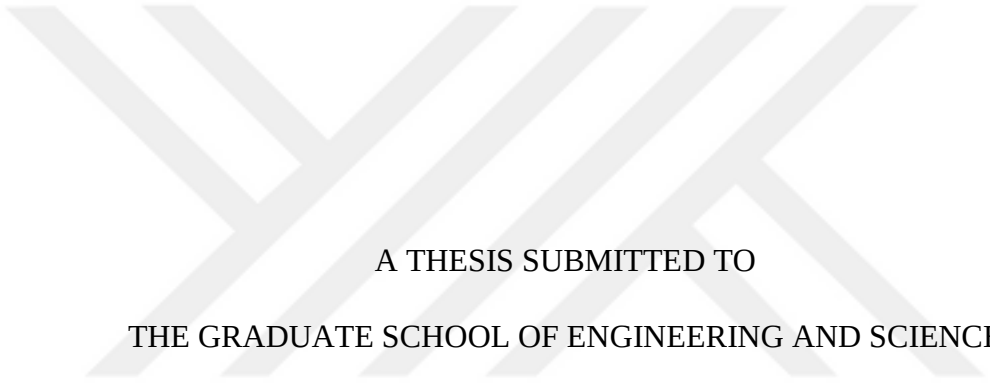


MIRNA BASED IDENTIFICATION OF PROSTATE CANCER BY INVESTIGATION OF URINARY EXOSOMES



A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
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IN
MOLECULAR BIOLOGY AND GENETICS

By
Naz Bozbeyoğlu
August 2020

**MIRNA BASED IDENTIFICATION OF PROSTATE CANCER BY
INVESTIGATION OF URINARY EXOSOMES**

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August 2020

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.



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ABSTRACT

MIRNA BASED IDENTIFICATION OF PROSTATE CANCER BY INVESTIGATION OF URINARY EXOSOMES

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M.Sc. in Molecular Biology and Genetics

Advisor: İhsan Gürsel

August 2020

Prostate cancer is one of the most incident cancer subtypes with high mortality rate. Currently, diagnosis of prostate cancer is based on rectal examination, Prostate Specific Antigen (PSA) testing and biopsy. Normal range of PSA is defined as 0-4 ng/ml and individuals with higher PSA levels are considered as potential prostate cancer patients. However, PSA fluctuates as a result of many factors and it is shown to increase with age. Thus, PSA testing causes significantly high false positive results and many healthy men have biopsy unnecessarily due to high PSA levels or they even get overtreated. This situation has huge psychological as well as financial effects on these people. Herein, we investigated the diagnostic potential of urinary exosomal microRNAs (miRNA) in prostate cancer. Rather than investigation of cellular miRNAs, we focused on exosomal miRNAs because of high integrity of exosomes and their abundance in many biofluids including urine. Development of a sensitive diagnostic method from urine would be advantageous because accurate diagnosis of prostate cancer would be possible by a non-invasive procedure. miRNAs are the small non-coding RNAs and they suppress expression of target genes via degradation of mRNA or post-translational regulation. miRNAs have high potential as cancer biomarkers because they can act as tumor suppressor and repress oncogenic gene expression or function as oncogenic miRNA and suppress tumor suppressor gene expression. For this reason, we identified several candidate exosomal tumor suppressor and oncogenic miRNAs and continued our study with the most potent ones. At the beginning of the study, we validated that we efficiently isolated exosomes via several techniques such as flow cytometry, Dynamic Light Scattering (DLS), Nanoparticle Tracking Analysis (NTA) and Transmission Electron Microscopy (TEM). Then, we studied differential expression of candidate miRNAs in prostate cancer PC-3 cell line. Similar to our literature search findings, we found that expression levels of miR-107, miR-139, miR-145 and miR-204 were significantly lower in PC-3 exosomes in comparison to healthy urinary exosomes. On the other hand, oncomiRs; miR-21-5p, miR-375-5p and miR-574-

3p were upregulated in PC-3 cell line exosomes. Of note, expression of another candidate miRNA; miR-30a was almost the same in PC-3 exosomes with healthy controls. We used these results as preliminary data and collected urine specimens from 17 prostate cancer patients. We firstly analyzed their PSA level-age and PSA Level- Gleason Score correlations. Our analyses revealed that PSA level was increasing with age and PSA was not correlated with Gleason Score. We concluded that PSA was being affected by several reasons in addition to tumor formation and it was not correlated with disease progression. Again, this was a finding which supported that a more sensitive diagnosis method than PSA testing was necessary for prostate cancer. When we detected expression levels of candidate miRNAs in urinary exosomes of prostate cancer patients and healthy controls, we observed that miR-107, miR-139, miR-145 and miR-204 were downregulated whereas miR-375-5p was upregulated in patients' urinary exosomes. For miR-21, there was a very slight upregulation and miR-30a-5p and miR-574-3p levels were almost the same with healthy controls. Further, we wondered the diagnostic potential of these miRNAs in our patient cohort and we performed Receiver Operator Characteristic (ROC) Curve analysis for them. When they were used as combination, our miRNAs had 77% (AUC=0.7731) accuracy in distinguishing patients from healthy controls. After that, we examined miRNA dysregulations in patients with different PSA levels with the hypothesis that they may have different miRNA expression profiles. We saw that expression of miRNAs in exosomes patients with PSA<10 ng/ml and PSA=10-15 ng/ml were very similar with our expectations; downregulation of tumor suppressor and upregulation in oncogenic miRNAs whereas patients with PSA>15 ng/ml had a unique profile and all miRNAs were downregulated. Due to sample size limitations, we only tested diagnostic potential of candidate miRNAs in exosomes of patients with PSA<10 ng/ml and observed that AUC was 0.8500 so we could discriminate patients and healthy controls with 85% accuracy by using our candidate miRNA panel in testing. Taken together, our findings indicated that candidate urinary exosomal tumor suppressor and oncogenic miRNAs which we suggested in thesis are very potent in diagnosis of prostate cancer with differential expressions in prostate cancer patients with different PSA levels and this study opens the way for development of a non-invasive prostate cancer diagnosis method.

Keywords: Exosome, miRNA, urine biomarker, prostate cancer, diagnosis

ÖZET

ÜRİNER EKSOZOMLARIN İNCELENMESİ İLE PROSTAT KANSERİNİN MİRNA TABANLI TANIMLANMASI

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Moleküler Biyoloji ve Genetik, Yüksek Lisans

Tez Danışmanı: İhsan Gürsel

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Prostat kanseri, görülme sıklığı ve ölüm oranı en yüksek olan kanser tiplerinden biridir. Günümüzde prostat kanseri teşhisi, rektal muayene, Prostat Spesifik Antijen (PSA) testi ve biyopsiye dayanmaktadır. Normal PSA aralığı 0-4 ng / ml olarak tanımlanır ve bundan daha yüksek PSA seviyeleri olan kişiler potansiyel prostat kanseri hastaları olarak kabul edilir. Ancak PSA seviyesi birçok faktörün bir sonucu olarak değişkenlik gösterebilir ve yaşla birlikte arttığı gösterilmiştir. Bu nedenle, PSA testi, önemli ölçüde yanlış pozitif sonuçlara neden olur ve birçok sağlıklı erkek, yüksek PSA seviyeleri nedeniyle gerekmediği halde biyopsi yaptırır ve hatta tedavi görür. Bu durumun bu insanlar üzerinde çok büyük psikolojik etkilerinin yanı sıra finansal etkileri de vardır. Biz bu çalışmada, prostat kanserinde üriner eksozomal mikroRNA'ların (miRNA) tanısal potansiyelini araştırdık. Hücresel miRNA'ları araştırmak yerine, eksozomların yüksek bütünlüğü ve idrar dâhil birçok biyolojik sıvı içerisindeki bolluğu nedeniyle eksozomal miRNA'lara odaklandık. İdrardan hassas bir teşhis yönteminin geliştirilmesinin avantajlarından biri prostat kanserinin doğru teşhisinin invazif olmayan bir yöntemle mümkün olabilmesidir. miRNA'lar küçük kodlamayan RNA'lardır ve mRNA degradasyonu veya post-translasyonel düzenleme yoluyla hedef genlerin ifadesini bastırırlar. miRNA'lar, kanser biyobelirteçleri olarak yüksek potansiyele sahiptir çünkü tümör baskılayıcı olarak hareket edip onkojenik gen ifadesini bastırabilirler veya onkojenik miRNA olarak davranıp tümör baskılayıcı gen ifadesini baskılayabilirler. Biz bu bilgilerden yola çıkarak, birkaç aday eksozomal tümör baskılayıcı ve onkojenik miRNA belirledik ve çalışmamıza en güçlü miRNA adaylarıyla devam ettik. Çalışmanın başında, eksozomları verimli bir şekilde izole ettiğimizi akış sitometrisi, Dinamik Işık Saçılımı Spektrometresi (DLS), Nanopartikül İzleme Analizi (NTA) ve Transmisyon Elektron Mikroskobu (TEM) gibi çeşitli tekniklerle doğruladık. Daha sonra prostat kanseri PC-3 hücre hattında aday miRNA'ların ekspresyonunu inceledik. Literatür araştırma bulgularımızla uyumlu olarak, miR-107, miR-139, miR-145 ve miR-204 ekspresyon seviyelerinin, sağlıklı üriner

ekzozomlara kıyasla PC-3 ekzozomlarında anlamlı derecede düşük olduğunu bulduk. Diğer yandan miR-21-5p, miR-375-5p ve miR-574-3p PC-3 hücre hattında yukarı regüle edildi. Diğer bir aday miRNA olan miR-30a seviyesi için PC-3 ekzozomları ve sağlıklı ekzozomlarda neredeyse hiçbir fark gözlenmedi. Bu önbulguların ardından miRNA seviyelerinin prostat kanseri hastalarındaki değişimlerini görmek için 17 prostat kanseri hastasından idrar örnekleri topladık. İlk olarak bu hastaların PSA düzeyi-yaş ve PSA düzeyi-Gleason Skor korelasyonlarını analiz ettik. Analizlerimiz PSA düzeyinin yaşla arttığını ve PSA'nın Gleason Skoru ile korele olmadığını ortaya koydu. PSA'nın tümör oluşumuna ek olarak farklı çeşitli nedenlerden etkilendiği ve hastalığın ilerlemesi ile ilişkili olmadığı sonucuna vardık. Yine bu, prostat kanseri için PSA testinden daha duyarlı bir tanı yönteminin gerekli olduğunu destekleyen bir bulguydu. Prostat kanseri hastalarının ve sağlıklı kişilerin idrar ekzozomlarında aday miRNA'ların ekspresyon seviyelerini tespit ettiğimizde miR-107, miR-139, miR-145 ve miR-204'ün azaldığını gözlemledik, miR-375-5p ise hastaların üriner ekzozomlarında yukarı regüle edildi. miR-21 seviyelerinde çok az bir artış vardı ve miR-30a-5p ve miR-574-3p seviyeleri sağlıklı ve hastada neredeyse aynıydı. Ayrıca, bu miRNA'ların hasta grubumuzdaki tanısal potansiyelini merak ettik ve bunlar için Alıcı İşletim Karakteristiği (ROC) analizi gerçekleştirdik. miRNA'larımız kombinasyon olarak kullanıldıklarında hastaları sağlıklılardan ayırt etmede % 77 (AUC = 0.7731) doğruluğa sahipti. Daha sonra, farklı PSA seviyelerine sahip hastaların farklı miRNA ekspresyon profillerine sahip olabileceği hipotezi ile miRNA düzensizliklerini inceledik. PSA<10 ng/ml ve PSA=10-15 ng/ml olan hastaların ekzozomlarında miRNA ekspresyonunun beklentilerimizle çok benzer olduğunu gördük; çoğu tümör baskılayıcı miRNA'nın aşağı regülasyonu ve onkogenik miRNA'nın yukarı regülasyonu söz konusuydu. PSA>15 ng/ml olan hastalar kendilerine özgü bir profile sahipti ve tüm miRNA'ların seviyeleri hastalara göre düşüktü. Örnek sayısının az olması nedeniyle, aday miRNA'ların tanısal potansiyelini yalnızca PSA<10 ng/ml olan hastaların ekzozomlarında test ettik ve AUC'nin 0.8500 olduğunu gözlemledik. Bu, aday miRNA'larımızı kullanarak hastaları ve sağlıklıları % 85 doğrulukla ayırt edebildiğimiz anlamına gelmektedir. Sonuç olarak, bu çalışmadan elde edilen veriler, tezde önerdiğimiz aday üriner ekzozomal tümör baskılayıcı ve onkogenik miRNA'ların, prostat kanseri tanısında potansiyellerinin çok yüksek olduğunu ve buna ek olarak farklı PSA düzeylerine sahip hastalarda regülasyonlarının farklı olduğunu göstermiş ve bu çalışma invazif olmayan prostat kanseri tanı yöntemi geliştirilmesinin yolunu açmıştır.

Anahtar Sözcükler: Ekzozom, miRNA, idrar biyobelirteci, prostat kanseri, tanı

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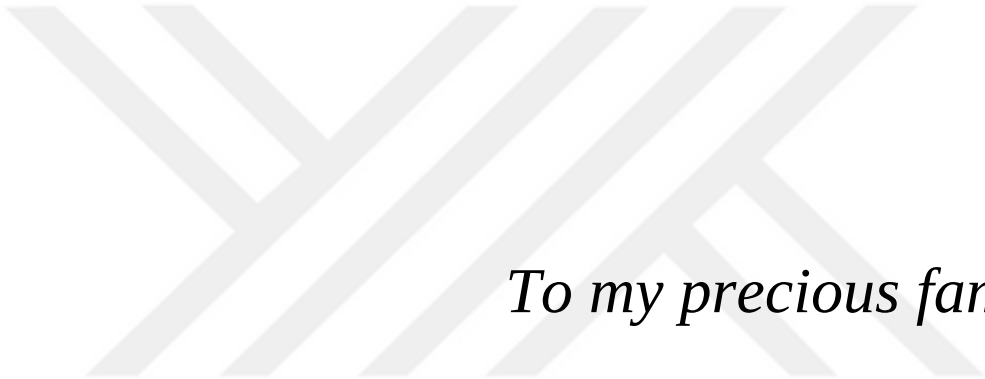
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To my precious family...

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Abbreviations

AGO2	Argonaute 2
AKT	Protein Kinase B
ALIX	ALG-2 Interacting Protein X
ANOVA	Analysis of Variance
APAF1	Apoptotic Peptidase Activating Factor 1
AR	Androgen Receptor
ASC	Adipose-Derived Stromal Cells
AUC	Area Under Curve
BCA	Bicinchoninic Acid
BCL-XL	B-cell Lymphoma-Extra Large
BCL-2	B-Cell Lymphoma 2
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CDK	Cyclin-Dependent Kinase
CT	Cycle Threshold
CTL	Cytotoxic T-Lymphocyte
CXCR4	Chemokine Receptor Type 4
DC	Dendritic Cell
DGCR8	DiGeorge Syndrome Critical Region 8
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic Acid
DNMT1	DNA (Cytosine-5)-Methyltransferase 1

DPBS	Dulbecco's Phosphate Buffered Saline
ERG	ETS-Related Gene
ERSPC	European Randomized Study of Screening for Prostate Cancer
ESCC	Esophageal Squamous Cell Carcinoma
ESCRT	Endosomal Sorting Complexes Required for Transport
EV	Extracellular Vesicle
FACS	Fluorescence-activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration
FOXD1	Forkhead Box D1
GS	Gleason Score
GTP	Guanosine Triphosphate
HCV	Hepatitis C Virus
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
IL	Interleukin
ILV	Intraluminal Vesicle
LNCaP	Lymph Node Carcinoma of the Prostate
MEM	Minimum Essential Medium
MET	Hepatocyte Growth Factor Receptor (HGFR)
MFI	Mean Fluorescence Intensity
MIF	Macrophage Migration Inhibitory Factor
MYBL2	MYB Proto-Oncogene Like 2

mRNA	Messenger RNA
miRNA	microRNA
MTDH	Metadherin
mTOR	Mammalian Target of Rapamycin
MV	Microvesicle
MVB	Multivesicular Bodies
NEAA	Non-Essential Amino Acid
NF- κ B	Nuclear Factor Kappa B
NIH	National Institute of Health
OncomiR	Oncogenic microRNA
OSCC	Oral Squamous Cell Carcinoma
PBS	Phosphate Buffered Saline
PC-3	Prostate Cancer Cell Line
PCA-3	Prostate cancer gene 3
RT-qPCR	Quantitative Reverse Transcription PCR
PCa	Prostate Cancer
PDCD4	Programmed Cell Death Protein 4
PD-L1	Programmed Death-Ligand 1
PE	Phycoerythrin
PEG	Polyethylene Glycol
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
PSA	Prostate-Specific Antigen
PTA	Phosphotungstic Acid

PTEN	Phosphatase and tensin homolog
RICTOR	Rapamycin-Insensitive Companion of Mammalian Target of Rapamycin
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acid
RNase	Ribonuclease
ROC	Receiver Operating Characteristic
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SEN1	Sentrin Specific Protease
SIRT1	Sirtuin 1
SIX1	Sineoculis Homeobox Homolog 1
snRNA	Small Nuclear RNA
SOX5	SRY-Box Transcription Factor 5
SPSS	Statistical Package for the Social Sciences
SYBR	Synergy Brands
TEM	Transmission Electron Microscopy
TMPRSS2	Transmembrane Protease, Serine 2
TP53	Tumor Protein P53
TRBP	RNA Binding Protein
TSPAN8	Tetraspanin 8
UA	Uranyl Acetate
WHO	World Health Organization

Chapter 1

Introduction

1.1 Cancer

Cancer is caused by abnormal cell growth and division and it is the second leading cause of death in 2018 [1]. There are many reasons of cancer and some of them are gene mutations, age, cellular stress (e.g. oxidative stress), diet and environmental factors such as radiation [2]. Although there are many checkpoints for regulation of cell division during cell cycle, when there are mutations in genes which regulate actions of these checkpoints, cell division continues in an uncontrolled manner and leads to tumor formation. One of the most important checkpoint is G1 checkpoint which is controlled by p53 gene so mutation in this gene cause tumorigenesis [3].

Several cancer subtypes have very high incidence and mortality rates. There were 18 million new cancer cases and 9.6 million deaths in 2018 worldwide [4]. In addition to its high incidence rate, cancer has high costs for the patients as well as governments. About \$87.8 billion was spent for cancer-related health services in U.S. Moreover, patients paid roughly \$4 billion for their cancer treatments [5]. These facts make cancer researches important and vital. Thus, American Cancer Society has funded \$420.2 million for cancer researches in 2020 [6].

1.1.1 Prostate Cancer

Prostate cancer is one of the major cancer types that cause mortality and it is the second most incident cancer type in men worldwide. According to World Health Organization (WHO), prostate cancer is the second most diagnosed cancer type in men with 1.3 million diagnoses in 2018 and the fourth most diagnosed cancer type in total sex-independent cases [7]. Prostate cancer is classified based on factors such as aggressiveness, grade of the cancer and age of the patient. Mainly, prostate cancer classes can be listed as aggressive and nonaggressive prostate cancer (PCa), high and low-grade PCa, early onset

and indolent PCa (occurring after age of 55) [8]. The class and grade of the prostate cancer has a big role in prognosis of the disease and mortality because patients who are diagnosed in early stages have higher survival rates.

Development of prostate cancer is multifactorial which means that it is dependent on more than one condition; both genetic and environmental factors have essential roles as well as diet and age. According to American Cancer Society, 6 in 10 prostate cancer patients are 65 or older. Additionally, average diagnosis age is 66 and cases under age of 40 are rare [9]. Correlation of prostate cancer with age can be described with change in testosterone:oestrogen ratio with age. In men, serum testosterone level declines as the age gets older but oestrogen level is constant. It causes a change in testosterone:oestrogen ratio which is supposed to be one of the main reasons of prostate cancer. Testosterone is an androgen which binds to androgen receptors (AR). As testosterone level decreases, AR level increases to stabilize the AR-dependent signaling which causes DNA damage and development of prostate cancer consequently [10]. In addition to this, environmental factors such as radiation and cellular stress cause cells to divide in an uncontrolled manner and may cause prostate cancer.

1.1.1.1 Diagnosis of Prostate Cancer

Diagnosis of prostate cancer, especially early detection of it, is important for good prognosis of the disease. In addition, not only late diagnosis causes mortality but also overdiagnosis, which means diagnosis of a person as prostate cancer when he is healthy, and overtreatment; treatment of an individual as a result of screening whereas he would not have any symptom without treatment have huge impact [11]. Serum Prostate Specific Antigen (PSA) level and biopsy are currently used for diagnosis of prostate cancer. First step in diagnosis is checking the PSA level if it is out of the normal/expected range. Secondly, biopsy is performed if PSA level is out of range and grade of the cancer is identified. However, for some patients despite the fact that they have high PSA levels, the biopsy results show that there is no tumor in prostate or benign hyperplasia is detected so the person with high PSA level comes out to be healthy. This is because PSA level can change depending on many factors in addition to cancer so it can be misleading [12].

1.1.1.1.1 Prostate-Specific Antigen (PSA)

Prostate-specific antigen (PSA) is a protein, kallikrein-like serine protease, produced by both normal and malignant prostate glands. This protein is produced by epithelial cells of prostate so it can be said that PSA is an organ-specific marker. On the other hand, production of PSA is dependent on many factors in addition to prostate cancer, so it is not a cancer-specific marker [13]. Besides, normal PSA level for healthy controls is not clear since it can fluctuate over time. Prostatitis, which is inflammation of prostate gland and urinary tract infection are diseases that increase PSA levels. Even biopsies are found to increase PSA level. These situations can lead to PSA level increase for reasons other than cancer and overdiagnosis of cases. On the other hand, benign prostate hyperplasia drugs such as finasteride and dutasteride cause decrease in PSA level and may result in misdiagnosis of prostate cancer. PSA level below 4 ng/ml is accepted as in normal range and above 4 ng/ml is considered as suspicious but biopsies are needed for the final decision [14].

There are many researches supporting that there are significantly many patients which are either misdiagnosed or overdiagnosed for prostate cancer and they have reduced life quality followed by high mortality. In one study, overdiagnosis of prostate cancer is investigated and it is found that 67% of total diagnosis cases is overdiagnosed [15]. In another study, when European Randomized Study of Screening for Prostate Cancer (ERSPC) results were evaluated, 4.1% of patients were found to be overtreated and the researchers conclude that PSA testing in Europe was associated with decrease in the life time of patients so it was not recommended for diagnosis [16].

1.1.1.1.2 Biopsy and Gleason Score

The second step in the diagnosis of prostate cancer after PSA test is identification of the prostate cancer grade by biopsy. Gleason Score is the score that pathologists give to the tumor parts obtained from 2 different sites. They decide on this according to how much tumor looks like a healthy tissue, so how much it is differentiated [17]. Gleason Score (GS) for each tumor part ranges from 1 to 5 and they are added for finding the GS of 2

sites. Gleason Score 1 is very similar to healthy tissues so that well differentiated whereas 5 is very poorly differentiated. Gleason scores can be 6 (3+3), 7 (3+4) or (4+3), 8 (4+4), 9 (4+5 or 5+4) and 10 (5+5) for 2 tumor sites. It is the most commonly used grading system for prostate cancer and score under 6 means the tissue is so similar to healthy tissue whereas score higher than 7 means that the cells are poorly differentiated and they look very different from healthy cells. After Gleason Scores for 2 tumor sites are summed up, they are graded according to the Gleason Grading System. The grading system for prostate cancer is as the following; Grade 1 if Gleason Score is 6, Grade 2 if Gleason Score is 7 (3+4), Grade 3 if Gleason Score is 7 (4+3), Grade 4 if Gleason Score is 8 (4+4) and Grade 5 if Gleason Score is 9 or 10. Gleason Grading is important for both diagnosis and prognosis of prostate cancer [18].

1.1.1.2 Therapy of Prostate Cancer

There are main therapy options in prostate cancer; radical prostatectomy, radical radiotherapy, conservative management, immunotherapy, hormonal therapy and chemotherapy (docetaxel). Radical prostatectomy means removing the tumor from the body by surgery. On the other hand, radical radiotherapy is the method that high-energy X-rays are used and it is a non-surgical therapy. On the other hand, conservative management is watching the disease progression and healing the symptoms rather than treating it [19].

Immunotherapy has been one of the best choices for therapy of prostate cancer in some patients and its efficiency depends on the cancer stage and history of the patient. Prostate has many tumor-associated antigens which can be targeted for vaccines. This is the reason that prostate cancer is a good cancer type for developing therapeutic cancer vaccines. In addition, development of prostate cancer is relatively slow so there is time for development of anti-tumor immune response in the body which is an advantage for cleaning the tumor. However, prostate cancer cells can prevent immune response by making the antigen presentation inefficient by downregulating human leukocyte antigen class I [20].

Sipuleucel-T (Dendreon Corp.) is the therapeutic vaccine approved by the US Food and Drug Administration (FDA). Phase III researches show that it is most efficient on early

prostate cancer because there was an increase in overall survival of patients with early prostate cancer. The mechanism of this therapeutic vaccine is not understood completely and it is known that tumor sizes or PSA levels are not affected but overall survival increases. For high-risk prostate cancer patients, combinational therapies such as chemotherapy and hormonal therapy (enzulatamid) combinations are applied frequently [21].

