Research Article

Increased level of miRNA 30b-3p in patients with prostatic hyperplasia and testosterone with high-level of prostate-specific antigen

Wasnaa Jumaa Mohammad[1*], Ban Hussein Hameedi[2] and Noor Alhuda Khalil Iberahim[3]

[1*]*Microbiology and Immunology Department of Basic Science, College of Nursing, University of Baghdad, Iraq.*
[2]*Department of Zoology, Department of Basic Science, College of Nursing, University of Baghdad, Iraq.*
[3]*Department of Biochemistry, Department of Basic Science, College of Nursing, University of Baghdad, Iraq.*

Abstract

Background: Prostate cancer (PCa) is the most common causing cancer-related in death in men and lack of reliable diagnostic tool. MicroRNAs are small molecules single-stranded RNA that affecting protein expression at the level of translation and dysregulation can dramatically affect cell metabolism. However, the using of circulating miRNAs as diagnostic biomarkers for diagnosis of PCa is still unknown.
Methods: Ten patients with prostatic hyperplasia with high-level of PSA and 10 healthy controls were conducted in this study. The reverse transcription of miRNA based on quantitative polymerase chain reaction (qPCR) were used for evaluating the dysregulation of miRNA 30b-3p and using of ELISA to evaluate the level of prostate-specific antigen (PSA) and testosterone hormone.
Results: Circulating miRNA 30b-3p level was increased in patients with prostatic hyperplasia with higher level of PSA as compared with healthy controls. Also, the testosterone hormone was increased in those patients as compared with normal level of testosterone in healthy individuals.
Conclusion: The serum miRNA 30b-3p level increased in patients with hyperplasia in prostate and may be one of potential biomarker for diagnosis of PCa.

Keywords: Prostate cancer, Hyperplasia, ELISA, Prostate-Specific Antigen, miRNA 30b-3p, Testosterone.

1. Introduction

Prostate cancer (PCa) is the most commonly diagnosed new solid cancer and the second most common causing cancer-related death in men in the United States (1). The prostate-specific antigen (PSA) concentration in the blood is a test approved by the US food and drug administration as an aid to the early detection of PCa (2). Screening with PSA has been widely used to detect PCa for decades in the United States and many industrialized nations, but it continues to be controversial. Until a few years ago, the American cancer society and the American Urological Association recommended PCa screening for men at average risk who are 50 years or older and have an expectancy of life at least 10 years, after the patient and physician discuss the risk and benefits of screening and intervention (3).

Prostate-specific antigen (PSA) is a member of the human kallikrein of serine proteases family with the PSA gene (KLK3) encoding for the protein hk3 (4).

All kallikreins are serine proteases that are produced as pre and proenzymes and all have conserved positioning of the aspartate/histidine/serine catalytic triad. Of the kallikreins that have been characterized, most have trypsin-like proteolytic activity. PSA however, differs from the rest of family in that it has achymotrypsin-like substrate specificity, cleaving after hydrophobic residues. The major physiological proteolytic of substrates for PSA appears to be the gel forming proteins in freshly ejaculated semen, semenogelin I (Sg I) and semenogelin II (Sg II) are synthesized and secreted by seminal vesicles (5). The identification of PSA processing protease may also yield insights into why varying ratios of different molecular forms of PSA are present in human serum in patients with normal prostates compared to those with

benign prostatic hyperplasia (BPH) or prostate cancer(6).

PSA presenting in the serum in a number of different forms, all forms are enzymatically inactive. These can be classified into two general categories: complex PSA (i.e. initially enzymatically active but now inactive due to binding to serum protease inhibitors) and free PSA (i.e. unbound, never active PSA) (7).

Approximately 65-95% of PSA in the serum is PSA bound to ACT (PSA-ACT) and this is the predominant form of PSA in the serum in men with normal prostate, BPH or prostate cancer. The remaining PSA consists of free PSA forms (5-35%) that are enzymatically inactive (either as incorrectly processed pro-PSA or PSA protein that has been proteolytically cleaved) and, therefore unable to complex with serum protease inhibitors. Antibodies are available that bind selectively to free, un-complex PSA and these form the basis of clinical testing measuring the ration of free PSA to the total PSA. This ratio decreases in patients with prostate cancer (i.e. decreased free and/ or higher total PSA consistent with higher amounts of active PSA entering blood serum) and increasingly used to differentiate BPH from prostate cancer in men with serum PSA (8).

