

Comprehensive cataloging of miR-363 as a therapeutic & non-invasive biomarker of prostate cancer

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Background & objectives: Overcoming the challenge of early diagnosis of prostate cancer (PCa) by exploring molecular biomarkers is urgently needed. With this objective, this study was designed to explore the biomarker and therapeutic potential of miRNA (miR)-363-3p in PCa pathogenesis.

Methods: Total participants (n=188) were enrolled, and blood and tissue samples were collected from individuals categorized into the control group (n=55), benign prostate hyperplasia (BPH) group (n=60), PCa group (n=48), and castration-resistant PCa (CRPC) group (n=25). MiR expression profiling was carried out using quantitative polymerase chain reaction (qPCR), and biomarker analysis was conducted employing receiver operating characteristics (ROC) curve. The miR-363 target genes were predicted by *in silico* tools like Target Scan and starBasev 2.0 and its expression was validated by qPCR and association among them was established by using the STRING database.

Results: The results showed that the tumour-suppressive nature of miR-363-3p in both PCa tissues and serum were significantly higher than the control with a greater area under curve (AUC) was 0.969 (sensitivity: 85%; specificity 100%) and 0.988 (sensitivity: 97.5%; specificity: 87.5%), respectively. The targetome analysis of miR-363-3p revealed five target genes-*NRAS*, *E2F3*, *PTEN*, *MDM2*, and *CCNE2* which were strongly associated with cell division and proliferation. The expression analysis of the target genes showed a significant tumour-suppression of *PTEN* gene and significant upregulation of oncogenic genes such as *NRAS*, *E2F3*, *MDM2*, and *CCNE2*.

Interpretation & conclusions: Collectively, the findings of this study suggest that miR-363-3p may be a potential biomarker in differentiating individuals with PCa and CRPC from healthy controls. The miR-363-3p triggers various oncogenic genes (*MDM2*, *NRAS*, *E2F3*, *CCNE2*) and tumour suppressor genes (*PTEN*) that are actively involved in PCa progression and development.

Key words Biomarker- microRNA - prostate cancer

Cancer statistics 2020 reports that among males worldwide, prostate cancer (PCa) ranks as the second

most common cancer, after lung cancer, and the fifth most cause of cancer-related death¹. In Asian countries

like India, it is observed that over time there has been an increase in the global incidence of up to 9.3 per cent new cases, and a 13.9 per cent mortality rate with a 5.8 per cent prevalence rate². The heterogeneous nature with the decelerated tumour growth rate of PCa in due course of time causes its early detection difficult³. Despite the availability of modern diagnostic procedures for prostate cancer (PCa), such as serum prostate specific antigen (PSA) level measurement, Digital Rectal Examination (DRE), and Gleason biopsy, these methods have been reported to produce inaccurate results at times, leading to false positive mistakes⁴. These can hence lead to misdiagnosis and over-treatment causing several complications of invasive methods. Therefore, to overcome the burden, researchers all around the world are focused on alteration caused by PCa at the genomics and proteomics level. Molecular biomarkers that can objectively and selectively measure the pathological state of the disease have been studied immensely⁴. MicroRNA (miR) is one of the possible biomarkers discussed in the present study since these can sensitively and precisely detect the early stage of PCa⁵.

MiRs are highly conserved non-coding regions that are post-transcriptionally involved in many biological processes like apoptosis, proliferation, differentiation, division, *etc.* These are 20-22 base pair long and target multiple genes to form complex networks⁶. These play an important role in PCa initiation and development. Several studies indicate their expression as a tumour suppressor or oncogenic depending upon the microenvironment these are present in PCa⁷. Their ambiguous nature of expression along with multiple target genes makes it suitable for therapeutic purposes in the management of PCa.

The miR-363 is found within a cluster of miR-106-92a. The dysregulation of miR-363 has been connected to tumour-suppressing effects in various cancers according to published studies⁸⁻¹¹. Accumulating evidence shows that miR-363-3p plays a role in PCa tumour genesis by targeting genes like DKK3¹². However, only a few studies indicate its expression in PCa and its later stages. Hence, this study was undertaken to explore the biomarker potential of miR-363-3p in both solid and liquid biopsies and compare them with their clinicopathological parameters. Furthermore we undertook to perform computational analysis of miR-363-3p targetome to predict and validate the target genes significantly involved in PCa progression and development.

Material & Methods

This study was undertaken at the department of Biochemistry, King George's Medical University (KGMU), Lucknow, and samples were taken from department of Urology, KGMU, Lucknow, after obtaining the ethical approval from the Institutional Ethical Committee (IEC). The study was conducted between December 2021 to September 2023. Voluntary informed and written consent was obtained from all the study participants to collect tissue and blood samples.

