Scientific Research Council of Alanya Alaaddin Keykubat University

INVESTIGATION OF CANDIDATE miRNAs AS POTENTIAL BIOMARKERS IN THE THE EARLY DIAGNOSIS OF PROSTATE CANCER AND BENIGN PROSTATE HYPERPLASIA

Project number

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Project Team

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The Clinical Research Ethics Committee of the Alanya Alaaddin Keykubat University Faculty of Medicine

(Ethical Approval No. 10354421-2020/16-24)

(Ethical approval date : 13.02.2020)

Objective and Scope

Prostate cancer (PCa) is one of the most common cancers in men and the second highest cause of cancer-related deaths (Vanacore et al, 2017; Siegel et al., 2019). Early diagnosis of cancer is very important for the clinical evaluation of the patient and selective and effective treatment. Currently, PCa is diagnosed by serum prostate-specific antigen (PSA) measurements, digital rectal examination (DRE) and pathologic evaluation of prostate biopsies performed under transrectal ultrasonography guidance (Bhagirath et al., 2018). PSA value should be measured at least twice for biopsy decision. Although PSA is a guide in the diagnosis of PCa, its level can also be increased in benign conditions such as benign prostatic hyperplasia (BPH) and prostatitis (Abramovic, 2020). Nevertheless, the free/total (f/t) PSA ratio of 4-10 ng/ml is recommended to differentiate between BPH and prostate cancer (Bhagirath et al., 2018; Vanacore et al., 2017; Siegel et al., 2019). However, the PSA level does not provide reliable results and is lower than the determined value in some cancer patients or considerably higher in some BPH patients. This leads to a large number of patients being biopsied unnecessarily or some patients being missed (Abramovic, 2020; Szelisk et al., 2018). More specific, sensitive, and reliable biomarkers are needed in the clinic to aid pre-treatment decision-making, especially to better distinguish malignant from non-malignant conditions, as well as aggressive from non-aggressive tumors (Abramovic, 2020).

MiRNAs are short non-coding RNA sequences of 19-22 nucleotides in length that regulate the expression of various genes post-transcriptionally. MiRNAs can suppress gene expression by targeting several genes, thus acting as tumor suppressors or oncogenes (Cowland et al., 2007; Kanwal et al., 2017). In recent years, miRNAs have been nominated as reliable biomarkers that help us make accurate diagnoses of many diseases such as cancer (Kanwal et al., 2017). Although there are various studies on miRNAs detected in tissues and sera of patients with PCa, there is still very little data on which miRNAs can be used as biomarkers. Moreover, there are conflicting results in the literature regarding various miRNAs, whether they are downregulated or upregulated (Aghdam et al., 2019; Moya et al., 2019; Xu et al., 2015; Zhu et al., 2015).

This study aimed to investigate the roles of miR-134-5p, miR-149-5p, miR370-3p, miR-107, and miR-221 expression levels on the diagnosis and severity of the disease in patients diagnosed with PC and BPH. In addition, it was aimed to compare some clinical features such as serum PSA and Gleason Score with serum miRNA levels and to determine the relationship between them. The study will contribute significantly to the literature by evaluating these miRNAs, which have been shown to play a role in cell proliferation in various types of cancer (Qi et al., 2019; Su et al., 2017; Zhao et al., 2017) and whose role in PC has not yet been fully elucidated, and will shed light on the evaluation of new diagnostic biomarkers.

2. Material Methods

2.1. Study Design and Patients

The study included 20 PCa patients and 17 BPH patients who are clinically and pathologically confirmed, aged between 40-70 years, with serum PSA levels above 4 ng/mL, who presented to the urology clinic between January 2021 and December 2022. Additionally, 20 healthy volunteers who visited the urology outpatient clinic for routine check-ups were included in the study as the control group. Patients who had undergone drug therapy or surgery for prostate cancer had chronic inflammatory or infectious diseases, were hospitalized within the past year for a chronic illness, or had another known malignancy were excluded from the study.

The Clinical Research Ethics Committee of the Alanya Alaaddin Keykubat University Faculty of Medicine granted ethical approval (Approval No. 13.02.2020/16-24), and informed consent was obtained from all participants.

