Research Article

Circulating microRNA (143, 21) as biomarker for prostate cancer

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ABSTRACT

Background: Prostate cancer (PCa) is the second utmost widespread cancer in men and major male cancer in the economically developed countries. Although, several tumors of prostate mature gradually are restrained in the prostate without reducing the patient's life quality. While other tumors are violent, spread rapidly and thus should be diagnosed at initial stages. Regardless of its false negative and false positive rates, test of the prostate-specific antigen is only extensively accessible PCa test diagnostics and screening. Therefore, the establishment of predictive biomarkers capable of distinguishing between indolent and aggressive PCa would reduce the risk of overdiagnosis and overtreatment. One promising approach is the utilization of microRNAs (miRNAs) in the diagnosis and prognosis of PCa patients. **Objective:** The aim of present study is to examine whether the PCa, and BPH, patients have distinctive concentration of miRNA-21 and miRNA-143, which might lead to discover noninvasive diagnostic biomarkers with high sensitivity and specificity. **Materials and Methods:** A case–control study has been conducted and based on three groups, first group include 50 patients with PC. The second group was 50 patients who have benign hyperplasia who were observation in Thi-Qar Oncology Center who were included in this study, whereas, the third group contained 50 healthy (non-BHO and non-PCa) volunteers. Venipuncture was used to collect samples of blood from these group's volunteers. The collection of 3 ml blood was carried out in plain non-EDTA tubes to clot. Further, centrifuges were utilized to separate the serum and then stored at –20°C which was further used to identify free miRNA-143 and miRNA-21 qPCR. **Results:** The levels of miR-21 and mi-143 in the control group were constant and equal to 1. miR-21 was significantly highest in prostatic cancer and followed by BPH, 6.40 versus 2.28, respectively, and the level of significance was high ($P < 0.01$). While miR-143 was significantly lowest in prostatic cancer 0.12 when compared with both control and BPH groups ($P < 0.001$); though, no significant variance was found among the miR-143 of control group and BPH group having a ratio of 1 versus 1.11 ($P = 0.140$), correspondingly. As soon as, the analysis of receiver operator characteristic curve was done and outcomes showed that the miR-21 cutoff value was >4.513 with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy levels of 88.0%, 90.0%, 89.8%, 88.2%, and 89.0%, respectively, while miR-143 cutoff value was ≤0.264 with sensitivity, specificity, PPV, NPV, and accuracy levels of 78.0%, 98.0%, 79.6%, 96.1%, and 88.0%, respectively. **Conclusion:** We can get a diagnostic biomarker with high sensitivity and specificity, using a combination of these two miRNA and miRNA-21 which show high sensitivity, and miRNA-143 which show high specificity (88.0 and 89.0), respectively, and area under curve (0.88 and 0.92), respectively. This could possibly complement one another in an indicative test while enhancing sensitivity when related to the miRNAs individually.

KEY WORDS: Biomarker, Micro RNA (143, 21), Prostate cancer

INTRODUCTION

Regardless of the controversial usage as screening tool, the circulating prostate-specific antigen (PSA) is the utmost communal yet non-invasive biomarker for the prostate cancer (PCa) detection at present. In reality, the PSA blood levels are frequently increased in males having benign situations, for instance, benign prostatic hyperplasia, infection of urinary tract, and prostatitis. The test of PSA is a low specificity test as it is particularly for the prostate gland than that of PCa.[1] The screening on the basis of PSA blood levels causes a great prevalence for lower risk PCa while, most of them need no treatment. Thus, there is a necessity to look for some innovative and better quality biomarkers for PCa diagnosis. Moreover, being the fragment of huge non-coding genome, the small microRNAs (miRNAs) of about 18–22 nucleotides are non-coding RNAs which post-transcriptionally regulate the gene expression.[2] These have regulatory functions on the

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cell's molecular signaling pathway as well as can modulate the approximate 30–50% protein-coding gene expression in human beings.[3,4] In addition, it was confirmed that the differential expression profiles of miRNA in PCa can be definitely interrelated to its clinical expression. Finally, accruing data recommend the miRNAs are auspicious potential biomarkers which could be utilized for PCa detection.[5] For the identification of PCa, miRNAs consist of numerous properties of to be exceptional biomarkers, for example, their utilization in prognosis and monitoring of cancer, circulation in body fluids (non-invasive sampling way), and deregulated expression in PCa. Hence, these mark the miRNAs an appropriate tool for prognostic and diagnostic marker identification.

