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INVESTIGATION OF SPECIFIC ONCOMIR MICRORNAS THAT MODIFY METHYLATION PATTERNS ONTO EFFECTION OF PROSTATE CANCER

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ONAY SAYFASI

Biruni Üniversitesi Sağlık Bilimleri Enstitüsü Moleküler Biyoloji ve Genetik Anabilim Dalında Afshan BABADZE tarafından hazırlanan "Investıgatıon Of Specıfic Oncomır Mıcrornas That Modıfy Methylatıon Patterns Onto Effectıon Of Prostate Cancer" adlı tez çalışması aşağıdaki jüri tarafından YÜKSEK LİSANS tezi olarak kabul edilmiştir.

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Statement

I hereby declare that all information in this thesis has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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SYMBOLS AND ABBREVIATIONS

4Kscore	Four Kalikrein Score
αHCG	α chorionic gonadatropin
AJCC	American Joint Committee on Cancer
AMACR	Alpha-methyl-CoA Racemase
APC	Adenomatous Polyposis Coli
bp	Base Pair
BPH	Benign Prostatic Hyperplasia
BRCA2	Breast Cancer Type 2 Susceptibility Protein
caC	Carboxylcytosine
CHEK2	Checkpoint Kinase 2
CIMP	CpG island methylator phenotype
CpG	Cytosine base located adjacent to a guanine base
DNA	DeoxyriboNucleic Acid
DRE	Digital Rectal Examination
ERG	Transcriptional Regulator ERG
ETV1	ETS Variant 1
ETV4	ETS Variant 4
FDA	Food and Drug Administration
FFPE	Formalin-Fixed, Paraffin-Embedded
GOLPH2	Golgi Membrane Protein 1
GS	Gleason Score
GSTP1	Glutathione S-Transferase Pi 1
HBG1	γ-globin
HOXB13	Homeobox B13
mC	Methylated cytosine

miRNA	MicroRNA
Mi-PS	Mi Prostate Score
MRI	Magnetic Resonance Imaging
PCa	Prostate Cancer
PCA3	Prostate Cancer Antigen 3
PCR	Polymerase Chain Reaction
PHI	Prostate Health Index
PSA	Prostate-specific Antigen
RARB2	Retinoic Acid Receptor Beta
RASSF1A	Ras Association Domain Family member 1
RNA	RiboNucleic Acid
ROC	Receiver Operating Characteristic
RP	Radical Prostatectomy
SNV	Single Nucleotide Variants
TMPRSS2	Transmembrane Protease, Serine 2
TNM	Tumour, Node, Metastasis
tPSA	Total PSA
UICC	International Union Against Cancer

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INVESTIGATION OF SPECIFIC ONCOMIR MICRORNAS THAT MODIFY METHYLATION PATTERNS ONTO EFFECTION OF PROSTATE CANCER

Afshan

1.ABSTRACT

Prostate Cancer (PCa) is the second most common cause of cancer-related deaths in men. Although the etiopathogenesis of PCa has not been clearly elucidated, the evidences has been obtained that epigenetic factors have been implicated in the onset and progression of cancer for the last 10 years.Hypermethylation of genes has been shown to be associated with PCa. MicroRNA (miRNA) is a single-stranded, small non-coding molecules containing about 22 nucleotides. miRNA plays a critical role in the genetic pathogenesis of many types of cancer and mostly human cancer types. DNA methylation is known to be involved in cancer formation, progression and metastasis. Thus, DNA hypermethylation in CpG islands is a marker in the early detection of cancer that can be used as an indicator. Epigenetic mechanisms can be used as a marker in the diagnosis of the disease as well as in the treatment process. It is suggested that epigenetic changes such as DNA methylation and histone modification may be an effective strategy in the treatment of cancer by targeting. In current thesis, promoter methylation statuses and expression changes ofhsa-mir-192, hsa-mir-512-5p, hsa-miR-513a-2 andhsa-mir-572 were analyzed by methylation specific PCR and real time PCR respectively in prostate cancer tumor tissues. Subsequently, here was shown the gene-specific promoter methylation changes that was generated intumor tissues ofmiRNAs, which were shown to be significant. In this work, the possible tumor suppressor potentials ofmiRNAs have been shown.

Keywords: microRNA, methylation, prostate cancer, biomarkers

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2.INTRODUCTION

2.1.The Prostate Gland

The prostate is a gland located in the male reproductive system just below the mesentery and in front of the rectum. This walnut-sized gland surrounds a part of the urethra and produces fluid that is part of the sperm (Bhavsar A *et al*. 2014). The prostate is arranged into an apex, a base, and anterior and posterior surfaces (Figure 2.1). It is also encapsulated by connective tissue that mostly consists of smooth muscle fibers and elastic connective tissue (Bhavsar A *et al*. 2014). The prostate gland can grow to 40 grams in older men (Kgatle *et al*. 2016).

The prostate has three zones: the peripheral, central, and transition zone (Figure 2.1) (Salman *et al*. 2015, Bhavsar *et al*. 2014, Shen *et al*. 2010). About 70% of the peripheral zone contains the glandular tissue within the prostate. In contrast, the glandular tissuepercentage of central zone consists is 25% and of transition zone consists is 5%. Prostate cancer (PCa) is most commonly found within the peripheral zone, besides it Benign Prostatic Hyperplasia (BPH) is often found in the transition zone (Bhavsar *et al*. 2014, Shen *et al*. 2010).

In male individuals, as the age progresses, the prostate can grow and block the urethra or bladder. This condition, which known as benign prostatic hyperplasia (BPH), can cause sexual and urine production problems. Although there is no cancer, surgery may be necessary (Prostate Cancer Treatment, 2002). Prostate cancer begins with uncontrolled development and proliferation of abnormal sperm-secreting prostate gland cells. If not treated, lymph nodes, bones and other parts of the body can metastasize. Most patients remain asymptomatic in the early stages of disease. However, during the later stages of the disease, various urinary symptoms such as dysuria, hematuria, hematospermia, pain in the pectoral region and swelling of the pelvis are seen (Catalona *et al*. 1994, Barry *et al*. 2009). Symptoms of BPH or other problems in the prostate may resemble symptoms of prostate cancer (Prostate Cancer Treatment, PDQ Adult Treatment Editorial Board, 2002).

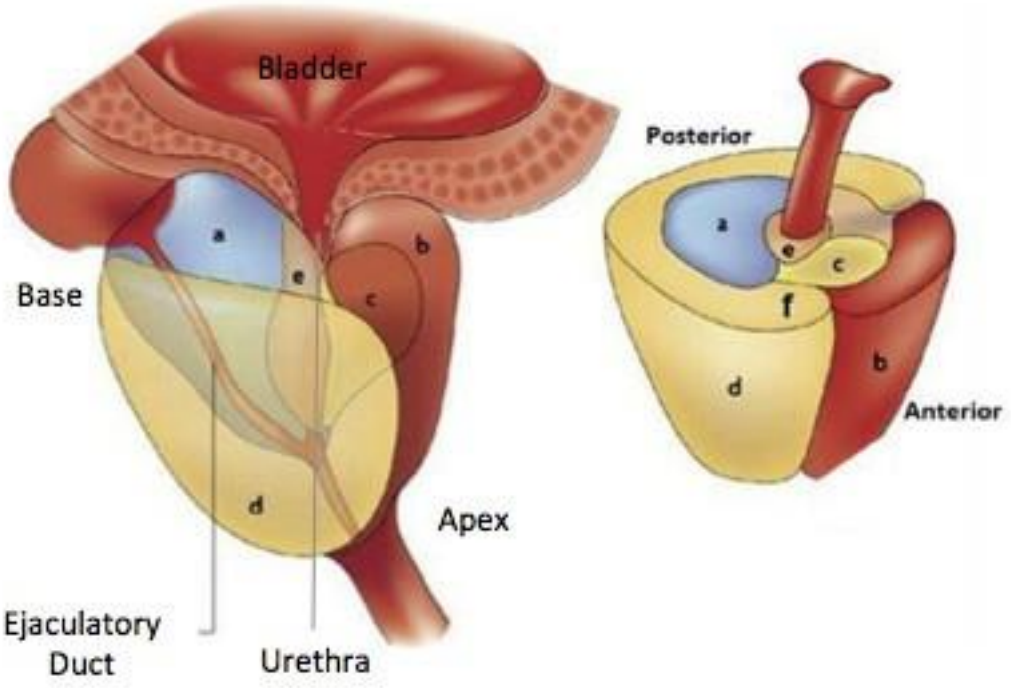

Figure 2.1. The Prostate. The prostate is divided into a)the central zone, b)anterior fibromuscular stroma, c)transition zone, d&f)peripheral zone and e)periurethral gland (De Marzo *et al*. 2007).