1.2. Extracellular Vesicles

Extracellular vesicles are vehicles which facilitate the communication between cells and regulate many biological mechanisms. Since they carry nucleic acids, lipids and proteins, they have been important for investigation of intercellular communication in both prokaryotes and eukaryotes. While some of these physiological pathways are understood, some of them are still unclear. Extracellular vesicles can be isolated from many types of biological fluids such as urine, plasma, saliva, breast milk and amniotic fluid. According to their sizes, EVs are divided into two main groups; microvesicles and exosomes [22].

Sizes of microvesicles can range from 100 nm to 1000 nm whereas exosomes are the smallest extracellular vesicle (EVs) with size of 30 nm-150 nm. Exosomes can be secreted from a cell as a part of normal conditions or as a result of a stimuli. For this reason, they are known to have roles in development and progression of many diseases including cancer [23]. In normal conditions, cargos of EVs, is very susceptible to degradation in the extracellular space if they were not encapsulated in EVs. For example, RNAs are one of the most sensitive molecules due to presence of RNase. However, EVs prevent degradation of RNAs and this characteristic of EVs makes them effective targets for therapeutic and diagnostic applications which will be outlined in Sections 1.3.2 and 1.3.3 [24].

1.2.1 Biogenesis of Exosomes

Exosomes are originated in endosomes and they firstly arise as intraluminal vesicles (ILVs) in multivesicular bodies (MVB); so that late endosomes. Destination of ILVs depends on fusion of MVB with lysosomal compartment. If a MVB fuses with lysosomal

compartment, its contents will be degraded whereas other MVBs will fuse with plasma membrane and result in exosome secretion. Endosomal V0V1-ATPase and some GTPases such as Rab GTPases are shown to have role in fusion with plasma membrane [25]. Contents of endosomes are known to be internalized by endocytosis into the MVBs and either degraded by lysosomes or secreted inside exosomes by exocytosis. (see Figure 1.1)

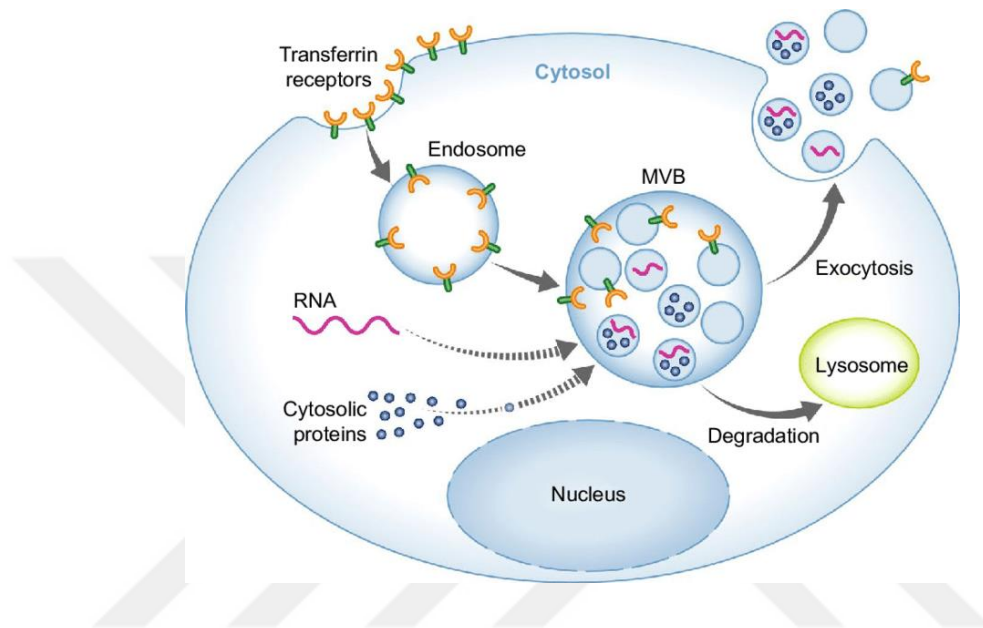


Figure 1.1 Biogenesis of exosomes [26].

In exosome biogenesis, proteins in ESCRT (endosomal sorting complexes required for transport), lipid rafts enriched in ceramide, tetraspanin-enriched microdomains and ALIX, syntenin and syndecans also have essential roles. ALIX, syntenin and syndecans form a complex, link with ESCRT system and facilitate the endocytosis of nucleic acids and proteins into the exosomes [27]. CD9, CD63 and CD81 are tetraspanins found on endosomal membrane. The first biological event occurring on endosomal membrane is enrichment of the membrane with these tetraspanins in formation of ILVs (see Figure 1.2). They are also the surface markers of exosomes which can be used in characterization of exosomes. In comparison to exosomes, microvesicles (MVs) do not follow the endosomal pathway and they directly bud off from the plasma membrane [28].

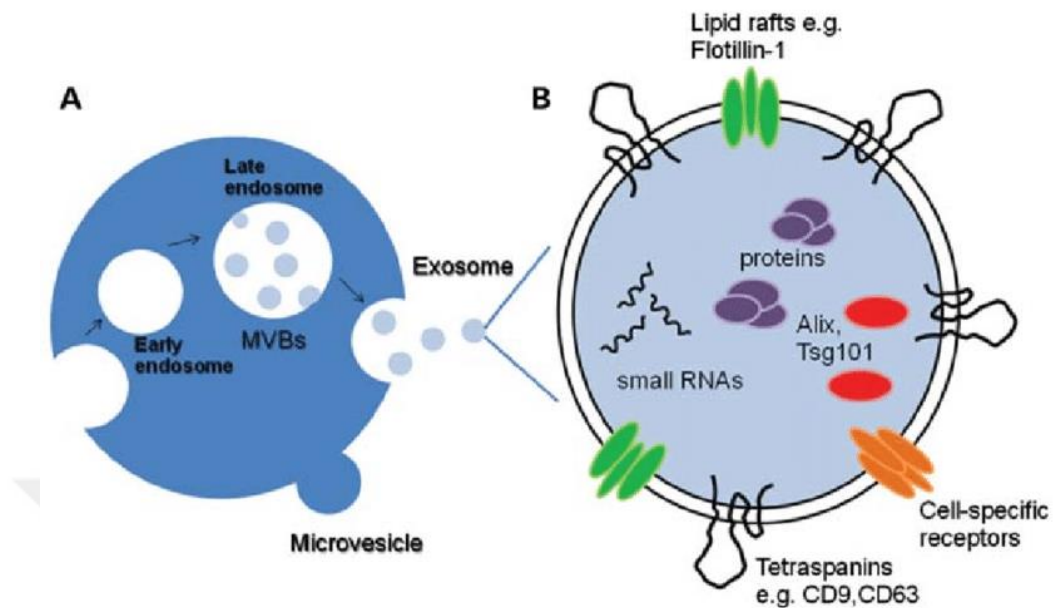


Figure 1.2 Exosome biogenesis. (A) Comparison of microvesicle and exosome biogenesis. (B) Representation of common exosomal markers [29].

1.2.2 Exosomes in Health and Disease

Exosomes circulate in the body fluids and change activity and phenotype of recipient cells. They have important roles in diseases including autoimmune diseases, inflammation, cardiovascular diseases and metabolic disorders [30-32]. Exosomes also involve in viral infections. For instance, non-enveloped virions were detected in exosomes of Hepatitis C Virus (HCV)-infected cells and these virions were shown to be entering target cells [33]. Obesity was also shown to be related with exosomes and exosome number was found to be increasing in obesity [34]. Cancer is another disease that exosomes regulate. In cancer, tumorigenesis leads to increased exosome secretion [35]. Moreover, exosomes involve in tumor growth tumor microenvironment remodeling, cell migration and immune evasion [36-39].

1.2.3 Diagnostic Applications of Exosomes in Cancer

When exosomes were first recognized, they were defined as ‘trash bags’. However, their roles in intracellular communication has been investigated and it is shown that contents of exosomes can be good targets for diagnosis of diseases including cancer because they carry nucleic acids, proteins and lipids which give important information about the cells that they are released from. Biological fluids are the places that exosomes accumulate so by isolation and investigation of exosomes, diagnostic markers can be discovered as well as markers of disease progression [40].

Pancreatic cancer is one of the cancer types that exosomes are used for diagnosis and determination of metastasis. Macrophage migration inhibitory factor (MIF) was suggested to be upregulated in exosomes of pancreatic cancer patients. Also, surface proteins CD44v, CD44, MET, Tspan8 and CD133 which are the markers of pancreatic cancer initiating cells were detected in pancreatic cell originated exosomes [41]. Another cancer type with high mortal rate; lung cancer has also potential of being diagnosed by exosomal contents. Tumor cell-derived exosomes promote angiogenesis, invasion and proliferation in lung cancer. According to the studies made with healthy controls and lung cancer patients, candidate exosomal microRNAs (miRNAs) for early diagnosis of lung cancer were detected [42]. Additionally, exosomes were also shown to have role in apoptosis of tumor cells in breast cancer [43].

Since exosomes can protect miRNAs against RNase degradation and they regulate the expression of genes, exosomal miRNAs are one of the diagnostic markers of cancer. These miRNAs regulate the expression of genes playing role in pathways such as angiogenesis and apoptosis. miR-34a, miR-182 and miR-375 are some of the candidate miRNAs for diagnosis of prostate cancer [44]. In addition to miRNAs, some mRNA biomarkers from urine were also detected in prostate cancer such as PCA-3 and TMPRSS2:ERG [45]. However, there is no exosomal diagnosis kit used for prostate cancer so that overdiagnosis of prostate cancer cases is still ongoing as detailed in Section 1.1.1.

1.2.4 Therapeutic Applications of Exosomes in Cancer

Exosomes are efficient therapeutic agents for many diseases including pulmonary hypertension, diabetes and cancer [46]. Effects of exosomal therapy has been researched for various diseases and about 90 exosomal therapy studies are in clinical trial phases in 2020 according to NIH Clinical Trials database [47]. Exosomal therapy in cancer is one of the most studied field due to high incidence and mortality rates of cancer. In mouse models, Zitvogel et al showed that exosomes derived from tumor antigen peptide-stimulated DCs primed tumor-specific cytotoxic T-lymphocytes (CTL) and resulted in delay of tumor growth and tumor elimination [48]. This research was one of the leading studies about exosomal therapy and many similar ones have been conducted for different cancer subtypes.

Delivery of cargos to the target cells by loading of this cargo into exosomes has been one of the most preferred ways of exosomal therapy. Let-7a miRNA was loaded into exosomes of embryonic kidney cells and reduction in size of breast cancer cells was observed [49]. Additionally, Phosphatase and tensin homolog (PTEN) mRNA was loaded into mouse embryonic fibroblasts and glioma cell proliferation decreased [50]. Another example of exosomal therapies in cancer subtypes was reduction of the activity of Bcl-xL by adipose-derived stromal cells (ASCs) derived exosomal miR-145 and induction of prostate cancer cell apoptosis [51]. In addition to RNA delivery via exosomes, chemical drugs were also used as chemotherapeutic agents. For prostate cancer therapy, exosomes were loaded with Paclitaxel and decrease in prostate cancer cell growth was shown [52].

1.3 microRNAs

microRNAs are small, single-stranded, non-coding RNAs which are 22-25 nucleotides long [53]. They target mRNAs and either cleave or deadenylate the mRNAs or repress the translation [54]. By these ways, they facilitate gene silencing and they are one of the main regulators of biological processes. They have role in every cellular activity and are very important in maintaining cell homeostasis, differentiation and development [55]. They also play a vital role in development of many diseases including cancers,

autoimmune diseases such as rheumatoid arthritis, cardiovascular diseases, neurological disorders and liver diseases [56].

1.3.1 Biogenesis of microRNAs

There are 2 types of miRNAs; intragenic and intergenic miRNAs. Half of the currently discovered miRNAs are intergenic which means that they are found and processed from either introns or exons of the gene; but mostly from the introns. The other half of the miRNAs are intragenic and they are transcribed from a different gene regulated by a different promoter [57]. The most common pathway for biogenesis of miRNAs is the canonical pathway. In the canonical pathway, primary microRNAs (pri-miRNAs) transcribed by RNA Polymerase II are capped and polyadenylated. Mature miRNA sequences are found in the stem of a hairpin on the pri-miRNA [58]. Then, they are processed to single hairpins; pre-miRNAs, by the microprocessor complex. Microprocessor complex consists of two components; DiGeorge Syndrome Critical Region 8 (DGCR8) and Drosha which is a ribonuclease III enzyme. Pre-miRNAs are transported to the cytoplasm by Exportin 5/Ran GTP complex and the complex cleaved by RNase III endonuclease, Dicer and RNA Binding Protein, TRBP [59]. The length of the miRNA is the mature miRNA length after this step. Argonaute (Ago2) proteins load functional strand of the miRNA into the RNA-induced silencing complex (RISC) and lead it to the target mRNA (see Figure 1.3) [60]. Other biogenesis pathways are Drosha-independent and Dicer-independent pathways but they are not as common as the canonical pathway.

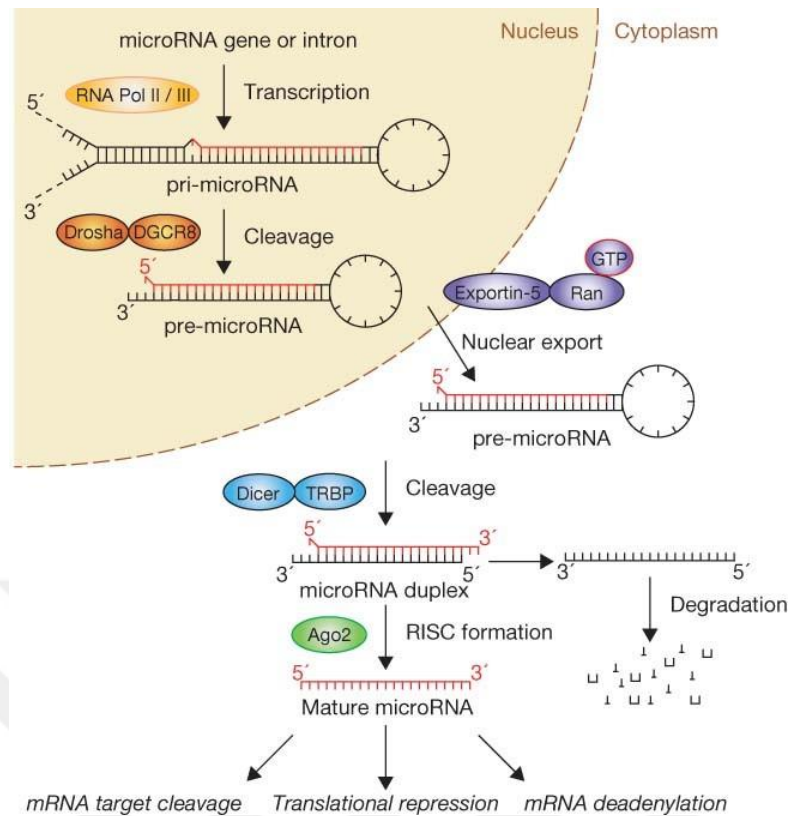


Figure 1.3 Canonical pathway of microRNA biogenesis [61].

1.3.2 microRNAs and Cancer

MicroRNAs are shown to be good biomarkers and therapeutic agents for cancer [62]. Besides, dysregulation of some miRNAs are found to be the key player in progression of several cancer types. Cellular processes such as proliferation, growth, cell death, activation of invasion and metastasis are regulated by microRNAs [63]. miRNAs can function as oncogenes or tumor-suppressors. If a miRNA functions as an oncomiR, it targets a tumor suppressor gene and decreases its expression for inducing cell proliferation and repressing apoptosis. On the other hand, if it acts as a tumor suppressor, it targets an oncogene and reduces its expression [64]. The first study that investigated role of microRNAs in human cancers is from Calin et al. They revealed that miR-15 and miR-16 were downregulated in chronic lymphocytic leukemia [65]. After this study in 2002, many researches about miRNAs as cancer hallmarks have been conducted.

Studying microRNAs in cancer researches for diagnostic or therapeutic reasons has high potential and many advantages. First of all, microRNAs can be isolated from many biological fluids such as blood and urine, saliva which makes the noninvasive biomarker testing methods possible [66]. Secondly, miRNAs are very stable due to their small size so they make the technically challenging samples such as formalin fixed samples easier [67]. Additionally, microRNAs are found free in the circulation as well as encapsulated in extracellular vesicles. EV miRNAs give more information about tumor such as the tumor microenvironment. Moreover, microRNAs serve as biomarkers even for the grade of cancers. Finally, miRNAs are tissue-specific so that every tumor has different microRNA profile depending on the tissue type which make the profile specific for each tissue and more reliable for comparison with healthy profile [68].

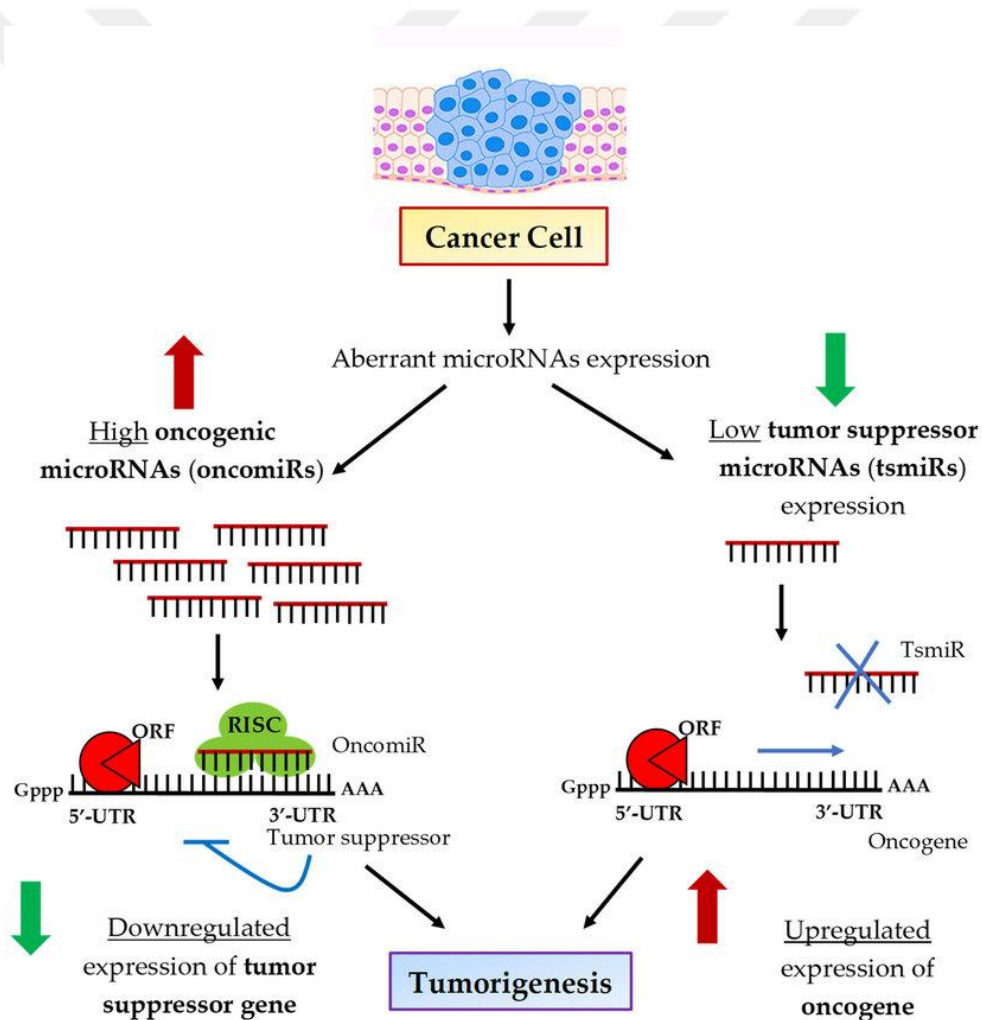


Figure 1.4 Mechanisms of tumor suppressor and oncogenic miRNAs [69].

1.3.2.1 microRNAs as Cancer Biomarkers

Tumor-suppressor and oncogenic properties of microRNAs make them good biomarker candidates. Differential expression of miRNAs results in changes of gene expression. There are hundreds of different targets for miRNAs and miRNA expression differs in each tissue; which means that different cancer types have different miRNA profiles [70]. Especially due to the advances in high-throughput studies in last years, miRNA biomarker panel researches for almost every cancer type has increased tremendously [71].

There are many promising researches and evidences about discovery of miRNA biomarkers for several cancers by examination of blood specimens. Heneghan and colleagues conducted a study for determining miRNA biomarkers of breast cancer. They reported that miR-195 expression was increased in cancer cohort [72]. Moreover, miR-215, miR-299-5p, and miR-411 expression levels was found to be downregulated by van Schooneveld et al [73]. Additionally, Konishi et al revealed that miR-21 concentration was significantly higher and miR-375 expression was significantly lower in esophageal squamous cell carcinoma when compared to healthy controls [74]. Besides, miR-486 was found to be a potential biomarker for non-small cell lung cancer and its expression was significantly increased in patients [75]. For gastric cancer, miR-204 level was significantly lower in the cancer cohort when compared to healthy donors [76]. Further, it has been documented that miR-145 levels were lower than the healthy controls as well as miR-204-5p levels [77, 78].

Although miRNA biomarker discovery from blood is more common, urinary biomarker researches gathered speed because it is a non-invasive method and has high potential for being used as biomarker specimen. Erbes et al stated that miR-21-5p, miR-125b-5p, miR-155-5p, and miR-451-5p were differentially expressed in urine of breast cancer patients [79]. Indeed, in bladder cancer patients, it was observed that miR-10b and miR-34b were upregulated compared to controls [80]. Furthermore, Andreu et al revealed that miR-375 level was significantly lower in high-grade bladder cancer patients. On the other hand, miR-146a was significantly upregulated in low-grade bladder cancer patients [81]. For prostate cancer, a study pointed up diagnostic power of miR-222-3p*miR-24-3p/miR-30c-5p biomarkers in cell free urine for prostate cancer diagnosis [82].

1.3.2.2 microRNAs in Prostate Cancer Diagnosis

Prostate cancer has been one of the most studied cancer subtype for biomarker discovery due to its high prevalence and mortality rate. When low reliability of PSA testing is also taken into consideration, increase in researches about this field is inevitable [83]. Huge progress has been made in miRNA biomarker in recent years and promising evidences showed that microRNAs are strong biomarker candidates for prostate cancer diagnosis just like other cancer subtypes [84]. By loss-of-function and gain-of-function experiments in human cancer cells, mouse xenografts, transgenic mouse models and knockout mouse models, it was shown that miRNAs have essential roles in tumor formation and prognosis of cancer [85]. Besides, advances in technology led to improvement in studies with high number of prostate cancer patient and healthy volunteers so number of researches which are statistically more reliable increased. Several miRNA profiling studies have been conducted for prostate cancer as well as high throughput profiling analysis in last 15 years [86-89]. Figure 1.5 shows the mechanism of actions of several miRNAs in prostate cancer.

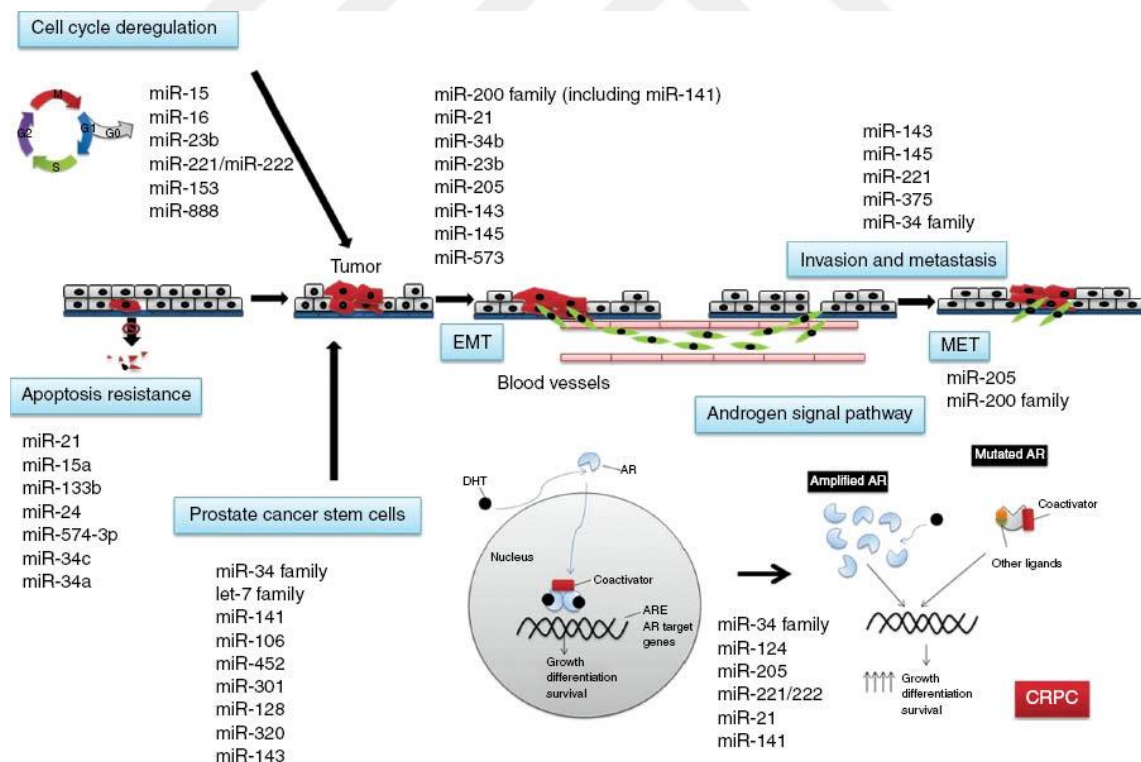


Figure 1.5 Roles of miRNAs in prostate cancer development [90].