MicroRNAs are small molecules single-stranded of RNA that affecting protein expression at the level of translation, and dysregulation can dramatically affect cell metabolism. Comparison of 736 microRNAs expression levels between the poorly metastatic SV40T immortalized prostate epithelial cell line P69 to its highly tumorigenic and metastatic subline M12 identified 231 miRs that were overexpressed (9).

MicroRNAs represent non-coding RNAs that are important for stem cell development in embryonic state and epithelial to mesenchymal transition (EMT). Tumor cells hijack EMT and stemness to grow and metastasize to distant organs including bone. Tumor microenvironment and tumor cells interaction with stromal fibroblasts at the primary and metastatic sites and this interaction could lead to tumor growth, EMT and bone metastasis (10).

The role of RNAs in non-coding state in EMT and metastasis of cancer cells remains poorly understood. Recent studies highlight the directive functions of non-coding RNAs including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) in modulating cancer cell growth, survival EMT and metastasis (11).

In contrast to lncRNA, microRNAs are small (20-23 nucleotides) non-coding ribonucleic acid (RNA) molecules which bind to complementary sites in the messenger RNAs (mRNAs) of their target genes, thereby inducing the post-transcriptional silencing of genes. It is predicted that miRNAs might regulate up to one-third of all genes (12).

The presumed number of unidentified miRNAs is large. Currently about, 1881 miRNAs are identified in *Homo sapiens* (human) based on miR base. MicroRNAs are located throughout the genome including intergenic regions and in the introns of both protein-coding and non-coding genes (13,14).

Testosterone is one of the principal androgen in circulation, while dihydrotestosterone (DHT) is the primary nuclear androgen and the most potent androgen in tissue. the adults males circulation contain, roughly 44% of testosterone bounding with high affinity to sex hormone binding globulin (SHBG), 54% bounding with low affinity to albumin and only 1-2% of testosterone exists in a free (unbound) state. About 25% of DHT in the circulation is secreted by the testis while most (65%-75%) arises from conversion of testosterone in peripheral tissue (15).

The prostate is a major site in men with no testicular DHT production from testosterone. In circulation free testosterone enters prostate cells by method of passive diffusion. In 1941 Charles Huggins shows that blocking the production of testosterone slows the growth of prostate cancer. Sixty-five years after Huggins findings', we are not much further ahead; we are still blocking testosterone to slow the growth of prostate cancer, especially in cases when cancer has spread. Additional evidence that testosterone is involved in causing prostate cancer comes from different sources. Rats were given high doses of testosterone develop prostate cancer (16). The men who are castrated prior to puberty do not produce testosterone do not develop prostate cancer. Men with cirrhosis of liver, a condition that increases their female sex hormone, which suppresses testosterone have lower incidence of prostate cancer (17).

2. Material and Methods

Ten patients who attended the urology department between January to March 2017 in teaching hospital of medical city in Baghdad and underwent PSA values. The patients aged between 48 to 65 years with prostate hyperplasia and PSA levels between 6-15ng/ml. Ten individuals enrolled in this study aged between 50 to 60 years with normal values of PSA as control group. There are some exclusion criteria for the patients used: first, the age above 75 years excluded, urinary infection excluded, bladder stones and catheterization were excluded.

2.1 Selection of miRNA

hsa-miR-30b-3p MIMAT0004589
hmiR-30b-3p RT primer
hmiR-30b-3p-F

CUGGGAGGUGGAUGUUUACUUC

GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC GAAGTA
GTTCTGGGAGGTGGATGTT

hsa-miR-30d-5p MIMAT0000245

hmiR-30d-5p RT primer

hmiR-30d-5p-F

UGUAAACAUCCCCGACUGGAAG

GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC CTTCCA
GTGTGTAAACATCCCCGAC

2.2 RNA isolation

Total RNA was isolated from patient's serum and control using Trizol Reagent (Life Technologies/Invitrogen) according to the instructions.

Messenger RNA Specific RT-qPCR: cDNA synthesis was performed with Tetro Reverse Transcriptase kit (Bioline Ltd.) according to the manufacturer's recommendation. Quantitative PCR was performed using real-time PCR (ABI PRISM 7900HT, Applied Biosystems). PRMT1 (Mm0048135_g1) TaqMan gene expression assay was used and quantified by the comparative DCT method and normalized to GAPDH (Mm99999915_g1) expression.

