort that includes 55 healthy volunteers, 50 BPH subjects, and 43 PCa subjects chosen according to the inclusion and exclusion set. The healthy volunteers for the study recruited were age-matched with the BPH and PCa groups. PSA tests and histopathological evaluation of prostate biopsy during TURP and TRUS were certain methods used in the distinct differentiation of BPH from PCa. The blood samples were collected in a plain vial for serum isolation. The vials were centrifuged at 2000 rpm for 10 mins, and the serum isolated was kept in 1000 μl Qiazol reagent at −80°C.

2.2. MiRNA preparation and storage

The microRNA was isolated from serum using the miRNAeasy serum/plasma kit, Qiagen, and the mirVana™ miRNA Isolation Kit, Invitrogen, according to the manufacturer's recommendation. The microRNA was converted into cDNA using the MiRNA-X miRNA First-Strand Synthesis Kit from Takara as per the manufacturer's recommendation and stored at −80°C for expression analysis.

Table 2
Patient characteristics of the study

Clinical characteristics		Screening cohort ($n = 60$)		Validation cohort ($n = 148$)		
		mPCa	Control	Prostate cancer	Benign prostate hyperplasia	Control
		($n = 28$)	($n = 32$)	($n = 43$)	($n = 50$)	($n = 55$)
Age	Mean	70.5	51.4	72.8	55.7	50.4
	Range	61–85	40–65	61–85	48–76	43–62
PSA (ng/ml)	02–10	2 (7.1%)	27 (84.3%)	1 (2.3%)	39 (78%)	55 (100%)
	11–20	9 (32.1%)	5 (15.6%)	10 (23.2%)	11 (22%)	0
	>20	17 (60.7%)	0	32 (74.4%)	0	0
Prostate volume (cc)	Mean	52.8	32	42.9	36.5	25.4
	Range	(20–120)	(22–61)	(26–95)	(24–86)	(17–48)
Gleason score	7	16 (57.1%)		36 (83.7%)		
	≥7	12 (42.8%)		7 (16.2%)		
Tumor stage	T2a	6 (21.4%)		15 (34.8%)		
	T2b or T2c	14 (50%)		10 (23.2%)		
	≥T3	8 (28.5%)		8 (18.6%)		
Lymph node status	N_X	6 (21.4%)		7 (16.2%)		
	N_0	12 (42.8%)		30 (69.7%)		
	N_1	10 (35.7%)		6 (13.9%)		
Metastasis	M0	0 (0%)		37 (86.1%)		
	M1	28 (100%)		6 (13.9%)		

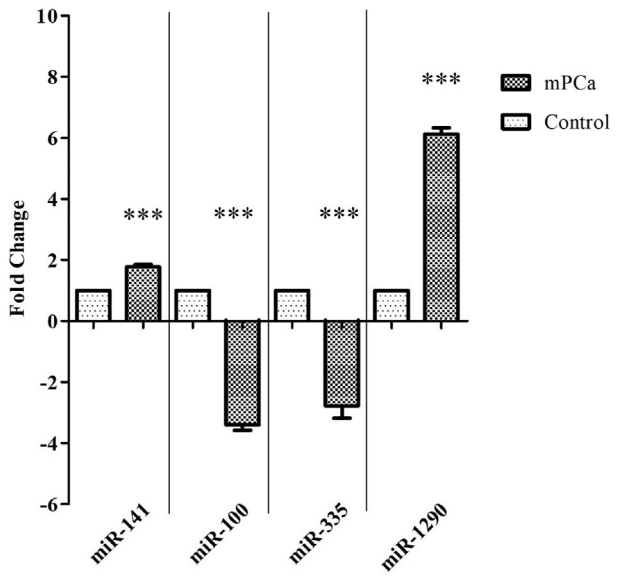

Fig. 2. The quantitative expression analysis of miRNA 141, miRNA 100, miRNA 335, and miRNA 1290 between mPCa and the control group in the screening cohort.

2.3. Quantitative analysis of miRNA by qPCR

The quantitative real-time PCR (qPCR) analysis of microRNA was carried out using TB Green Advantage® qPCR Premix, Takara. The sequence of reverse and forward primers for Real-Time PCR is listed in Table 1. The cDNA was used as templates in the qPCR analysis by using the fast real-time PCR System (7500HT, ABI, Applied Biosystems). The reaction volume is 25 μl per reaction under RT-PCR conditions where denaturation is 95°C-10 sec qPCR ×40 cycles at 95°C-5 sec, 60°C-20 sec, and dissociation curves 95°C-60 sec, 55°C-30 sec and 95°C-30 sec was performed at this standard condition. For normalization, the expression of U6 was used as the internal control. The Cycle of threshold (Ct) value of each sample was calculated by using the 2-dd CT method for fold change expression of microRNAs.