1.3.2.2.1 Tumor Suppressor microRNAs

Tumor suppressor microRNAs have oncogene targets and they reduce cancer cell proliferation, inhibit metastasis and increase survival. In cancers, downregulation of tumor-suppressor miRNAs is expected [91]. When taken into account that locations of more than 50% of miRNA genes are in cancer-associated genomic regions or in fragile sites, tumor-suppressor microRNA studies have gained speed for diagnosis and treatment of cancer [64]. In prostate cancer, miR-145 was detected as a tumor-suppressor miRNA which targets SENP1 (Sentrin Specific Protease) gene and causes growth arrest in PC-3 cell line [92]. Besides, Guan et al revealed that miR-218 targets RICTOR which is the component of mTOR pathway and prevents angiogenesis in prostate cancer [93]. miR-152 is another tumor suppressor miRNA which binds to 3'-UTR of DNA (cytosine-5)-methyltransferase 1 (DNMT1) and decreases its expression level which might be responsible for aggressiveness of prostate cancer [94]. In our study, expression of 5 oncomiRs; miR-30a-5p, miR-107, miR-139-5p, miR-145-5p and miR-204-5p were investigated and their functions are explained in more detail in the following sections.

1.3.2.2.1.1 hsa-miR-30a-5p

One of the candidate tumor suppressor miRNAs in prostate cancer is hsa-miR-30a-5p which is found on Chromosome 6 [95]. In general, this miRNA is downregulated in many cancer subtypes which means it functions as a tumor suppressor miRNA [96]. One of its targets is SIX1, a gene which has role in development and inhibits cell apoptosis in organ development [97]. Other examples of miR-30a-5p targets are MYBL2, FOXD1, and SOX4 which also induce cell proliferation [98].

Downregulation of miR-30a in prostate cancer patients was shown by Xu et al [99]. This finding was supported by the research conducted by Zhao et al and Zhu et al [97]. When exosomal hsa-miR-30a-5p expression was examined, it was found to be responsible for development of some cancer subtypes such as ovarian cancer and oral squamous carcinoma [100, 101]. However, role of exosomal miR-30a, especially urinary exosomal miR-30a dysregulation, in prostate cancer is yet to be understood.

1.3.2.2.1.2 hsa-miR-107

hsa-miR-107 is found on Chromosome 10 and it has only one mature sequence [102]. miR-107 plays a vital role in many cancers including colorectal cancer, gastric cancer, pancreatic cancer and hepatocellular carcinoma. miR-107 was found to be having a dual role in cancer; so it can be a tumor suppressor in some cancer types whereas it was upregulated and functioning as an oncomiR in other cancers. For instance, although its overexpression was shown to induce migration and invasion in hepatocellular carcinoma, it had a tumor suppressor effect on colorectal, pancreatic cancer and non-small cell lung cancers [103-106].

There is a contradiction about dysregulation of hsa-miR-107 in prostate cancer. There are researches which claim it can act as an oncomiR whereas some studies demonstrate tumor suppressor role of miR-107 in prostate cancer. Zhang et al showed that miR-107 targeted Cyclin E1 and represses cell proliferation in prostate cancer so it acted as a tumor suppressor [107]. However, Lodes et al stated that miR-107 was upregulated in prostate cancer patients compared to healthy controls [108]. The diagnostic ability of miR-107 in prostate cancer is not well studied and needs to be further investigated.

1.3.2.2.1.3 hsa-miR-139-5p

hsa-miR-139-5p is a 23 nucleotides long miRNA found on Chromosome 11 [109]. Its tumor suppressor effects have been studied in many researches of different cancer subtypes including breast cancer, parathyroid carcinoma and esophageal squamous cell carcinoma (ESCC). These studies revealed that hsa-miR-139-5p was downregulated in cancer [110-112].

In prostate cancer, miR-139 was also found to be behaving as a tumor suppressor. In the study which aims identification of proliferation inhibitory mechanism of hsa-miR-139-5p in LNCaP prostate cancer cell line, it was shown that cyclin D1 level was decreased by miR-139 activity so miR-139 inhibits cell proliferation by targeting Notch1 and interferes with the cell cycle [113]. Similarly, in another study, Nom et al indicated that growth inhibition effect of hsa-miR-139-5p in prostate cancer was due to downregulation of activated AKT and cyclin D1 and upregulation of the p21 which is a CDK inhibitor [114]. Progression of prostate cancer was found to be regulated by miR-139 too. In

prostate cancer, miR-139 was found to inhibit SOX5 expression and TWIST was downregulated. By this way, miR-139 repressed Epithelial-Mesenchymal Transition [115]. In the literature, there is no research about the role of exosomal hsa-miR-139-5p in prostate cancer although there are many researches which support the tumor suppressor effects of it on prostate cancer cells.

1.3.2.2.1.4 hsa-miR-145-5p

hsa-miR-145 is a miRNA found on the Chromosome 5 [116]. Almost all studies aiming determination of the role of hsa-miR-145-5p in tumorigenesis and progression of several different cancers ended up with the same conclusion; miR-145-5p was downregulated in cancer and it was a future-promising biomarker candidate. In breast cancer cells, miR-145 was downregulated and acted as a tumor suppressor [117]. Likewise, miR-145 level decrease in ovarian cancer was reported by Hang et al and they claimed downregulation of miR-145-5p in cancer cells and also their derived exosomes leads to ovarian cancer development [118].

Effects of miR-145-5p in prostate cancer is similar to other cancer types. In LNCaP prostate cancer cells, overexpression of miR-145-5p resulted in inhibition of proliferation and invasion and also facilitated early apoptosis of the cells [119]. In addition, Pan et al found that miR-145-5p targeted Metadherin (MTDH) in prostate cancer cells. This gene was shown to be upregulated in several tumor cells and it was responsible from proliferation, and migration of tumor cells [120]. In another study, overexpression of miR-145-5p caused SOX2 repression and inhibition proliferation of prostate cancer cells [121]. There are a few researches about exosomal miRNA in different cancer subtypes. However, there is no research about correlation of urinary exosomal miR-145-5p and prostate cancer until today.

1.3.2.2.1.5 hsa-miR-204-5p

miR-204-5p is the last tumor suppressor miRNA candidate in this study and it is found on the Chromosome 9 [122]. In the literature, there are evidences about tumor suppressor effects of miR-204-5p in different cancer types. Overexpression of miR-204-5p caused repression of proliferation, migration and viability in human and murine breast cancer cells [123]. Similarly, the expression of miR-204-5p was lower in oral squamous cell carcinoma (OSCC) cells and it was claimed that miR-204-5p targeted chemokine receptor type 4 CXCR4 [124]. Moreover, miR-204-5p was shown to be a good biomarker candidate for non-small cell lung cancer [125].

Role of hsa-miR-204-5p has been investigated and its tumor suppressor activities and its mechanisms have been reported. In prostate cancer cells, apoptosis was induced by miR-204-5p. In the same study, researchers clarified the mechanism of apoptosis inhibition by concluding that miR-204-5p was targeting BCL2 in these cells [77]. Besides, miR-204 targeted SIRT1/p53 pathway in doxorubicin-treated prostate cancer cells and enhanced mitochondrial apoptosis by this way [126]. Supporting this finding, Wu et al claimed that miR-204 expression level decreased in chemoresistant prostate cancer tissues [127]. miR-204-5p plays an important role in metastasis in prostate cancer as well as tumorigenesis. miR-204-5p was shown to be targeting NF- κ B signaling in prostate cancer and repressing bone metastasis [128]. Diagnostic ability of urinary exosomal miR-204-5p in prostate cancer is yet to be identified.

1.3.2.2.2 Oncogenic microRNAs

Oncogenic microRNAs, also known as oncomiRs are key players in tumor formation, carcinogenesis and metastasis [129]. They generally target mRNA of tumor-suppressor genes and lead to RNA degradation or translational repression via binding to the 3' untranslated region of these mRNAs [130]. miR-221/miR-222 are oncogenic microRNAs which have been examined by several studies and it was concluded that they facilitate cell cycle progression at G-S phase in prostate cancer [131-133]. Another common oncomiR cluster is MiR-106b/miR-25 which was found to be targeting caspase 7 mRNA [134, 135]. In addition, miR-18a and miR-32 were found to be candidate biomarkers for aggressive prostate cancer and they might be a target for drug development [136, 137].

Not only patient researches but also preliminary cell lines studies have vital role in biomarker discovery. One good example of these studies is by Seashols-Williams et al which includes high-throughput sequencing and miRNA panel analysis. According to the authors, miR-9 was an oncomiR validated by miRNA panel, RT-qPCR as well and high-throughput sequencing analysis. This study was conducted on tumorigenic and metastatic subline M12 as well as other prostate cancer cell lines DU-145, PC-3 and human P69 prostate cell line as a control [138]. In our study, expression levels of 3 oncomiRs; miR-21-5p, miR-375-5p and miR-574-3p were investigated and functions of these miRNAs are explained in the following sections.

1.3.2.2.2.1 hsa-miR-21-5p

miR-21 is one of the most frequently overexpressed microRNA in prostate cancer patients compared to healthy controls. This miRNA is located on the Chromosome 17 and has a length of 22 nucleotides [139]. Li et al stated that tissue samples obtained from prostate cancer patients had higher miR-21 expression than healthy controls and it was significantly associated with stage of the cancer, Gleason score and biochemical recurrence [140]. Moreover, another study made with PC-3 cell line revealed the mechanism of oncogenic property of miR-21. miR-21 targets and inhibits the expression of tumor suppressor gene PTEN and promotes cell division and cancer progression [141]. Metastasis is also related with miR-21. miR-21 attenuates expression of tumor suppressor PDCD4 (programmed cell death) which is an inhibitor of prometastatic factor. [142] In addition to researches made on blood samples for miR-21, urine is also a good specimen for diagnosis of prostate cancer. In prostate cancer patients' urine samples, miR-21 was found to be upregulated in prostate cancer patients in comparison to benign prostate hyperplasia [143].

Exosomal miR-21 was also found to be overexpressed in prostate cancer. Agaoglu et al showed that miR-21 level was higher in serum exosomes of prostate cancer patients with metastasis compared to patients with localized tumor [144]. A study revealed that miR-21 suppresses APAF1 (apoptotic peptidase activating factor 1) and PDCD4 expression levels in order to to inhibit apoptosis [145]. Despite the fact that there are some researches about exosomal miR-21 dysregulation in prostate cancer, especially urinary miR-21

deregulation and its mechanism should be investigated in more detail in order to translate this biomarker into clinic.

1.3.2.2.2.2 hsa-miR-375-5p

Another oncomiR investigated in this study, hsa-miR-375-5p is found on Chromosome 2. Hsa-miR-375 has 2 mature strands; hsa-miR-375-5p and hsa-miR-375-3p [146]. Although hsa-miR-375-3p is more abundant than hsa-miR-375-5p, miR-375-5p was examined in this study. There are evidences that less abundant strand of miRNAs can also be functional and may have roles in diseases [147-149].

hsa-miR-375 was found to be dysregulated in many cancer types including prostate cancer [150-152]. miR-375 has dual role in cancer so it was shown to be acting as tumor suppressor as well as oncogenic miRNA in different cancer types such as liver cancer, breast cancer and colon cancer [153-155]. In prostate cancer, many researches supported the hypothesis that miR-375 was acting as an oncomiR and upregulating genes which promote cell proliferation. [156-158] Exosomal miR-375 was also studied in prostate cancer. Lie et al found that there was an increase in miR-375 in LNCaP cell-derived exosomes [159]. According to another study conducted by Zhan et al, exosomal miR-375 level in serum samples was also associated with metastasis in prostate cancer [160]. Diagnostic potential of urinary exosomal miR-375-5p is yet to be studied.

1.3.2.2.2.3 hsa-miR-574-3p

hsa-miR-574-3p which has length of 22 nucleotides is found on Chromosome 4 [161]. This microRNA was shown to have tumor-suppressor or oncogenic effects in different cancer subtypes such as osteosarcoma, ovarian cancer and breast cancer [162-164]. In prostate cancer, it functions as an oncogene according to both high-throughput and RT-qPCR results. In both serum and urinary cells of prostate cancer patients, miR-574-3p level was found to be significantly higher than healthy controls [165]. In serum samples of prostate cancer patients, fifteen miRNAs including miR-574-3p were found to be upregulated at the end of the microarray analysis. This result was complementary with

prostate cancer cell line 22Rv1 data [108]. miR-574-3p has important tumor suppressor targets including TP53 targets (TP53TG3, TP53TG3B, TP53TG3D) [166].

When extracellular vesicles of prostate cancer patients were investigated, miR-574-3p level was shown to be a good biomarker candidate in prostate cancer patients as a result of investigation of it in serum microvesicles [167]. miRNAs found in extracellular vesicles, especially in urinary exosomes, are not well studied in prostate cancer and need to be further examined.



1.4. Aims and Outline of the Study

Exosomes, which are the smallest members of extracellular vesicles with 30-150 nm diameter size, carry microRNAs and dysregulation of these miRNAs play role in cancer development. Tumorigenesis, tumor microenvironment remodeling and metastasis are examples for effects of exosomal miRNAs. Studies with exosomal miRNAs isolated from several biological fluids such as blood and urine prove their efficiency in both diagnosis and therapy of different cancer types including prostate cancer. Prostate specific antigen (PSA) is the current biomarker used for diagnosis of prostate cancer. However, PSA level was shown to be altered due to many reasons in addition to prostate cancer so it can be misleading in several cases. Thus, a new biomarker research for prostate cancer research would be invaluable and it has become a necessity.

In this study, we focused on the diagnostic potential of urinary exosomal miRNAs in prostate cancer. The first aim of the study was investigation of candidate exosomal miRNA levels in PC-3 cell line which is a prostate cancer cell line and comparison of them with healthy urinary exosomal miRNA levels. The second aim of the study was to reveal differences of candidate miRNAs relative expressions in prostate cancer patient and healthy urinary exosomes. miRNA level changes in patients would make these miRNAs potential exosomal biomarkers for prostate cancer. Another aim of the study was to identify differential expression of candidate miRNAs in urinary exosomes of prostate cancer patients with different PSA levels and comparison of them between each other. Finally, we aimed to test the diagnostic ability of candidate urinary exosomal miRNAs and compare the efficiency of them when they were used as single and as combination for discrimination of patients and healthy controls.

For the purposes stated above, first of all, exosomes were isolated from supernatants of PC-3-cell lines by ultracentrifugation. Besides, morning urine specimens were collected from 14 healthy male individuals and urinary exosomes were isolated by precipitation with PEG. Exosome characterization was performed via flow cytometry, qNano, DLS and TEM. Then, miRNA was isolated from exosomes of PC-3 cell line and healthy controls. Finally, relative expressions of them were analyzed by RT-qPCR.

For the second purpose of the study, morning urine specimens were collected from 17 prostate cancer patients with their consent. Exosome isolation was performed with the same procedure of healthy urinary exosome isolation with PEG. Following miRNA

isolation and RT-qPCR, relative expressions of candidate miRNAs were calculated according to the efficiency-corrected CT method and expression levels of urinary exosomal miRNAs in prostate cancer patients and healthy controls were compared. Finally, in order to determine the diagnostic ability and diagnostic potential of miRNAs, ROC curve was generated for each miRNA and their different combinations and AUC values were analyzed as predictors as diagnostic potential of miRNAs.



Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Cell Culture Solutions

For preparation of cell culture media, RPMI1640 with phenol red (#21875-034), Sodium Pyruvate (100mM, #11360-070), MEM Non-Essential Amino Acids (100X, #11140-050), Penicillin-Streptomycin (10,000U/mL, #15140-122) were used from Gibco, Thermo Fisher Scientific (Waltham, MA, USA). HEPES Buffer (1M, #03-025-1B), FBS (#04-127-1A, Lot 1808243) was purchased from Biological Industries (Beit-Haemek, Israel). RPMI media was prepared by adding appropriate amount of FBS so that 10% of the total volume is FBS and Sodium Pyruvate, MEM Non-Essential Amino Acids (NEAA) , Penicillin-Streptomycin, HEPES were added to the media by diluting 1/100 each of them. 10X Trypsin without phenol red (2.5%, #15090-046) and DPBS (#14190-250) were also from Gibco, Thermo Fisher Scientific (Waltham, MA, USA).

2.1.2 Antibodies

In order to capture exosomes for exosome characterization purposes, purified exosome-specific antibodies were used. Purified human-specific antibodies used for exosome characterization were listed in Table 2.1

Table 2.1 Purified human-specific antibodies; their catalog numbers and clones.

Antibody	Company	Catalog Number	Clone
CD9	Biolegend	312102	HI9a
CD63	Biolegend	353014	H5C6

For flow cytometry analysis, fluorochrome conjugated antibodies were used. (Table 2.2)

Table 2.2 Fluorochrome-conjugated human-specific antibodies; their catalog numbers and clones.

Antibody	Company	Catalog Number	Clone
CD9-PE	Biolegend	312106	HI9a
CD63-PE	Biolegend	353004	H5C6
CD81-PE	Biolegend	349506	5A6
CD274 (PD-L1)-PE	Biolegend	329706	29E.2A3
Mouse IgG1, κ -PE (Isotype Control)	Biolegend	400112	MOPC-21
Mouse IgG2b, κ -PE (Isotype Control)	Biolegend	402204	27-35

RT-PCR and qPCR primers were synthesized by Oligomer Biotechnology (Ankara, Turkey). Primer sequences for RT-PCR, qPCR and their optimal conditions are listed in Table 2.3 and Table 2.4 respectively.

Table 2.3 RT-PCR primer list

miRNA	Primer	Sequence 5'-3'	Annealing Temperature
hsa-miR-21-5p	Stem-loop	GTCGTATCCAGTGCAGGGTCCGA GGTATTTCGCACTGGATACGACTC AACA	58°C
hsa-miR-30a-5p	Stem-loop	GTTGGCTCTGGTGCAGGGTCCGA GGTATTTCGCACCAGAGCCAACCT TCCA	58°C
hsa-miR-107	Stem-loop	GTCGTATCCAGTGCAGGGTCCGA GTATTTCGCACTGGATACGACTGA TAG	58°C
hsa-miR-139-5p	Stem-loop	GTTGGCTCTGGTGCAGGGTCCGA GGTATTTCGCACCAGAGCCAACAC TGGA	58°C
hsa-miR-145-5p	Stem-loop	GTTGGCTCTGGTGCAGGGTCCGA GGTATTTCGCACCAGAGCCAACAG GGAT	58°C
hsa-miR-204-5p	Stem-loop	GTCGTATCCAGTGCAGGGTCCGA GGTATTTCGCACTGGATACGACAG GCAT	58°C
hsa-miR-375-5p	Stem-loop	GTCGTATCCAGTGCAGGGTCC GAGGTATTTCGCACTGGATACG ACGGTTTG	58°C
hsa-miR-574-3p	Stem-loop	GTCGTATCCAGTGCAGGGTCC GAGGTATTTCGCACTGGATACG ACTGTGGG	58°C

Table 2.4 qPCR primer list

miRNA	Primer	Sequence 5'-3'	Annealing Temperature
U6 snRNA	Forward	GCTTCGGCAGCACA TATACTAAAAT	58°C
U6 snRNA	Reverse	CGCTTCACGAATTTG CGTGTCAT	58°C
hsa-miR-21-5p	Forward	TGGTTTGGTAGCTTA TCAGACTGA	58°C
hsa-miR-30a-5p	Forward	TGTGTTGTGTAAAC ATCCTCGAC	58°C
hsa-miR-107	Forward	TGTTTTTTTGAGCAG CATTGTACAG	58°C
hsa-miR-139-5p	Forward	GGTGTCTACAGTGC ACGTG	58°C
hsa-miR-145-5p	Forward	GGTTTTTTTGTCCAG TTTTCCCAG	58°C
hsa-miR-204-5p	Forward	TGTTTTTTTTTTTTTCC CTTTGTCATCCT	58°C
hsa-miR-375-5p	Forward	GTTTTTTTGCAGACGAG CCC	58°C
hsa-miR-574-3p	Forward	TGTGTCACGCTCATG CAC	58°C
Universal Reverse Primer	Reverse	GTGCAGGGTCCGAG GT	58°C

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Cell Culture of PC-3 cell line

See Section 2.1.1 for the ingredients of the solutions used in culturing of PC-3 cell line. As the cell culture media, RPMI 1640 with 10% FBS was used. Before collecting supernatant for exosome isolation, PC-3 cells were cultured in RPMI 1640 medium and after they were attached, supernatant was aspirated and changed with RPMI with 10% exo-free FBS containing RPMI 1640. They were passaged every two days when they are at 80-90% confluency. Cells were detached with 2X Trypsin for 3 minutes at 37°C. Cell counting was done by hemocytometer explained in Section 2.2.1.2. For exosome isolation, supernatant of confluent cells were collected and centrifuged at 1500xg for 10 minutes at 4°C and supernatant was snap-frozen and stored at -80°C.

2.2.1.2 Cell Counting by Hemocytometer

Hemocytometer is used for PC-3 cell line culture. Cell pellet is resuspended in cell culture media and 10 µl of cell suspension is added to the 90 µl of culture media. 100 µl trypan blue is added for distinguishing dead and live cells and 10 µl of the mixture is put onto the hemocytometer. Finally, the hemocytometer is observed under the light microscope and cell count is calculated as the following;

Total cell # = Counted cell # / 4 (chamber number) x 10⁴ x 20 (dilution factor) x 1 (ml, resuspension volume)

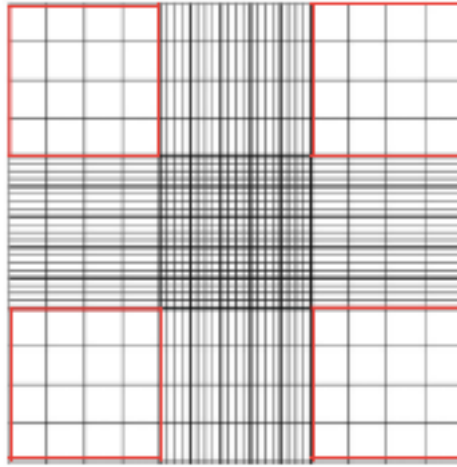


Figure 2.1 Representative figure of chambers of hemocytometer. Cells within the chambers designated with red lines are counted in order to determine the cell number.

2.2.2 Urine Sample Collection from Prostate Cancer Patients and Healthy Controls

Morning urine samples of 17 prostate cancer patients with adenocarcinoma were collected from Department of Urology, Bilkent City Hospital (Ankara, Turkey). In addition, morning urine specimens of 14 healthy men were also included into the study as the control group.

The study was approved by Institutional Review Board (Human Ethical Committee Approval #E1-20-905). Individuals were clearly informed about the research and they all provided informed consents.

2.2.3 Exosome Isolation from Urine

2.2.3.1 PEG8000 Preparation for Exosome Isolation

40% PEG8000 (Polyethylene glycol) (#P2139, Sigma Aldrich, Germany) solution was prepared by firstly adding 40 g of PEG into 150 ml distilled water. After rotating at 60°C, volume is completed to 200 ml and 40 g of PEG was added again. Solution is rotated at 60°C until it is completely clear. It was stored at 4°C.

2.2.3.2 Exosome Isolation from Urine by Precipitation with PEG

Urine was transferred to 50 ml falcon and centrifuged at 3000xg for 30 minutes at 4°C in order to get rid of apoptotic blebs. Supernatant was transferred into a fresh falcon and 2.5 ml 40% Polyethylene glycol (PEG) per 10 ml urine was added so that PEG was 1:5 diluted. It was vortexed vigorously and incubated for 1.30 hours while shaking at RT. Urine-PEG mixture was vortexed again and centrifuged at 4000xg for 1 hour at 4°C. Supernatant was aspirated and pellet was dissolved in 1X DPBS.

2.2.4 Exosome Isolation from Cell Culture Supernatant

Collected cell culture media was vortexed vigorously and centrifuged at 1500xg for 10 minutes. Pellet was discarded and supernatant was transferred into an ultracentrifuge tube. Volume was completed upto 35 ml with DPBS and centrifuged at 10000xg for 10 minutes. Pellet was discarded again and supernatant was transferred into a fresh ultracentrifuge tube. It was centrifuged at 30000xg for 30 minutes. After this step, pellet contains microparticles so that supernatant was transferred into a ultracentrifuge tube for exosome isolation. Supernatant was ultracentrifuged at 100000xg for 90 minutes. Supernatant was discarded by aspiration, pellet was completed to 35 ml and it was washed at 100000xg for 90 minutes. Finally, supernatant was discarded and pellet was dissolved in 100 µl DPBS.

2.2.5 Examination of Exosomal Protein Content

BCA Protein Assay Kit (#23225, Thermo Fischer Scientific, US) was performed according to the manufacturer's protocol.