2.2.Characteristics of Prostate Cancer

2.2.1.Epidemiology of Prostate Cancer

Prostate cancer is the second most common cause of cancer-related mortality in men after lung cancer with a higher incidence of the disease observed in developed countries (Center *et al*. 2012). The incidence has begun to increase worldwide since 1990. PCa is the third after lung and colon cancer in cancer-related deaths and responsible for 9% of cancer-related deaths (Jemal *et al*. 2006). Approximately 1,1 million people were diagnosed with prostate cancer in 2012, and about 70% of the cases occurred in developed regions (GLOBOCAN, 2012). The morbidity of prostate cancer varies between different regions of the world, with highest in North America and lowest in South Asia. In New Zealand and Australia, the highest incidence rate for age is 111,6 in 100,000 and the lowest is 3,7 in 100,000, while it is 4,5 in South Asia (Bashir *et al*. 2015, Torre *et al*. 2015, Hassanipour-Azgomi *et al*. 2016) It has been noted that prostate cancer has been detected in approximately 2,8 million men in the United States, and the incidence has increased over time (Gronberg, 2003, Vanagore *et al*. 2017). Men have a 1 in 8 chance of being diagnosed with PCa during their lifetime. Nevertheless only 1 in 27 is expected to die from Pca. This

is due to the majority of PCa cases that are indolent or slowgrowing in nature This is due to the majority of lazy or slowly growing PCa cases in nature. (Canadian Cancer Society, 2016). According to 2015 datas the prostate cancer incidence rate is 35 cases per 100,000 in Turkey (Zorlu *et al*. 2014).

2.2.2.Common Prostate Cancer Risk Factors

There are several established risk factors that may indicate the presence of PCa in men (Attard *et al*. 2016, Cuzick *et al*. 2016, Glass *et al*. 2013). Age is one of the powerful risk factor for having PCa. For men with older age, the risk of becoming PCa is higher, and the incidence rapidly increases after age of 70 (Attard *et al*. 2016). Ethnicity is also one of the established risk factors for PCa(Attard *et al*. 2016, Cuzick *et al*. 2016, Glass *et al*. 2013). The incidence of PCa in patients with different ethnicities is variable depending on the geographical location (Center *et al*. 2012). Notably, African American men have the highest incidence of PCa in the US (Thompson *et al*. 2006). This may be relatedto socioeconomic status, limited access to healthcare and late PCa screening for this population (Dale *et al*. 1996). Likewise, patients who have a family history of PCa are also considered to have a higher risk of developing PCa (Attard *et al*. 2016, Glass *et al*. 2013). This depends on the amount of relatives affected with the disease and the relationship of the patient to the affected relative. Furthermore, known genes disclose about 35% of the familial risk for PCa. (Lichtenstein *et al*. 2000). Genetic mutations in genes such as BRCA2 are found in families with higher rates of breast and ovarian cancer (Rebbeck *et al*. 2015). Relatively rare, men who are carriers of BRCA2 mutation are also more likely to develop PCa. (Kote-Jarai *et al*. 2011,Castro *et al*. 2013). In addition, other gene mutations that have also been associated with an increased risk of PCa include HOXB13 and 5 CHEK2 genes (Karlsson *et al*. 2014, Goh and Eeles, 2014).

2.2.3.Classifying Prostate Cancer

2.2.3.1.The Gleason Grading System

The main screening methods for diagnosing of prostate cancer are digital rectal examination (DRE), serum prostate-specific antigen (PSA) level measurement, and transrectal ultrasound-guided biopsy. The suspicion in the DRE shows prostate cancer alone, without its PSA level, in about 18% of all patients. Furthermore, in patients with PSA levels of 2 ng / mL, the positive predictive value of doubt in the DRE is 5-30% (Vanacore *et al*. 2017, Loeb and Catalona, 2007). Prostate cancer is classified using the Gleason scoring system according to its morphological characteristics (Gleason and

Mellinger, 1974, Munkley *et al*. 2017). The Gleason grading system that is used to diagnose prostate adenocarcinoma has emerged as a result of a study involving more than 2,900 patients from the 1960s to the 1970s. Donald Gleason elaborated on the histological growth patterns (grade) of prostate adenocarcinoma and performed a clinical correlation analysis such as staging and prognosis. Now, it is one of the strongest prognostic factors for PCa (Montironi *et al*. 2011, Lopez-Beltran *et al*. 2006). The Gleason classification system is used to classify prostate tumors based on their histological morphology and patient stratification based on their risk of having lazy or aggressive PCa (Figure 2.2). For ease of classification the prostate tumours are stratified into 5 different glandular patterns referred to as Gleason patterns (Montironi *et al*. 2011). Gleason models range from 1-5, and represent morphologies of the prostate gland depending on their differentiation. The lower Gleason patterns are well differentiated glands, more similar to the normal morphology of the prostate gland. Higher Gleason models represent poorly differentiated glands and indicate a high risk of PCa. Gleason 1 correlates with the best distinguished and most favorable prognosis. Gleason 5 correlates with the least clear and poor prognosis. The Gleason score, which is shown to be better correlated with the biological behavior of prostate adenocarcinoma, has been developed due to the presence of two or more Gleason patterns in many prostate adenocarcinomas. The sum of the primary and secondary patterns gives the Gleason score (Chen and Zhou, 2016). Gleason patterns 1 and 2 are no longer assigned to needle biopsies. The reason for this is poor reproducibility and poor correlation with the grade of radical prostatectomy (Gordetsky and Epstein, 2016, Cury *et al*. 2008). The Gleason 3 pattern consists of discrete fabrics of various sizes, such as branching (Gordetsky and Epstein, 2016, Steinberg *et al*. 1997). The Gleason 4 pattern contains poorly formed, fused cribriform glands (Gordetsky and Epstein, 2016,Baisden *et al*. 1999). The Gleason 5 pattern consists of tumor layers, individual cells, and cell cords (Gordetsky and Epstein, 2016, Robinson, and Epstein, 2010). Typically, tumors assigned with GS 6 or less are classified as low risk, whereas tumors assigned with GS 7 and GS 8 or higher are classified as medium and high risk, respectively (Lopez-Beltran *et al*. 2006). Multiple tumours (may) have different GS and reflect the multifocality of the disease (Arora *et al*. 2004, Ruijter *et al*. 1996). This makes it difficult to predict the progression of the disease at an early stage. In last years, the original Gleason classification system has been modified to address clinical dilemmas associated with patient risk stratification.

In 2014 was held the last conference of the International Society for Urological Pathology (ISUP). They discussed and proposed changes to the existing Gleason grading system, with intention to make clearer guidelines for assigning GS that would more accurately reflect the disease prognosis of PCa patients in order to make proper treatment decisions. An important modification was made to categorize the GS into 5 Grade Groups (Table 2.1) (Shen *et al*. 2013, Van Der Kwast *et al*. 2013, Matoso *et al*. 2016). This is based on evidence that PCa patients with GS 7 tumours in which Gleason pattern 4 is predominant in the area (GS 7 (4+3) have worse prognosis compared to PCa patients with GS 7 tumours in which Gleason pattern 3 is predominant in the area (GS 7 (3+4)) (Amin *et al*. 2011, Pierorazio *et al*. 2013). Also, patients with GS 8 tumours have better prognosis than GS 9-10 tumours (Epstein *et al*. 2016). As a result, the newly proposed classification system separates GS 7 (3 + 4) and GS 8 into their own categories (Table 2.1). Other recommendations offered by this new system are to combine large and small cribriform architectural patterns under the Gleason pattern 4 category, since both morphologies showed a higher probability of poor results (Figure 2.3) (Kweldam CF *et al*. 2014). In addition, GS 6 tumors are also classified under grade group 1 to clearly reflect its low risk prognosis (Shen *et al*. 2013, Matoso *et al*. 2016).