Study participants and sample collection: A total number of 188 participants who presented to the department of Urology, KGMU, Lucknow were undertaken to collect tissue samples. The individuals with age more than 40 years and pathologically confirmed for BPH and PCa by Gleason Score were enrolled for the study. The tissue samples were taken from participants with PCa (n=48) and BPH (n=60) undergoing TransRectal Ultrasound (TRUS) and Transurethral resection of the prostate (TURP), respectively. No tissues were collected from CRPC and healthy male participants. The tissue samples were maintained in RNAlater (Thermo Fischer Scientific, USA) by slicing them into 0.5 cm segments and submerging them in the solution. The blood samples collected from healthy males (n=55), CRPC (n=25), PCa, and BPH for serum isolation. Those suffering from any other chronic malignancy were excluded from the study. The plain vials underwent centrifugation at x2000 rpm for 10 min to separate serum, then stored in Qiazol reagent (Qiagen, Germany) at a 1:200 ratio at -80°C.

Isolation of miR and total RNA: MiR was extracted from serum as per the instructions provided by the manufacturer using the miRNAeasy serum/plasma kit (Qiagen, Germany) and the mirVana™ miRNA Isolation Kit (Invitrogen, USA)¹³. Following the instructions provided by Takara, the microRNA was transformed into cDNA using their MiRNA-X miRNA First-Strand Synthesis Kit (TaKaRa, Japan). Subsequently, it was stored at a temperature of -80°C in preparation for expression study. Using a manual organic extraction technique, total RNA was extracted from whole blood. This RNA is then transformed into complementary DNA using Applied Biosystems' High-Capacity cDNA Reverse Transcription Kit (ThermoScientific, USA).

Relative quantification of miR by real time-PCR: The TB Green Advantage® qPCR Premix (TaKaRa, Japan) and the iTaq™ Universal SYBR® Green Supermix

(Bio-Rad, USA) were used for the quantitative real-time-PCR (qRT-PCR) measurement of target genes' microRNA and relative mRNA expression. The sequence of primers of miR and mRNA used in this study for RT-PCR are listed in Supplementary Table. The qRT-PCR utilized the cDNA as a template. This was accomplished by means of an Applied Biosystems rapid real-time PCR equipment (7500HT, ABI, USA). Denaturation at 95°C for 10 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, 60°C for 20 sec, and a dissociation curve at 95°C for 60 sec, 55°C for 30 sec, and 95°C for 30 sec were carried out under these standard conditions¹³. U6 expression acts as an internal control for miR, whereas *GAPDH* served as an internal control for mRNA, to standardize the results. Utilizing the 2-dd Ct technique for fold change expression of miRs, the Cycle of threshold (Ct) value for every sample was computed.

MiR targetome analysis by in silico tools: For prediction of target gene prediction, we used software including TargetScan (https://www.targetscan.org/vert_72/), starBase v2.0 (<http://starbase.sysu.edu.cn/>), and miRDB (<https://mirdb.org/>) to locate specific genes targeted by miR^{14,15}. A number of factors were considered for selecting target genes, including the projected target efficacy (using the context+ scores technique), the location of the putative regulatory elements, the evolutionary conservation, and the risk of off-target effects. This assisted in reducing the amount of findings that were reported as false positives. The DAVID 6.8 Bioinformatic programme was utilized in order to carry out the pathway enrichment analysis for the Kyoto Encyclopaedia of Genes and Genomes (KEGG)¹⁶. Annotation, visualization, functional analysis, and integrated discovery were all performed using DAVID version 6.8 once the predicted target genes were put in. After doing an analysis of the molecular genes that were targeted, the KEGG pathway was carried out.

Statistical analysis: The data were subjected to analysis using the Statistical Package for Social Sciences version (SPSS) 21(IBM, USA) and GraphPad Prism 5.0 (Dotmatics, USA). Mann-Whitney U test was used to analyze non-parametric data. The biomarkers for CRPC, BPH, and PCa were identified using Receiver Operating Characteristic (ROC) curves. The area under the curve (AUC), sensitivity, and specificity were calculated using Youden's index. A result with a *P* value of less than 0.05 was deemed statistically significant.

Result

The demographic details of individuals enrolled in this study revealed a gradual increase in mean age with the pathogenesis of the prostate gland. Also, the PSA level was found to be >20 ng/ml only in PCa (56.2%) and CRPC (100%) group, while the PSA level between 11-20 ng/ml was found to be greater in PCa (39.6%) as compared to BPH (13.3%) group (Table I).