Pierce BCA Protein Assay ol. BSA standard found in the kit was serially diluted starting from 2000 µg/ ml to 2 µg/ ml. The standards were placed into the 96-well plate as 25 µl/well and samples as 5 µl/well. Working solution was prepared and added into the wells as 200 µl/well. Then, the plate was incubated in dark at 37°C for 30 minutes. The plate was cooled off at RT for 15 minutes and absorbance at 562 nm was measured with Synergy HT microplate reader. (Biotek, Winooski, VT, USA) Standard curve graph was drawn, the concentrations of the samples were calculated according to it and multiplied with 5.

2.2.6 Exosome Characterization

2.2.6.1 Flow Cytometry

Purified exosomal surface marker antibodies were used in order to coat carboxyl latex beads. (#C37282, Thermo Fisher Scientific) For 200 sample stainings, 20 µl latex bead was completed to 100 µl with DPBS and precipitated at 16000xg for 10 minutes. Supernatant was discarded and pellet was dissolved in the appropriate antibody so that the mixture had 1µl:1µg (bead:antibody) ratio. For exosome capture, purified anti-CD9 (#312102, Biolegend), anti-CD63 (#353014, Biolegend) and anti-CD81 (#349502, Biolegend) antibodies were used. 50 µl DPBS was added on the bead:antibody mixture and it was incubated while rotating for 30 minutes at room temperature. After that, the volume was increased to 500 µl with PBS and incubated overnight at RT. In the morning, bead and antibody solution was centrifuged at 16000xg for 10 minutes and dissolved in 1 ml 5% BSA solution for blocking. After incubation for 6 hours at RT, the mixture was

centrifuged again at 16000xg for 10 minutes and the pellet was resuspended in 200 μ l 1% BSA. Antibody-coated latex beads are ready to use after this step.

For staining, 1 μ g exosome was mixed with 1 μ l antibody-bead solution for PC-3 and 5 μ g exosome was mixed with 1 μ l antibody-bead solution for healthy urinary exosomes since PEG decreases binding efficiency of beads to exosomes. 50 μ l DPBS was added and the mixture was incubated at RT for 30 minutes while rotating. Upon incubation the volume was increased to 500 μ l with DPBS and the mixture was incubated overnight at RT while slowly rotating. After overnight bead-exosome incubation, solution was precipitated at 10000xg for 5 minutes and supernatant was discarded. anti-human CD9-PE (#312106, Biolegend), CD63-PE (#353004, Biolegend), CD81-PE (#349506, Biolegend) antibodies and their isotype controls; mouse IgG1, κ PE (#400112, Biolegend) and mouse IgG2b, κ PE (#402204, Biolegend) were incubated with bead-exosome solution at a concentration of 1 μ g/ml dye in 100 μ l final volume for 1 hour at RT in the dark. Finally, upon the incubation, 1 ml DPBS was added and centrifuged at 10000xg for 10 minutes. The pellet was resuspended in 100 μ l of FACS Buffer and analyzed with NovoCyte 3000 flow cytometer.

2.2.6.2 Nanoparticle Tracking Analysis (qNano)

In order to identify particle numbers and particle size dispersity of PC-3 exosomes, iZON qNano Gold (Izon Science Ltd, Christchurch, New Zealand) was used. PC-3 exosome sample isolated by ultracentrifugation was diluted 1:10 with DPBS respectively. NP150 nanopore which targets particles with 70-420 nm diameter was used and stretch of the nanopore was set to 47.00 mm. CPC100 beads were used for calibration. Particle numbers were started to be recorded when the current was stabilized.

2.2.6.3 Dynamic Light Scattering (DLS)

PC-3 and healthy/patient urinary exosomes were diluted 1:50 with DPBS. Zetasizer Nano ZS (Malvern, Worcestershire, UK) was used for determination of size distributions. During the measurements, Refractive Index (RI) was set to 1.38.

2.2.6.4 Transmission Electron Microscopy (TEM)

In sample preparation, exosomes are diluted 1:10 with DPBS. For positive staining, 2% uranyl acetate (UA) with pH:5.4 (#22400, EMS, Switzerland) is used whereas 2% phosphotungstic acid (PTA) with pH:7 (#19500, EMS, Switzerland) is used for negative staining. For this purpose, 2% uranyl acetate is prepared in 70% ethanol and 2% phosphotungstic acid (#19500, EMS, Switzerland) is prepared in boiled distilled water. Then, these solutions are filtered with 0.22 μ m filters. For each grid, 5 μ l diluted exosomes are taken and dropped on the nickel grid with 200 meshes. For placement of exosomes on the grid surface, grids with exosome droplets are incubated at room temperature for 10 minutes. Excess droplets are taken with paper and PTA, UA, and 3 distilled water drops are prepared for each grid. Grid surfaces with exosomes are placed on PTA and UA drops for 1 minute respectively for staining. Grids are protected from light since they are light-sensitive. Following the staining protocol, grids are washed on distilled water drops and excess water is discarded with paper again. Grids are left for drying out and dry grids are examined by using TEM (JEOL-JEM1400, Japan) at 80 kV with CCD camera (Gatan Inc., Pleasanton, CA, USA) with digital attachment. Sizes of exosomes are measured by using image analysis program, DigitalMicrograph TM, found in the camera system.

2.2.7 Determination of miRNA Levels in Exosomes

2.2.7.1 Exosomal miRNA Isolation

Exosomal miRNA was isolated by using the Hybrid-R miRNA Isolation Kit (#325-150, Geneall). The manufacturer's instructions for small RNA (miRNA) isolation were followed. Briefly, 500 µl RiboEx solution found in the kit is added onto exosomes and centrifugation steps in the protocol are followed. Isolated miRNA is stored at -80°C.

2.2.7.2 cDNA Synthesis

RevertAid RT Reverse Transcription Kit (#K1691, Thermo Fisher Scientific) was used for cDNA synthesis. For each reaction, equal amount of RNA was used. The only difference was that instead of random primers, stem-loop primer with 20 pM concentrations was used for each miRNA in different cDNA tubes. (Table 2.3). Synthesized cDNAs were then stored at -80°C.

2.2.7.3 qPCR

qPCR reactions were performed with LightCycler 480 SYBR Green I Master Mix by following steps in Table 2.6 (#04887352001, Roche Life Science). 10 pM miRNA-specific forward and universal reverse primers were used for each miRNA. Reagents and volumes/well of these reagents for qPCR are listed in Table 2.5. Primer information is given in Table 2.4.

Table 2.5 Reagents and volumes of them used in qPCR.

Reagent	Volume/tube
LightCycler 480 SYBR Green I Master Mix	5 μ l
Nuclease-free water	3 μ l
10 μ M Forward Primer	0.5 μ l
10 μ M Reverse Primer	0.5 μ l
cDNA	1 μ l
Total Volume	10 μ l

Table 2.6 qPCR conditions used in the experiments.

qPCR Step	Temperature	Duration	Cycle Number
Initial Denaturation	95°C	5 minutes	1
Denaturation	95°C	15 seconds	45
Annealing	58°C	20 seconds	
Elongation	72°C	20 seconds	
Extension	72°C	5 minutes	1
Hold	4°C	∞	∞

Protocol for isolation and analysis of urinary exosomal miRNAs were shown in Figure 2.2.

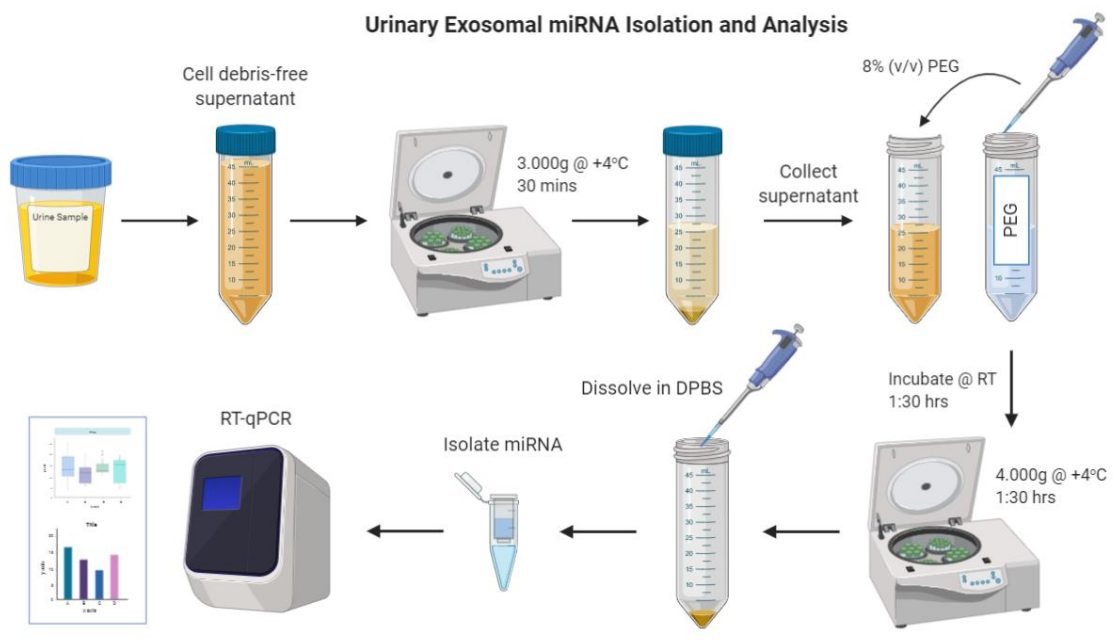


Figure 2.2 General scheme summarizing isolation and analysis of the urinary exosomes.

2.2.8 ROC Curve Generation

Receiver Operating Characteristic (ROC) Curve for each miRNA was generated with Graphpad Prism 6 Software. For drawing ROC curves in order to test diagnostic ability of miRNAs when they are used as a combination, binary logistic regression was performed with SPSS and then calculated predicted probabilities were used in ROC Curve analysis in Graphpad.

2.2.9 Statistical Analyses

Non-parametric t-test was performed with Graphpad Prism 6 Software (Graphpad Software Inc., San Diego, CA, USA) for comparison of relative expressions of miRNAs in healthy urinary and PC-3 exosomes. For statistical analysis of miRNA expressions of

healthy and patient urinary exosomes, Mann-Whitney test was used since the data was not normally distributed according to Column Statistics. In order to compare miRNA levels in exosomes of patients with different PSA levels and healthy exosomes, ANOVA and Multiple Comparisons tests were used in Graphpad. Finally, in order to calculate predicted probabilities for miRNA to be used in ROC curve generation, binary logistic regression was performed with SPSS (IBM Corp., Armonk, NY, USA).



Chapter 3

Results

3.1 Characterization of PC-3 and Urinary Exosomes

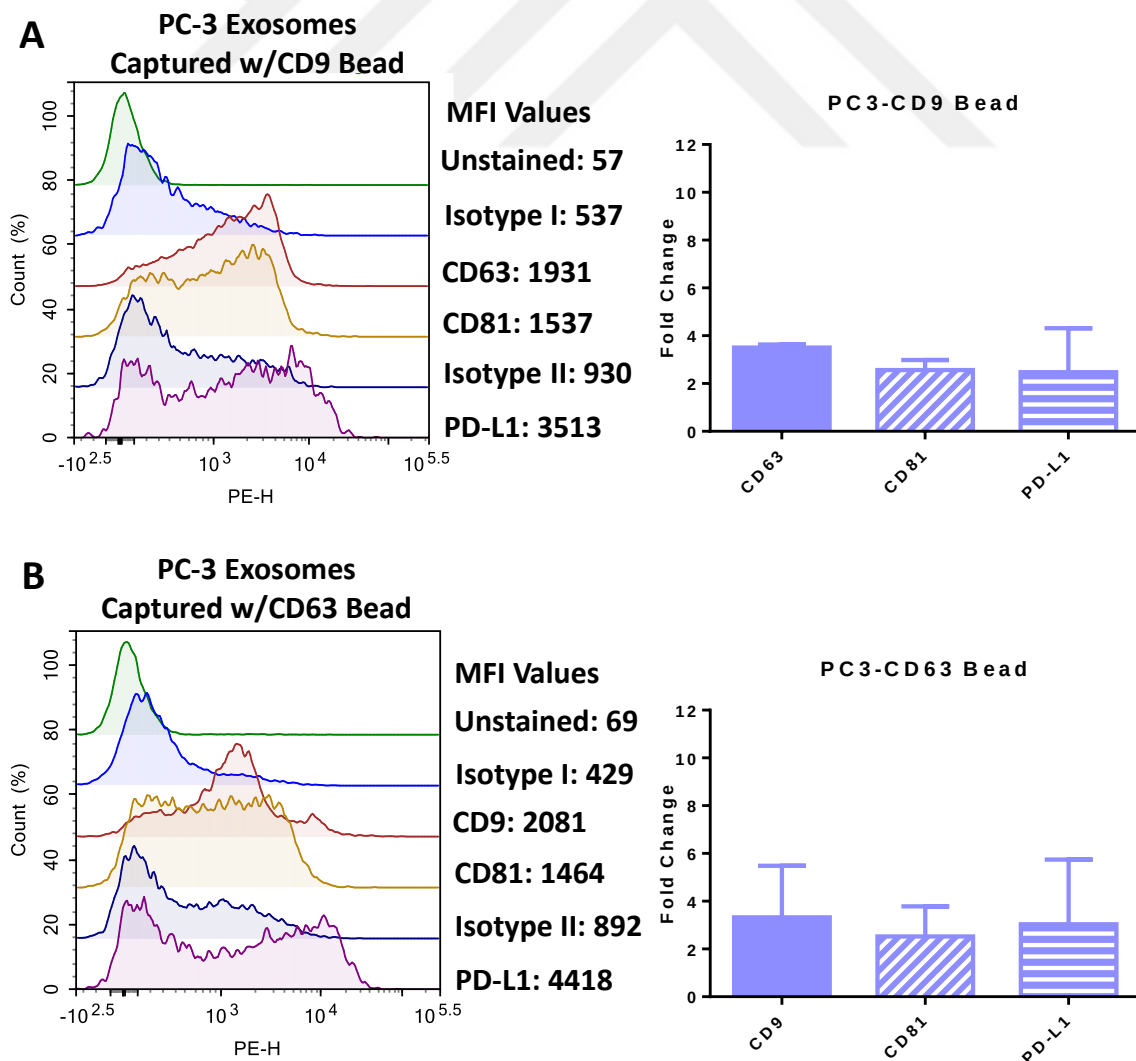
3.1.1 Surface Protein Characterization

Tetraspanin proteins such as CD9, CD63 and CD81 are known to be found in the membrane of the exosomes and they can be used as exosome surface markers [168]. Therefore, we wanted to validate that PC-3 exosomes isolated by ultracentrifuge and urinary exosomes isolated by precipitation with PEG carry these tetraspanins. Since healthy and patient exosomes were isolated by the same method, we assumed that if healthy exosomes could be stained for the exosome markers, patient exosomes would also be positive for them. For investigation of surface proteins in exosomes via flow cytometry, exosomes should be captured with carboxyl latex beads because of their small sizes. For this reason, we captured PC-3 and healthy exosomes with CD9 and CD63 beads and stained the exosomes with CD9, CD63 and CD81 antibodies as described in Section 2.2.6.1. This experiment was made as duplicates and representative histograms and mean fold change bar graphs were shown in Figure 3.1.

When PC-3 exosomes were captured with CD9 and CD63 beads, mean fluorescence intensities (MFI) of CD9, CD63 and CD81 were found to be higher than isotype control I (mouse IgG1, κ -PE) which means that PC-3 exosomes were positive for these 3 surface markers. Similarly, healthy urinary exosomes also had higher MFI values than isotype controls for these 3 markers in their surface. To be more specific, when PC-3 exosomes were captured with CD9 bead, it had 3.5 and 2.6 folds higher MFI values compared to isotype for CD63 and CD81. Additionally, CD9 and CD81 fold changes were 3.3 and 2.5 respectively when PC-3 exosomes were captured with CD63 bead (Figure 3.1A and B). MFI value fold changes of healthy urinary exosomes when captured with CD9 bead were found to be 10.4 when stained with CD63 and 2.1 when stained with CD81. When these

exosomes were captured with CD63 bead, CD9 and CD81 MFI fold changes were 2.15 and 1.8 respectively (Figure 3.1C and D) .

Programmed death ligand 1 (PD-L1) is an immunomodulatory protein which binds to the PD-1 receptor and represses the activation of T cells and regulates immunological homeostasis. In cancer, PD-L1 binding to PD-1 on activated T-cells facilitates immune escape. Recent studies have shown that PD-L1 expression was high in tumor cells. PD-L1 expression in PC-3 is constitutive and healthy urinary exosome should also contain PD-L1 in its surface [169]. We hypothesized that both PC-3 exosomes and healthy urinary exosomes should carry PD-L1. In order to check exosomal PD-L1 expression in cancer cell line PC-3 and healthy urinary exosomes, we stained CD9/63 beads-captured PC-3 and healthy urinary exosomes with PD-L1-PE antibody. PC-3 and healthy urinary exosomes had both higher MFI values than isotype II (mouse IgG2B, κ -PE), for CD9 and CD63 captured exosomes. Of note, when we compared PDL-1 MFI fold changes in CD9 and CD63 bead-captured PC-3 and healthy exosomes, there was not a significant difference (Figure 3.1).



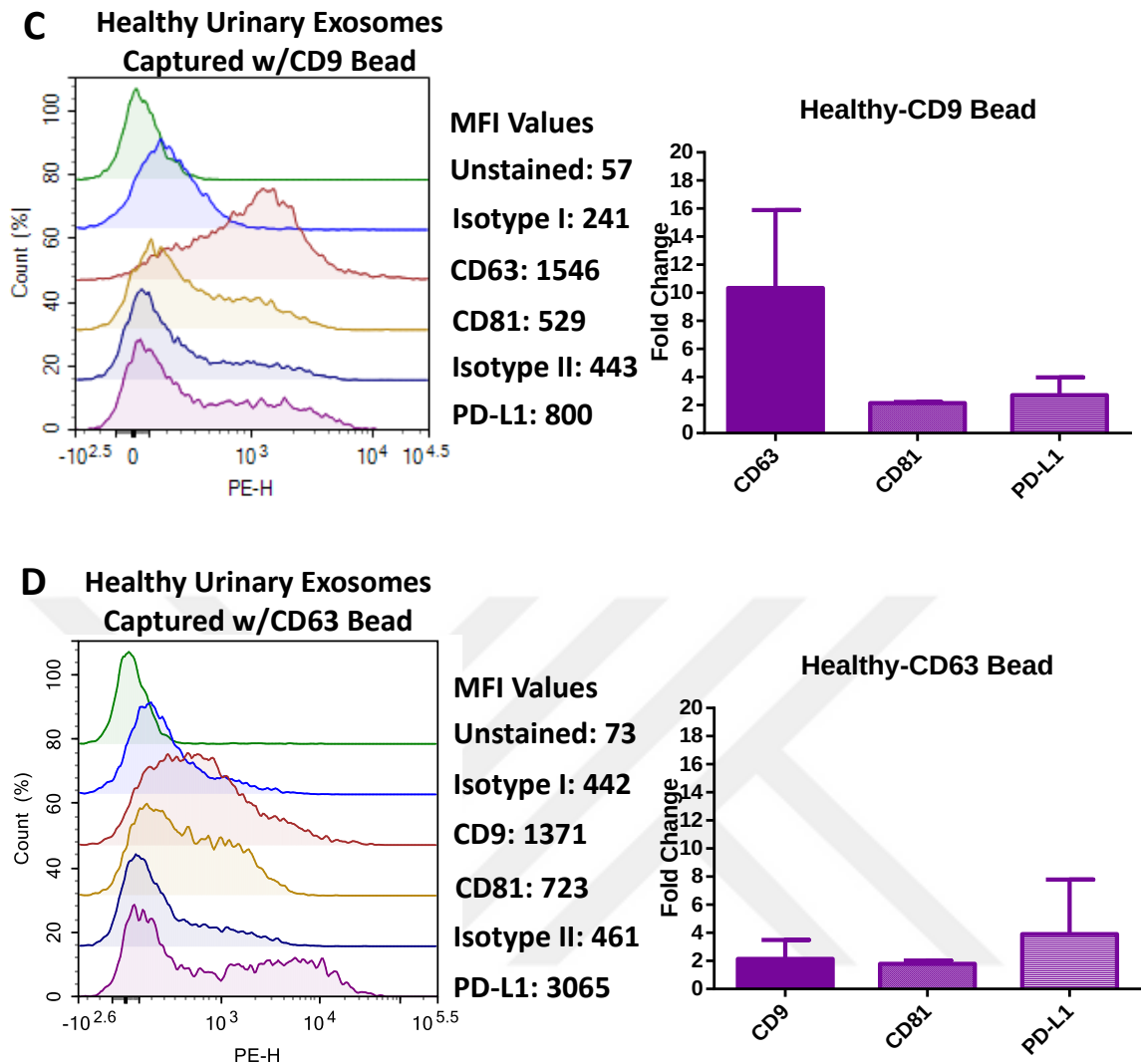


Figure 3.1 Presence of exosome-specific markers and PD-L1 in PC-3 exosomes and healthy urinary exosomes. Representative histograms and mean fold change bar graphs of presence of surface marker tetraspanins; CD9, CD63 and CD81 in (A) PC-3 cell line exosomes isolated by ultracentrifuge and captured with CD9 bead and (B) CD63 bead. (C) Healthy urinary exosomes isolated by precipitation with PEG and captured with CD9 bead and (D) CD63 bead. Exosomes were captured with CD9/63 beads and then stained for exosome-specific markers and PD-L1.

3.1.2 Size Distribution of Exosomes

Size distribution of PC-3 exosomes was investigated via iZON qNano Gold (Section 2.2.6.2), and dynamic light scattering (DLS) (Section 2.2.6.3). According to qNano results, mean diameter of PC-3 exosomes was 119 nm and concentration of the exosomes was detected as 3.96×10^{11} particles/ml. Mode of the particle diameter for PC-3 exosome was found to be 88 nm (Figure 3.2A). Similarly, in DLS results, PC-3 exosome mean diameter was 132.5 nm and single peak was observed for sizes of particles when these exosomes. Three reads were taken for each sample in DLS and representative graphs are depicted in Figure 3.2B, C, D.

Particle sizes of healthy and patient urinary exosomes were also detected via DLS. Similar to PC-3 exosomes, large population of the particles in the healthy and patient urinary exosomes had diameters close to 100 nm. However, diameters of urinary exosomes were smaller than PC-3 exosome diameters when the large population of exosome samples were taken into consideration. (Figure 3.2B, C, D). Since peaks with higher than 200 nm were observed due to characteristic of PEG to make clumps of exosomes, mean diameter size would not be appropriate for analysis of exosome sizes isolated by PEG. Rather, taking sizes of the large population of the sample into consideration would be accurate.

We also wanted to visualize the PC-3 and healthy/patient urinary exosomes. For this reason, we performed Transmission Electron Microscopy (TEM) with these samples. TEM images confirmed that PC-3, healthy and patient urinary exosomes had sizes in exosome range which is 30-150 nm (Figure 3.3). Although backgrounds of the healthy and patient urinary exosomes' TEM images were crowded because of PEG, exosomes could have been selected. TEM images revealed that PC-3 exosomes formed clumps and the representative image shows the clump formed by many exosomes. The same thing was true for patient urinary exosomes; they were also observed as a clump in contrast to healthy urinary exosomes which were placed as single exosomes (Figure 3.3).