2.4. Insilico targetome analysis of miRNA

Online target prediction software Targetscan, MiRwalk 3.0, and miRDB were used to find Online target prediction software Targetscan, MiRwalk 3.0, and miRDB were used to find specific target genes targeted by miRNA. Target genes were nominated according to predicted target efficacy (context + scores method), positioning

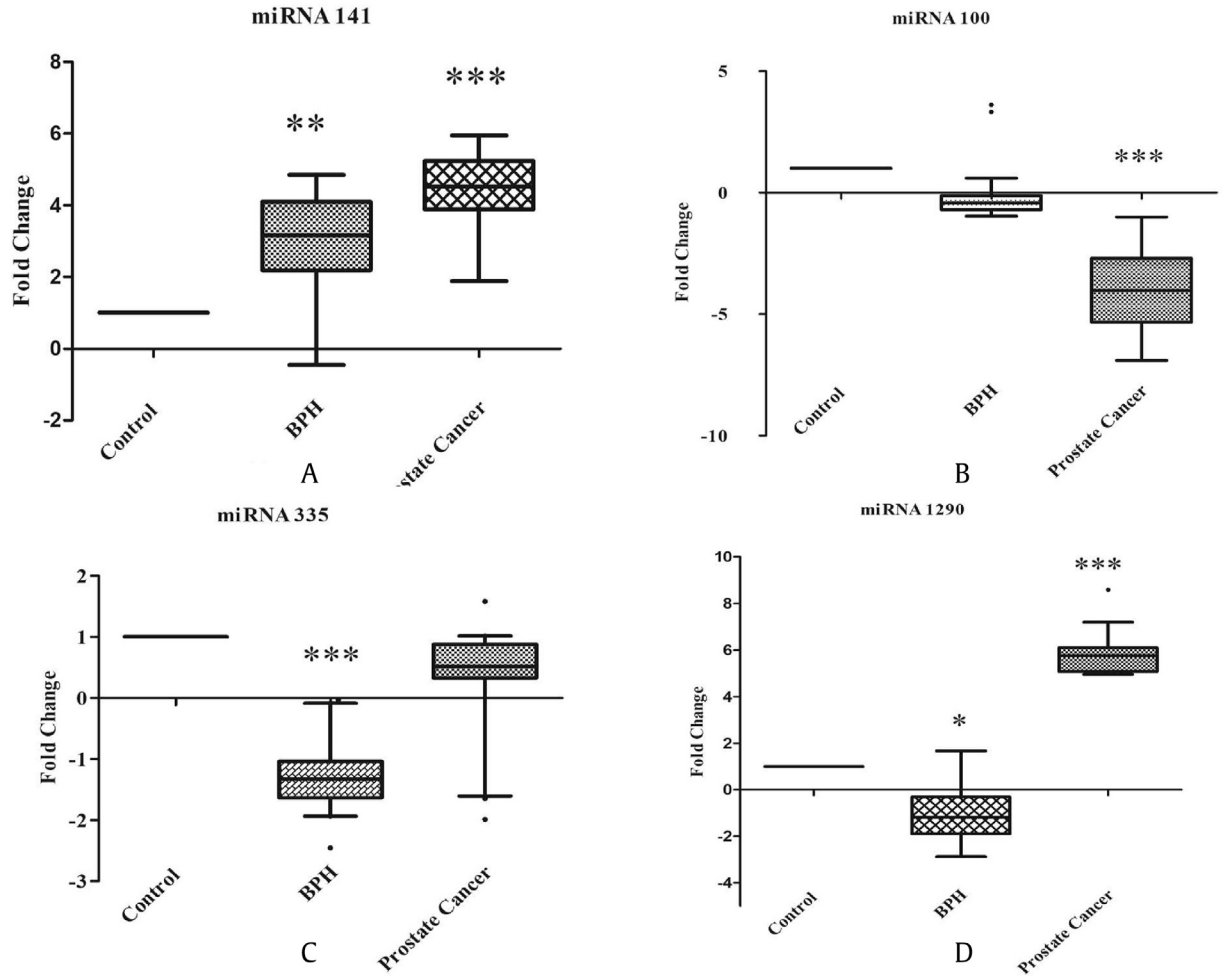

Fig. 3. Box plot for the quantitative expression of (A) miRNA 141, (B) miRNA 100, (C) miRNA 335, and (D) miRNA 1290 in the serum of BPH and PCa patients.