Quantitative validation of miR expression in tissue and serum: As reported previously, we reported miR-363-3p to have significantly downregulated in PCa as compared to BPH *via* microarray technique¹⁷. In the present study, a comparison between BPH and PCa tissue samples showed significant ($P<0.0001$) down regulation of miR-363 with a 2.99 fold change decrease (Fig. 1A). Similarly, expression of miR-363 in the serum of healthy control, BPH, PCa, and CRPC was found to be significantly ($P<0.0001$) down regulated with a 4.91, 6.11, and 6.06 fold change decrease, respectively (Fig. 1B). On applying the t-test, between the BPH and PCa, and BPH and CRPC groups, it was seen that PCa and CRPC were significantly ($P<0.0001$) down regulated with 1.19 and 1.14 fold change decrease, respectively, as compared with BPH.

Association of miR-363 with clinicopathological parameters: The expression of miR-363 was correlated with clinicopathological parameters like Gleason Score, prostate volume, and PSA level. It was found that the miR-363 expression in the serum of the PCa group was negatively and significantly correlated with Gleason Score ($P<0.05$) and prostate volume ($P<0.01$) (Table II). Likewise, the Gleason Score was negative and significantly ($P<0.01$) correlated with miR 363-3p in the CRPC serum group (Table II). This observation suggests that as the Gleason Score increased and the disease progressed, miR-363 expression decreases. While other parameters including lymph node distribution showed no significant association with miR-363 expression in BPH, PCa, and CRPC (Fig. 2).

Diagnostic potential of miR-363: According to ROC curve analysis, miR-363 covered a highly significant ($P<0.0001$) area in both PCa tissue (0.969; sensitivity: 85%; specificity 100%) as well as serum (0.988; sensitivity: 97.5%; specificity: 87.5%) (Fig. 3A & B). Similarly, for CRPC, miR showed a significant ($P<0.0001$) AUC with 0.894, 85 per cent sensitivity, and 87.5 per cent specificity (Fig. 3B). However, for BPH, miR has the least AUC (0.144) but is significant

Table I. Participants' characteristics of the study

Clinical characteristics	CRPC (n=25), n (%)	Prostate cancer (n=48), n (%)	Benign prostate hyperplasia (n=60), n (%)	Control (n=55), n (%)
Age (yr)				
Mean	73.9 (70-81)	68.5 (51-70)	51.4 (40-65)	41.6 (40-50)
PSA (ng/ml)				
02-10	0	2 (4.2)	52 (86.7)	55 (100)
11-20	0	19 (39.6)	8 (13.3)	0
>20	25 (100)	27 (56.2)	0	0
Prostate volume (ml)				
Mean	-	52.8	42.9	32
Gleason Score				
7	0	22 (45.8)	-	-
≥7	25 (100)	26 (54.2)	-	-
Tumour stage				
T2a	2 (8)	13 (27.1)	-	-
T2b or T2c	10 (4)	29 (60.4)	-	-
≥T3	13 (52)	6 (12.5)	-	-
Lymph node status				
N ₀	8 (32)	18 (37.5)	-	-
N+	17 (68)	30 (62.5)	-	-

N0/N+, lymph node absent/present

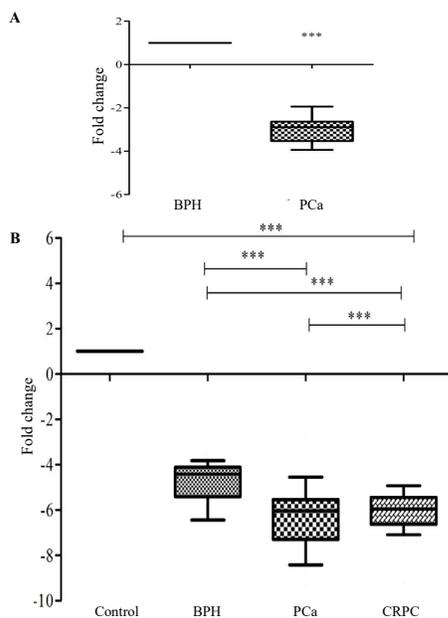


Fig. 1. The expression profiling of miR-363 in (A) prostate cancer (PCa) tissue (n=48) as compared to the Benign Prostate Hyperplasia (BPH) tissue (n=60) and (B) BPH (n=60), prostate cancer (n=48), and Castration-Resistant Prostate Cancer (CRPC) serum (n=25) as compared to the healthy control serum (n=55), respectively. The miR-363-3p was significantly downregulated in both tissue and serum ($P^{***}<0.0001$).

($P=0.002$) and has 87.5 per cent sensitivity and 10 per cent specificity (Fig. 3B).