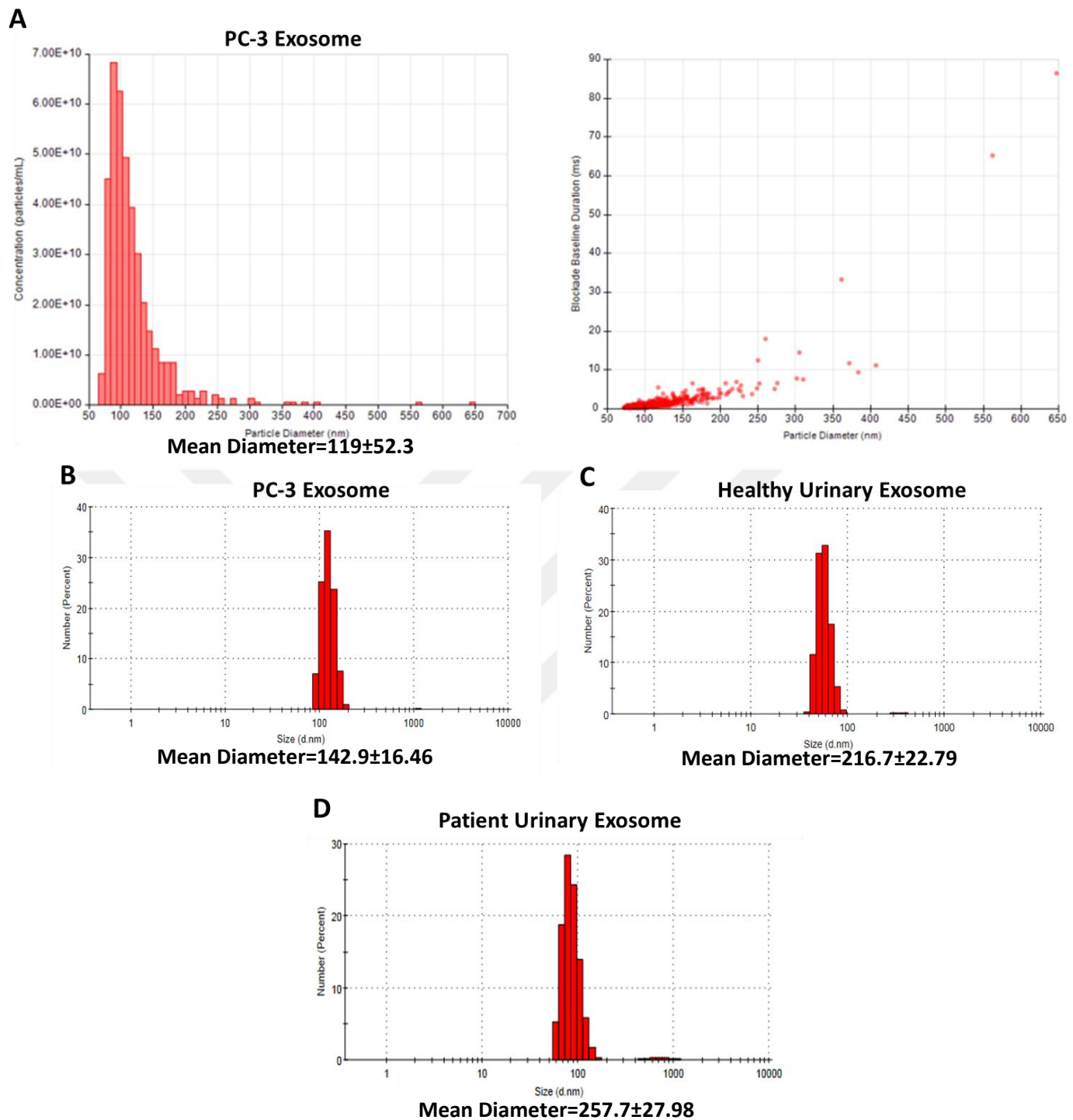


Figure 3.2 Size distributions of PC-3 exosomes, healthy and patient urinary exosomes. Mean particle diameter and particle concentration of (A) PC-3 cell line exosomes investigated via qNano. Representative size distributions of DLS reads for (B) PC-3 cell line exosomes, (C) healthy urinary exosomes and (D) patient urinary exosomes examined. The data are representative of 3 reads for DLS. Sizes of exosomes are represented under the graphs as Average $\pm$ SD.

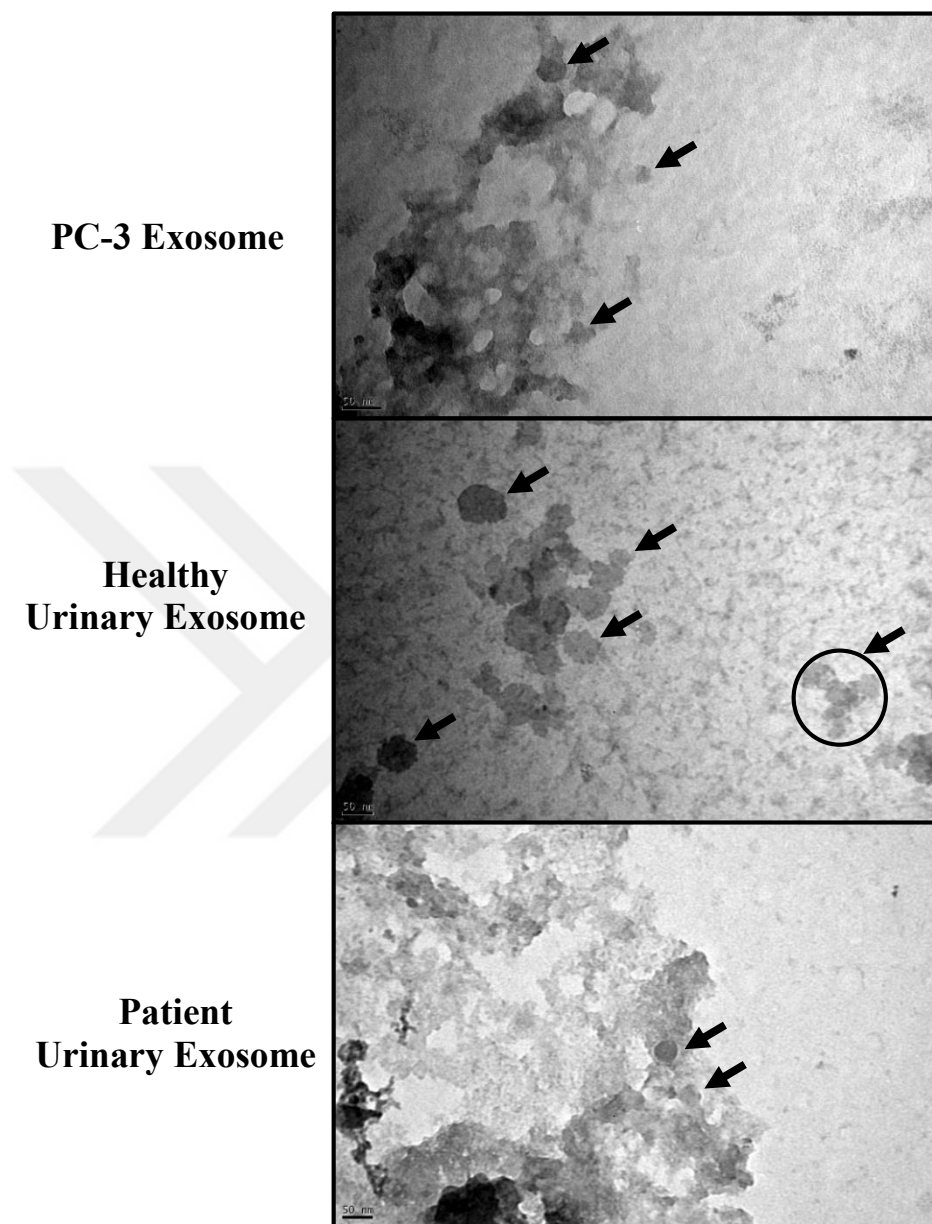


Figure 3.3 . Representative Transmission Electron Microscopy (TEM) images of PC-3, healthy urinary and patient urinary exosomes. Exosomes were stained with uranyl acetate (positive stain) and phosphotungstic acid (negative stain) and investigated via TEM

Exosome diameters were measured on TEM images as described in Section 2.2.6.4 and shown in Table 3.1. The smallest mean width and length diameters belonged to patient urinary exosomes followed by PC-3 exosomes and healthy urinary exosomes respectively. The same pattern was observed in median diameters of exosomes. They all had mean diameters in the range of exosomes which is 30-150 nm as well as median, minimum and maximum diameters which confirms efficient exosome isolation.

Table 3.1 Exosome diameters (in width and length scales) of PC-3 exosomes, healthy and patient urinary exosomes according to TEM images. PC-3 and healthy/patient exosomes were visualized via TEM and their diameters were measured.
Exo=Exosome

		Mean (nm)	Median (nm)	Minimum (nm)	Maximum (nm)	Standard Deviation (nm)
PC-3 Exo	Width	65.4	66.9	34.8	90.4	16.0
	Length	66.0	67.6	51.7	77.2	10.5
Healthy Exo	Width	93.3	91.8	49.8	146.6	25.0
	Length	70.0	70.1	57.2	82.6	10.4
Patient Exo	Width	49.1	46.3	34.6	86.4	13.0
	Length	42.7	42.7	42.2	43.2	0.5

3.2 Determination of miRNA Levels in PC-3 Exosomes

Candidate miRNA levels of prostate cancer cell line PC-3 was firstly compared with healthy exosomal miRNA levels. Since PC-3 is a prostate cancer cell line with metastasis to bone, PC-3 experiments might give a good preliminary data before making experiments on patient urine specimens [170]. In this aspect, morning urine specimens from 14 healthy individuals were collected and urinary exosome was isolated by precipitation with PEG. (Section 2.2.2 and Section 2.2.3.2) From PC-3 cell line supernatant, exosome was isolated by ultracentrifugation method (Section 2.2.4) Then, miRNAs were isolated and their relative expressions were determined by RT-qPCR by using efficiency-corrected CT method. (Section 2.2.7) miRNAs can be investigated as tumor-suppressor miRNAs expected to be downregulated in PC-3 cell line and oncogenic miRNAs expected to be upregulated in PC-3 cell line compared to healthy urinary exosomes. Candidate miRNAs were selected by analysis of the literature. (Section 1.3.2.2) At first, more than 10 candidate miRNAs were picked as candidates. However, some of them were eliminated from our panel due to low primer efficiency. From the remaining miRNAs we continued our analyses with the most efficient and future-promising candidates. PC-3 exosomal miRNA level detection experiments were performed as triplicates.

When tumor-suppressor miRNAs in prostate cancer were examined, the data showed that hsa-miR-107, hsa-miR-139-5p, hsa-miR-145-5p were significantly downregulated as expected. ($p \leq 0.05$) (Figure 3.4B, C and D). Moreover, the data revealed that the most downregulated miRNA was has-miR-204-5p with the significance $p \leq 0.01$ (Figure 3.4E). On the other hand, no significant difference was observed in hsa-miR-30a-5p expression in comparison to healthy controls (Figure 3.4A).

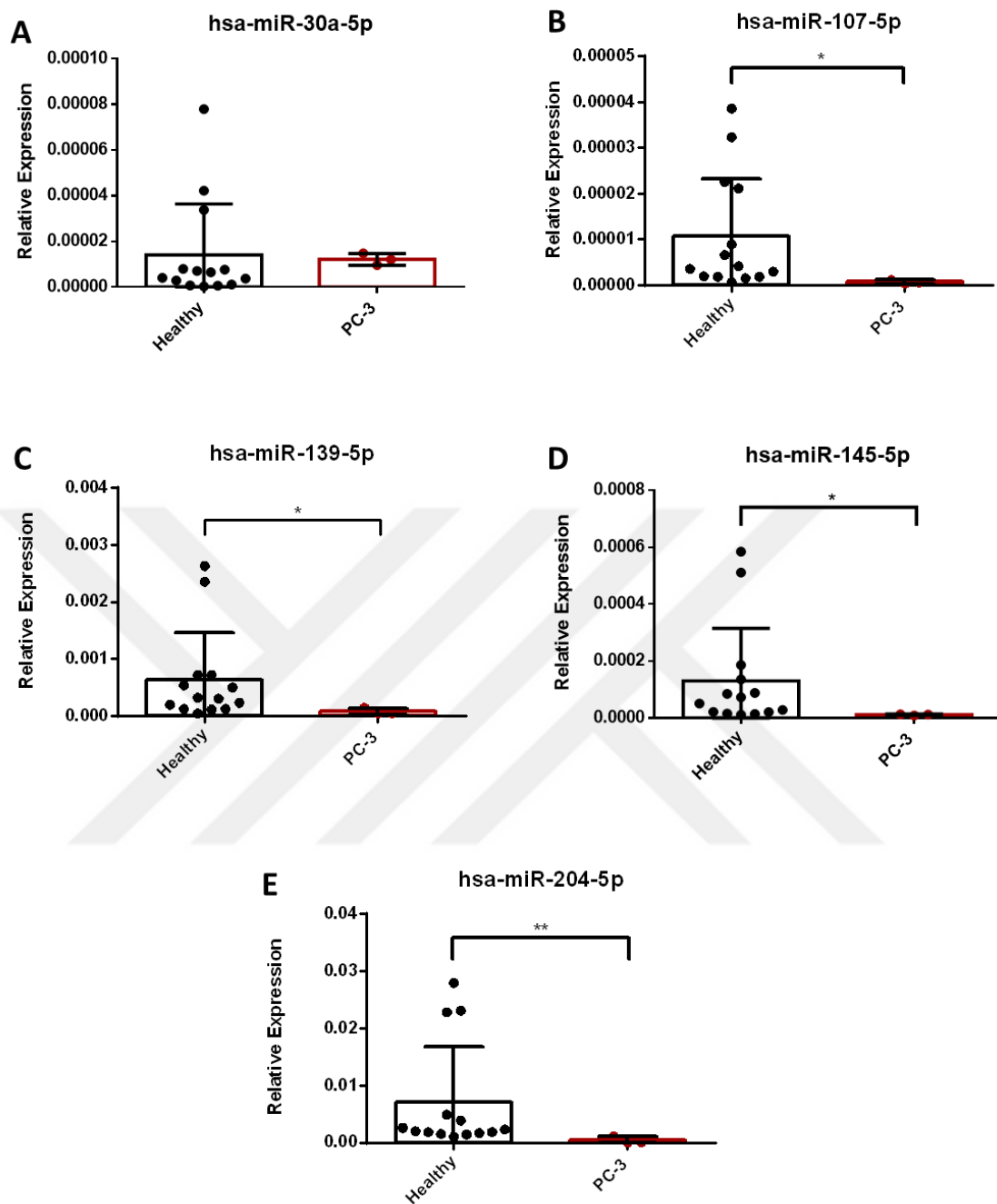


Figure 3.4 Expressions relative to U6 snRNA of tumor suppressor microRNAs in healthy control urinary exosomes and PC-3 exosomes. Levels of (A) hsa-miR-30a-5p (B) hsa-miR-107 (C) hsa-miR-139-5p (D) hsa-miR-145-5p and (E) hsa-miR-204-5p in healthy urinary exosomes (n=14) and PC-3 exosomes (n=3) were detected by RT-qPCR. The experiment was performed as duplicates. * $p \leq 0.05$ ** $p \leq 0.01$

Changes of oncogenic miRNAs were also important as potential biomarkers so we analyzed expression levels of candidate prostate cancer oncogenic miRNAs in PC-3 and healthy urinary exosomes. hsa-miR-21-5p and hsa-miR-375-5p were significantly upregulated ($p \leq 0.01$) (Figure 3.5A and B). Another oncogenic miRNA, hsa-miR-3-574-3p was also upregulated but no significance could be detected statistically (Figure 3.5C).

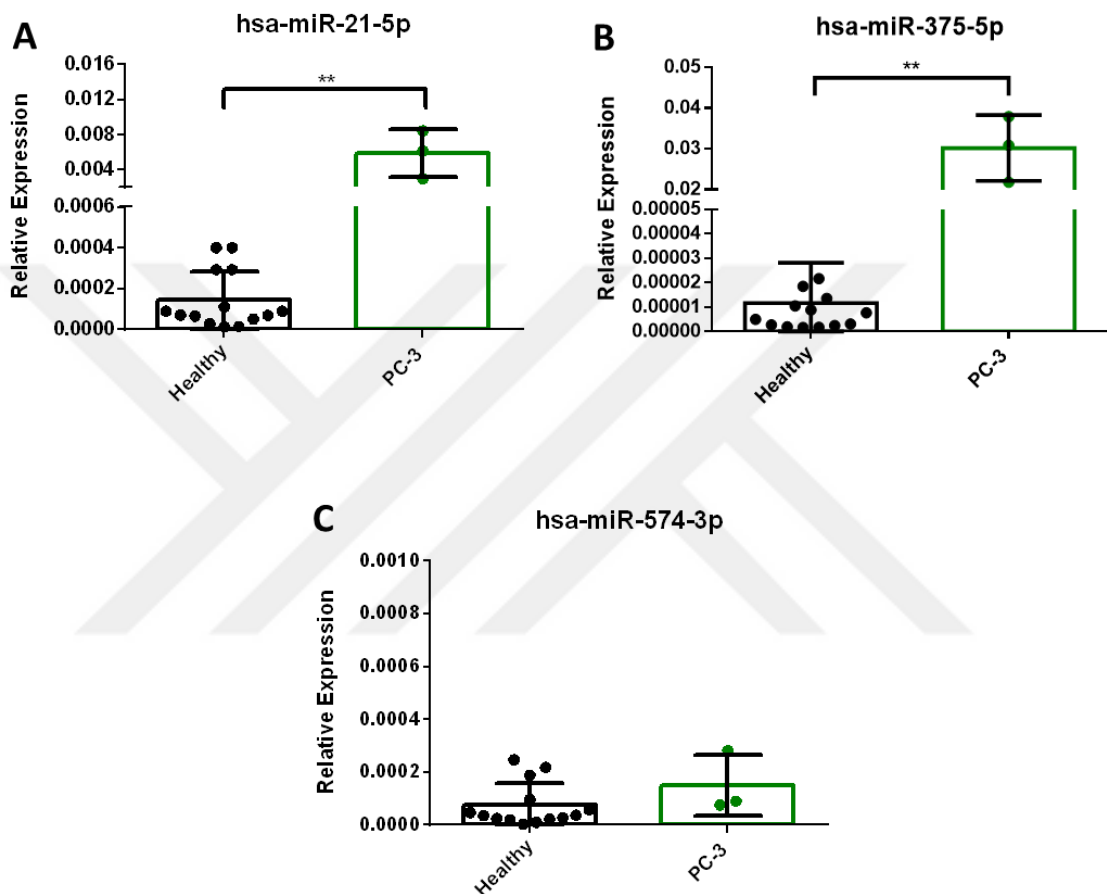


Figure 3.5 Expressions relative to U6 snRNA of oncogenic microRNAs in healthy control urinary exosomes and PC-3 exosomes. Levels of (A) hsa-miR-21-5p (B) hsa-miR-375-5p and (C) hsa-miR-574-3p in healthy urinary exosomes (n=14) and PC-3 exosomes (n=3) were detected by RT-qPCR. The experiment was performed as duplicates. ** $p \leq 0.01$

3.3 Determination of miRNA Levels in Prostate Cancer Patients' Exosomes

3.3.1 Cohort Profile in This Study

After miRNA deregulation studies made with in PC-3 exosomes, future promising results were obtained and we started to collect prostate cancer patients' urine samples in order to isolate exosomes and identify their miRNA profile. (Section 2.2.3) For this reason, morning urine samples of 17 prostate cancer patients were obtained. Before the experimental procedures, age, total PSA level (ng/ml) and Gleason Score information of these patients were used in order to study correlations of these 3 parameters with each other.

Profile of the patients in this study is described in Table 3.2. Age distribution of patients were similar; 6 patients with ages between 60-65, 7 patients with ages between 65-70 and 5 patients with ages between 70-75 were participating our study. Total PSA levels (ng/ml) and Gleason Scores were also given in the table.

Table 3.2 Profile of the patients in this study. Age, total PSA level (ng/ml) and Gleason Score information of the patients in our cohort.

Age	Total PSA Level (ng/ml)			Gleason Score			
	<10	10-15	>15	6	7	8	9
60-65	5	1	0	2	2	1	1
65-70	5	0	2	1	5	1	0
70-75	0	4	1	3	1	1	0

There were many researches about correlation of age and PSA level of the patients so we wanted to start with analyzing age-PSA correlation in our cohort [171-173]. We observed that, in general, as the age increases, PSA levels of our patients increase. PSA levels of patients with 66-70 ages and other 2 groups of patients (60-65 and 71-75 ages) had no significant difference. However, patients with ages of 60-65 had significantly lower PSA values compared to patients with ages of 71-75 (Figure 3.6A).

Next, we analyzed if PSA was an efficient biomarker for the progression of prostate cancer so we tested the correlation of PSA with Gleason Score. There was almost no correlation between these 2 parameters in our cohort with $R^2=0.0285$ (Figure 3.6B).

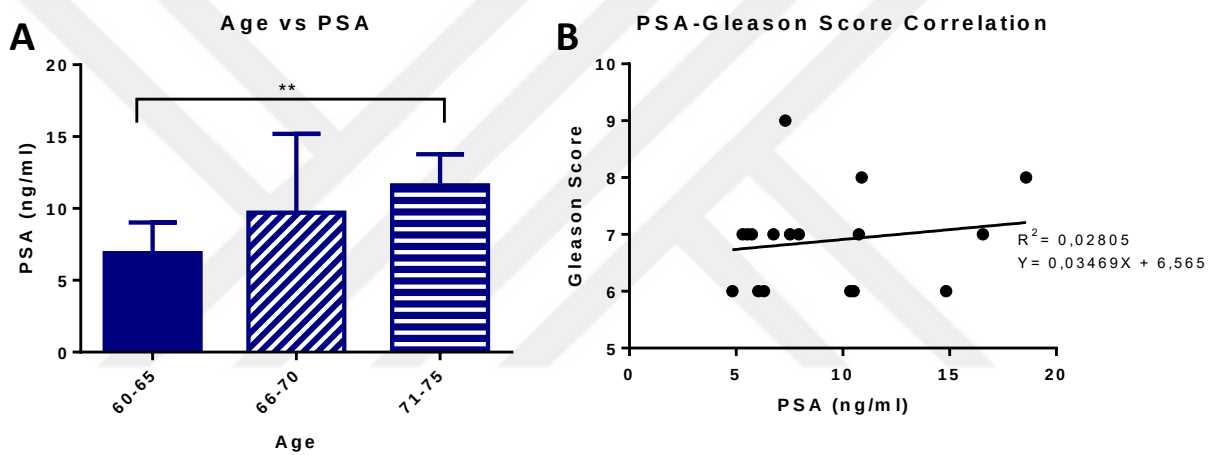


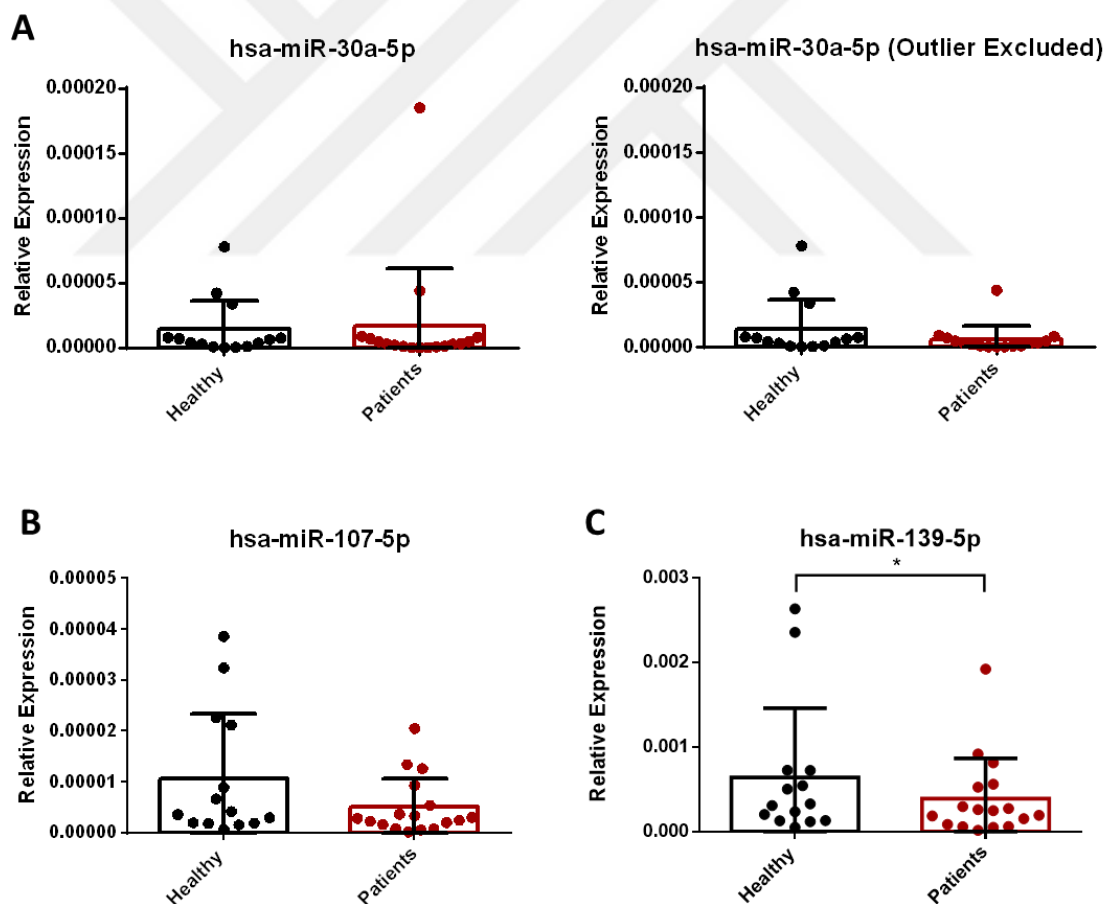
Figure 3.6 Age-PSA and PSA-Gleason Score Correlations of the patients. (A) Correlation of PSA levels and ages of the patients (n=17). $p \leq 0.01$ (B) PSA and Gleason Score correlation of the patients (n=17). $**p \leq 0.05$

3.3.2 miRNA Levels in Patients' Urinary Exosomes

For the purpose of determining candidate miRNA levels in urinary exosomes, exosome was isolated from urine specimens of 17 prostate cancer patients with adenocarcinoma and 14 healthy controls. (Section 2.2.2.2) Then, miRNA levels were detected by RT-qPCR. (Section 2.2.6) Again, as in the PC-3 cell line studies, at the beginning of the patients' study, we had more candidate miRNAs but some of them were eliminated due

to low primer efficiency and reliability. Same tumor suppressor and oncogenic miRNAs were investigated with PC-3 exosomes for patients.

In expression of tumor suppressor miRNAs, similar pattern was observed with PC-3 cell line (Compare Figure 3.7 and 3.4). hsa-miR-107 and hsa-miR-204-5p were downregulated in prostate cancer patients although no significance was detected statistically (Figure 3.7B and E). On the other hand, hsa-miR-139-5p and hsa-miR-145-5p were significantly downregulated in patients with $p \leq 0.05$ and $p \leq 0.01$ respectively (Figure 3.7C and D). Almost no downregulation was observed in hsa-miR-30a-5p similar to PC-3 cell line. However, one outlier was identified for miR-30a and when it was excluded, downregulation in hsa-miR-30a-5p was observed (Figure 3.7A). Patient and healthy urinary exosomal miRNA level detection experiments were performed as duplicates in all experiments.