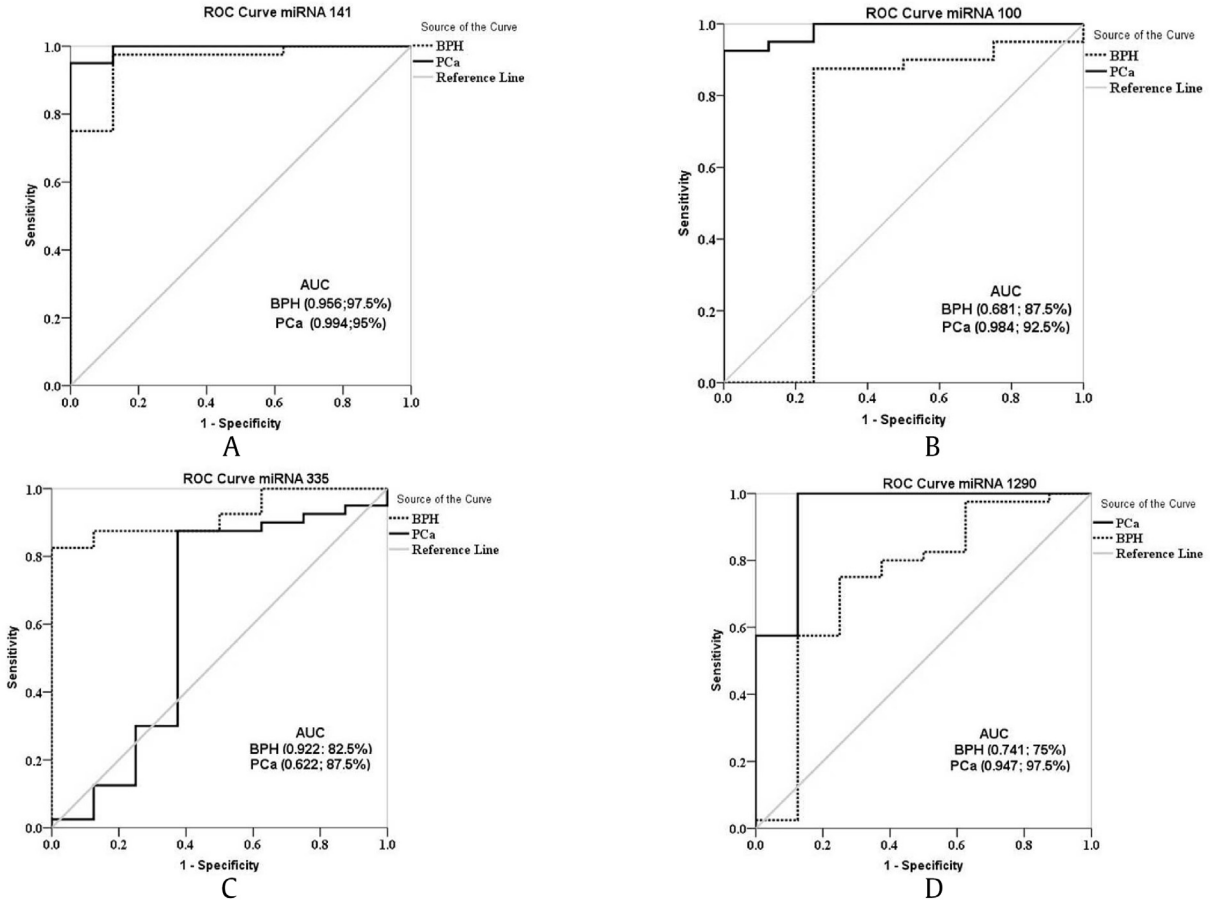

Fig. 4. ROC curve analysis of individual (A) miRNA 141 (B) miRNA 100 (C) miRNA 335 (D) miRNA 1290 for BPH and PCa groups.

of the potential regulatory elements, evolutionary conservation, and probability of off-target. This helped minimize the number of false-positive results. The Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway enrichment analysis was done using DAVID 6.8 Bioinformatics.[6] Predicted target genes were pasted in DAVID v6.7 for annotation, visualization, integrated discovery, and functional analysis. The analysis of molecular genes targeted was followed by Cytoscape 3.9.1.[7]

2.5. Statistical analysis

The data were statistically analyzed using Statistical Package for Social Sciences (SPSS) version 21 and GraphPad Prism 5.0. For the analysis of non-parametric data, we have applied the Mann-Whitney U test. Receiver operating characteristic (ROC) curves were used to determine the biomarkers for BPH and PCa by calculating the area under the curve (AUC), sensitivity, and specificity using Youden's index. The $P < 0.05$ was considered a significant result.

3. Results

3.1. Clinicopathological details of samples

The patients were enrolled in both screening and validation cohorts. The demographic details reveal that the mean age of mPCa and PCa group was 70.5 and 72.8, respectively, while for the control group and BPH group was 51.4 and 55.7, respectively. The range varied vastly between intragroup in both cohorts, showing an indolent growth of cancer progresses with age. Looking over the PSA level, it was observed that the control (84.3%) and BPH (78%) groups have low PSA levels (2–10 ng/ml) as compared to mPCa (60.7%) and PCa (74.4%) group (>20 ng/ml) (Table 2).