Targetome analysis of miR-363: *In silico* targetome analysis of miR-363 to predict target genes involved in PCa pathogenesis was performed. The Venn diagram (Supplementary Fig. 1) shows the 257 commonly targeted genes. All the common target genes were used for KEGG pathway analysis. Upon analyzing the enrichment scores, significant P value and the number of genes involved, it was seen that maximum target genes were part of regulation of actin cytoskeleton, PCa, PI3-Akt pathway, followed by other pathways like FoxO signaling pathway, Hippo signalling (Fig. 4A). Also, among several pathways, it was found that a handful number of target genes like *CCNE2*, *E2F3*, *PTEN*, *MDM2*, and other were directly involved in PCa pathway as seen in the enrichment graph and KEGG map (Fig. 4B; Supplementary Fig. 1). All the genes were evaluated further by ENCORI database, to evaluate their *in silico* correlation and binding with miR-363-3 ($P<0.5$) using Pan-Cancer and miR-mRNA interaction, respectively. We found that out of six genes, five genes have a significant correlation with miR-

Table II. Association of clinicopathological parameters with miR-363 expression

Clinicopathological parameters	BPH		PCa		CRPC
	Serum	Tissue	Serum	Tissue	Serum
Gleason Score			-0.31*	-0.293	-0.395**
PSA level	-0.177	-0.065	-0.071	0.0013	0.128
Prostate volume	0.128	-0.243	-0.37**	0.045	

P>0.05; **>0.01

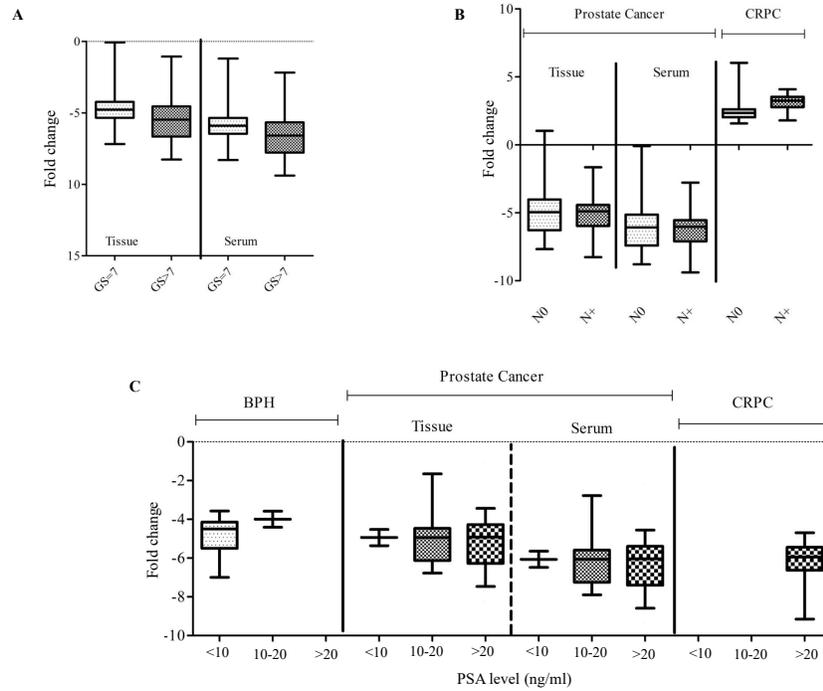


Fig. 2. The relative expression (fold change) of miR-363-3p with (A) Gleason Score (GS) in PCa group, (B) lymph node (N) distribution in PCa and CRPC groups, and (C) PSA level in BPH, prostate cancer and CRPC. PSA, prostate specific antigen.

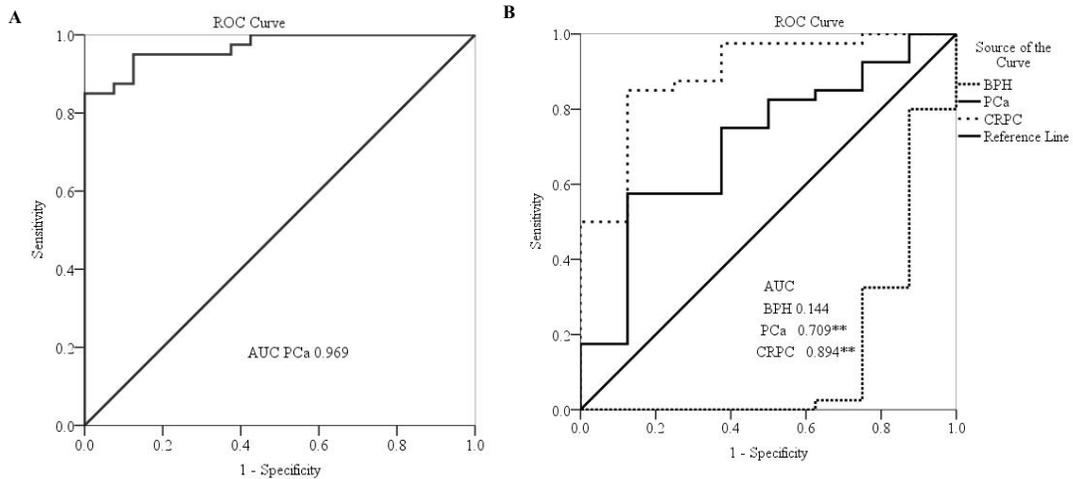


Fig. 3. The biomarker analysis of miR-363 in (A) prostate cancer tissue, (B) BPH, prostate cancer, and CRPC serum. The AUC lying between 0.8 to 0.9 is considered excellent and denotes high potential as diagnostic biomarker since it can differentiate between BPH, PCa and CRPC from healthy controls.