MATERIALS AND METHODS

Patient Group and Sample Collection

This case–control study comprised three groups, first consisted of 50 PCa patients who were under observation at Thi-Qar Oncology Center. The oncology specialists involved in this research supervised these patients from January 2018 to February 2019. The second group was 50 patients who have benign hyperplasia, this group has been collected from urosurgical department.

Whereas, the third group was contained 50 healthy (non-PCa and non-BPH) volunteers. Venipuncture was used to collect samples from all groups; 3 ml blood was collected in plain tubes without EDTA. The blood was permitted to clot to separate the serum through 5 min centrifugation at 13000 rpm. The separated serum was then gathered in Eppendorf tubes and stored at –20 for further usage in miRNA-143 and miRNA-21 qPCR.

Total RNA Extraction

The TRIzol® reagent kit (Bioneer, Korea) was used to extract the total RNA as per the instruction of the company. Further, nanodrop spectrophotometer (THERMO, USA) was used to check the extracted genomic DNA. Thus, purity of DNA and concentration of DNA was estimated by reading the absorbance at 260/280 nm.

Stem-loop RT-qPCR

The expression analysis of 143miRNA and 21miRNA was quantified using stem loop RT-qPCR which was further normalized with GAPDH housekeeping genesis normal samples, blood patients, and serum using the technique of real-time PCR. This method was done according to the described method of.[6]

Primers and Probes

GAPDH gene primers and probes

The primer three designs online and NCBI-GenBank database were used to design the GAPDH gene probes and primers. Macrogen Company, Korea provided all these primers which are given in the table below:

Gene	Sequence
GAPDH primer	F TCAGCCGCATCTTCTTTTGC
	R TTAAAAGCAGCCCTGGTGAC
GAPDHprobe	FAM-CCAGCCGAGCCACATCGCTC-TAMRA

miRNA primers and probes

The design of probes and primers for 143miRNA and 21miRNA was done in recent research to select the sequence of miRNA while utilizing miRNA primer design tool by the sanger center miRNA database registry. Macrogen Company, Korea delivered these probes and primers as given in the table below:

Primer	Sequence
hsa-miR-21 RT primer	GTTGGCTCTGGTGCAGGGTCCGAGG TATTCGCACCAGAGCCAACTCAACA
hsa-miR-21 Primer	F GTTTGGTAGCTTATCAGACTGA
	R GTGCAGGGTCCGAGGT
hsa-miR-21 Probe	FAM-TCAGTCTGATAAGCTA-MGB

Data Analysis of qRT-PCR

The outcomes of q RT-PCR data for housekeeping gene and miRNA were examined with the assistance of relative quantification levels of gene expression (fold change). For this, the ΔCT method was preferred while utilizing the reference termed by Livak and Scmittgen[7] as following equations:

$$\text{Ratio (reference/target)} = 2^{\text{CT(reference)}-\text{CT(target)}}$$

RESULTS

The Expression Level of miR-21 and miR-143 in Patients and Control Groups

The miR-21 and miR-143 fold change protein expressions are non-normally distributed quantitative variables according to the Kolmogorov–Smirnov normality test; therefore, these variables were expressed in the form of median and interquartile range as indexes of central tendency and dispersion, respectively, instead of the comparable indexes mean and standard deviation used for normally distributed data.