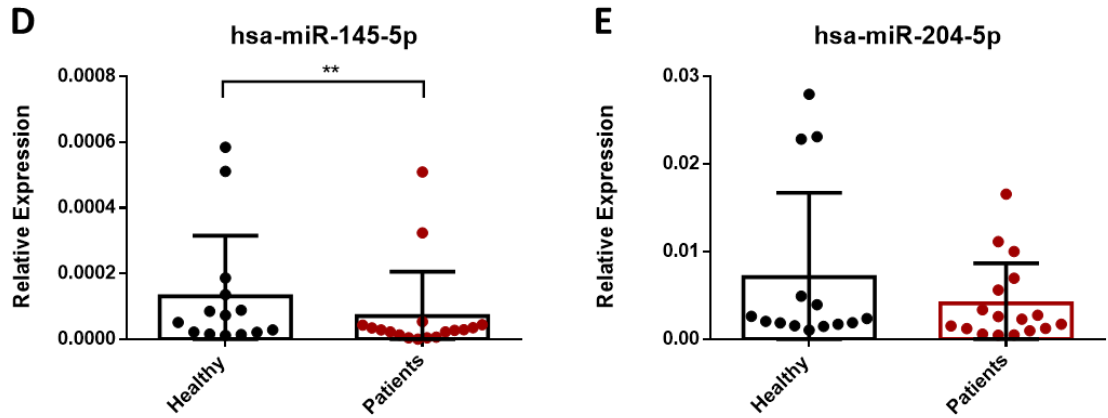


Figure 3.7 Expressions relative to U6 snRNA of tumor suppressor miRNAs in healthy control urinary exosomes and patient urinary exosomes. Levels of (A) hsa-miR-30a-5p (B) has-miR-107 (C) hsa-miR-139-5p (D) hsa-miR-145-5p and (E) hsa-miR-204-5p in healthy (n=14) and prostate cancer patient urinary exosomes (n=17) were detected by RT-qPCR. The experiment was performed as duplicates. * $p \leq 0.05$ ** $p \leq 0.01$

When the oncogenic miRNA levels were compared between prostate cancer patients and healthy controls, the highest upregulation was observed at hsa-miR-21-5p and has-miR-375-5p (Figure 3.8A and B). miR-574-3p was almost not upregulated.

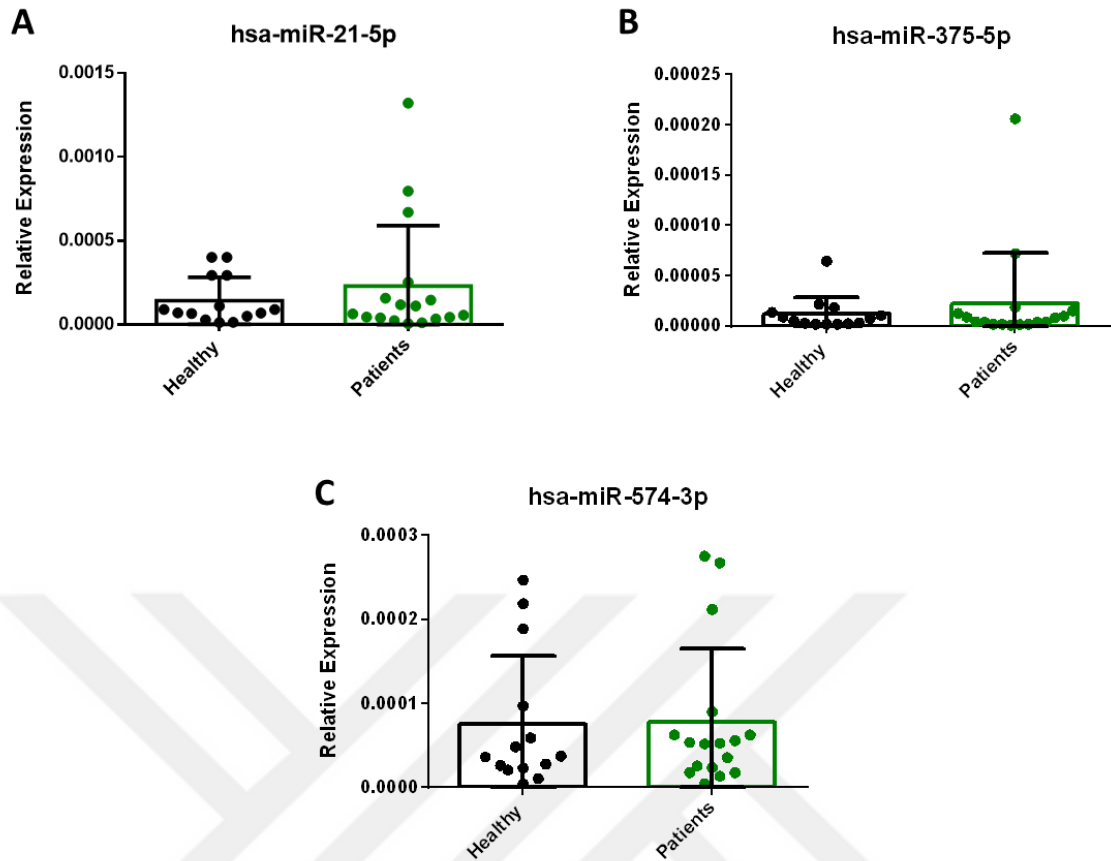


Figure 3.8 Expressions relative to U6 snRNA of oncogenic miRNAs in healthy control urinary exosomes and patient urinary exosomes. Levels of (A) hsa-miR-21-5p (B) hsa-miR-375-5p and (C) hsa-miR-574-3p in healthy (n=14) and patient urinary exosomes (n=17) were detected by RT-qPCR. The experiment was performed as duplicates.

Following identification of relative expression levels in PC-3 exosomes and healthy/patient urinary exosomes, we wondered if fold changes of the candidate miRNAs were similar for PC-3 and patients' urinary exosomes and if miRNA up/downregulation patterns were compatible with our hypothesis; upregulation for oncomiRs and downregulation for tumor suppressor miRNAs. For this reason, we calculated fold changes for each miRNA and compared them for PC-3 and patients' urinary exosomes. Of note, for hsa-miR-30a-5p, outlier in prostate cancer patients was included so n=14 for healthy controls and n=17 for prostate cancer patients.

For hsa-miR-30a-5p expression, there was almost no change in expression of miR-30a-5p in PC-3 and patients' comparison to healthy controls. Other tumor-suppressor miRNAs;

hsa-miR-107, hsa-miR-139-5p, hsa-miR-145-5p and hsa-miR-204-5p were downregulated in both PC-3 cell line exosomes and prostate cancer patients' urinary exosomes (Figure 3.9). For oncogenic miRNAs, we concluded that they were all upregulated in PC-3 exosomes and only expression of miR-574-3p was not upregulated in patient urinary exosomes but other oncomiRs were upregulated whereas miR-21 was very slightly upregulated. Especially hsa-miR-21-5p and hsa-miR-375-5p were upregulated with high fold changes in PC-3 exosomes. In general, fold changes of miRNA expressions were higher in PC-3 exosomes than patients' exosomes (Compare Figure 3.9A and B). To sum up, expression up/downregulation patterns of miRNAs in PC-3 exosomes and prostate cancer urinary exosomes were similar.

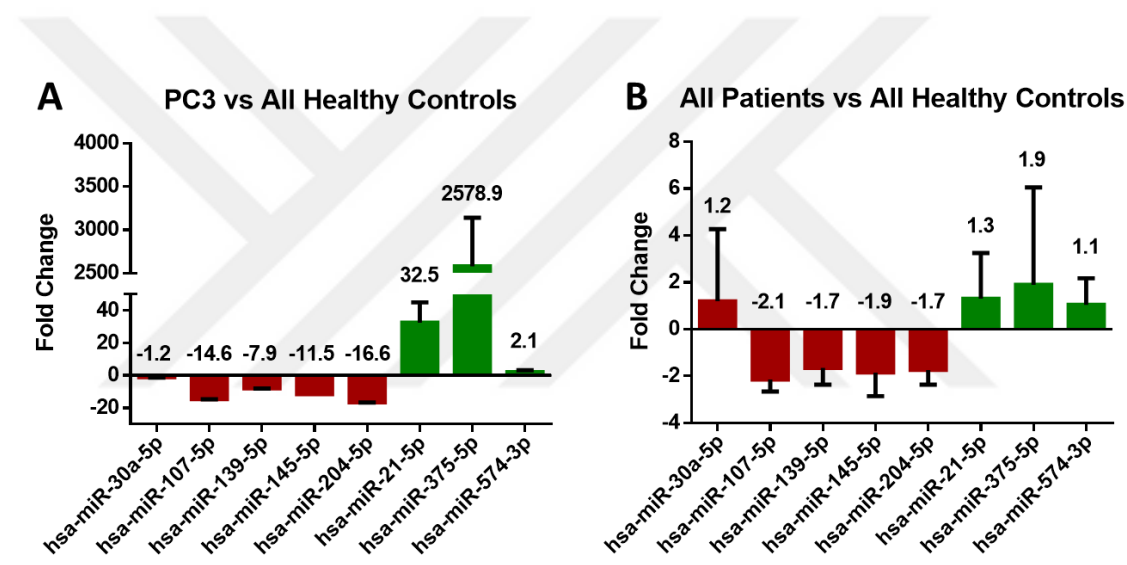


Figure 3.9 Fold changes of relative expression levels of candidate miRNAs for PC-3 and patients' urinary exosomes. Fold changes of miRNA levels for (A) PC-3 exosomes (n=3) vs healthy urinary exosomes (n=14) (B) Patient (n=17) vs healthy urinary exosomes (n=14).

3.3.3 Diagnostic Ability of Candidate miRNAs

Expression up/downregulation of candidate miRNAs were identified. However, we wanted to know if these miRNAs were effective and sufficient in discriminating patients from healthy controls in our cohort. In order to test the diagnostic ability of our candidate miRNAs, Receiver Operating Characteristic (ROC) Curve was drawn for miRNAs. (Section 2.8) At first, the miRNAs were investigated in 2 classes like in their expression analyses; tumor-suppressor and oncogenic miRNAs. Then, combination of these miRNAs were analyzed for their diagnostic ability by firstly calculating predicted probabilities by binary logistic regression for each miRNA and then using predicted probabilities for generating a ROC Curve. (Sections 2.7 and 2.8) In ROC Curve, y-axis (Sensitivity%) corresponds to true-positive rate whereas x-axis (100%-Specificity%) corresponds to false-positive rate. So that as sensitivity and specificity of the test increases, curve gets closer to the y-axis and 90° shape [174]. Area Under Curve (AUC), is the scale measure of the candidate biomarker performance and increases as the potency of the biomarker increases [175]. AUC values for each miRNA and their combinations were calculated.

When tumor-suppressor miRNAs in prostate cancer (expected to be downregulated in prostate cancer) were investigated separately, the most potent candidate miRNA was found to be hsa-miR-107 with AUC of 0.65 and the least potent miRNA was has-miR-30a-5p with AUC of 0.5756 Moreover, when these tumor-suppressor miRNAs were examined as a combination, so if all these tumor suppressor miRNAs were used in testing, AUC was calculated as 0.6345 (Figure 3.10A). So that if the tumors-suppressor miRNAs were used as a combination in our cohort, we would be able to distinguish patients from healthy controls with 64% accuracy. Then, oncomiRs were analyzed in terms of diagnostic ability. AUC values of them were very close to 0.5 and they almost did not change if they are used as a combination (Figure 3.10B). For oncomiRs, we can say that testing of expression of these oncomiRs only for diagnosis of prostate cancer would not give accurate results in our cohort.

Finally, power of combination of all miRNA candidates (including tumor suppressors and oncomiRs) except for hsa-miR-30a-5p were investigated for diagnosis of prostate cancer (Figure 3.10B). hsa-miR-30a-5p was excluded because it was not downregulated in patients in contrast to our expectation (Figure 3.9). When the tumor suppressor miRNAs and oncomiRs were used as a combination, AUC was 0.7731 with $p\text{-value} \leq 0.01$ so if we

used our miRNA panel excluding miR-30a, we were able to distinguish patients from healthy individuals with 77% accuracy in our cohort.

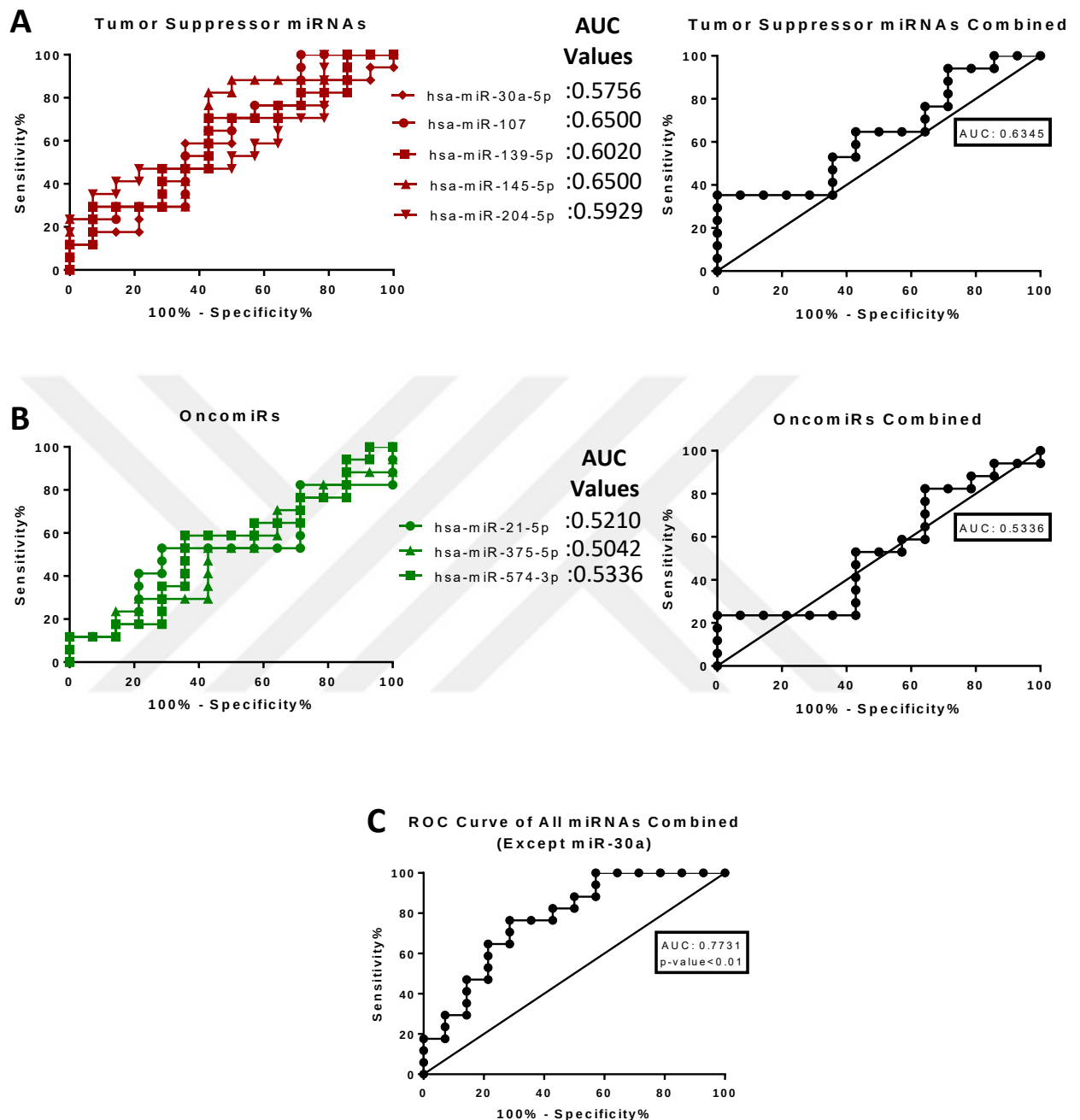
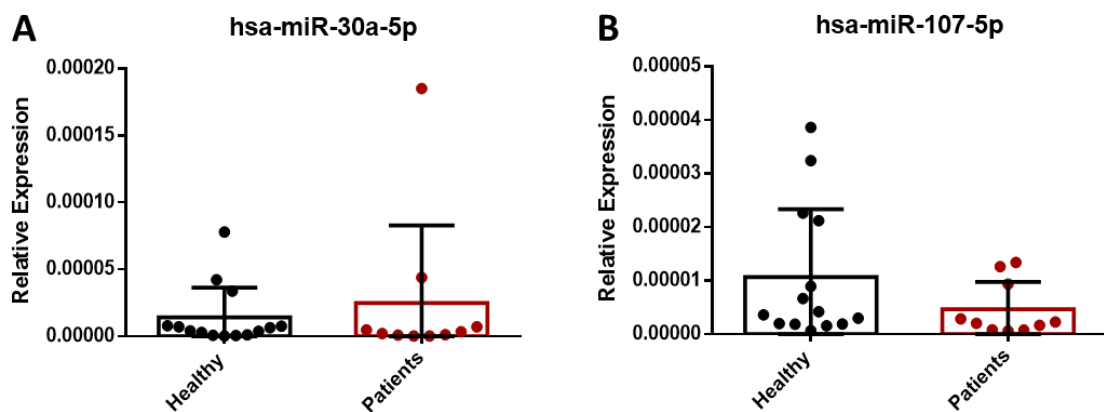


Figure 3.10 Diagnostic ability of candidate miRNAs. ROC Curves of (A) Tumor suppressor (B) Oncogenic miRNAs in prostate cancer patients as single and as combination. (C) Diagnostic ability of combination of all candidate miRNAs (tumor suppressor and oncogenic miRNAs) as a combination except for miR-30a. n=14 for healthy controls and n=17 for patients.

3.3.4 miRNA Levels in Urinary Exosomes of Patients with Different PSA Levels

Detection of miRNA deregulation in prostate cancer patients led us to study patients in groups according to their PSA levels because miRNA up/downregulation in patient exosomes with different PSA groups would cause different miRNA panels for different PSA groups. For this purpose, we divided our 17 prostate cancer patients in 3 groups; patients with PSA<10 ng/ml, patients with PSA=10-15 ng/ml and PSA>15 ng/ml.

In urinary exosomes of patients with PSA<10 ng/ml, all tumor suppressor miRNAs in prostate cancer except for hsa-miR-30a-5p were shown to be downregulated (Figure 3.11). This result is complementary with tumor suppressor miRNA expression data when patients were not grouped according to their PSA levels (Compare Figure 3.11A and 3.7A).



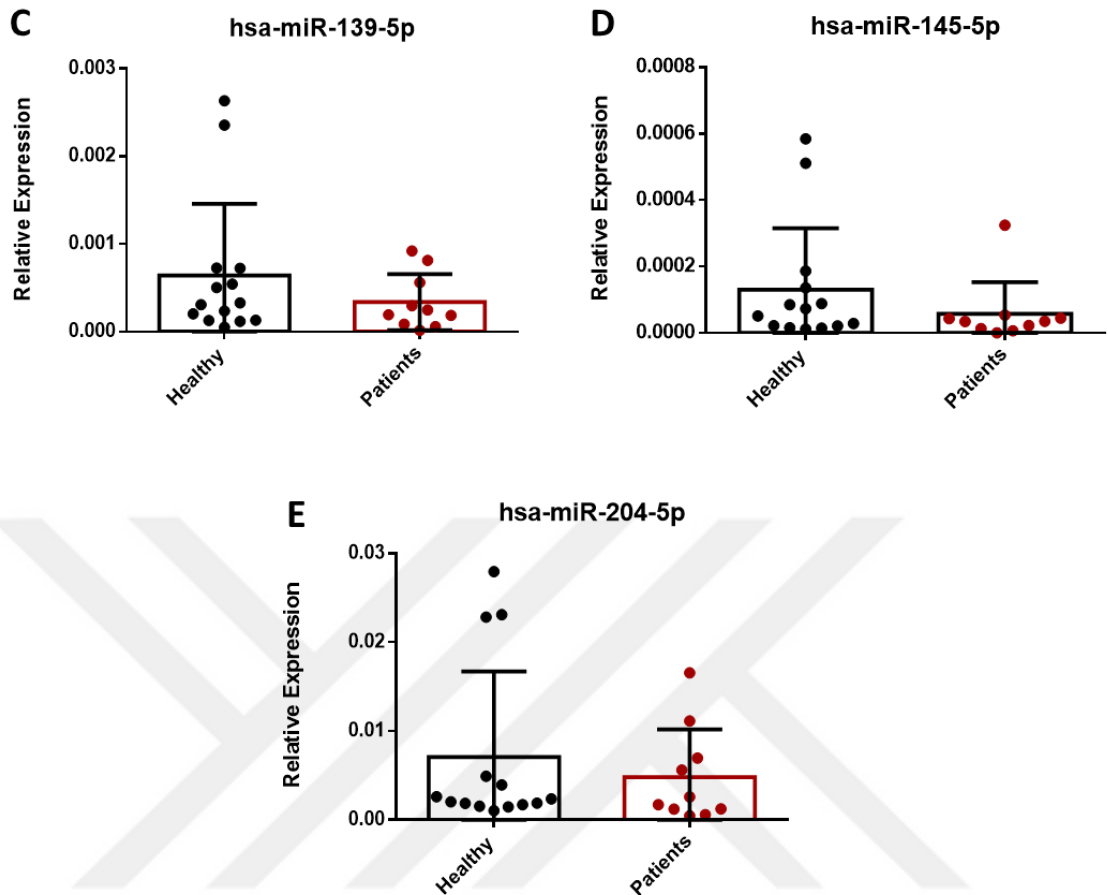


Figure 3.11 Expressions relative to U6 snRNA of tumor suppressor miRNAs in healthy control urinary exosomes and exosomes of patients with PSA levels lower than 10 ng/ml. Levels of (A) hsa-miR-30a-5p (B) has-miR-107 (C) hsa-miR-139-5p (D) hsa-miR-145-5p and (E) hsa-miR-204-5p in healthy urinary exosomes (n=14) and patient urinary exosomes (n=10). were detected by RT-qPCR. The experiment was performed as duplicates.

Candidate oncomiRs were also examined and RT-qPCR results revealed that miR-21 and miR-375-5p were upregulated (Figure 3.12A and B). On the other hand, no change in expression of hsa-miR-574-3p was observed (Figure 3.12C).

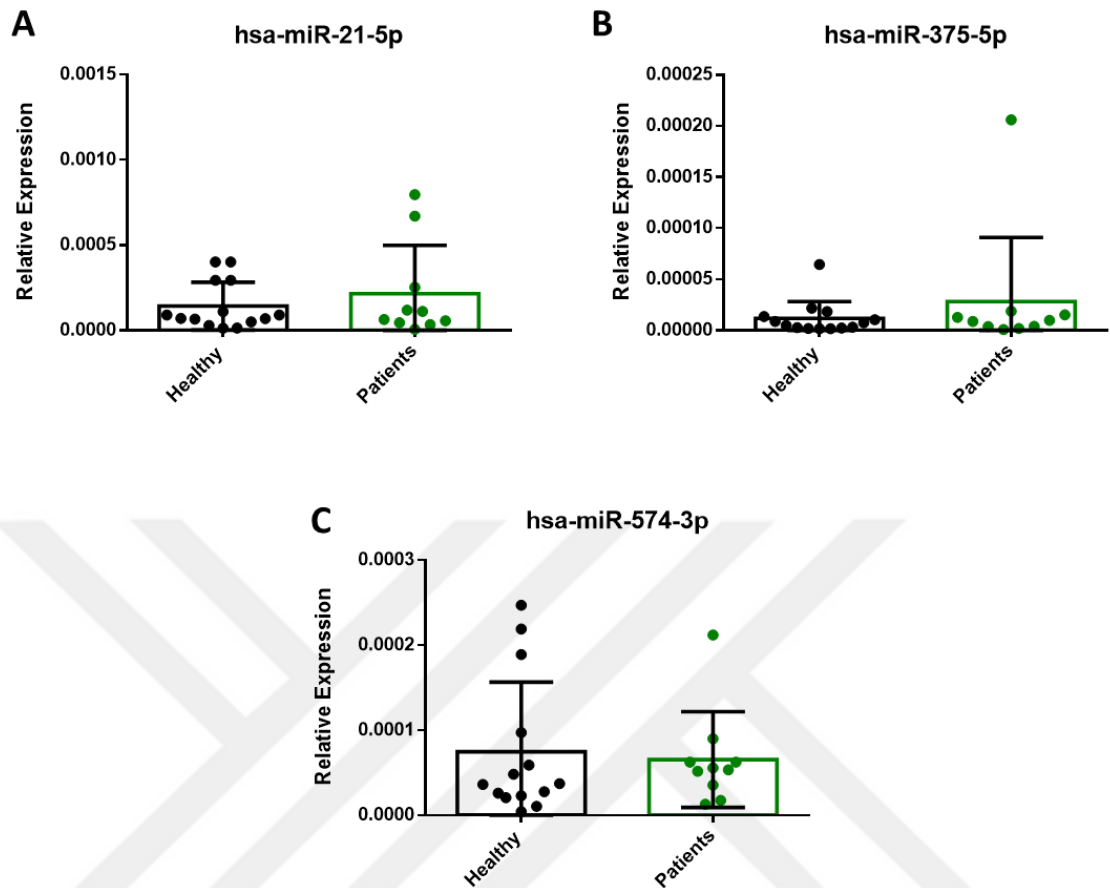


Figure 3.12 Expressions relative to U6 snRNA of oncogenic miRNAs in healthy control urinary exosomes and exosomes of patients with PSA levels lower than 10 ng/ml. Levels of (A) hsa-miR-21-5p (B) hsa-miR-375-5p (C) hsa-miR-574-3p in healthy urinary exosomes (n=14) and patient urinary exosomes (n=10) were detected by RT-qPCR. The experiment was performed as duplicates.