3.2. Quantitative expression analysis of microRNA in clinical samples

The analysis of miRNA expression in the screening cohort (tissue samples) between mPCa ($N = 28$) and normal ($N = 32$) groups revealed significantly upregulated expression of miRNA 141 and miRNA 1290 with 1.76-fold and 6.12-fold change increase, respectively. While the other two miRNAs, miRNA 100 and miRNA 335, were significantly downregulated with a 3.4- and 2.77-fold change decrease, respectively (Fig. 2). Comparison of miRNA levels in serum from PCa ($N = 43$), BPH ($N = 50$) cases, and healthy controls ($N = 55$) revealed that miRNA 141 was significantly upregulated in both BPH ($P = 0.003$) and PCa ($P = 0.0003$) groups (Fig. 3A). The expression of miRNA 100 was reported to decrease significantly in PCa ($P = 0.0002$) and insignificantly in BPH ($P = 0.87$) (Fig. 3B). The expression of miRNA 335 was found to be downregulated significantly in BPH ($P = 0.0002$), and there was no significant fold change in the PCa group when compared to controls (Fig. 3C). The miRNA 1290 expression in serum was found to be significantly downregulated by 1.09-fold in the BPH ($P = 0.03$) group, whereas it was significantly upregulated by 5.77-fold in the PCa ($P < 0.0001$) group (Fig. 3D).

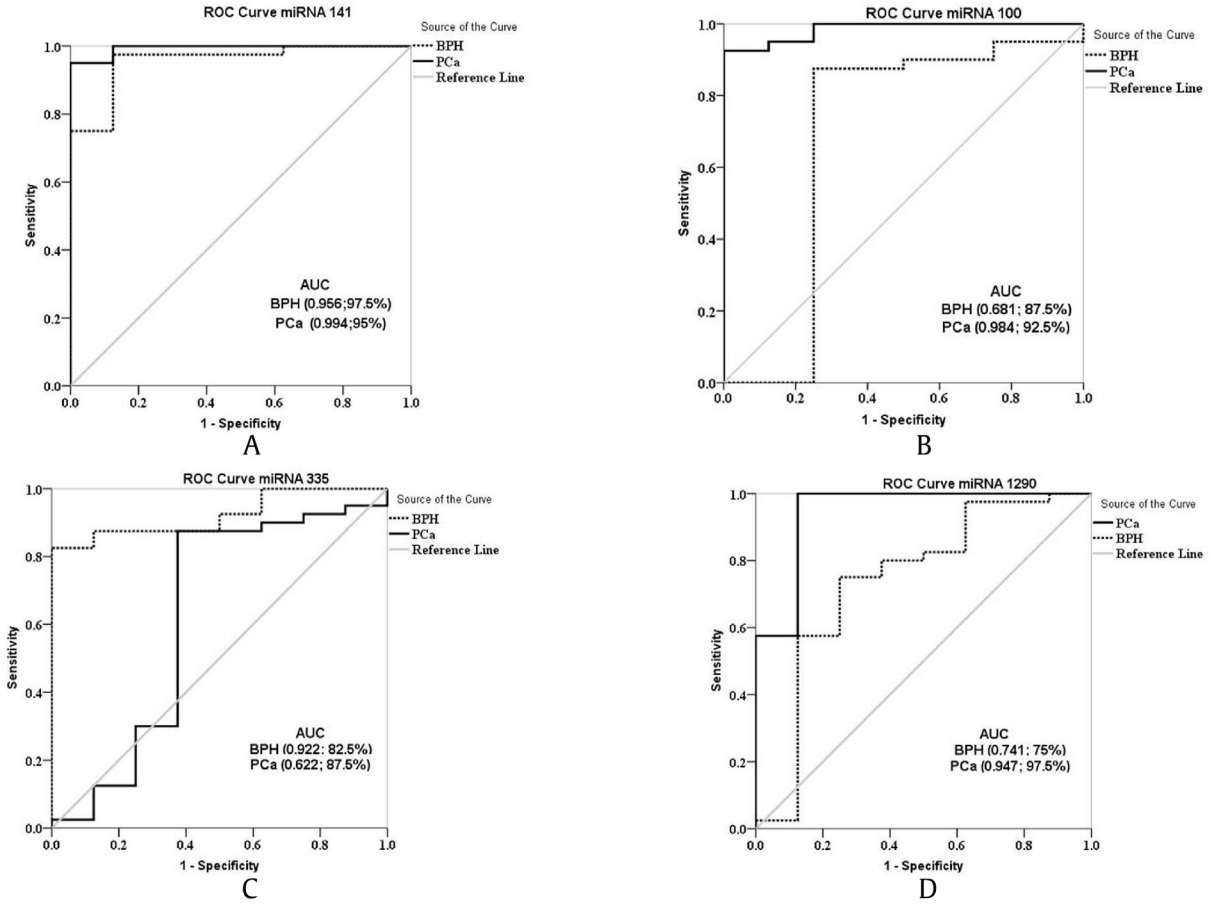

Fig. 5. ROC curve analysis of combination (A) all four miRNAs in both groups (B) signature panel of three, miRNA 100, miRNA 141, and miRNA 1290 for PCa group (C) panel of miRNA 335 and miRNA 141 for BPH group.