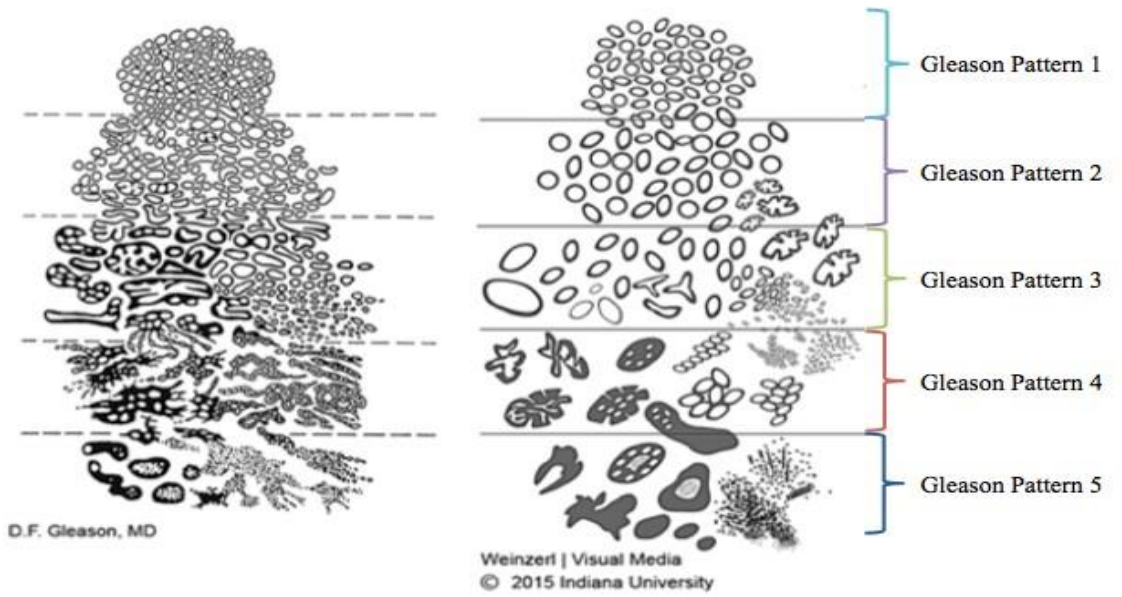

Figure 2.2. The Gleason Scoring System Schematic. Original (left, 1966 & 1967) vs. Modified (right, 2014) (Epstein *et al*. 2016).

Table 2.1. 2014 Gleason Grading System for Prostate Cancer Tumours. Each Grade Group corresponds to a Gleason score from the original Gleason scoring system.

Grade Group (2014)	Gleason Score	Histological Definition
1	3+3 = 6	Well-formed glands; glands are not fused
2	3+4 = 7	High composition of well-formed glands with lesser composition of poorly formed or fused glands
3	4+3 = 7	High composition of poorly formed or fused glands, lesser composition of wellformed glands
4	8	Poorly formed or fused glands with some areas lacking glands
5	9-10	Mainly composed of areas lacking glands, with a lesser component of poorly formed or fused glands

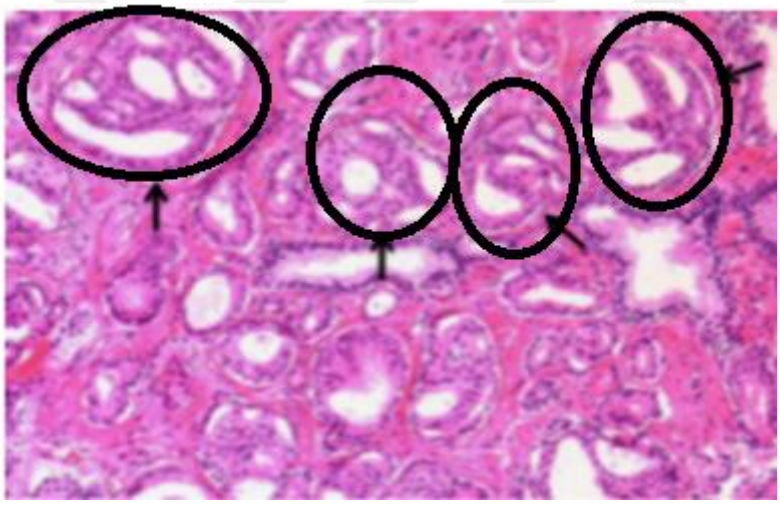

Figure 2.3. Histological representation of cribriform glands (gleason pattern 4). Cribriform glands are indicated by the black arrows(Epstein *et al*. 2016).

2.2.3.2.Clinical and Pathological Staging

The tumour, node and metastasis (TNM) staging system was developed in 1992 by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) to help in the separation of low, medium or high risk patients with prostate cancer (Amin MB *et al*. 2017). Shortly, this system discloses primary tumor spread (T) to the lymph nodes (N) and whether distant metastases have been formed or not (M).It classifies prostatic tumours based on their extension into surrounding or remote areas throughout the body (Table 2.2) (Andreoiu and Cheng *et al*. 2010, Cheng L *et al*. 2012). Both clinical and pathological TNM staging is performed in patients with PCa to describe the predicted

disease prognosis (Cheng L *et al*. 2012). Clinical staging is performed before the treatment and reflects evidence of tumor spread using imaging and other modalities (i.e. magnetic resonance imaging (MRI), transrectal ultrasonography (TRUS)) or digital rectal examinations (DREs) (Cheng L *et al*. 2012, Oon SF *et al*. 2011). Conversely, pathological staging is usually performed after radical prostatectomy; wherein the entire organ can be assessed histologically for tumor growth in the prostate or outside the prostate. (Ehrlich *et al*. 1982). The TNM stage consists of a tumor component (T-stage) that describes the spread of the tumor in the prostate gland or to adjacent structures (Table 2.2) (Amin MB *et al*. 2017, Cheng L *et al*. 2012). T1 tumors are neither palpable nor detectable during visualization, but can be detected by biopsy. T2 prostate tumors are large enough to be detected by DRE or TRUS and are confined within the prostate.T3 tumors break through the 'capsule' of the prostate and can invade the seminal vesicles.Tumors invading other organs, such as the pelvic wall or rectum, are arranged by T4. TNM also includes a lymph node and distant metastasis component (N and M stages respectively) that explain whether a tumor metastasizes to regional lymph nodes or distant organs (Table 2.2). If regional (pelvic) lymph nodes are affected the N classifier is 1, otherwise it is 0 (not affected) or X (not assessed). Distant metastases are explained by M=1, if no metastases can be found M is 0.

The same scoring system is used for clinical and pathological staging. However, the "p" is placed in front of the stage category in order to represent a pathological staging. In general, TNM is used to predict the outcome of disease and guide treatment decisions (Andreoiu and Cheng *et al*. 2010, Cheng L *et al*. 2012).However, clinical staging poorly predicts the pathological stage determined after RP.This is because of multifocality of PCa. As a result, PCa is usually represented by clinical stage when compared with the final pathological stage(Montironi *et al*. 2011, Andreoiu and Cheng *et al*. 2010, Cheng L *et al*. 2012). Classification systems that accurately predict the course of the disease for PCa patients without the need for RP will be ideal for early and potentially more effective treatment.

Table 2.2. TNM staging according to the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) as of 2016

Stage	Clinical	Pathological
Tx	**Primary tumour cannot be assessed**	-
T0	**No evidence of primary tumour**	-
T1 (a-c)	**Clinically inapparent tumour** **- T1a:** Tumour found in < 5% of resected tissue	-

	- T1b: Tumour found in > 5% of resected tissue **- T1c:** Tumour upon needle biopsy	
T2 (a-c)	**Tumour palpable and confined to prostate** **- T2a:** Tumour spread within < ½ of one lobe **- T2b:** Tumour spread within > ½ of one lobe, but not both lobes **- T2c:** Tumour spread to both lobes	**Organ-confined tumour** (No sub-classification)
T3 (a-b)	**Extraprostatic tumour that does not invade adjacent structures** **- T3a:** Extraprostatic extension **- T3b:** Tumour invades seminal vesicles	**Extraprostatic Extension** **- pT3a:** Extraprostatic extensions or invasion into bladder neck **- pT3b:** Seminal vesicle invasion
T4	**Invasion of adjacent structures (except seminal vesicles): external spincter, rectum, bladder, levator muscles and/or pelvic wall**	**Invasion of rectum, levator muscle or pelvic wall**
Nx	**Regional Lymph nodes not assessed**	**Regional lymph nodes not sampled**
N0	**No regional lymph node metastasis**	**No positive regional lymph nodes**
N1	**Metastasis in regional lymph nodes**	**Metastasis in regional lymph nodes**
M0	**No distant metastasis**	**No distant metastasis**
M1 (a-c)	**Distant Metastasis** **- M1a:** Non-regional lymph nodes **- M1b:** Bone metastasis **- M1c:** Other sites with or without bone metastasis	**Distant Metastasis** **- M1a:** Non-regional lymph nodes **- M1b:** Bone metastasis **- M1c:** Other sites with or without bone metastasis