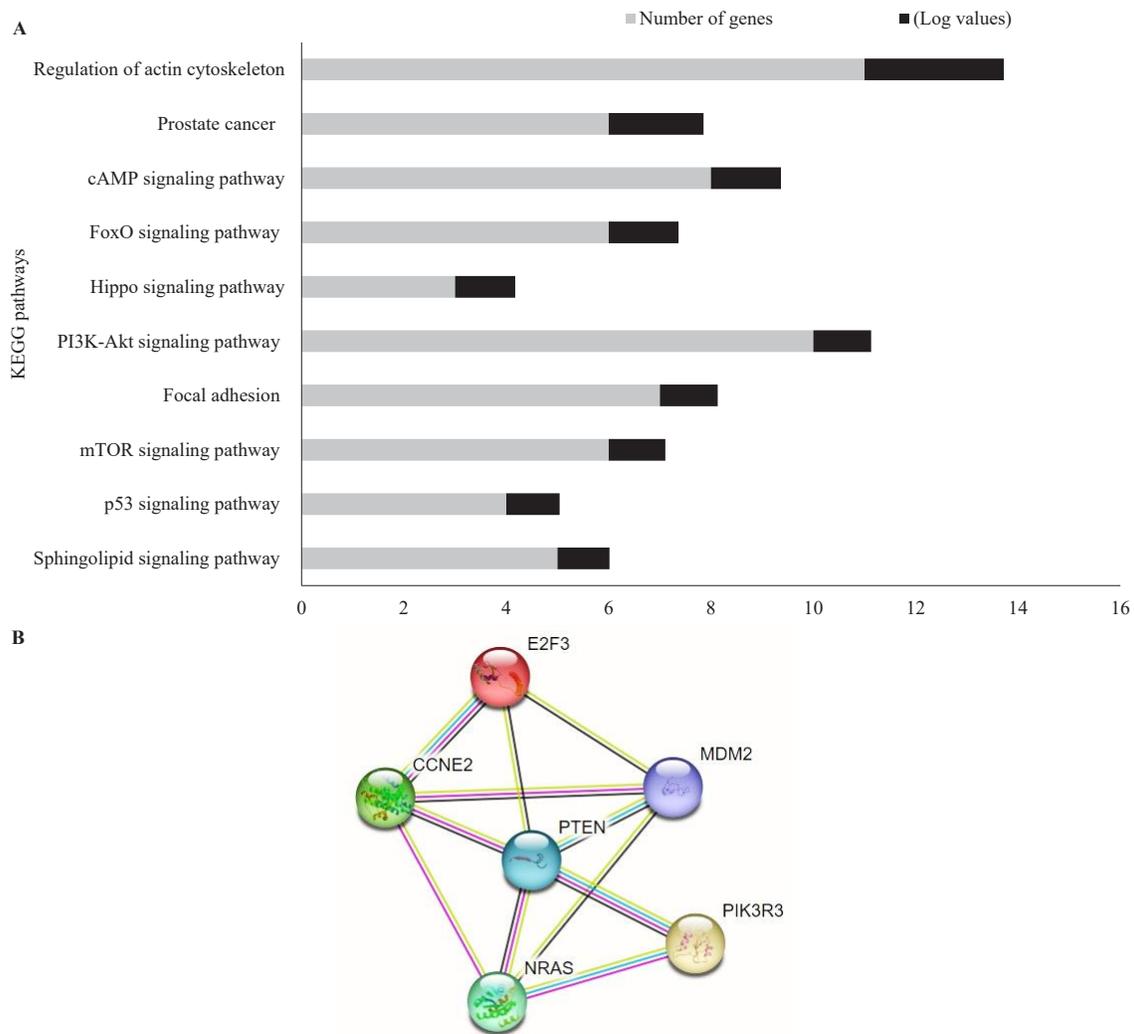


Fig. 4. (A) KEGG pathway analysis of miR-363 target genes as predicted by *in silico* tools. Potential target genes are involved in prostate cancer pathway. **(B)** The target genes (*E2F3*, *MDM2*, *CCNE2*, *PTEN*, and *NRAS*) association using String database with a enrichment value 0.00171. The stronger association and combination is shown by more number of colored line. KEGG, Kyoto encyclopedia of genes and genome.

363-3p. However, *PIK3R3* did not have a significant correlation with miR-363-3p (Supplementary Fig. 2).