The levels of miR-21 and mi-143 in control group were constant and equal to 1. miR-21 was significantly highest in prostatic cancer and followed by BPH, 6.40 (7.65) versus 2.28 (1.69) in control group, respectively, and the level of significance was high ($P < 0.01$), as shown in Table 1 and Figure 1 miR-21 was significantly lowest in prostatic cancer 0.12 (0.18) when compared with both control and BPH groups ($P < 0.001$); however, there was no significant

Table 1: Comparison of miR-21 and miR-143 protein expression among study groups

miR	Control *n*=50	BPH *n*=50	Prostatic cancer *n*=50	*P*1	*P*2	*P*3
miR-21						
Median (IQR)	1 (---)	2.28 (1.69)	6.40 (7.65)	<0.001	<0.001	<0.001
Range		0.76–19.35	2.70–93.96	HS	HS	HS
miR-143						
Median (IQR)	1 (---)	1.11 (0.63)	0.12 (0.18)	0.140	<0.001	<0.001
Range		0.11–4.30	0.03–1.90	NS	HS	HS

N: number of cases, BPH: Benign prostatic hyperplasia, IQR: Interquartile range, *P*1: Control versus BPH, *P*2: Control versus carcinoma, *P*3: BPH versus carcinoma, HS: Highly significant at *P*≤0.01, NS: Not significant at *P*>0.05

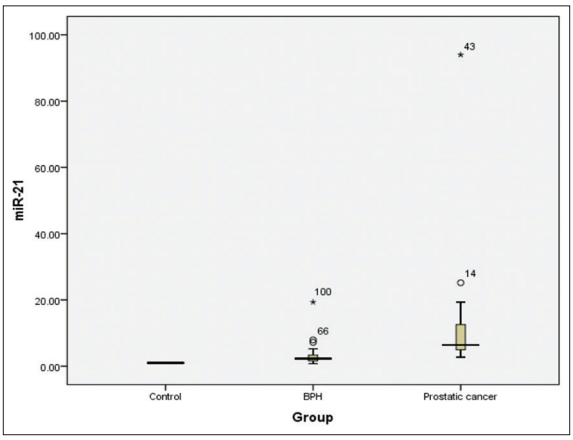

Figure 1: Box plot showing comparison of miR-21 among control and study groups

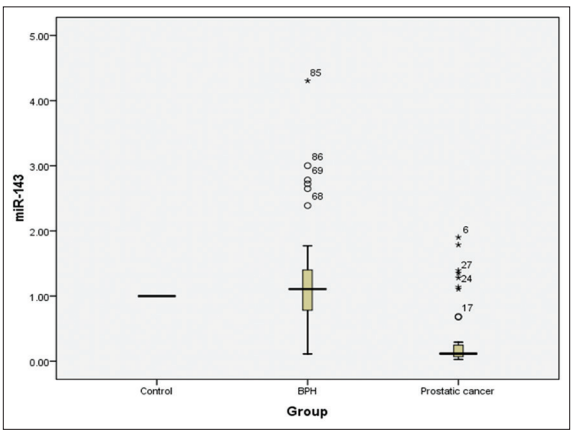

Figure 2: Box plot showing comparison of miR-143 among control and study groups

difference in mrR-21 between BPH and control groups, 1.11 (0.63) versus 1 (---) (the value of fold change in all control cases equals (1); therefore, IQR can be calculated and was written as (---), respectively (*P* = 0.140), as shown in Table 1 and Figure 2.

To evaluate the miR-143 and miR-21's cutoff value as well as to predict the prostatic cancer as diagnostic tests or adjuvant diagnostic tests, receiver operator characteristic (ROC) curve analysis was carried out and the results are shown in Tables 2 and 3 and Figures 3 and 4; the miR-21 cutoff value was >4.513 with sensitivity, specificity, positive predictive value (PPV),