Table 3
Correlation between clinical parameters and miRNA expression in the Validation Cohort

miRNA	Group	PSA level		Gleason score	
		rho value	*P*	rho value	*P*
miRNA 141	BPH	−0.161	0.301		
	PCa	0.978	0.978	**0.336***	**0.034**
miRNA 100	BPH	−**0.383*****	**0.003**		
	PCa	0.390	0.390	−0.142	0.383
miRNA 1290	BPH	−**0.650*****	**0.004**		
	PCa	0.097	0.097	−0.139	0.394
miRNA 335	BPH	−0.243	0.491		
	PCa	0.079	0.432	−0.028	0.864

BPH, Benign Prostate Hyperplasia; PCa, Prostate Cancer; rho, Spearman's rank correlation coefficient. *$P < 0.05$; ***$P < 0.001$.

3.3. Diagnostic test analysis

The sensitivity and specificity of circulating miRNAs in serum as predictive non-invasive biomarkers of BPH and PCa were tested by ROC analysis. According to the ROC curves, among the individual miRNAs in the PCa group, miRNA 141, miRNA 100, and miRNA 1290 have the largest value for area under curve (AUC) as 0.994 ($P < 0.0001$; Sensitivity 95%), 0.984 ($P < 0.0001$; Sensitivity 92.5%), and 0.947 ($P < 0.0001$; sensitivity 97.5%), respectively. (Figs. 4A, 4B, 4D). For the BPH group, miRNA 141 and miRNA 335 have the largest values for AUC at 0.956 ($P < 0.0001$; Sensitivity 97.5%) and 0.922 ($P < 0.0001$; sensitivity 82.5%), respectively (Figs. 4A, 4B).

Further, we have made attempts to construct a panel of miRNAs that may have more power in distinguishing BPH and PCa from healthy individuals. For instance, combining expression of all miRNAs was seen as insignificant for BPH (AUC-0.57; $P = 0.211$) and highly significant for PCa (AUC-0.682; $P = 0.001$) (Fig. 5A). However, it was noteworthy to comprehend the strength of the miRNA panel consisting of 3 significantly upregulated miRNA, i.e., miRNA 141, miRNA 1290, and miRNA 100- have a greater and significant AUC value of 0.81 ($P < 0.001$) (Fig. 5B). Similarly, for the BPH group, miRNA panel consisting of miRNA 141 and miRNA 335 had greater AUC values of 0.77 ($P = 0.001$) (Fig. 5C).

3.4. Association of clinicopathological parameters with miRNA expression

Correlating the groups, we found that only miRNA 141 is positively and significantly correlated to the Gleason Score; thus, as the disease progresses, the Gleason Score and miRNA 141 expression increase. On the contrary, the expression of miRNA 100 and miRNA 1290 are negatively and significantly correlated with PSA levels in the BPH group. Thus, by increasing the PSA level, the miRNA 100 and miRNA 1290 expression decreases in BPH patients (Table 3).

The comparative analysis among the intragroups in the PSA level reveals that miRNA 141 is significantly linked with increasing PSA. MiRNA 141 was seen as significantly upregulated as the PSA level increased from the low-Gray-high level, while miRNA 335 was found to be significantly downregulated in the PSA Gray zone and upregulated in the high PSA level (Fig. 6A).

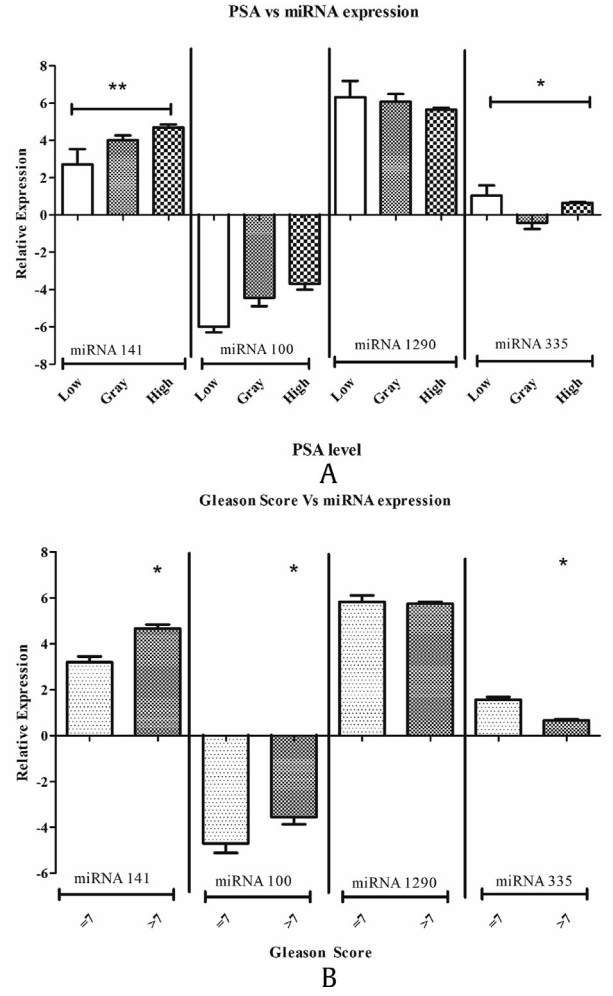

Fig. 6. (A) Comparative analysis between intragroup PSA levels (Low PSA – below 4 ng/ml; Gray PSA level; 4–10 ng/ml; High PSA level-above 10 ng/ml) in the Prostate Cancer group. (B): Comparative analysis between intragroup Gleason Score (GS) (GS = 7; GS > 7) in the prostate cancer group.