Gene expression: The expression analysis of *NRAS*, *E2F3*, *CCNE2* and *MDM2* showed a significant up-regulation with 5.46, 5.26, 3.11 and 3.3 fold change increase, respectively, in PCa tissues as compared with BPH tissues (Fig. 5A). On the contrary, a significant down-regulation of *PTEN* gene, a tumour suppressor was observed with a 5.61 fold change decrease in PCa tissues as compared with BPH tissues (Fig. 5A).

The gene expression in serum samples for the PCa group revealed a significant upregulation of *NRAS*, *E2F3*, *CCNE2* and *MDM2* with 2.32, 6.81, 2.66, and

5.46 fold change increase, respectively, and significant downregulation of *PTEN* with 2.93 fold change decrease as compared to the healthy males (Fig. 5B-F). Similarly, significant ($P < 0.0001$) pattern of upregulation of *CCNE2* (3.46 fold change), *E2F3* (7.14 fold change), *MDM2* (0.22 fold change), and *NRAS* (5.51 fold change) and down-regulation of *PTEN* (4.27 fold change) for CRPC group was observed as compared with healthy males (Fig. 5B-F).

The association between miR-363-3p and target genes indicates that *CCNE2* was notably and positively associated with *CRPC* out of the five genes. Heat map revealed that the target genes exhibited the highest negative correlation with *NRAS*, *CCNE2*, *E2F3*, and