2.2.3.3. Aciner adenocarcinoma

Invasive malign epithelial tumor containing secretory cells. Prostate adenocarcinoma involves a spectrum ranging from well differentiated gland structures, which can be difficult to distinguish with benign glands, to poorly differentiated tumors of which prostate origin is difficult to understand. Morphological features of prostate adenocarcinoma are nuclear anaplasia, invasion, and structural defect. Invasion can be seen in the form of irregular glands, individual irregularities outward from the glands and also in the form of perineural invasion. Structural dysfunction may be in the form of small back to back glands, combined glands, cribriform structures, cords and solid islands (Eble *et al*. 2004, Mostofi *et al*. 1993). The prostate adenocarcinoma (AAC) is divided into seven

histologic subtypes by the World Health Organization (WHO), including atrophic, pseudohyperplastic, foamy, mucinous, signet ring cell, oncocytic and lymphoepithelioma-like. These histological subtypes may be found alone, or may be accompanied by classical AAC (Eble *et al*. 2004). 68 cases are reported in the literature that rarely seen in prostate (Ahn *et al*. 1991, Pedro *et al*. 2006). Extraprostatic spread of these tumors is frequent, but distant organ metastases are not a frequent finding (Minei *et al*. 2001, McKenney *et al*. 2004). Macroscopic identification of prostate adenocarcinoma in radical prostatectomy specimens is difficult, it requires a microscopic examination. However, in grayish yellow color, the borders are seen as a hard area that can not be completely distinguished from the surrounding tissue. Microscopically, prostate adenocarcinoma exhibits a broad spectrum; from anaplastic tumor to well differentiated neoplasm, which is difficult to distinguish from nonneoplastic gland (Rosai and Ackerman 2004). Most prostate adenocarcinomas arise from one or more pattern-forming acini. The diagnosis depends on the combination of structural and cytologic symptoms. The features seen in the light microscope are usually adequate for diagnosis (Bostwick *et al*. 2003). In gland-forming carcinomas, glands are more crowded than prostate tissue. Glands in the prostate adenocarcinoma typically grow indiscriminately. Glands are placed perpendicular to each other. Another pattern of infiltrating structure is the presence of small atypical glands between large benign glands. The difference between glandular differentiation, cribriform-shaped formations, unified glands, distorted gland structures and benign glands, becomes more evident based on the structural pattern. Indifferentiated prostate cancer is characterized by solid islands, cell cords, and isolated individual cells (Eble *et al*. 2004).

2.3.Epigenetics

New opportunities for cancer diagnosis and screening can arise from the identification of epigenetic changes associated with cancer. Epigenetics was first introduced by Conrad Waddington in 1942 (El Hajj *et al*. 2017). Epigenetics refers to inherited changes in gene expression that are not based on the underlying DNA sequence (Henikoff and Matzke, 1997). DNA is compressed into a chromatin structure with the nucleosome in the eukaryotic nucleus. The histone octamer contains two elements of the nuclear histone (H3, H4, H2A and H2B) (Luger *et al*. 1997, Chen *et al*. 2014). Packing of DNA into chromatin creates a potential barrier to the factors that use DNA as a template (Chen *et al*. 2014). Each normal somatic cell contains the same genome consisting of about three billion base pairs. Numerous epigenetic modifications regulate transcriptional access to genes of a

particular cell type and developmental stage. There are essentially three changes that regulate the epigenetic mechanisms of gene expression and the chromatin structure. These are; DNA methylation, histone covalent modification, which are the main protein components of chromatin and miRNAs (Chen *et al*. 2014).

Epigenetic arrangements in the form of DNA methylation patterns and histone modifications regulate gene regulation (El Hajj *et al*. 2017, Jaenisch and Bird, 2003). DNA methylation means the covalent attachment of a methyl group to the 5 position of the cytosine pyrimidine ring (Bestor, 2000) and represents a relatively stable and conserved signal which makes it an attractive option for epigenetic studies. The most pronounced epigenetic modification of DNA is methylation, which occurs primarily at the carbon 5 'position of the cytosine in the context of CpG dinucleotides. Most of the CpG isoform and promoter region are demethylated while genomic non-coding regions usually cause genomic instability. During differentiation, development or disease processes, supporting methylation is associated with transcriptional silencing (El Hajj *et al*. 2017, Smith and Meissner, 2013, Weber and Schubeler, 2007). Changes in chromatin structure occur via post-translational modifications of the histone such as acetylation, methylation and phosphorylation. These modifications can change the conformation of chromatin between an open, transcriptionally active form known as euchromatin and a condensed, transcriptionally inactive form known as heterochromatin (Jenuwein and Allis, 2001).

2.3.1.DNA methylation

DNA methylation plays an important role in many different biological processes such as embryonic development, transcription, chromatin formation, X chromosome inactivation, genomic imprinting, genomic instability and carcinogenesis (Cheung *et al*. 2009). Methylation is the only known epigenetic modification of DNA. In addition, post-translational histone modifications are also epigenetic changes, such as DNA methylation (Kouzarides, 2007). Generally, methylation occurs in 5-cytoplasm. Many domains of the genome include CpG dinucleotides (Figure 2.4.). These domains are termed CpG islands and are usually found in the promoter regions of genes in the human genome and contain 70% CpG nucleotides (Saxonov *et al*. 2006). In normal somatic cells, most of the CpG islands are usually found in unmethylated form. The abnormal hypermethylation of CpG islands of some tumor suppressor genes is associated with tumorigenesis. Although the cause of abnormal methylation is unknown, it is suggested that alteration or degradation of the regulation of DNA methyltransferases or some chromatin binding proteins are

responsible for this. Recent studies have highlighted DNA methylation as a marker that can be used in the early detection of cancer in biological specimens such as serum, plasma or urine (Laird, 2003). For example, hypermethylation of 31% of the GSTP1 gene in the prostate cancer and 25% of the APC tumor suppressor gene in the esophageal adenocarcinoma can be detected in the plasma as DNA methylation markers in both tumors (Chuang, 2003).