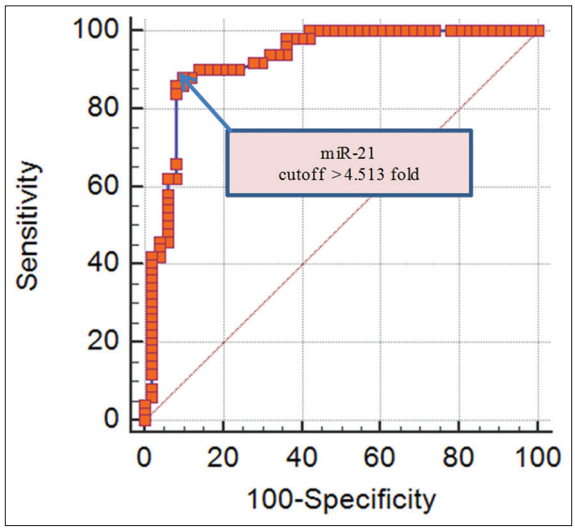

Figure 3: Receiver operator characteristic curve analysis for the calculation of miR-21 possible diagnostic cutoff value

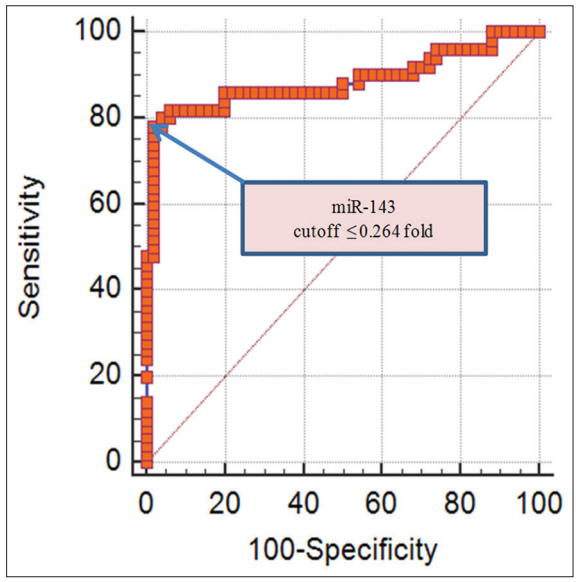

Figure 4: Receiver operator characteristic curve analysis for the calculation of miR-143 possible diagnostic cutoff value

negative predictive value (NPV), and accuracy levels of 88.0%, 90.0%, 89.8%, 88.2%, and 89.0%, respectively. The miR-143 cutoff value was ≤0.264 with sensitivity, specificity, PPV, NPV, and accuracy levels of 78.0%, 98.0%, 79.6%, 96.1%, and 88.0%, respectively.

Table 2: Sensitivity and specificity of miR-21 level (>4.513-fold) in prediction of prostatic cancer

miR-21 level (fold)	Prostatic cancer *n*=50	BPH *n*=50
>4.513	44 (TP)	5 (FP)
≤4.513	6 (FN)	45 (TN)
Sensitivity %	88.0	
Specificity %	90.0	
PPV %	89.8	
NPV %	88.2	
Accuracy %	89.0	
AUC (95% CI)	0.923 (0.853–0.967)	

PSA: Prostatic specific antigen, BPH: Benign prostatic hyperplasia, CI: Confidence interval, AUC: Area under curve, NPV: Negative predictive value, PPV: Positive predictive value, TN: True negative, FP: False positive, TP: True positive

Table 3: Sensitivity and specificity of miR-143 level (≤0.264-fold) in prediction of prostatic cancer

miR-143 level (fold)	Prostatic cancer *n*=50	BPH *n*=50
≤0.264	39 (TP)	1 (FP)
>0.264	11 (FN)	49 (TN)
Sensitivity %	78.0	
Specificity %	98.0	
PPV %	79.6	
NPV %	96.1	
Accuracy %	88.0	
AUC (95% CI)	0.885 (0.806–0.940)	

PSA: Prostatic specific antigen, BPH: Benign prostatic hyperplasia, TN: True negative, FP: False positive, TP: True positive, FN: False negative, NPV: Negative predictive value, CI: Confidence interval, PPV: Positive predictive value

DISCUSSION

miR-21 was significantly highest in prostatic cancer and followed by BPH, 6.40 (7.65) versus 2.28 (1.69), respectively, and the level of significance was high ($P < 0.01$)., miR-143 was significantly lowest in prostatic cancer 0.12 (0.18) when compared with both control and BPH groups ($P < 0.001$); however, there was no significant difference in mrR-143 between BPH and control groups, 1.11 (0.63) versus 1 (---), respectively, ($P = 0.140$).