Similarly, the expression of miRNA 141 was significantly regulated in patients with Gleason Score >7 as compared to Gleason Score = 7. On the contrary, miRNA 100 and miRNA 335 expression were significantly downregulated with increasing Gleason Score (Fig. 6B).

3.5. *Insilico targetome analysis*

Further, an attempt to reveal the target genes of miRNA 141, miRNA 100, miRNA 335, and miRNA 1290 was carried out through target prediction analysis and their suggestive role in PCa. The target genes were predicted based on seed sequence alignment by using miRDB and TargetScan (Table 4). We performed gene set enrichment and KEGG pathway analysis of genes targeted by each panel, the first panel-PCa panel consisting of miRNA 141, miRNA 100, and miRNA 1290, and the second panel-BPH panel consisting of miRNA 141 and miRNA 335. It was carried out by the DAVID 6.8 functional annotation tool. The KEGG pathways involved were selected with an adjusted *P*-value <0.05. The targeted pathways revealed a significant involvement of targeted genes in several pathways like P13-Akt signaling, FoxO signaling, the p53 pathway, MAPK signaling, mTOR signaling, autophagy, the Wnt signaling pathway, the Hippo signaling pathway, etc., which directly or indirectly are involved in PCa pathogenesis (Fig. 7) Besides these pathways, we observed certain target genes directly involved in prostate cancer like E2F3, CCND2, CCNE2, EGFR, BCL2, KRAS, MDM2, CREB5, CHUK, CCNE2, etc., which form a complex network of genes with a confidence level >0.7 and enrichment score ppi 2.55E-15 as seen in Cytoscape 3.9.1 (String App) (Fig. 8).

4. Discussion

Circulating non-invasive miRNAs have been a trending topic of controversial debate as biomarkers for PCa, for the past decade. They serve as diagnostic platforms by using “liquid biopsy” like serum to determine the staging of PCa. They play an ambiguous role as both tumor suppressors and oncogenes, depending upon their micro-tumor environment, and regulate by attaching themselves to mRNA at 3′-UTR or 5′-UTR ends during post-transcriptional modification to either suppress or trigger one or several genes. Hence, the present study evaluates the expression profiling and biomarker potential of miRNA 141, miRNA 1290, miRNA 100, and miRNA 335 in the serum of BPH, PCa patients, and healthy controls. Moreover, we have attempted to explore and analyze the targetome of each miRNA that is involved in PCa progression.

MiRNA 141 is located at chromosome 12p13.31, a highly evolutionary conserved region, and is a part of the miRNA 200 family, which plays a crucial role in the EMT process.[8,9] In serum analysis, miRNA 141 was significantly upregulated in both the BPH and PCa groups with a 3.07- and 4.41-fold increase, respectively, as compared with the control, thus displaying its oncogenic expression. There was a significant 1.37-fold increase in expression from BPH to PCa. According to a recent finding, it was reported that circulating miRNA 141 is an oncogenic miRNA that was found to be strongly associated with the detection of aggressive metastases in PCa.[10–13] The reason behind its oncogenic behavior was believed to be the downregulation of the AR co-repressor by promoting AR transcriptional activity.[14] Besides PCa, miRNA 141 dysregulation was also reported to play a significant role in the carcinogenesis of the breast, ovarian, gastric, colorectal, and nasopharyngeal carcinomas.[15–17] These studies reveal the involvement of miRNA 141 in various biological processes like migration, invasion, and proliferation that help in tumor development and progression.

MiRNA 100 is located at chromosome 11q24.1,[18] which is linked to gene-rich chromosome and disease-rich chromosome 11 and is certainly involved in multiple diseases and cancer. It belongs to the miRNA 99 family.[19] As disease progresses from indolent to metastasis stage, the present study reveals significant downregulation of miRNA 100 in PCa with a 3.98-fold decrease. While a major

Table 4
Genes targeted by panel of miRNA as predicted by Targetscan and miRDB

Panel	MiRNA	Target genes
PCa	miRNA 141 miRNA 100 miRNA 1290	E2F3, CCND2, CCNE2, EGFR, GLS, GFR2, IRS2, TGFB2, ZEB1, CBL, BCL2, KRAS, MDM2, CREB5, CHUK, CCNE2, EGFR, GSK3B, IGF1, FGFR3, FZD8, MTOR, TAOK1, BMPR2, IGF1R, NTRK3, SLC44A1
BPH	miRNA 141 miRNA 335	BCL2L2, DKK1, ID4, MYC, CRKL, TUG4, ZEB2, SP1, TNC, MAPK1, E2F3, CCND2, CCNE2, EGFR, GLS, GFR2, IRS2, TGFB2, ZEB1, CBL

BPH, Benign Prostate Hyperplasia; PCa, Prostate Cancer.