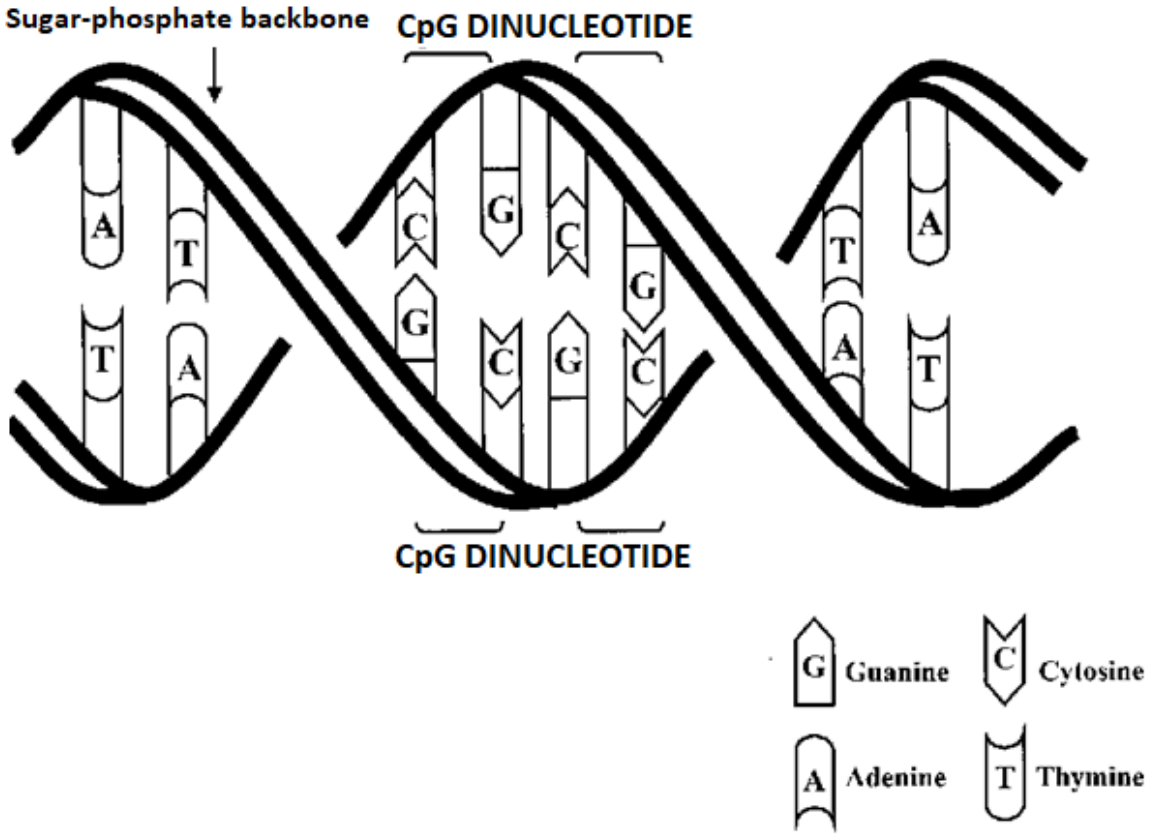

Figure 2.4. DNA backbone and CpG dinucleotides. DNA double helix and CpG dinucleotide pairs. DNA structure with opposing base pairs arranged on a double helix sugar–phosphate backbone. CpG dinucleotide pair units that are the sites for possiblemethylation are outlined.

2.3.2.Molecular mechanism of DNA methylation

DNA methylation is the result of enzyme-induced chemical modification. As a result of covalent attachment of a methyl (-CH3) group to the 5 carbon atom on the cytosine base cytosine nucleotide is getting methylated. This event is regulated by a group of enzymes called DNA methyltransferases. The methyl group is provided by S-adenosyl methionine (SAM). After methylation, S-adenosyl methionine (SAM) is converted to S-adenosyl

homocysteine (SAH). SAH is converted back to SAM through the folate-cobalamin pathway (Figure 2.5).

There are five types of DNA methyltransferase genes, DNMT1, DNMT2, DNMT3L, DNMT3A, DNMT3B. These developmentally regulated genes play a critical role in the realization and regulation of DNA methylation. DNMT1 is responsible for the regulation of cytosine methylation. Some of the epigenetic markers on the DNA are transferred from progeny to progeny exactly. These epigenetic markers need to pass to the offspring parental.During DNA replication in the offspring cells, the parental features are transferred to the cells by the DNMT1 enzyme. During this transfer, the DNMT1 protein in cooperation with the MECP2, which is a methyl-CpG binding protein, methylates the semi-methylated DNA, thereby allowing the parental features to be transferred to the offspring cells (Kimura and Shiota, 2003).It is known that DNMT1 plays a role in the suppression of some target genes by interacting with many different proteins such as transcription factors (STAT3, HP1), histone modifying molecules (HDAC1, HDAC2) (Robertson *et al*, 2000, Rountree *et al*. 2000, Smallwood *et al*. 2007, Zhang *et al*. 2005). The DNA methyltransferase-3 proteins (DNMT3) are responsible for de novo methylation of CpG islands, i.e. the newly formed methylation of previously unmethylated bases. DNMT3A is a protein responsible for parental imprinting. Imprinted genes are methylated in each parental allele and are expressed as monoallergenic.

Although DNMT3A, DNMT3B and DNMT3L genes are thought to lack of any methyl transfer activity, it is suggested that DNMT3B is responsible for the methylation of centromeric satellite repeat sequences, and that mutations in the DNMT3B 13 gene lead to ICF (centromeric heterochromatin instability) syndrome (Jeanpierre *et al*. 1993).

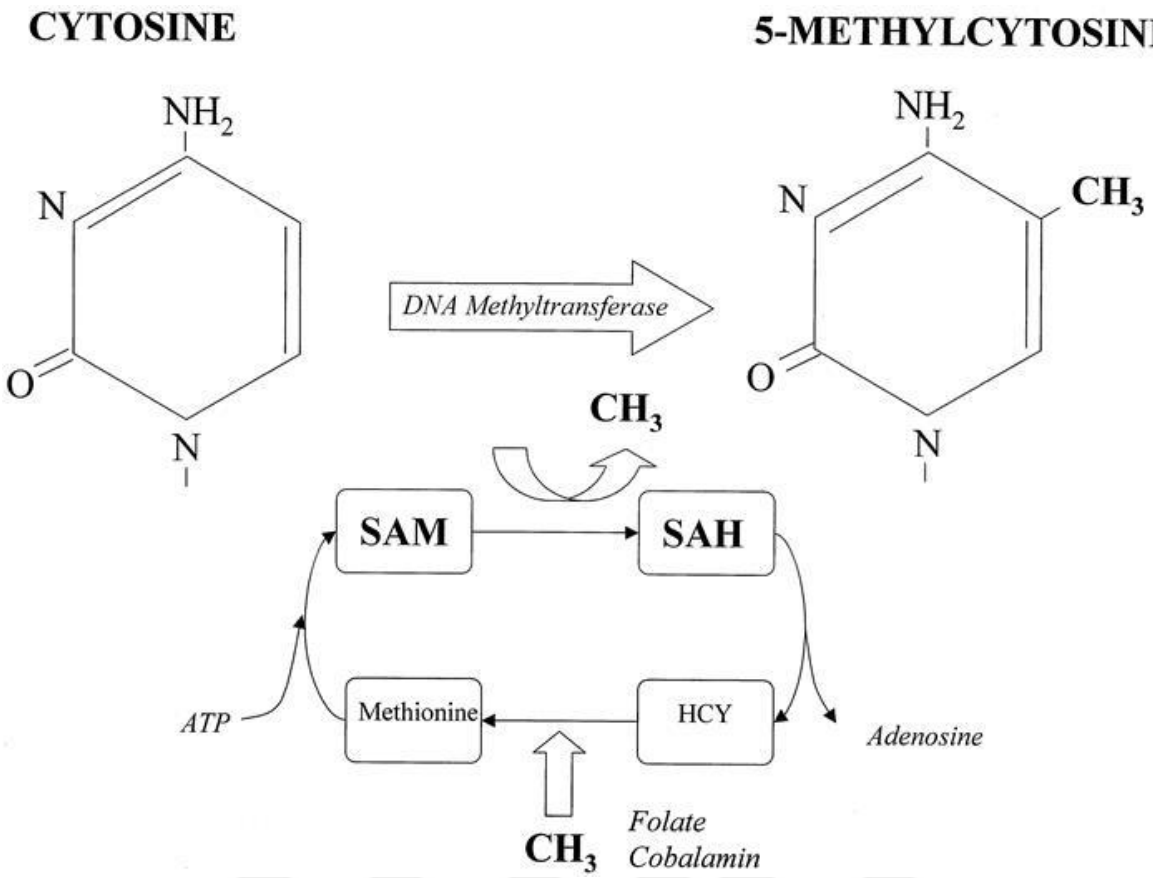

Figure.2.5. Methylation cycle. Methylation of the 5-carbon on the cytosine residue is executed by the DNA methyltransferase enzyme, which uses a methyl group from S-adenyl methionine (SAM). This is converted to S-adenyl homocysteine (SAH), which is then broken down to homocysteine (HCY) and adenosine. SAM is reconstituted from HCY by methionine. Folate and cobalamin are required for and provide the methyl groups for this reaction.

2.4.DNA methylation changes in cancer

Abnormal patterns of DNA methylation are one of the most common changes found in cancer (Esteller *et al*. 2001, Esteller, 1999, Feinberg, 1999, Feinberg, and Vogelstein, 1983, Feinberg, and Vogelstein, 1983, Fleisher *et al*. 1999, Irizarry *et al*. 2009, Issa, 2004). Cancer cells exhibit a global loss of DNA methylation in addition to a gain of methylation in some CpG islands (Issa, 2004). These changes provide tumour cells with a growth advantage by increasing their genetic instability and allowing them to accrue progressive changes that support their continued proliferation and metastasis (Robertson, 2005).