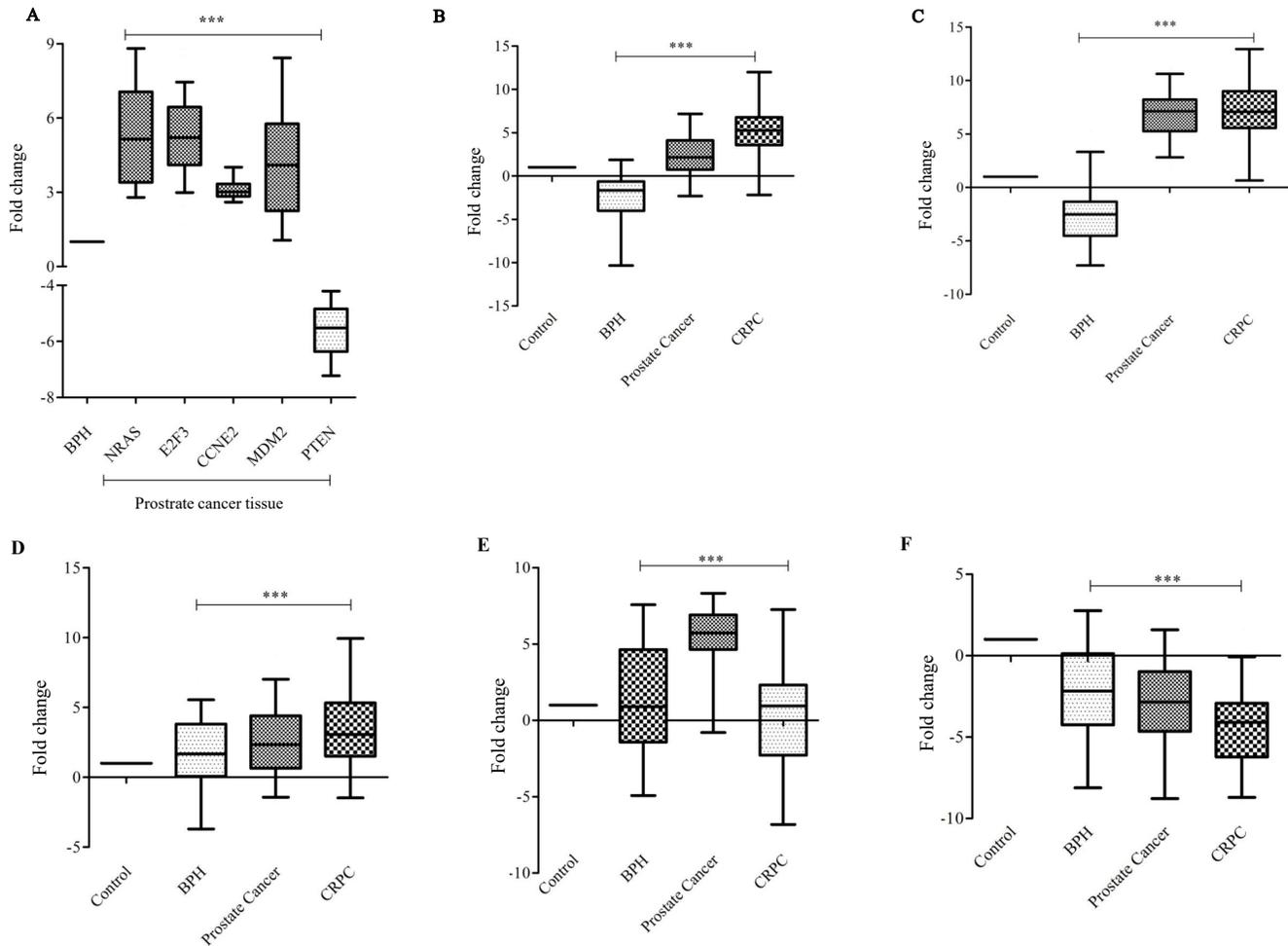


Fig. 5. The expression analysis of Targetome of miR-363. **(A)** Target genes in prostate cancer tissue (n=48), **(B)** *NRAS*, **(C)** *E2F3*, **(D)** *CCNE2*, **(E)** *MDM2*, and **(F)** *PTEN* in BPH (n=60), prostate cancer (n=48), and castration resistant prostate cancer (CRPC) (n=25) as compared with healthy control (n=55). The *NRAS*, *E2F3*, *CCNE2*, and *MDM2* gene was significantly upregulated in both tissue and serum while *PTEN* was significantly down-regulated (significant values $P^{***} < 0.0001$).

a slightly lower correlation with *MDM2*. Conversely, *PTEN* showed a positive correlation with miR-363 in BPH, PCa, and CRPC serum (Fig. 6). This suggests that as the expression of miR-363-3p decreased during PCa progression, *PTEN* expression also decreased simultaneously.

Discussion

The decelerated growth rate of tumours and lack of precise and accurate diagnostic methods for PCa require advances in the early detection of PCa. Recently, there has been a lot of research ongoing to evaluate the aberrant expression of miRs to explore the biomarker potential. The dysregulation of miR has commonly been seen in many cancers, as both oncogenic and tumour suppressors, depending upon

the tumour microenvironment. Exploring the role of miR in PCa aims to improve diagnostic accuracy by identifying specific miR signatures that can differentiate these conditions from healthy individuals. Our prior investigation identified the diagnostic potential of miR-141, miR-335, miR-1290, miR-100, miR-4510, miR-183, and miR-329 as key biomarkers supported by bioinformatic analysis and statistical validation^{13,18}. Besides these miRs, the microarray profiling on the PCa and BPH tissue samples revealed miR-363-3p as one of the significantly downregulated miR¹⁷. In this study, the expression profiling of miR-363-3p on an independent set of tissue and serum and validated the significant down regulation of miR-363-3p.

The miR-363 is found on the Xq26.2 chromosome within a cluster of miR106-92a. This cluster has two

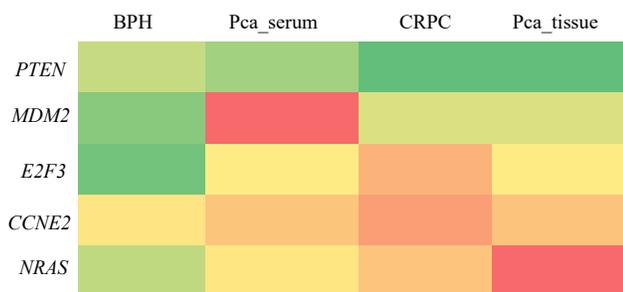


Fig. 6. The heatmap of correlation of miR-363 with target gene expression (*NRAS*, *E2F3*, *CCNE2*, *MDM2*, *PTEN* and *PIK3R3*). Red indicates the lowest value, orange indicates mid percentile at 50 and green indicates the highest value (Significance at $P^* > 0.05$).

autosomal paralogs miR-17-92 and miR-106b-25, that show oncogenic behaviour in various cancer¹⁹. This cluster consists of miR-106a, miR-18b, miR-19b-2, miR-20b, miR-92a-2 and miR-363²⁰. Unlike five miRs having their seed sequences in oncogenic expression, miR-363-3p has been found to be dysregulated with tumour-suppressive effects on various cancers like breast, colorectal, and others^{21,22}. Although there have been only a few studies exploring the role of miR-363-3p in PCa, some studies suggest a significant down regulation of miR-363-3p in both tissue as well as serum²³. Similarly, this study showed a significant ($P < 0.0001$) down-regulation of miR-363-3p in PCa and CRPC with 1.19 and 1.14 fold change decrease as compared with BPH. Moreover, the ROC curve analysis of miR suggested good biomarker potential because with significant AUC, high sensitivity, and high specificities. Furthermore study also showed miR-363-3p having significant non-invasive biomarker potential to distinguish PCa and CRPC from healthy controls.