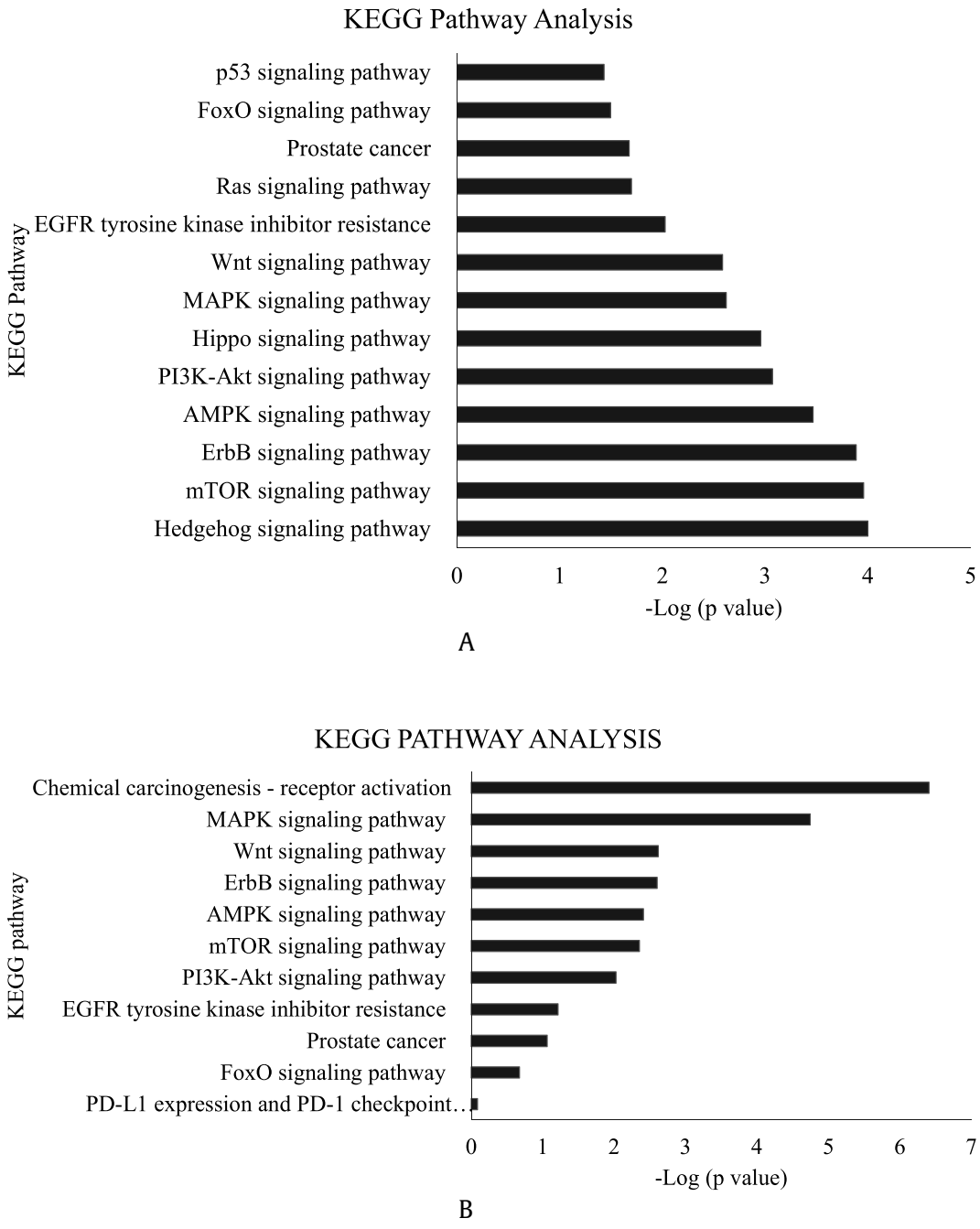

Fig. 7. KEGG pathway enrichment analysis of genes targeted by (A) the PCa panel of miRNA (B) the BPH panel of miRNA.

decrease of 3.75-fold change between the BPH and PCa groups was seen exhibiting tumor suppressor behavior. It is reported in various studies to express itself as both oncogenic like gastric cancer and tumor suppressor like hepatocellular carcinoma (HCC), pancreatic adenocarcinoma, adrenocortical cancer, bladder cancer, ovarian cancer, cervical cancer, PCa, and colorectal cancer.[20–26]

MiRNA 335, transcribed on chromosome 7q32.2, was found to be significantly downregulated in serum, with a 1.27-fold decrease in healthy individuals. Several studies have reported their down-regulation in various cancers like prostate, ovarian, thyroid, breast, and pancreatic and their upregulation in very few, like gastric cancer and endometrial cancer.[27–30] The expression profiling of miRNA 335 in PCa tissue, as reported by Xiong et al,[27] was found to be significantly downregulated as compared to respective controls.