2.3 Reverse transcription and real-time PCR

A total of 4μL of purified RNA (from a total volume of 100μL eluate) was reverse-transcribed using the miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon). A total of 0.5μL of UniSp6 RNA was spike-in the RT mix for the quality control of cDNA synthesis. The total reaction volume was 10μL. Primers for mature sequences of selected miRNAs were purchased from Exiqon (microRNA LNA™ PCR primer set). No-template controls were included. All PCR assays (total reaction volume: 20μL) were run on an iCycler iQ™

Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). SYBR Green master mix was used (miRCURY LNA™ Universal RT microRNA PCR SYBR® Green Master Mix; Exiqon). PCR amplification efficiencies were calculated for each individual miRNA using the following equation:

$$E = (10^{-1/\text{slope}} - 1) \times 100 \qquad (1)$$

The efficiency threshold was calculated at $\pm 10\%$ across a 10-fold dilution series across five points. Candidate housekeeping genes were selected on the basis of literature data and manufacturer's recommendations. After stability assessment assays, data were normalized against the mean between miR-191-5p and miR-425-5p Ct values as follows:

$$\Delta Ct = -(Ct_{\text{target}} - Ct_{\text{miR-191-5p+miR-425-5p}}) \qquad (2)$$

Values are reported as the mean $\pm$ SD.

3. Results

Table (1) show the level of expression of microRNA in patients with hyperplasia in prostate with high-level pf PSA and control group which show elevated level of miRNA in patients compared with healthy control.

Table 1. The level expression of miRNA 30b-3p in patients and control group.

Groups	Samples	RNU	miR30-b	DCt	DDCt	miR30-b	miR30-b
Patient	1	32.2	39.1	6.9	1.61	0.33	
	2	31.5	37.7	6.2	0.91	0.53	
	3	31.5	41.6	10.1	4.81	0.04	
	4	32.5	37.7	5.2	-0.09	1.06	
	5	31.7	34.5	2.8	-2.49	5.62	1.51
	6	32	37.6	5.6	0.31	0.81	
	7	31.4	37.7	6.3	1.01	0.50	
	8	31.2	37.9	6.7	1.41	0.38	
	9	31.6	34.7	3.1	-2.19	4.56	
	10	31.3	36.2	4.9	-0.39	1.31	
Control	11	31.6	35.5	3.9	-1.39	2.62	
	12	31.1	35.3	4.2	-1.09	2.13	
	13	30.1	37	6.9	1.61	0.33	
	14	30.5	34.4	3.9	-1.39	2.62	
	15	31.1	37.1	6	0.71	0.61	
	16	30.5	35.9	5.4	0.11	0.93	1.43
	17	31.6	35.1	3.5	-1.79	3.46	
	18	30	35.5	5.5	0.21	0.86	
	19	30.3	37.1	6.8	1.51	0.35	
	20	30.3	37.1	6.8	1.51	0.35	

Table 2. Compare between patients and control in parameters.

The Group	No.	Mean ± SE		
		miR30-b ()	PSA ()	Testosterone ()
Patients	10	1.514 ± 0.61	7.860 ± 0.28	8.520 ± 0.49
Control	10	0.870 ± 0.21	1.540 ± 0.28	3.800 ± 0.50
T-test	---	1.362 NS	3.202 **	1.468 **
P-value	---	0.334	0.0006	0.0001

** ($P < 0.01$), NS: Non-significant.

Table 3. Correlation coefficient between parameters.

Parameters	Correlation coefficient-r	Level of sig.
miR30-b & PSA	0.38	*
miR30-b & Testosterone	0.36	*
PSA & Testosterone	0.70	**

* ($P < 0.05$), ** ($P < 0.01$).

Table (2) show the comparison between patients and the control group in miRNA, PSA, and testosterone hormone which shows significant difference in PSA, and testosterone ($p < 0.01$) in patients compared with healthy control.

Table (3) show the correlation between the parameters which show significant difference between miRNA and PSA ($p < 0.05$), also a significant difference with testosterone ($p < 0.05$) and a significant difference of PSA with testosterone ($p < 0.01$).

4. Discussion

The dysregulation of miRNAs has been widely identified in different kinds of human cancers and demonstrating their important roles during cancer development and progression. The mechanisms that possible underlying the importance of miRNAs is uncovering in the pathogenesis of human cancers may lead to the development of miRNA based therapeutic strategies or diagnostic/prognostic biomarkers [18].