The clinical diagnosis of patients included in the study was conducted at the Urology Outpatient Clinic of Alanya Alaaddin Keykubat University Training and Research Hospital, with pathological assessments performed in the Pathology Laboratory of the same institution. Serum PSA levels were determined using the ELISA method, and miRNA expression analyses were performed using quantitative real time polymerase chain reaction (qRT- PCR) following miRNA isolation from peripheral blood samples.

2.1.1.Study Criteria

Inclusion Criteria:

- For all groups, between 40-70 years of age.
- Prostate cancer group: Patients with positive digital rectal examination (DRE) results, serum PSA level above 4 ng/mL and a confirmed pathological diagnosis of Prostate cancer.
- BPH group: Patients with a PSA level over 4 ng/mL and a negative DRE result who were clinically and pathologically diagnosed with BPH.
- Control: Healthy volunteers who applied to the urology outpatient clinic for routine check-ups and whose PSA value was below 4 ng/ml.

Exclusion Criteria:

- To have undergone drug therapy or surgery for prostate cancer,
- To have chronic inflammatory or infectious diseases,
- Were hospitalized within the past year for a chronic illness,
- To have another known malignancy,
- Being outside the age limit of 40-70.

2.2. Blood Samples and PSA Levels

Two milliliters of peripheral venous blood were collected from the volunteers included in the study into EDTA tubes. Serum PSA immunoassays evaluated for clinical examinations were determined on the automated immunoassay analyser.

2.3. miRNA Expression with Quantitative Real-Time PCR Reaction (qRT-PCR)

miRNA was extracted from the peripheral blood samples of volunteers using the Hybrid-R^{TM} miRNA İsolation Kit and applied the following steps according to the manufacturer's instructions. Subsequently, according to the manufacturer's instructions, miRNA was converted to cDNA using the miR cDNA Synthesis Kit. A total of 20 µl volumes of reactions containing miRNA-specific stem-loop primers were performed for each sample.

The expression levels of miR-107, miR-134-5p, miR-149-5p, miR-370-3p, miR-221-5p were measured by quantitative real-time PCR using miRqGreen MasterMix and the reaction was performed according to the manufacturer's instructions. Expression levels of each miRNA were calculated using the comparative cycle threshold (ct) method according to the formula $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). The fold change ratio of each miRNA was determined and compared with that of the control.

2.4. Statistical Analysis Plan (SAP)

All statistical analysis and graphs were performed using GraphPad Prism-5 (version 9.0) and SPSS 21.0 software. The normal distribution of the data was evaluated with the Shapiro-Wilk test. The results were expressed as mean±standard deviation (x±SD) for continuous variables such as age that showed normal distribution and as median (Interquartile range [IQR]) for continuous variables that did not show normal distribution, such as PSA levels. Categorical variables were compared using the chi-square test.

miRNA (miR-107, miR-134-5p, miR-149-5p, miR-370-3p, miR-221-5p) expression levels did not show normal distribution, and statistical analyses were performed using the Kruskal-Wallis or Mann Whitney-U test. The relationship between miRNAs and serum PSA levels and Gleason scores was analyzed using the Spearman correlation test.

The role of miRNAs as biomarkers in prostate cancer was investigated by drawing the receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC), cut-off point, sensitivity and specificity for each miRNA was calculated. Statistical significance was taken as $p < 0.05$.

3. Important Dates

Ethical approval date：13.02.2020

Study Start and Completion: June 1, 2020- March 2, 2023

Patient registration start and completion: 26.01.2021-05.12.2022

The project duration lasted 2 years and 9 months in total.

The project's final report was presented to the Scientific Research Council of Alanya Alaaddin Keykubat University on 02.03.2023. The final report was accepted at the board meeting dated 08.03.2023.

4. References

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Informed Consent Form (ICF):

1. INFORMED CONSENT FORM PREPARED FOR PATIENT VOLUNTEERS

INFORMATION:
Research Name; Investigation of Candidate miRNAs as Potential Biomarkers in the Early Diagnosis of Prostate Cancer and Benign Prostatic Hyperplasia

Purpose of the Research: It is thought that miRNAs may be a new biomarker in distinguishing prostate cancer (PCa) and benign prostatic hyperplasia (BPH). The aim of our study is to determine the roles of the expression levels of a group of miRNAs thought to play a role in proliferation in patients diagnosed with PCa and BPH on the diagnosis and severity of the disease. The miRNAs to be determined will be evaluated as candidate biomarkers that can be used in the early diagnosis of PCa.