According to above data, both miRNA-21 and miRNA-143 seem to have a role in prostate tumorigenesis, dysplasia, and transformation from BPH to malignant tumor concerning miRNA-21. Moreover, Yang *et al*. designated that miR-21 consists the properties of proto-oncogene in the PCa's development and incidence procedure as well as it has stimulating impact on the development and occurrence of the tumors.[8] Thus, they thought that miR-21 performed a significant function in the regulation of PCa's invasion, proliferation, and other procedures.[9] Thus, to acknowledge the miRNA's mediating pathway, different researchers tested this theory through their research, in which the transfection of analog of miR-21 was carried out in invasive cell numbers, PCa cells, and relevant expression of genes were investigated. The outcomes deliberated that the transfection of miR-21 analog can escalate the invasive cell number, viability of PCa cells and can up regulate the expression of genes Bcl-2 (anti-apoptotic). Moreover, these persists the pro-proliferation genes MMP9 and MMP2. Thus, it shows a significant role of miR-21 in promoting the invasion and proliferation of PCa.[10,11]

In addition, the common observations of cancers highlight the inactivation of p57Kip2. The regulation of their gene expression deals with an alternative complexity layer by miRNAs. Lately, several miRNAs (miR-21, miR-92, miR-25, and miR-221/222) testified the downregulation of p57Kip transcript's expression.[12]

The identification of the progressive BPH signatures was done by the evaluation of profiles of miRNA-143 expression in control group and BPH. Several differentially articulated miRNAs were identified using RT-qPCR. The conclusions of the current study on 50 healthy male and 50 BPH patients exposed non-significant alteration among the BPH and control groups' miRNA-143 expression level. Moreover, another research's microarray profile information discovered the deregulation of miRNAs also containing miRNA-143 in the BPH group, these further specify their involvement in the PCa and BPH pathogenesis. Furthermore, Viana *et al.* described the involvement of miR-143 in the BPH pathogenesis by hindering target genes which contain MAP kinase 3, GTPase, KRAS proto-oncogene, and MAP kinase 4. This controversy with recent research may be relevant to the technical issues or sample size.[13,14]

Besides, this study revealed the downregulation of miR-143 in tissues of PCa in comparison to their consistent non-tumor tissues. More studies indicated the suppressed growth of cancer cells by miR-143. Particularly, former work established that miR-143 employs its role in the PCa while aiming HK2 which is glycolysis' key enzyme. In addition, they further observed the significant effects of glucose usage through the axis miR-143/HK2 in PCa. Dependable on the former reports, our attained quantitative real-time PCR's data revealed the downregulation in the PCa on comparison to the control group and BPH group. Although, in PCa, the potential clinical importance of miR-143 and expression profiles is proved by requiring more clinical specimens.

On the other hand, the mediated effect of miR-143 was essentially a variant mechanism which was because of the several targeted regulations through miR-143 in several tumor cells. The miR-143 subdue the growth of cancer cells in colorectal cancer by inhibiting the translation of KRAS while targeting HK2 and regulating DNMT3A.[15]

The miR-143's direct targets have been established in polo-like kinase 1, cytokinesis 1 protein regulators, topoisomerase 2A, BCL2, and liposarcoma cells.[16] Likewise in another research pf PCa, it was verified about the HK2 as miR-143's direct target in PCa that represented as a HK2 knockdown which repeated the miR-143's inhibitory effect on the cancer cell growth of PC3.[17] Moreover, the HK2 binding to the membrane of mitochondria has showed to encourage the effect of Warburg, which is a phenomenon categorized through augmented flux of glucose and production of lactate in the oxygen occurrence.[18]

ROC curve analysis shows that the miR-21 cutoff value was >4.513 with sensitivity, specificity, PPV, NPV, and accuracy levels of 88.0%, 90.0%, 89.8%, 88.2%, and 89.0%, respectively. The miR-143 cutoff value was ≤0.264 with sensitivity, specificity, PPV, NPV, and accuracy levels of 78.0%, 98.0%, 79.6%, 96.1%, and 88.0%, respectively.