2.4.1.Global genomic hypomethylation

The loss of DNA methylation is the first epigenetic change in cancer cells (Feinberg, and Vogelstein, 1983). Global genomic hypomethylation is largely due to the loss of

methylation in the repetitive DNA sequences (Esteller, 2008), and is universally seen in several cancers, as well as some pre-malign adenomas (Ehrlich, 2002). Furthermore, the degree of hypomethylation was associated with disease severity and metastatic potential (Feinberg, and Vogelstein, 1983, Esteller, 2008).

Depending on the cancer, global DNA hypomethylation has many functional effects.By weakening transcriptional repression, DNA hypomethylation can facilitate chromosomal instability, which is another distinguishing feature of tumor cells (Robertson, 2005). Experiments in which methylation was depleted showed that loss of DNA methylation leads to aneuploidy and chromosomal rearrangements (Karpf and Matsui, 2005), which are believed to be mainly due to the loss of methylated cytosines in centromeric or pericentric regions. (Eden *et al*. 2003).

2.4.2.Single-locus DNA hypomethylation

DNA hypomethylation is mainly described to occur in satellite DNA of centromeric regions, Alu regions and long interspersed elements (Ehrlich, 2009). Hypomethylation in the coding sequences is also observed in cancer (Ehrlich, 2002). In a recent study, it was found that CpG islands could be methylated normally in somatic tissues (Strichman-Almashanu *et al*., 2002), and hypomethylation of these islands in cancers could activate nearby genes (Feinberg and Tycko, 2004). Hypomethylation was also found in genes whose activation contributes to tumor formation (Feinberg and Tycko, 2004). There are several genes that are activated by hypomethylation in cancer, including oncogenes such as leukemia homeobox proto-oncogenes (HOX11) (Watt *et al*. 2000), v-myc myelocytomatosis viral oncogene homolog (C-MYC) in colorectal cancer (Sharrard *et al*. 1992), and v-Ha-ras Harvey rat sarcoma viral oncogene homolog (HRAS) in melanoma (Feinberg, and Vogelstein, 1983), as well as non oncogenes such as trefoil factor 1 (pS2) in breast cancer, which is implicated in the control of cell proliferation (Martin *et al*. 1997), and carbonic anhydrase 9 (MN/CA9) in renal cell carcinoma (Cho *et al*. 2001). Furthermore, hypomethylation in genes can disrupt genomic imprinting through the activation of normally silent alleles, and there is a large series of cancers that exhibit such imprinting loss (Feinberg, 1999).

2.4.3.DNA hypermethylation

The hypermethylation of DNA in cancer occurs simultaneously with global genomic hypomethylation.Hypermethylation of DNA frequently occurs in the CpG islands of gene

promoters and in most cases is realted with transcriptional silencing (Esteller, 2008, Rauch *et al*. 2008). It is estimated that an average of 600 of about 45,000 CpG islands in the genome is hypermethylated in cancer (Costello *et al*. 2000). Hypermethylation of promoters is an important mechanism for inactivation of tumour suppressor genes (Herman and Baylin, 2003), and aberrant hypermethylation and downregulation have been observed in genes involved in the cell cycle, DNA repair, cell signaling, chromatin remodeling, transcription, and apoptosis for almost every type of tumour (Robertson, 2005).

Studies have found that CpG hypermethylation patterns occur in cancer-specific fashion (Costello *et al*. 2000), in both sporadic and hereditary cancers of the same tumor type (Esteller *et al*. 2001).In these studies, it was found that cancer-related DNA methylation changed according to the type of cancer being investigated (Esteller *et al*. 2001, Costello *et al*. 2000). It has been suggested that this may be due to different growth selective pressures or individual CpG island sensitivities for each tumor type (Costello *et al*. 2000). Promoter hypermethylation in certain CpG islands may provide a selective advantage for the survival of a specific cell type (Esteller, 2002). Therefore, the reason for certain genes to be downregulated in one type of cancer versus another is because there are important cellular consequences for the lack of expression of that gene that promotes the growth of tumors of a particular tissue (Esteller, 2002). Hypermethylated genes known in different cancers include glutathione S-transferase P (GSTP1) in prostate cancer (Wu *et al*. 2011), breast cancer 1, early onset (BRCA1) in breast and ovarian cancers (Esteller *et al*. 2001, Esteller *et al*. 2000), and mutL homolog 1, colon cancer, nonpolyposis type 2 (hMLH1) in gastric, colorectal, and endothelial cancers (Fleisher *et al*. 1999, Esteller, *et al*. 2001, Herman *et al*. 1998)

2.4.4.CpG island methylator phenotype

One theory shows that there is a CpG island methylator phenotype (CIMP) in human cancers. This theory was developed from studies in colorectal cancer and revealed a subset of cancers that showed a 3-5 fold increase in the frequency of aberrant hypermethylation in multiple loci, and this pattern of methylation in a cluster of genes was not seen in the remaining cases (Toyota *et al*. 1999). According to this theory, CIMP cancers are biologically unique compared to other cancers with differences in genetics, histology, barepathology and clinical features (Issa, 2004). However, this is still a controversial concept without a consensus in the selection of genes involved in a panel to distinguish CIMP cancers from other types (Feinberg and Tycko, 2004).

2.5.Diagnostic Biomarkers for Prostate Cancer

2.5.1.What are Biomarkers?

A common target of many PCa diagnostic tests is to accurately identify patients with PCa prior to or upon biopsy while minimizing the false positive and false negative results.Biomarkers are characterized as factors that can be measured to indicate normal or abnormal biology in a patient or to indicate a response to therapeutic interventions (Biomarkers Definitions Working Group, 2001). They can also be classified as diagnostic, prognostic, predictive and therapeutic biomarkers to reflect their intended clinical use.Genetic, epigenetic and proteomic changes have been studied in patients with PCa who demonstrate a strong potential as diagnostic biomarkers for early detection of the disease (Falzarano *et al*. 2015, Verma *et al*. 2011, Hatakeyama *et al*. 2016). The most clinically relevant and useful diagnostic biomarkers are those that can be detected in biological samples, such as urine, serum and biopsy tissue, which are obtained through non-invasive or minimally invasive procedures.

2.5.2.Genetic Diagnostic Biomarkers

2.5.2.1.Chromosomal Alterations

Genetic changes in PCa tumors are often associated with a gain or loss of gene expression (Barbieri *et al*. 2013). More specifically, chromosomal rearrangements often arise for the disregulation of gene expression. Chromosomal anomalies include amplifications (chromosome 8q), deletions (chromosome 8p), inversions and translocations (Qian *et al*. 1995, Taylor *et al*. 2010). One of the most common chromosomal rearrangements observed in PCa involves deletion on chromosome 21 to create a TMPRSS2:ERG gene confluence (Tomlins SA *et al*. 2005, Demichelis F *et al*. 2007, Winnes M *et al*. 2007). TMPRSS2 is an androgen-regulated gene which encodes Transmembrane Protease, Serine 2 and is located upstream of the ERG gene, which is a part of the ETS gene family of transcription factors. As a result of this gene fusion, ERG expression is increased and causes aberrant expression of downstream target genes that promote cell motility and carcinogenesis (Tomlins SA *et al*. 2005, Winnes M *et al*. 2007). TMPRSS2: ERG gene fusion occurs in approximately 50% of PCa patients, mainly due to heterogeneity of the disease. It is also reported that TMPRSS2 fuses with other members of the ETS gene family, such as ETV1 and ETV4 (Winnes M *et al*. 2007).

TMPRSS2:ERG gene fusion is assumed to occur during the early development of PCa with data indicating that lower levels were found in a more aggressive disease (Scheble VJ *et al*. 2012). Thus, TMPRSS2: ERG participates as a diagnostic biomarker for early diagnosis of prostate cancer. The studies examined the efficacy of the synthesis of the TMPRSS2: ERG gene to create patients with PCa using urinary deposits (Hessels D *et al*. 2007). Specificity of this biomarker is high (93%), but sensitivity is low (37%) (Hessels D *et al*. 2007). Combining the biomarker TMPRSS2: ERG with other promising diagnostic biomarkers can improve overall detection rate.