Duration of the Research; Approximately 2 years
Total Number of Volunteers Participating in the Research; 20 volunteers for each groups (60, total)

Method to be Followed in the Research; Patients who come to the outpatient clinic with suspected prostate cancer and who decide to undergo surgery as a result of clinical evaluation will use 2 ml of blood sample taken in an EDTA tube and tissue samples for clinical diagnosis. The study will be conducted with your blood taken on the first day (before biopsy; baseline). While your blood is taken for routine laboratory tests, an additional tube of blood will be taken for this study. You may feel some pain when the needle is inserted or removed during the blood sample collection. You may feel slight dizziness after the procedure. For tissue samples, paraffin blocks will be obtained from the tissues obtained after the biopsy in the Pathology Laboratory and a diagnosis will be made with routine pathological examinations. No additional medication or medical intervention will be applied for the study. Therefore, the study does not have any side effects on you.
You are free to participate in this study. You can accept it at the beginning and then change your mind and leave the study without giving any reason.
Your records that will reveal your identity will be kept confidential and will not be disclosed to the public, and your identity will remain confidential even if the research results are published.

Person Who Can Be Reached 24/7 During the Research Period Name / Surname / Phone:

Dr. Ali AKKOÇ Phone: …….

VOLUNTEER CONSENT
I have read all the explanations in the Informed Volunteer Consent Form. The written and verbal explanation regarding the research, the subject and purpose of which are specified above, was made to me by the physician named below. I know that I am participating in the research voluntarily, that I can withdraw from the research at any time with or without a reason, and that I can be excluded from the research by the researcher regardless of my own will.

I agree to participate in the research in question with my own consent, without any pressure or coercion.

Name / Surname / Signature / Date of the Volunteer
Name / Surname / Signature / Date of the Person Making the Explanations
Name / Surname / Signature / Date of the Person Witnessing the Consent Process, if necessary
Name / Surname / Signature / Date of the Legal Representative, if necessary

2. INFORMED CONSENT FORM PREPARED FOR HEALTHY VOLUNTEERS

INFORMATION:
Research Name; Investigation of Candidate miRNAs as Potential Biomarkers in the Early Diagnosis of Prostate Cancer and Benign Prostatic Hyperplasia

Purpose of the Research: It is thought that miRNAs may be a new biomarker in distinguishing prostate cancer (PCa) and benign prostatic hyperplasia (BPH). The aim of our study is to determine the roles of the expression levels of a group of miRNAs thought to play a role in proliferation in patients diagnosed with PCa and BPH on the diagnosis and severity of the disease. The miRNAs to be determined will be evaluated as candidate biomarkers that can be used in the early diagnosis of PCa.

Duration of the Research; Approximately 2 years
Total Number of Volunteers Participating in the Research; 20 volunteers for each groups (60, total)

Method to be Followed in the Research; In order to compare the blood values of healthy individuals with PC and BPH patients, a 2 ml peripheral blood sample will be taken once into EDTA tubes from you (healthy volunteer) who is of similar age and gender to our patients. You may feel some pain when the needle is inserted or removed during the blood sample collection. You may feel slight dizziness after the procedure. Expression levels of a group of miRNAs thought to play a role in PC and BPH will be studied from the samples taken. No additional medication or medical intervention will be applied for the study. Therefore, the study does not have any side effects on you.
You are free to participate in this study. You can accept it at the beginning and then change your mind and leave the study without giving any reason.
Your records that will reveal your identity will be kept confidential and will not be disclosed to the public, and your identity will remain confidential even if the research results are published.

Person Who Can Be Reached 24/7 During the Research Period Name / Surname / Phone:

Dr. Ali AKKOÇ Phone: …….

VOLUNTEER CONSENT
I have read all the explanations in the Informed Volunteer Consent Form. The written and verbal explanation regarding the research, the subject and purpose of which are specified above, was made to me by the physician named below. I know that I am participating in the research voluntarily, that I can withdraw from the research at any time with or without a reason, and that I can be excluded from the research by the researcher regardless of my own will.

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