The second group of patients was the ones with PSA levels between 10-15 ng/ml. In this patient group, all tumor-suppressor miRNAs were downregulated as we expected (Figure 3.13). Interestingly, the most downregulated miRNA was hsa-miR-30a-5p with a very small standard deviation in contrast to patients with PSA<10 ng/ml (Compare Figure 3.13A with 3.11A)

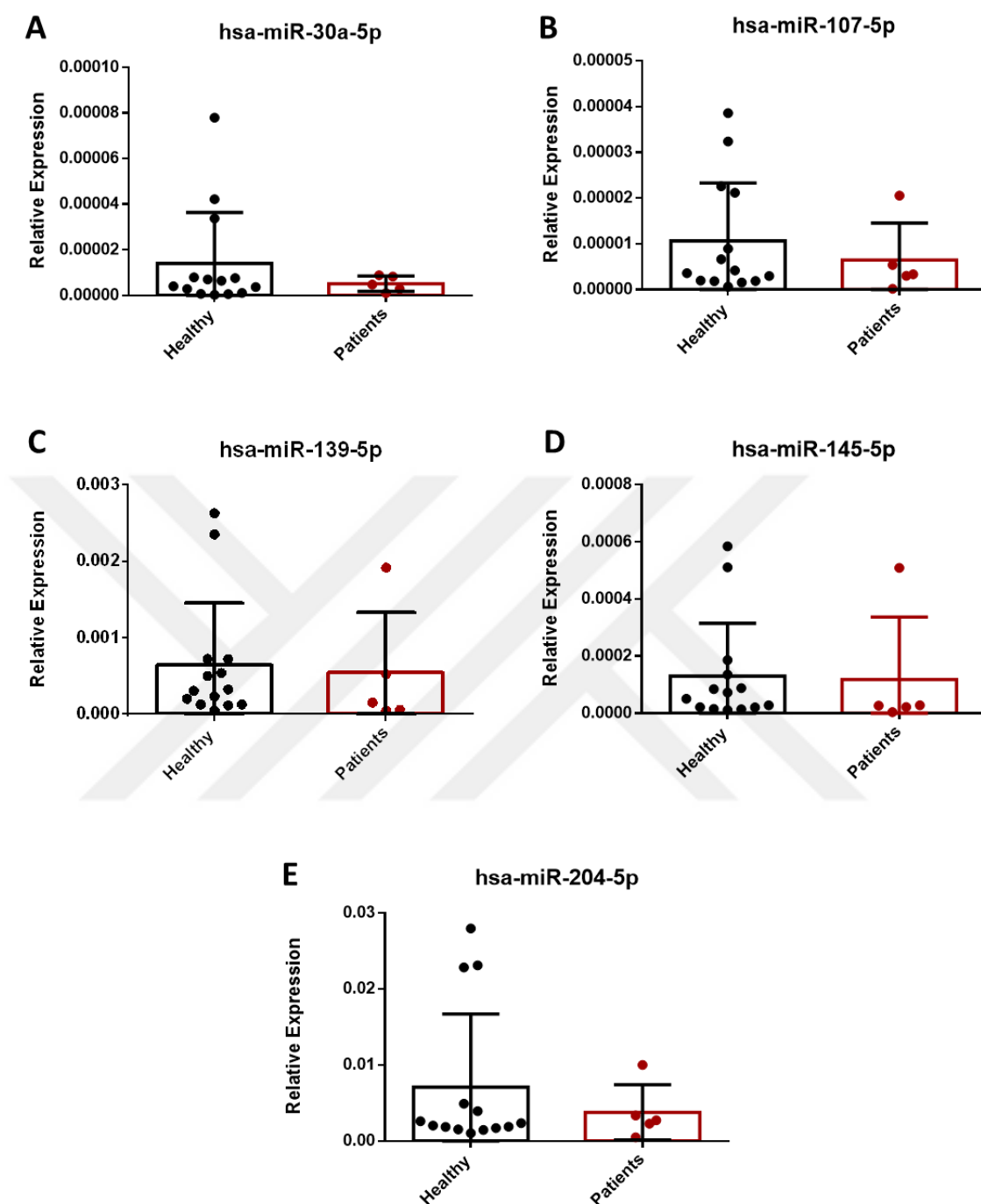


Figure 3.13 Expressions relative to U6 snRNA of tumor suppressor miRNAs in healthy control urinary exosomes and exosomes of patients with PSA levels between 10-15 ng/ml. Levels of (A) hsa-miR-30a-5p (B) has-miR-107 (C) hsa-miR-139-5p (D) hsa-miR-145-5p and (E) hsa-miR-204-5p in healthy urinary exosomes (n=14) and patient urinary exosomes (n=5) were detected by RT-qPCR. The experiment was performed as duplicates.

In exosomes of prostate cancer patients with PSA levels between 10-15, all candidate oncomiRs were upregulated compared to healthy exosomes. Standard deviations of oncomiR relative expression levels were high probably due to small sample size with n=5 for patients (Figure 3.14).

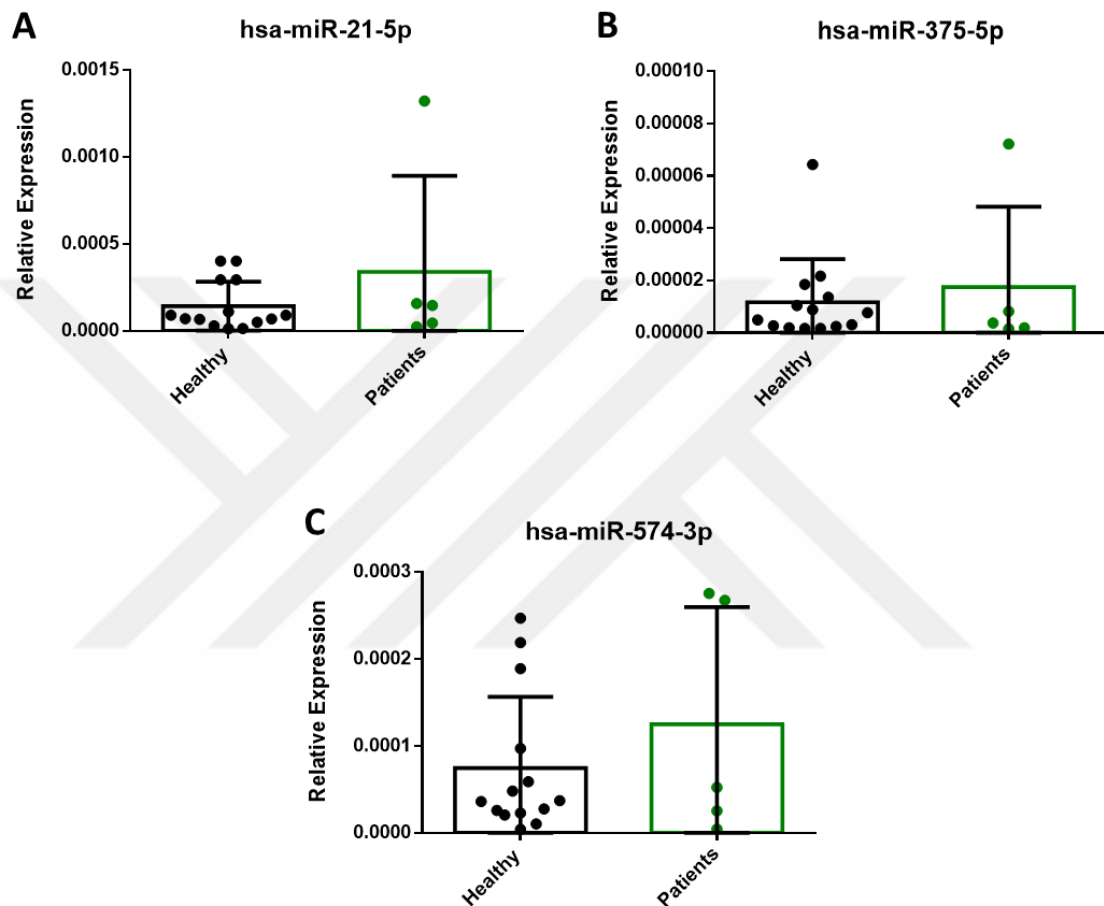


Figure 3.14 Expressions relative to U6 snRNA of oncogenic miRNAs in healthy control urinary exosomes and exosomes of patients with PSA levels between 10-15 ng/ml. Levels of (A) hsa-miR-21-5p (B) hsa-miR-375-5p (C) hsa-miR-574-3p in healthy urinary exosomes (n=14) and patient urinary exosomes (n=5) were detected by RT-qPCR. The experiment was performed as duplicates.

Finally, we investigated candidate tumor suppressor and oncogenic miRNAs' expressions in patients with PSA>15 ng/ml. The data revealed that all tumor suppressor miRNAs were downregulated in urinary exosomes of patients with PSA>15 (Figure 3.15).

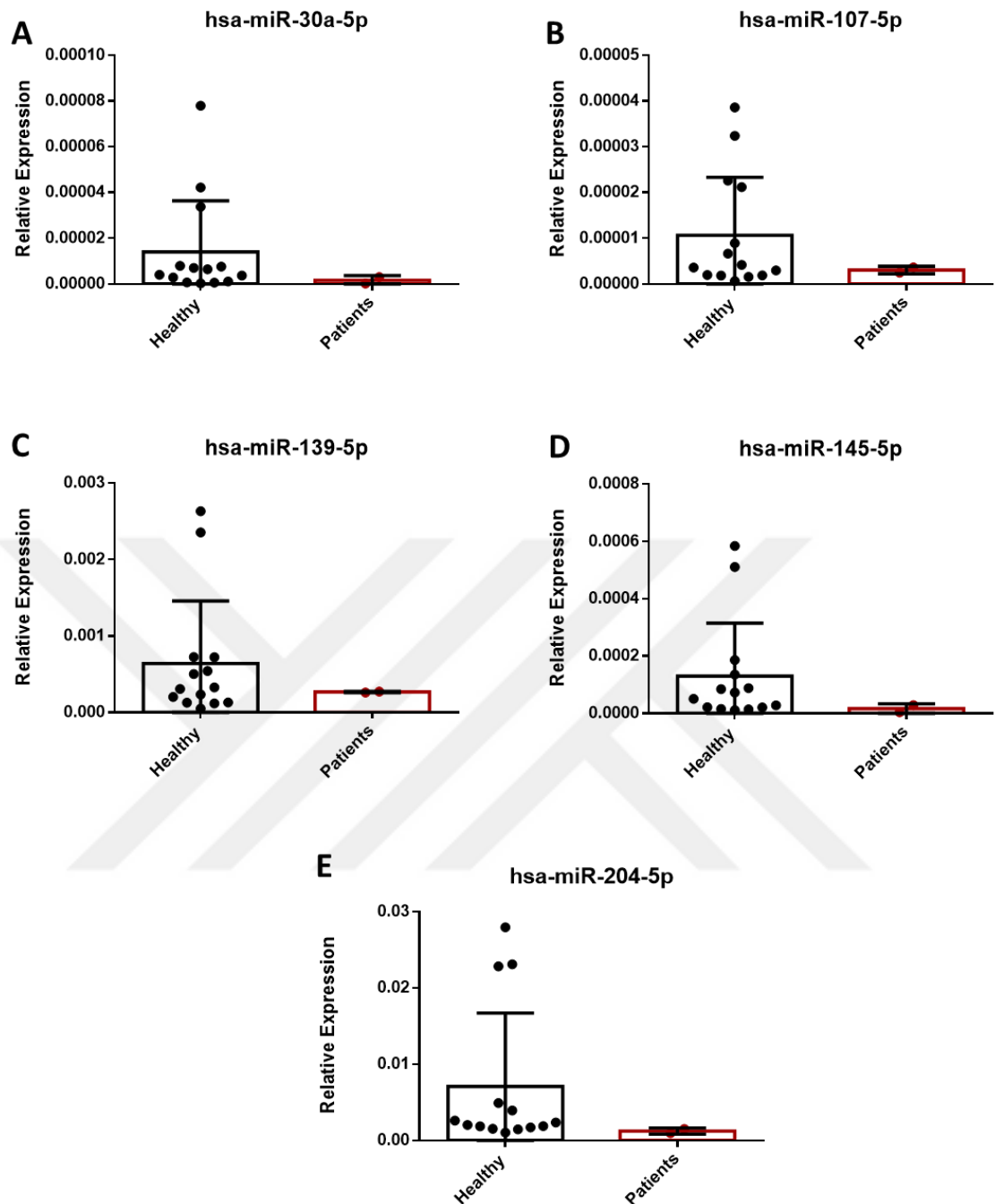


Figure 3.15 Expressions relative to U6 snRNA of tumor suppressor miRNAs in healthy control urinary exosomes and exosomes of patients with PSA levels higher than 15 ng/ml. Levels of (A) hsa-miR-30a-5p (B) has-miR-107 (C) hsa-miR-139-5p (D) hsa-miR-145-5p and (E) hsa-miR-204-5p in healthy urinary exosomes (n=14) and patient urinary exosomes (n=2) were detected by RT-qPCR. The experiment was performed as duplicates. * $p \leq 0.05$

Surprisingly, the same result was valid also for oncomiRs; all miRNAs including tumor suppressor and oncogenic miRNAs were downregulated (Figure 3.16). In contrast to patients with PSA<10 ng/ml, but similar to patients with PSA=10-15 ng/ml, hsa-miR-30a-5p was downregulated so it can also be a good biomarker for prostate cancer patients with PSA>10 (3.15A).

Importantly, although 2 patients were available with PSA>15 ng/ml, their standard deviations were too low for both tumor suppressor and oncogenic miRNAs (Figures 3.15 and 3.16). Moreover, although significance could not be shown for these patients, statistical analysis showed that if there were more patients in this group, expression changes would be significant. (Data not shown)

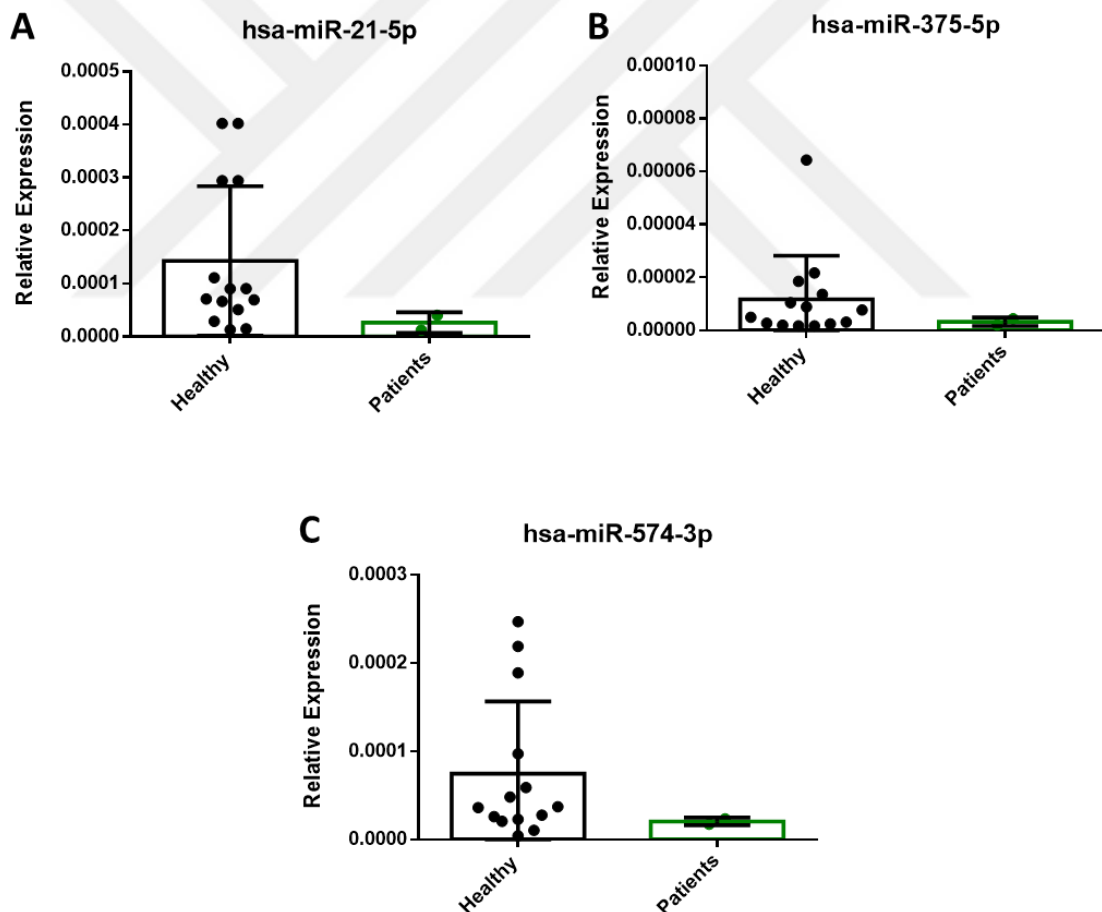


Figure 3.16 Expressions relative to U6 snRNA of oncogenic miRNAs in healthy control urinary exosomes and exosomes of patients with PSA levels higher than 15 ng/ml. Levels of (A) hsa-miR-21-5p (B) hsa-miR-375-5p (C) hsa-miR-574-3p in healthy

urinary exosomes (n=14) and patient urinary exosomes (n=2) were detected by RT-qPCR. The experiment was performed as duplicates.

Fold change bar graphs for candidate miRNA expression levels of patients with different PSA levels were drawn. These graphs showed that the most compatible patient group with our expectation (downregulation for tumor-suppressor miRNAs and upregulation for oncomiRs in prostate cancer patients) was the ones with PSA level between 10-15 ng/ml. In this patient group, all miRNAs were regulated as in our hypothesis except for miR-145. Level of miR-145 in exosomes of patients with PSA=10-15 ng/ml was not different from healthy controls (Figure 3.17B). The second most compatible patient group to our miRNA panel was the patients with PSA<10 ng/ml (Figure 3.17A). Interestingly, all miRNAs were downregulated for patients with PSA levels higher than 15 so they have a unique pattern of candidate miRNA expression.

According to fold change expression graph for patients with PSA<10 ng/ml, miRNA expression pattern was very similar to the graph in which patients were not grouped according to PSA levels. (Compare Figures 3.9A and 17A). Moreover, one miRNA expected to be upregulated in patients, hsa-miR-375-5p, was more upregulated in exosomes of patients with PSA<10 ng/ml than other two groups of patients with 2.4 folds (Compare Figures 3.17A and B,C).

For patients with PSA levels between 10-15 ng/ml range, all miRNAs had expected pattern of miRNA expressions except for miR-145. It can be said that hsa-miR-30a downregulation was striking because it was upregulated in patients with PSA<10 ng/ml (Compare Figures 3.17A and B). For miR-107 and miR-139, all groups were downregulated. miR-204 downregulation was observed in every group but upregulation of miR-21 was very obvious in only the group with PSA=10-15 ng/ml (Figure 3.17B). Finally, miR-574-3p was upregulated in only this group so this can be a biomarker for this group.

Sample size of group of patients with PSA>15 ng/ml (n=2) was too low and all miRNAs were downregulated. However, it was clearly understood that these patients have a unique miRNA regulation pattern with downregulation of all candidate miRNAs with high fold changes and small standard deviations (Figure 3.17C).

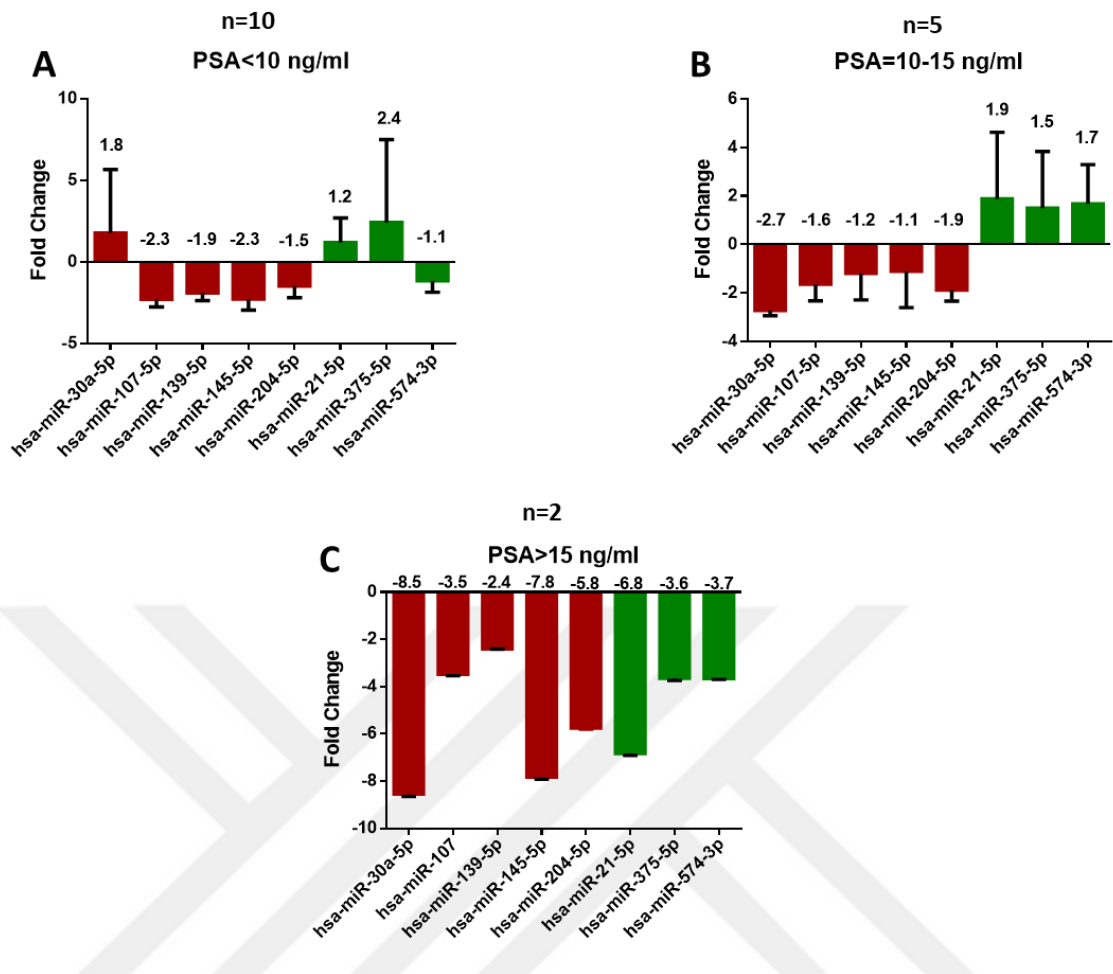


Figure 3.17 Fold changes of relative expression levels of candidate miRNAs for prostate cancer patients with different PSA levels. Fold changes of miRNA levels for (A) lower than 10 ng/ml (n=10) (B) between 10-15 ng/ml (n=5) and (C) higher than 15 ng/ml vs healthy urinary exosomes (n=14).

3.3.5 Diagnostic Ability of Candidate miRNAs for Patients with PSA Level Lower Than 10 ng/ml

After investigation of candidate miRNAs' relative expressions for patients with different PSA levels, we wondered the diagnostic ability of our candidates for these patients with different PSA groups. Therefore, we decided to perform ROC Curve analysis for each group of patients. The challenge was the small sample size for each group, and especially for patients with PSA=10-15 ng/ml (n=5) and PSA>15 ng/ml (n=2). That's why ROC Curve analysis is made only for patients with PSA<10 ng/ml (n=10).

In tumor suppressor candidate miRNAs, hsa-miR-107 had the highest and hsa-miR-30a-5p had the lowest AUC values. It can be interpreted as hsa-miR-107 had the highest power or ability to distinguish patients with PSA<10 ng/ml in our cohort from healthy individuals whereas hsa-miR-30a-5p was the least potent tumor suppressor candidate to descry patients. Besides, when tumor suppressor miRNAs were combined and diagnostic ability was tested again, AUC value increased to 0.7214 from 0.58-0.65 range for each miRNA when they are not combined (Figure 3.18A). All oncomiRs had low AUC values and it only increased from 0.52-0.57 range when miRNAs were used as single to 0.6017 when oncomiRs are combined (Figure 3.18B).

When all tumor suppressor and oncogenic miRNAs were combined to distinguish patients from healthy individuals, AUC value was found to be 0.8929 so our candidate miRNAs could discriminate patients with PSA<10 ng/ml from patients with almost 90% accuracy when all miRNAs were used in testing. In this patient cohort, relative expressions of miR-30a and miR-574-3p were in contradiction with our expectations (Figure 3.17A). For this reason, these 2 miRNAs were excluded from ROC Curve test and AUC was found to be 0.8500 and 0.8571 when miR-30a and both miR-30a/miR-574-3p were excluded from testing respectively (Figure 3.18C).