2.5.2.2.Long non-coding RNAs

Only 2% of transcribed RNAs encode proteins, while the remaining RNAs are called non-coding RNAs (ncRNAs) (International Human Genome Sequencing, 2004, Zhang *et al*. 2017). ncRNAs do not have a valid open reading frame, and they do not have the ability to code a protein (Consortium *et al*, 2007, Carninci *et al*. 2005, Ponting and Belgard 2010, Li *et al* 2017). They are grouped into two groups, short ncRNAs and long ncRNAs. Short ncRNAs are 200 nucleotides, which include RNAs that interact with piwi, small nucleolar RNAs (snRNA), M and others (Zhang *et al*. 2017, Michalik *et al*. 2014). RNAs longer than 200 nucleotides are called lncRNA (Li *et al*. 2017, Sana *et al*. 2012, Kapranov *et al*. 2007). There are a wide variety of ncRNAs in this group, including intergenic ncRNAs, long intronic ncRNAs, and pseudogen RNAs (Li *et al*. 2017, Sana *et al*. 2012). Long non-coding RNAs are transcribed by RNA Polymerase II and are thought to be regulatory elements that do not encode proteins. Prostate Cancer Antigen 3 (PCA3) is a long non-coding RNA molecule that is expressed only in the prostate by the DD3 gene (Bussemakers MJG *et al*. 1999). Especially PCA3 has no known function and is over expressed in PCA, which makes it a promising diagnostik marker. In 2012, the Food and Drug Administration (FDA) approved the use of PCA3 as a diagnostic test called Progensa PCA3 for men over the age of 50 years to help in the decision to conduct the prostate biopsy exams (Deras IL *et al*. 2008). The PCA3 test involves the calculation of the PCA3 score based on the PCA3 and PSA mRNA levels in post-DRE urine. The PCA3 score has been reported to exceed the PCa display capacity of tPSA by biopsy (Falzarano *et al*. 2015, Capoluongo E *et al*. 2014). Patients with a PCA3 score> 25 are advised to perform an initial or repeat biopsy examination in order to confirm the diagnosis of Pca (Capoluongo E *et al*. 2014).

2.5.3.Epigenetic Diagnostic Biomarkers

2.5.3.1.DNA Methylation Biomarkers

DNA methylation is an epigenetic modification that occurs when a methyl group is added to the 5' carbon of a cytosine base located adjacent to a guanine base (CpG) (Jurkowska RZ *et al*. 2011, Deaton A and Bird A, 2011). Regions rich in CpG-dinucleotides (> 50%) are called CpG Islands and are often associated with the promoter region of genes. It has been reported that changes in DNA methylation patterns are related with the development of various cancer types (Sproul D and Meehan RR, 2013, Mikeska T and Craig JM, 2014). Typically, there is a global loss of methylation, accompanied by gene-specific methylation enrichment in the gene promoter zones, including tumor suppressor genes and oncogenes, respectively. In particular, in PCa, hypermethylation of DNA has been intensively studied and the hypermethylation of key genetic players, including GSTP1, APC and RASSF1A, have been reported by many groups (Strand SH *et al.* 2014, Steiner I *et al* 2010, Liu L *et al*. 2002, Jerónimo C *et al*. 2011). These genes have been studied individually and in panels for their ability to serve as biomarkers for the diagnosis and prognosis of PCa (Blute ML *et al*. 2015, Rouprêt M *et al*. 2007, Trock BJ *et al*. 2012, Brikun I *et al*. 2014, Troyer DA *et al*. 2009).

Identification of aberrantly methylated genes as potential PCa biomarkers has aroused great interest because of the stability of DNA, which can be easily detected in urine, serum and biopsy samples (Mikeska T and Craig JM, 2014). GSTP1 is the most commonly hypermethylated gene in PCa tumors and is found in about 90% of all cases (Van Neste L *et al*. 2012), making it a promising biomarker for PCa detection. Similarly, promoter hypermethylation of APC, RASSF1A and RARB2 methylation has been extensively studied and is often found in PCa (Liu L *et al*. 2002, Chen Y *et al.* 2013). Several hypermethylated genes have been combined to improve the diagnostic ability of any individual methylation biomarker for detection of PCa (Brikun I *et al*. 2014, Vasiljević N *et al.* 2011, Møller M *et al*. 2017). In addition, genome-wide methylation studies have also been performed to identify novel differentially methylated genes outside of promoter regions that can also increase PCa diagnosis (Yang B *et al*. 2013, Devaney JM *et al*. 2015, Bhasin JM *et al*. 2015). Because of the many proposed methylation biomarkers, further validation is necessary before implementing them into clinical practice.

2.5.3.2.MicroRNA

miRNAs are a class of conserved, small, noncoding RNAs that are responsible for posttranslational, epigenetic regulation of gene expression, ranging in length from 19 to 25 nucleotides (Malumbres M, 2013, Oliveto *et al*. 2017, Celano *et al*. 2017). The first miRNA lin-4 was discovered in 1993 by Victor Ambros and Gary Ruvkun (Monteleone and Lutz, 2017, Lee *et al*. 1993). They bind to the 3 'coding region of the target mRNA and inhibit the expression of multiple target genes (Lin and Gregory, 2015, Masuda *et al*. 2017). A single miRNA molecule can regulate the expression of over 200 transcripts (Malumbres M, 2013). For this reason, dysregulation of a single miRNA may have a significant effect on cancer development. Today, more than 2500 types of miRNA have been detected in humans (Masuda *et al*. 2017, Michael *et al*. 2003). Recent studies have shown that miRNAsplay important roles in many critical biological processes including development, proliferation, differentiation, apoptosis, tumor formation, signal transduction, organ development, and hematopoietic lineage differentiation (Bartel, 2009, Huang *et al*. 2011). In PCa, the expression of miRNA is changed in PCa and was studied on several biological samples, such as urine, tissue and serum (Bertoli G *et al*. 2016). Some of the most common dysregulated miRNAsreported in PCa include hsa-mir-192, hsa-mir-572, hsa-mir-7a, hsa-mir-21, hsa-mir-99a, hsa-mir-141, hsa-mir-145, hsa-mir-200c, hsa-mir-221 and hsa-mir-375. Many of these miRNAsare observed to be dysregulated across multiple types of samples. In some studies miRNAs have been shown to be downregulated in tumors whereas other studies have shown that miRNAs in tumors are generally upregulated. (Vanacore *et al*. 2017, Lu *et al*. 2005). For example, it was reported that let-7a and hsa-mir-21 are upregulated, and hsa-mir-145 and hsa-mir-192 are downregulated both in tissues and in blood samples in patients with PCa compared to healthy control patients (Wach S *et al*. 2012, Kelly BD *et al*. 2015, Sandeep K. *et al*.2016). This characteristic makes them ideal biomarkers for early detection of PCa. In addition, multiple miRNAs have also been combined in a biomarker panel to improve the ability of detecting of individual miRNAs (Haldrup C *et al*. 2014, Moltzahn F *et al*. 2011, Chen Z-H *et al*. 2012). Although the miRNA biology area is relatively new, miRNAs serve as a promising source of biological information that can be used as evidence for the diagnosis of PCa. In spite of the fact that most of the complexity of miRNA is not fully clarified in both prostate cancer and cancer development in general, the importance of miRNA has been shown in several key oncogenic pathways in prostate cancer. Especially in prostate cancer, regulation of

miRNAs with androgen receptor signaling pathways and miRNAs on this pathway attracts attention. Interaction of miRNAs with other key signaling pathways in prostate cancer, such as PTEN / ACT signaling pathways, is also discussed (Catto *et al*. 2011, Jackson *et al*. 2014). Studies have shown that miRNAs differentially expressed in tumor tissues, function as prognostic and therapeutic biomarkers for prostate cancer in body fluids such as plasma, serum or urine (Goto *et al*. 2015).

2.5.4.Proteomic Diagnostic Biomarkers

Protein expression can be altered in PCa through numerous mechanisms such as abnormal miRNA-NA expression, chromosomal rearrangements, DNA methylation, and histone modifications. Abnormally expressed proteins have been discovered/suggested as diagnostic biomarkers for early detection of PCa (Alinezhad S *et al*. 2016, Kristiansen G *et al*. 2008, Uetsuki H *et al*. 2005). As noted previously, PSA codes for a glycoprotein which is over-expressed in PCa. (Salman *et al*. 2015, Stamey *et al*. 2004, Balk, 2003). Although it is not PCa specific, is the most widely used biomarker for PCa screening, despite recommendations against its use. GOLPH2 is a golgi membrane antigen that is observed to be up regulated in about 90% of patients with PCa (Kristiansen G *et al*. 2008, Wei S *et al*. 2008). In addition to its high sensitivity, it can be tested in urine and becomes a promising protein biomarker for detection of PCa (Laxman B *et al*. 2008). Other overexpressed proteins in PCa include Alphamethyl-CoA racemase (AMACR) and Early Prostate Cancer Antigen (EPCA), all of which have also been proposed as potential diagnostic biomarkers for PCa detection (Alinezhad S *et al*. 2016, Kristiansen G *et al*. 2008, Uetsuki H *et al*. 2005).