In the fact that current cancer detection test considering have their own limitations, the using of miRNAs as promising biomarkers for diagnosis and prognosis of cancer has aroused intense research interest. Additionally to the distinctive pattern of miRNA expression also serves as markers of important histopathologic features such as tumor stage, proliferative capacity and vascular invasion [19]. The Real-time PCR which is used to validate the expression of miRNAs discovered during high throughput arrays and studies the expression of individual miRNAs [20].

Recent evidence suggests that miRNA profiles from tissue sources as well as from circulating body fluids could be good tools for prognostic and diagnostic purposes. MiRNAs profiles not only can distinguish between tumors of different developmental origin but also possess prerequisites to be considered useful noninvasive detection biomarkers. First, exceptionally, they are stable in different clinical samples such as formalin-fixed, paraffin-embedded tissue, blood, serum and urine. Second, they can be measured quantitatively reliable in small amounts of samples by real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). Third, they are resistant to endogenous ribonuclease activity as well as variation in temperature and PH. In addition, among different species making the use of animals for preclinical studies feasible, they are highly conserved [21].

miRNA 30b-3p regulate androgen receptor in prostate biology and in the development of prostate cancer (PCa) [22].

Different studies have examined the expression of miRNA in serum or plasma in purpose to develop a blood-based diagnostic procedure for development and diagnosis of PCa.

In the present study, the serum level expression of miRNA 30b-3p can distinguish from healthy controls which show increased expression of miRNA30b-3p in patients with high-level of PSA.

Mitchel *et al*., diagnose in his study that serum level of miRNA-141 can distinguish PCa patients from healthy control which support the potential role of this miRNA as a diagnostic marker of PCa [23].

Mahn *et al*., study the oncogenic miRNAs in circulation and found miRNA-269 has sensitivity of 89% and specificity of 56% [24].

Chen *et al*., examined a panel of 5 serum miRNAs (let-7c, let-7e, miR-30c-622 and 1285) significantly different in PCa patients when compared with healthy control group. All miRNAs could distinguish PCa from healthy controls individually [25].

Selth *et al*., [26] studied on mouse model having PCa as a tool to discover serum miRNAs that could be used in clinical setting. miRNA (miR-141, -298, -346, and 375), these miRNAs among 45 miRNAs elevated significantly in mouse with metastatic PCa compared to those in healthy controls.

In a study of 51 PCa patients in Turkey compared with 20 healthy controls. Investigated the miRNA-21, -41 and -221 levels in plasma of patients compared to healthy control, they found that miR-21 and miR-221 levels were higher significantly in prostate patients than controls [27].

In our study which is the first study in Iraq that distinguish the expression the level of miRNA 30b-3p in patients with hyperplasia in prostate with high-level of PSA, there is evidence that alteration in miRNA function plays a role in prostatic carcinogenesis. The miRNAs dysregulation effect on a number of critical cellular processes involved in this process, this process including but not limited to: the cell cycle stimulation, apoptosis avoidance and modulation of androgen receptor-mediated signaling (18).

Serum androgens may not accurately reflect the true androgenic environment within the prostate. Testosterone is the major circulating androgen.

In a different analysis of 18 prospective studies, Roddam and collaborative group reported on the endogenous hormone and prostate cancer no association between blood levels of total testosterone and prostate cancer risk cancer risk based on data from 3,886 men with prostate cancer and 6,438 controls. It is largest serum based study with the most elegant and comprehensive analysis to date to test a central hypothesis in prostate cancer etiology. It is not surprising that the pooled analysis did not find a positive link between circulating levels of total testosterone and prostate cancer risk since, individually, few of the 18 studies included in the pooled analysis reported a significant positive association (28).

From the study of the European prospective investigation into cancer and nutrition (EPIC) (with 643 case-control pairs) identified a significant inverse association of androstenedione concentration and risk for advanced prostate cancer, and weak positive association between free testosterone concentration and risk for total prostate cancer among men and risk for high grade disease (29).

Several different reasons which contribute to the mixed results from epidemiologic studies. First, the androgenic serum levels are indirect indicators of intraprostatic androgen levels and may not an accurate reflection of androgen action within the prostate. In addition, relatively large assay variation, intrapersonal variation, study population differences, and heterogeneity of prostate cancer in these studies to replicate results. Furthermore, genetic susceptibility in the androgen metabolic and signaling pathways may contribute to the effects that androgens have on prostate cancer. Another study have measured testosterone and DHT in individuals which show definitely that men with higher serum levels of testosterone have higher risk of prostate cancer.

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