Current conclusions have recommended the blood/urine based markers' potential for the urological malignancies to be an upright replacement for the already present invasive techniques, for example, cystoscopy. In addition, the conventional urine based marker is deficient of adequate specificity and sensitivity to illustrate the unmet necessity as an additional alternate of screening, preliminary diagnosis and follow-up analysis of prostate and bladder cancer.[19] Thus, miRNAs function to be capable human cancer biomarkers as a substitute to the methods of conventional diagnosis.

In consistent with present study, a previous study has been done to assess the expression level of three oncogenic miRNAs comprising miR-205-5p, miR-141-3p, and miR-21-5p in the samples of urine of two utmost prevailing malignancies of urology. They further confirmed the substantial upregulation for all calculated miRNAs. Moreover, miR-21-5p and miR-141-3p showed greatest level of expression in prostate and bladder cancer correspondingly.[20]

Remarkably, all of the three miRNAs contained no substantial variation in BPH in comparison to the normal which was also constant in our current research. Hence, it is useful to stratify the benign patients as of the invasive patients. Whereas, it is in patient's benefit if offered methodology eludes the agonizing cystoscopy for the differentiations of invasive and benign malignancies, which is an approachable objective.[20] Further, a recent research reported the miR-21's overexpression in the cancer tissues of urothelial cancer patient's upper urinary tract.[21] Most specifically, the exertion of predominant oncogenic function of miR-21-5p through cellular apoptosis's inhibition occurs due to the targeting of several significant tumor suppressor genes such as bax, Tap63, pten, and Fas ligand.[20]

The usage of ROC specificity/sensitivity analysis termed these miRNAs as valued PCa diagnostic tumor markers. At present, the specimens of biopsy and evaluation of PSA are extensively utilized in the diagnosis of PCa and BCa. Being an invasive process, it might impact the life's quality of the patients. Therefore, the diagnosis of clinical PCa is done by common PSA biomarker.[22] Yet, to making a definite PCa diagnosis, the PSA specificity is limited. Thus, for this situation miRNAs may be a suitable non-traumatic substitute. Another benefit of selected miRNAs compared to the PSA is the non-invasive ability of tumor related miRNAs to distinguish the individuals of BPH and PCa. This indicated to be an astonishing diagnostic achievement.

Another previously conducted study mentioned that themiR-21's sensitivity and specificity was 0.88 and 0.91, respectively, whereas, SROC's area under curve (AUC) showed 0.95 to present the upright miR-21's diagnostic ability. It further specified the miR-21 as a good PCa diagnostic biomarker.[23]

Concerning miRNA-143, sensitivity, and specificity previous studies have found that miRNA-143 is abnormally expressed in various cancers, indicating that miR-143 may serve as a novel biomarker for cancer diagnosis. However, the diagnostic value of miR-143 in different cancers remains inconsistent. Based on evidence-based medicine, for the first time, the present meta-analysis aimed to explore the accuracy of miR-143 in cancer diagnosis.[24] Overall results of pooled analysis were: Sensitivity 0.78 (95% confidence interval (CI) = 0.74–0.82), specificity 0.85 (95% CI = 0.81–0.88), positive likelihood ratio 5.17 (95% CI = 4.02–6.64), negative likelihood ratio 0.26 (95% CI = 0.21–0.30), diagnostic odds ratio 20.25 (95% CI = 14.21–28.87), and AUC 0.88 (95% CI = 0.85–0.91), which came in consistent with the present study.[24,25]

CONCLUSION

Accordingly, we can get diagnostic biomarker with high sensitivity and specificity, using a combination of two miRNA or panel of miRNA, such as miRNA-21 which show high sensitivity, and miRNA-143 which show high specificity (88.0 and 89.0), respectively, and AUC (0.88 and 0.92), respectively. These can possibly complement one another in the diagnosing tests and therefore, escalating the sensitivity while comparing to the miRNAs individually.

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