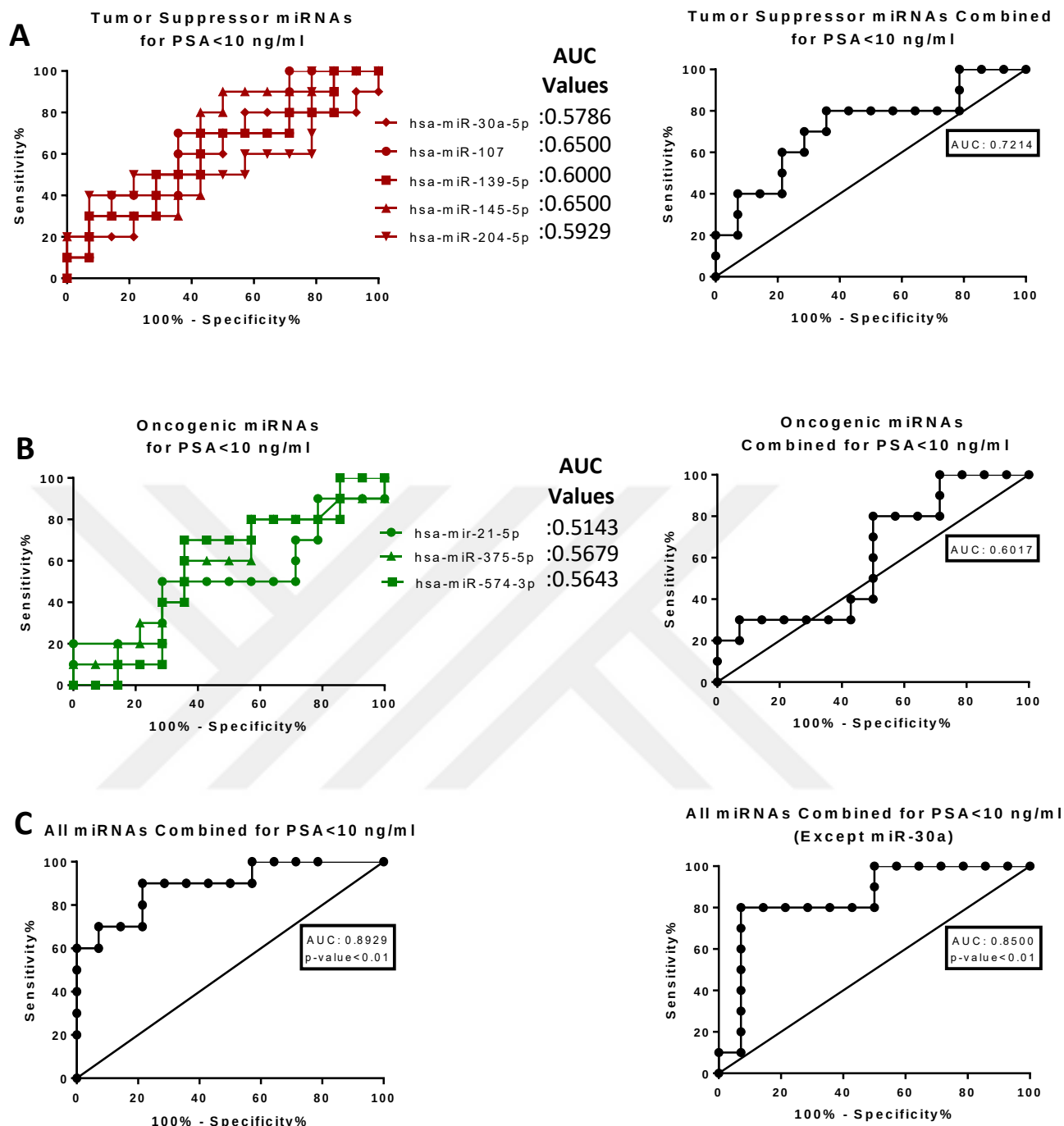


Figure 3.18 Diagnostic ability of candidate miRNAs in patients with PSA<10 ng/ml.

ROC Curves of (A) Tumor suppressor (B) Oncogenic miRNAs in prostate cancer patients as single and as combination. (C) Diagnostic ability of combination of all candidate miRNAs (tumor suppressor and oncogenic miRNAs) as a combination. n=14 for healthy controls and n=17 for patients.

Chapter 4

Discussion

Several cancer types have high incidence and mortality rates worldwide. Globally, cancer is the second cause of death independent of gender and 1 in 6 deaths is because of cancer [176]. According to analysis of American Cancer Society, there will be an estimated 1.8 million new cancer diagnosis and 606,520 deaths due to cancer in the United States in 2020 [177]. There are several reasons of cancer including age and genetics. However, some of the the most leading risk factors are high body mass index, lack of physical activity, tobacco, unbalanced diet and alcohol.

Prostate cancer is the fourth most incident cancer type among all cancer subtypes and the second most commonly occurring cancer in men. Its incidence rate is 1 in 9 men in the USA; so that about 1 in 9 men will have prostate cancer diagnosis in his life [9]. In 2018, 1,276,106 new prostate cancer cases were reported worldwide. Interestingly, developed countries had higher incidence compared to less developed countries [178]. When survival statistics of prostate cancer patients were investigated, 1 man in 41 was found to be dead due to prostate cancer [9]. In prognosis of prostate cancer, diagnosis stage of the cancer is very important. 5-year survival rates reveal that prostate cancer patients with metastasis had only 30% probability of 5-year survival whereas localized prostate cancer had almost 100% 5-year survival rate [179].

Tremendous effect of stage of the prostate cancer points out the importance of early and accurate diagnosis in prostate cancer. Prostate Specific Antigen testing from blood specimen is used for diagnosis of prostate cancer since 1986. However, PSA was found to be fluctuated due to several reasons such as age [14]. For instance, benign prostatic hypertrophy is observed in elder men and it leads to increase in PSA level [13]. In the developed world, routine PSA testing was performed for all adult men and led to an enormous increase in prostate cancer incidence [180]. PSA level limits were restricted in order to discriminate healthy and prostate cancer patients. PSA level of 0-4 ng/ml are

considered as healthy, 4–10 ng/ml is considered as a range for indication of about 25% risk of prostate cancer and PSA>10 ng/ml is considered as more than 50% risk [181].

PSA testing is successful in detection of early prostate cancer patients. On the other hand, it has a significant down-side. It has a very high false-positive rate which causes overdiagnosis and even overtreatment of individuals. According to one study which examined false positive rate of prostate cancer, researchers had an estimation about 60% of overdiagnosis due to PSA testing [182]. Similarly, in another research, 66% of PSA tested cancer patients were detected to be overdiagnosed [183]. Increase in PSA level due to age and overdiagnosis correlation was also studied. Overdiagnosis rate of men with age of 55 years was 27% whereas men with age of 75 years had 56% overdiagnosis [184]. Overdiagnosed individuals and many other men with high PSA levels got irrelevant biopsies which is an invasive procedure.

High false positive rate in PSA testing led to studies for discovery of alternative prostate cancer diagnosis methods. For this purpose, prostate cancer biomarker researches gained speed. Several proteins, RNA and DNA biomarkers were found as highly potent prostate cancer biomarkers such as PCA3 mRNA and Annexin A3 protein [185]. However, none of them could be as successful to be used in prostate cancer diagnosis in clinics worldwide yet. Accordingly, biopsies are still performed following the high PSA test result routinely and many patients get biopsies unnecessarily. When considering the huge need for a more sensitive and specific prostate cancer diagnosis method, researching novel biomarkers was inevitable.

With the aim of developing an alternative prostate cancer diagnosis method with higher sensitivity and specificity than PSA test, we investigated urinary exosomal microRNA profiles of prostate cancer patients. Exosomes are the smallest extracellular vesicles and they protect their cargos from degradation by nucleases [186]. Thus, examination of exosomal contents of prostate cancer patients would give enormous information about tumor. In addition, there are many studies about diagnostic potentials about miRNAs in different diseases including cancer [187-190]. For the reasons stated above, we hypothesized that urinary exosomal miRNAs would be differentially expressed in prostate cancer patients and healthy controls and this feature of exosomal miRNAs could be used in development of a prostate cancer diagnosis method.

In this thesis, expressions of 5 tumor suppressor and 3 oncogenic microRNAs were studied. Levels of these miRNAs were compared between healthy and prostate cancer patient urinary exosomes. Tumor suppressor miRNAs were hsa-miR-30a-5p, hsa-miR-107-5p, hsa-miR-139-5p, hsa-miR-145-5p and hsa-miR-204-5p. Expression of these miRNAs were expected to be downregulated in prostate cancer patients because they target oncogenic miRNAs and suppress their expressions. On the other hand, oncogenic miRNAs were hsa-miR-21-5p, hsa-miR-375-5p, hsa-miR-574-3p. In contrast to tumor suppressor miRNAs, their expressions were expected to be upregulated in prostate cancer patients due to their repressive effects on expressions of tumor suppressor genes [191]. Functions and mechanisms of the tumor suppressor and oncogenic miRNAs examined in this study are detailed in Section 1.3.2.2.1 and Section 1.3.2.2.2 respectively.

First of all, we characterized PC-3 (prostate cancer cell line) exosomes and healthy/patient urinary exosomes. After confirming that isolated exosomes contain exosomal markers on their surfaces by flow cytometry (Figure 3.1), we examined their size distributions. For PC-3 cell line exosomes, we could perform both qNano and DLS. However, for healthy/urinary exosomes we could only perform DLS because these exosomes were isolated by precipitation with PEG and PEG has characteristic of forming clumps which interfere with qNano readings. On the other hand, PC-3 was isolated by ultracentrifuge and qNano analysis showed that mean diameter of PC-3 exosomes were 119 nm (Figure 3.2A). According to DLS results, PC-3 exosomes had 132.5 nm mean diameter and large population of particles in healthy and patient urinary exosomes had diameters close to 100 nm (Figure 3.2B, C, D). The reason that we cannot tell the exact diameter is because clumps in these samples caused the Zetasizer to see the clumps as one particle and deviate the mean diameter. Similarly, TEM results showed that exosomes that we isolated had diameters in the range of exosome sizes (Table 3.1). Thus, we concluded that we could efficiently isolate exosomes both from urine and PC-3 cell line efficiently and continued with RT-qPCR experiments.

Before investigation of candidate microRNA levels in prostate cancer patients' urinary exosomes, we aimed to compare differences in expression levels of them in PC-3 cell line exosomes and healthy urinary exosomes. We had more candidate miRNAs at the beginning of the study. However, we eliminated some of them due to their low primer efficiencies or low reliabilities and continued our studies with the most promising ones. In tumor suppressor miRNAs, only miR-30a had almost no downregulation in its

expression but all other candidate tumor suppressor miRNAs were significantly downregulated in PC-3 exosomes. (Figure 3.4) On the other hand, in the literature, miR-30a was shown to be acting as a tumor suppressor in prostate cancer [192, 193]. There was a contradiction about role of miR-107 in prostate cancer the literature. Some studies revealed that it was acting as an oncomiR and some others indicated that it was a tumor suppressor miRNA [194] [195]. For this reason, we did not classify this miRNA as oncomiR or tumor suppressor firstly. After we observed that miR-107 was significantly downregulated in PC-3 exosomes, we hypothesized that it might have been acting as a tumor suppressor in prostate cancer. On the other hand, expression levels of all oncomiRs in PC-3 exosomes were higher than healthy urinary exosomes. miR-21-5p, miR-375-5p and miR-574-3p were upregulated. For miR-21-5p and miR-375-5p upregulation was significant ($p \leq 0.01$) (Figure 3.5). These results were mostly consistent with the literature. In the literature, our tumor suppressor miRNAs were found to be downregulated in different prostate cancer cell lines such as PC-3 and LNCaP [77, 107, 114, 119, 192]. Additionally, oncomiR candidates were also found by literature search and they were detected to be upregulated in prostate cancer cell lines except for miR-375-5p which was not studied in prostate cancer before [196]. Of note, most of these studies were not made on EV miRNAs. The abundant strand of miR-375 is miR-375-3p. However, in our preliminary experiments, we detected that miR-375-3p was not as reliable as miR-375-5p in our experimental setup due to fluctuations in its expression in PC-3 cell line. Also, primer efficiency for miR-374-5p was also much better so we went ahead with miR-375-5p and determined that it was significantly upregulated in PC-3 cell line.

After validating our candidate miRNAs in PC-3 exosomes, we proceeded through the second aim of the study; analysis of prostate cancer patients' urinary exosomes. To this end, we collected urine specimens from 17 prostate cancer patients with their consents. In the literature, we observed that one of the most important reasons for false positive results in PSA testing was increase in PSA with age [197]. We wondered if this correlation was accurate in our patient cohort and observed that PSA was significantly higher in 71-75 years old patients in comparison to 60-65 years old patients (Figure 3.6A). Then, we tested if PSA and Gleason Score were correlated in order to understand if PSA was a biomarker for stage of prostate cancer. No correlation was detected between these two variables (Figure 3.6B). Taken together, this was an evidence for increase in PSA level is due to reasons other than prostate cancer such as age, and prostate cancer

progression and stage was not correlated with PSA level. Age-PSA level correlation was also revealed by Putra et al [198].

Further, we examined expression levels of candidate miRNA levels in prostate cancer patients' exosomes. According to the evidences in the literature [92, 96, 103, 162] combined with our PC-3 findings, we suggested the same 5 tumor suppressor and 3 oncogenic miRNAs with the experiments of PC-3 exosomes. Similar to PC-3, in tumor suppressor candidates, only miR-30a had almost the same expression level in prostate cancer patients' urinary exosomes with healthy controls. However, one outlier was detected in the patients for miR-30a and when it was excluded, downregulation in miR-30a in patients' exosomes were observed (Figure 3.7). Moreover, RT-qPCR data reveals that our oncomiR candidates; hsa-miR-375-5p was upregulated in patients' exosomes, there was a very slight upregulation of miR-21 but no difference in levels of miR-574-3p was detected (Figure 3.8). These results were mostly consistent with the literature because we expected tumor suppressor miRNAs to be downregulated since they target oncogenic genes and oncomiRs to be upregulated in urinary exosomes because they target tumor suppressor genes [115, 193, 199-201]. The reason for not being able to detect any difference for some miRNAs such as miR-30a and miR-574 may be that we found our candidates by literature research. Most of the studies in the literature belongs to cellular miRNA expression levels and in the exosome studies, plasma urines were investigated generally which can have different miRNA expression levels from urinary exosomes. Off note, it is known that circulating plasma miRNAs are predominantly bound to AGO2 in addition to exosome associated counterparts. In this work we intentionally focused on the identification of exosome associated miRNA's. However, whether there are AGO2 associated miRNAs in the urine have not been explored. In the future, it is important to check for the existence of this species in the urine and if so, identify those miRNAs related to prostate cancer. We further aimed to detect differential expressions of the miRNAs in urine specimens because it is not an invasive method and we aimed to improve the validity of our study. Research concerning the roles of selected candidate miRNAs in prostate cancer and the changes in their expression levels were reported in several studies and were explained previously in Sections 1.3.2.2.1 and 1.3.2.2.2.

When expression levels of miRNAs were compared between PC-3 and prostate cancer patients' urinary exosomes, dysregulation of miRNAs were found to be very similar in PC-3 and patients' exosomes. The only exception was miR-30a with 1.2-fold

downregulation and 1.2-fold upregulation in PC-3 and patients' exosomes respectively (Figure 3.9). One reason of the high similarity rates between the expression levels of candidate miRNAs in exosomes of PC-3 cell line and urinary exosomal miRNAs extracted from prostate cancer patients could be due to the similarity of the tumor type. The patients' tumor was identified to be an adenocarcinoma and which is an identical tumor that the PC-3 cell line was developed [170]. Of note, in fold change graph (Figure 3.9B) plotted from patients vs healthy control data was plotted by including one of the outlier patient's miR-30a finding. Had we excluded this outlier data, then miR-30a comparison would also be as expected, downregulated in this figure (Figure 3.7A). Besides, in patients' urinary exosomes, all tumor suppressors were downregulated except for miR-30a and all oncogenic miRNAs were upregulated in PC-3 exosomes but miR-574-3p had no difference in expression when compared to healthy controls (Figure 3.9B). On the other hand, miR-574-3p was upregulated in PC-3 exosomes (2.1 fold) (Figure 3.9A).

Furthermore, we wondered if we would be able to discriminate patients from healthy controls in our cohort we used our candidate miRNAs. For this reason, we generated ROC curves in order to determine the diagnostic ability of these miRNAs. When tumor suppressor and oncogenic miRNAs were used separately in testing, their Area Under Curve (AUC) values were found to be lower than their combination (Figure 3.10A and B). So that we would be able to discriminate the patients from healthy controls more accurately if we used these miRNAs as combinations. When we tested the ability of our miRNAs when both tumor suppressor and oncogenic miRNAs were used in testing, AUC was calculated as 0.7731 which means that we were able to distinguish patients from healthy controls in our cohort with 77% probability when we checked the expressions of all our candidate miRNA levels. We were expecting this finding because adding more high potent biomarkers (variables) to the test would; so that combination of many efficient miRNAs would increase its performance.

Detection of miRNA level changes in prostate cancer patients with different PSA levels made us think that maybe some biomarkers were more accurate and efficient in a group of patients with a specific PSA level. If it was true, maybe PSA testing and then miRNA testing would be enough for diagnosis and biopsy would not be necessary in some group of patients or even only miRNA testing would be enough after a while. Thus, we grouped the patients in 3 groups; patients with PSA<10 ng/ml, PSA=10-15 ng/ml and PSA>15

ng/ml. For patients with PSA<10 ng/ml, expression of miR-30a was upregulated in patients as opposed to our expectation and levels of miR-21 and miR-574 were almost not different for healthy controls and patients. All other tumor suppressors were downregulated and oncomiRs were upregulated as we expected (Figures 3.11 and 3.12). However, it should be kept in mind that the same outlier was present in this group for miR-30a and we did not exclude it because of our sample size (n=10). Apparently, our miRNA panel was fitting to the patients with PSA=10-15 ng/ml. All tumor suppressor miRNAs were downregulated except for miR-145 (no change in expression level) and all oncomiRs were upregulated in this group of patients (Figures 3.13 and 3.14). Unfortunately, no significant difference was detected for any miRNA in exosomes of patients with PSA<10 ng/ml and PSA=10-15 ng/ml. This was probably due to low sample sizes of patients in these groups (n=10 and n=5 respectively). However, the miRNA patterns for these groups, especially for PSA=10-15 ng/ml group, was future-promising due to their good fit to our miRNA panel. The final patient group that we investigated expression level was PSA>15 ng/ml group. There was a unique expression pattern for this group. All candidate miRNAs including oncomiRs were downregulated. In addition, miR-145-5p was significantly downregulated in this patient group even though the sample size was very small (n=2). Interestingly, it was the patient group with the smallest standard deviations despite the fact that it had a very small sample size (Figures 3.15 and 3.16). If we had more patients in this group, we think that we would be able to observe statistically different expression levels almost for each miRNA because all tumor suppressor and oncogenic miRNAs had small p values and small standard deviations even though the sample size was very low.

Furthermore, we wanted to compare expression level patterns in patients with different PSA levels. For patients with PSA>15 ng/ml, all downregulated miRNAs as well as oncomiRs were downregulated with high fold changes. We concluded that this group needs to be further investigated with higher sample sizes of patients because miRNA expression pattern was different than other PSA groups. So that we compared miRNAs in PSA<10 ng/ml and PSA=10-15 groups. hsa-miR-30a, which is a tumor suppressor, was downregulated (2.7 fold) as we expected in PSA=10-15 ng/ml group. On the other hand, in PSA<10 ng/ml group, it was upregulated (1.8 fold) so miR-30a would be efficient in diagnosis of patients with PSA=10-15 ng/ml but not in PSA<10 ng/ml group. miR-107, miR-139 and miR-204 were all downregulated in both groups. Nevertheless,

miR-145 expression was almost no different from healthy controls in PSA=10-15 ng/ml group but 2.3 folds downregulated in PSA<10 ng/ml group so it would be a good biomarker for PSA<10 ng/ml group. In oncomiR candidates, miR-375-5p was upregulated in both groups whereas miR-21 and miR-574-3p were upregulated in only PSA=10-15 ng/ml group. So miR-21 and miR-574-3p is a potential miRNA biomarker for patients with PSA=10-15 ng/ml (Figure 3.17). In the literature, there is no research to date about determination of miRNA level dysregulations in prostate cancer patients with different PSA levels.

Finally, we attempted to test if our candidate tumor suppressor and oncogenic miRNAs had high potential diagnostic abilities for patients with PSA<10 ng/ml. To this end, we generated ROC Curve for this group of patients. We did not perform ROC Curve analysis for other 2 groups with PSA>10 ng/ml because of their sample size limitations. Similar to the ROC Curves when tumor suppressor and oncogenic miRNAs were examined separately, AUC values when all tumor suppressor miRNAs and oncomiRs were higher than their separate forms. Besides, when tumor suppressor miRNAs were combined, AUC value was higher than combined oncomiRs (Figures 3.10A, B and 3.18A, B). In patient group with PSA<10 ng/ml, downregulated miRNAs had a high potential when they were used as combination with AUC=0.7214 (Figure 3.18A). If we performed a diagnosis test on patients with PSA<10 ng/ml by using all of the candidate tumor suppressor and oncogenic miRNAs, we would be able to distinguish patients from healthy controls with 89% accuracy. However, miR-30a had an opposite regulation of our expectation from a tumor suppressor miRNA; it was upregulated (Figure 3.17A). For this reason, we tested our miRNA panel's diagnostic potential by excluding miR-30a and found out that AUC value was 0.85 (Figure 3.18C). This finding shows that miRNA panel without miR-30a would distinguish patients from healthy controls with 85% probability. This evidence is significant for this patient group because PSA=4-10 ng/ml is determined as a 'grey zone' in prostate cancer diagnosis [202]. For these individuals, biopsy is performed following the PSA test so they get an unnecessary invasive procedure if they are healthy and PSA level is fluctuated due to other reasons than prostate cancer. Accurate diagnosis may be possible by checking our biomarker miRNA levels in urinary exosomes explained in this study after a PSA test at the beginning. So that only urine test after PSA test would be enough for diagnosis and there will be no need to biopsies. After a while,

the ultimate goal of the study is to eliminate PSA testing and making sensitive and specific diagnosis of prostate cancer by only using our exosomal urinary miRNA panel.

For the future studies, increasing the sample size for both prostate cancer and healthy individuals would be important statistically. In addition, adding more miRNA candidates to the panel will be beneficial for increasing the sensitivity and specificity of the test. Moreover, *in silico* analyses can be performed to reveal the targets of these miRNAs and examining the reason of differences in miRNA expression patterns for patients with different PSA levels are important in order to understand functions of them in prostate cancer. Taken together, these findings are future-promising preliminary results for development of a prostate cancer diagnosis method by investigation of urinary exosomal miRNA expression levels.

In conclusion, our miRNA panel composed of 5 tumor suppressor and 3 oncogenic miRNAs has a promising diagnostic potential in prostate cancer. It has a 77% accuracy in discrimination of patients from healthy controls when miR-30a was excluded. Moreover, in patient groups with PSA<10 ng/ml, our miRNA panel had 85% accuracy in testing, again when miR-30a was excluded. Even though the dysregulations of these miRNAs needs to be further investigated in larger number of patients in order to be used in clinics, the findings of this thesis merits to conclude that urinary exosomal miRNA diagnosis procedure is highly potent for sensitive prostate cancer diagnosis when the miRNAs that we suggested are examined. In the lights of these findings, these results could be translated for diagnosis of other cancer subtypes such as bladder, cervix and ovary, probably with a different set of miRNA panels.

APPENDIX

Appendix A.1- Cell Culture Solutions

Exo-free RPMI-1640 Media

- 500 ml RPMI-1640 Media (with L-Glutamine at a final concentration 2mM)
- For 2% (Wash media): 10.64 ml; 10%: 58.14 ml FBS
- For exo-free RPMI media, heat-inactivated FBS was ultracentrifuged at 100000xg for 5 hours.
- For 2% (Wash media): 5.32 ml 10%: 5.8 ml HEPES (10mM)
- For 2% (Wash media): 5.32 ml 10%: 5.8 ml Non-Essential Amino Acids (1X was taken from 100X Stock Solution)
- For 2% (Wash media): 5.32 ml; 10%: 5.8 ml Na-Pyruvate (110µg/ml)
- For 2% (Wash media): 5.32 ml; 10%: 5.8 ml Penicillin/Streptomycin (50µg/ml)

Appendix A.2- Flow Cytometry

10X Phosphate Buffered Saline (PBS)

- 80 g NaCl
- 2 g KH₂PO₄
- 2g KCl
- 8.01 g Na₂HPO₄·2H₂O
- Complete to 1 L dH₂O

pH is set to 6.8. Then, PBS is autoclaved before use.

1X PBS

- 100 ml 10X PBS
- 900 ml dH₂O

pH is set to 7.4 before use.

FACS Buffer

- 5 g BSA
- 125 mg Na-Azide
- Complete upto 500 ml with 1X PBS.

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