The targetome analysis revealed that miR-363 was significantly involved in the many biological processes like cell cycle, proliferation, and cell survival *via* targeting *PTEN*, *MDM2*, *NRAS*, *CCNE2*, and *E2F3*, as predicted by *in silico* tools. These genes form a strong association with the genes in PCa network as seen in KEGG pathway map (map05215). There are three main phenotypes that describe cancer, namely, proliferation, migration, and invasive, which are evidently related to miR-363 as its functional role suggested by various studies^{8,24}. He *et al*²⁵ suggested miR-363 as tumour suppressor in osteosarcoma *in vitro* and *in vivo* by targeting *PDZD2* and *PCNA* affecting the tumour growth, apoptosis, and EMT phenotypes²⁵. Xie *et al*⁸, suggested the involvement of miR-363 in renal cell carcinoma by suppressing proliferation,

migration, and invasion by directly targeting *SIPRI*⁸. Conversely, study showed that upregulated miR-363 in PC3 cells induced cell proliferation through c-MYC, a downstream target⁹. Among the target genes of miR-363-3p, in this study, *PTEN* was significantly downregulated in PCa and CRPC due to its tumour suppressive properties. Loss of *PTEN* activity is evidently categorized as one of the most common driving events for PCa pathogenesis due to its 70 per cent incidence in PCa patients¹⁰. It is a major part of the PI3-AKT and mTOR pathways¹¹. It is involved in triggering signals to cells for proliferation, apoptosis, migration, adhesion, and angiogenesis. The other target genes like *E2F3*, *CCNE2*, *MDM2*, and *NRAS* were significantly upregulated in PCa and CRPC as compared to healthy controls.

CCNE2 is a cyclin that binds to Cyclin-dependent kinases (CDK) for the progression of the cell cycle²⁶. Our findings suggest that *CCNE2* and *E2F3* were significantly upregulated in PCa and CRPC. *CCNE2* and CDK2 phosphorylate retinoblastoma (RB1) are key factors triggering *E2F3*, a transcription factor. *E2F3* has been reported to be significantly upregulated in cancers by regulating proliferation and cell-cycle processes²⁶. This whole mechanism helps G1/S phase transformation during cell division. During PCa tumorigenesis, the *CCNE2* and *E2F3* gene anomalies cause their upregulation²⁷.

The next target gene *MDM2* is an oncogene, was also upregulated in PCa tissue and serum in this study. This gene forms an oncogenic-tumour suppressor relation with *PTEN*. It was previously observed that *PTEN* downregulates in aberrant conditions and PI3-AKT phosphorylates to activate AKT, which targets the localization and movement of *MDM2* in the nucleus²⁸. Inside the nucleus, *MDM2* regulates the negative feedback loop with p53. *MDM2* transcriptionally targets p53 and ubiquitinates p53 for proteasomal degradation. In this manner, cell cycle (G1 and G2 phase) arrest and apoptosis are disrupted leading to uncontrolled cell proliferation. p53 is generally expressed during metastasis or androgen-independent growth²⁹. The latter condition is CRPC where the cancer cell resists androgen depletion therapy and continues to express its activity. In an independent manner, *MDM2* is reported to interact with FoxO3A (a transcription factor for cell cycle regulatory proteins), epithelial-mesenchymal transition *via* E-cadherin and slug proteins, and pRB. The *MDM2* binds with pRB to trigger E2F transcriptional factors responsible for

G1/S phase progression that cause an effect on tumour growth. It is reported to be increased in PCa patients with radiotherapy²⁹. For therapeutics, it is suggested that as PTEN interacts with p53, MDM2 association with chemotherapy reverses to provide better treatment possibilities.

NRAS gene is a protooncogene that belongs to Ras family and is rarely found mutated in PCa, estimated at 0.41 per cent approximately³⁰. Our study indicates its upregulation as the disease progresses to the CRPC stage. NRAS and other Ras family members are activated by a series of signal transduction with GTPase as energy exchange. These activate RAF by phosphorylating it at Ser338 and Thr341. This complex initiates and triggers cellular proliferation and differentiation at the end³¹. Due to its rarity of mutation, there are only a few studies indicating their involvement in PCa. Other cancers like colorectal cancer have reported the synergist effect of EGFR-Ras in therapeutic interventions.

Overall this study highlights the tumour suppressive function of miR-363-3p and its potential as a non-invasive diagnostic biomarker in the progression of PCa from BPH to CRPC. The findings suggest a negative correlation between miR-363-3p levels and increasing Gleason Score as the disease advances. Additionally, there was an inverse relationship observed between miR-363-3p expression and serum PSA levels in PCa and BPH affected individuals. However, it is a larger sample size, a more robust association between miR-363-3p and clinicopathological parameters such as lymph node involvement and metastasis could be established. Apart from its association with clinicopathological factors, miR-363-3p also modulates the expression of various oncogenes (*MDM2*, *NRAS*, *E2F3*, *CCNE2*) and tumour suppressor genes (*PTEN*) that play crucial roles in disease progression, suggesting their potential as therapeutic targets for improved diagnosis and management of PCa.

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Conflicts of Interest: None.

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