MiRNA 1290 is a cancer-related miRNA transcribed on chromosome 1p36.13. Circulating miRNA 1290 was found to be significantly upregulated in the serum of PCa patients with a 5.77-fold change increase and downregulated in BPH patients with a 1.09-fold change decrease, thus indicating its oncogenic nature with disease progression to the metastasis stage. This could be evidently seen as there is a 6.87-fold increase from BPH to PCa, as indicated by the higher expression of miRNA 1290 in PCa as compared with BPH.[31] While there are few studies indicating its role in the CRPC stage suggesting the high levels of miRNA 1290 in circulating exosomal vesicles are associated with shorter overall survival in patients.[32] It was reported that miRNA 1290 in combination with miRNA 5100 may help in 99% diagnostic performance of PCa.[33]

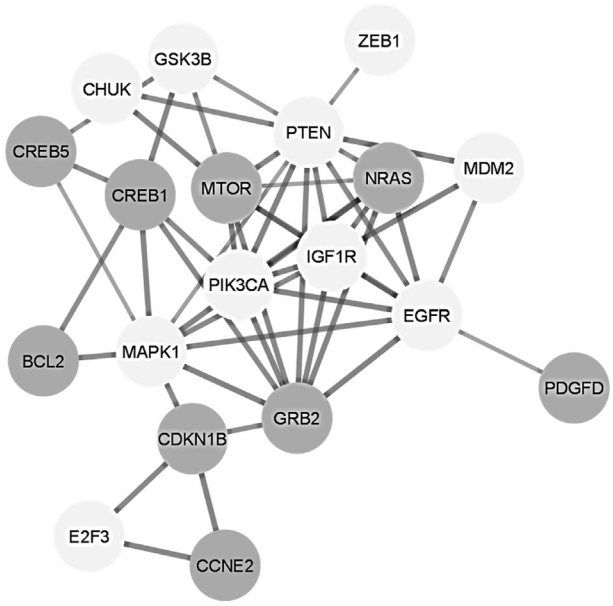

Fig. 8. Cytoscape 3.9.1: String app-the interaction of molecular targets of panel miRNA involved in PCa with confidence level >0.7 and an enrichment score of 2.55E-15.

Our study reveals that miRNA 141 and miRNA 1290 may act as oncogenic miRNAs and miRNA 100 and miRNA 335 as tumor suppressor miRNAs. Although the ROC curve analysis of each individual miRNA reveals good biomarker potential because they have a significant AUC, high sensitivity, and high specificity. Moreover, we tried to increase their efficacy by making panels of miRNA, which may give a wholesome picture of PCa for their precise diagnosis. A panel with the collaborative expression of miRNA 141, miRNA 100, and miRNA 1290 can significantly differentiate PCa from healthy controls since ROC curves show 98.3% sensitivity and 83.3% specificity. Likewise, the synergistic expression of miRNA 141 and miRNA 335 may be used for BPH diagnosis due to their 98.8% sensitivity. Their role in PCa progression was determined by additional Insilico analysis of the targeted targetome, including BCL2, E2F3, MDM2, NRAS, CREB1, CREB5, CHUK, EGFR, MTOR, ZEB1, PTEN, etc. The targetome indicates significant involvement of miRNAs in major pathways like P13-Akt signaling, FoxO signaling, the p53 pathway, MAPK signaling, mTOR signaling, autophagy, the Wnt signaling pathway, the Hippo signaling pathway, and various biological processes like migration, invasion, and proliferation that help in tumor development and progression. Further, exploring the interaction among these molecular targets using STRING suggests that a panel of miRNAs can be used not only to diagnose PCa and BPH in healthy controls but also, they may serve as therapeutic targets that directly alter the expression of significant genes involved in PCa pathogenesis.

5. Conclusion

In conclusion, our research adds to the increasing knowledge and evidence of dysregulated miRNA involvement in PCa initiation and progression. It helps in providing a more promising and noteworthy novel direction towards accurately identifying and characterizing the diagnosis of PCa by synergizing the combinatorial expressions of the circulating non-invasive panel consisting of miRNA 141, miRNA 1290, and miRNA 100. Moreover, upon establishing a relationship between the target genes and miRNA, it may help in developing therapeutic purposes for the better management of the disease. Further miRNA expression profiling in large populations may help in developing accurate methods for BPH and PCa diagnosis.

Ethics approval

All experimental protocols involving human participants were approved by the Ethics Committee of King George's Medical University (113th ECM II B-Ph.D/P8). Informed consent was received from all participants before enrollment. Written consent statements from patients who volunteered themselves were considered for study.

Conflicts of interest

The authors declare no competing interests.

Acknowledgment

The study was supported by ICMR-New Delhi, India (5/4/7-13/2019-NCD-II).

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