2.5.5.Diagnostic Biomarker Panels

Many of the PCa biomarkers mentioned earlier have been studied in combination to improve their ability to detect individual biomarkers.When biomarkers are combined, their diagnostic power is added and possible false positive and false negative results can be minimized.Recently, new biomarker panels have emerged that show strong diagnostic potential and perform better than the traditional PSA screening test (Falzarano *et al*. 2015). In addition to their intended use for PCa diagnosis, some biomarker panels may also provide prognostic information before performing any invasive procedure.Prospectively, combining biomarkers with the strongest diagnostic potential will have the greatest success in clinical practice to ensure patients that are given an early and accurate diagnosis.

2.5.5.1.Prostate Health Index

The Prostate Health Index (PHI) is a diagnostic test used to determine the likelihood of detecting PCa after biopsy in patients with serum PSA levels within the diagnostic gray zone and a negative DRE (Falzarano *et al*. 2015, Jansen FH *et al*. 2010, Lazzeri M *et al*. 2013). The PHI was approved by the FDA in 2012 and calculated a score based on tPSA,% fPSA, and -2proPSA levels.Compared to individual PSA measurements, it has a more predictive ability to distinguish between benign and malign states of the prostate gland in men aged 50 years and older, since in men with PCa are more likely to have a higher PHI(Jansen FH *et al*. 2010, Lazzeri M *et al*. 2013).

2.5.5.2.4Kscore

Currently, there are no reliable diagnostic tests that can distinguish between low and high-risk PCa during initial screening. However, 4-Kalikrein (4Kscore) was developed to determine the risk of having an aggressive PCa before initial or repeated biopsy tests (Braun K *et al*. 2016, Vickers AJ *et al*. 2010). 4Kscore is calculated based on an algorithm combining tPSA, fPSA, intact PSA and human kallikrein 2 levels in serum to create a probability of 0-100%. It will also consider clinical information of the patients, such as age, history of previous biopsy and positive or negative DRE. The 4Kscore is not approved by the FDA, but there is evidence that it has a strong potential as a pre-treatment prognostic marker to differentiate between aggressive and lazy PCa (Falzarano *et al*. 2015).

2.5.5.3.Mi-Prostate Score

As noted earlier, the TMPRSS2:ERG gene fusion occurs in about 50% of patients with PCa (Tomlins SA *et al*. 2005). Although it has a high specificity, it is limited by its low sensitivity (Hessels D *et al*. 2007). However, when combined with the detection of PCA3, the sensitivity was increased from 62% only for PCA3 to 73% when combined with TMPRSS2:ERG (Hessels D *et al*. 2007). A diagnostic test called "Mi-prostate Score" (Mi-PS) measures TMPRSS2:ERG levels and PCA3 levels in urine and serum PSA to create a score that predicts PCa within patients (Tomlins SA *et al*. 2016, Salami SS *et al*. 2013).In a recent study, the test was reported to have a sensitivity of 80% and a specificity of 90% (Salami SS *et al*. 2013).

2.5.6.Limitations of the Current Diagnostic Biomarkers

Many of the above-mentioned biomarkers have been proposed to improve the PCa detection (Verma *et al*. 2011). Ideally, biomarkers that are stable and easily detectable in biofluids (ie, urine, serum, whole blood) are suitable because they can be obtained from patients without performing invasive procedures. However, many clinical biomarkers have been proposed and require extensive validation in large multinational cohorts before being applied for widespread clinical use. For this reason, the current gold standard for PCa diagnosis is the histopathological examination of needle biopsy, which is limited by the ability to detect PCa due to sampling bias (Heidenreich *et al*. 2013, Stock *et al*. 2008, Patel and Jones, 2009). If no PCa is detected on the biopsy, there is no reliable diagnostic test that can confirm whether patients initially receiving a negative biopsy result are positive for PCa. Despite the emergence of many promising diagnostic biomarkers for PCa, markers that specifically identify patients with PCA with negative biopsy are warranted.

2.6.Aim of the thesis

In this study, methylation changes in hsa-mir-192, hsa-mir-512-5p, hsa-mir-513a-2 and hsa-mir-572 oncomir microRNAshave been studied in prostate cancer.It is aimed to analyze the promoter methylation status and expression changes of these microRNAs by methylation specific PCR (MSP) and real time PCR, respectively. Subsequently, the specific promoter methylation changes of miRNAs that shown to be significant will be investigated. In this study, possible tumor suppressor potentials of miRNAs will be shown.

3.MATERIALS AND METHODS

3.1.Required Devices

- Spectrophotometer
- Runik Thermal Cycler (SACEM)
- Real-Time Machine (Bio Rad)
- Horizontal Electrophoresis System (Major Science)
- Electrophoresis Power Supply
- Gel Screening System
- IncuBlock (Denville Scientific)
- Vortex Mixer (Stuart)
- Spectrafuge Mini Sentrifuge (Labnet)
- Microfuge Centrifuge (Beckman Coulter)
- Microwave (Altus)
- Big Centrifuge Machine (Nuve NF 1200)

3.2.Required Materials

- Quick-RNA FFPE Kit (Zymo Research)
- MiScript Reverse Transcription Kit (Qiagen)
- SensiFast Sybr Lo-ROX Kit (Bioline)
- Quick-DNA Miniprep Plus Kit (Zymo Research)
- EZ DNA Methylation-Gold Kit (Zymo Research)
- ZymoTaq qPCR Premix (Zymo Research)
- Universal Primer (Quanta Bio)
- p-Xylene (Merck)
- O'GeneRuler DNA ladder Mix (Thermo Scientific)
- UltraPure Agarose (Invitrogen)

- Tris Base (Fisher Bioreagents)
- Ethidium Bromide (AppliChem)
- Proteinase K
- ddH2O
- Ethanol %100
- Ethanol %75

3.3.Sample Collection

Tissue specimens obtained from 50 paraffin embedded tissue blocks with Adenomyomatous hyperplasia (benign protate tissues) diagnosis and 30 blocks with prostatic adenocarcinoma (malign prostate tissues) diagnosis were used as study material at Bezmialem Vakıf University from Medical Pathology Department's archive. All these materials were collected by the decision of ethics committee.For each sample, sections were takenfrom paraffin-embedded blocks in quantity of 100 micron for isolation of DNA and RNA and were transferred to 1,5 ml DNAseRNAse-free tubes.

Table 3.1. Ages and gleason grades of patients with Prostatic Aciner Adenocarcinoma

Patient numbers	Age	Gleason Grade	Percentage (%)
Patient 1	81	3+4	13%
Patient 2	73	4+3	80%
Patient 3	73	4+4	50%
Patient 4	69	4+5	40%
Patient 5	76	4+5	90%
Patient 6	79	4+5	90%
Patient 7	75	4+5	60%
Patient 8	74	3+4	60%
Patient 9	85	5+4	80%
Patient 10	57	4+5	40%
Patient 11	71	3+4	6%
Patient 12	69	3+4	10%
Patient 13	60	5+5	85%
Patient 14	79	4+3	35%
Patient 15	85	5+4	70%
Patient 16	69	5+4	75%
Patient 17	74	3+5	20%
Patient 18	62	3+5	50%
Patient 19	81	3+4	20%
Patient 20	61	3+5	25%
Patient 21	77	3+4	5%
Patient 22	79	4+5	25%
Patient 23	81	4+3	10%

Patient 24	77	3+4	21%
Patient 25	62	3+4	25%
Patient 26	68	4+3	30%
Patient 27	68	5+4	65%
Patient 28	75	4+3	18%
Patient 29	65	5+4	20%
Patient 30	80	3+4	15%

3.4.RNA Isolation

Benign and Malign tissue sections from each patient were obtained from formalin-fixed, paraffin-embedded (FFPE) blocks. RNAs were isolated from patient tissues that were collected after examination. All the samples were stored in Medical Pathology archive at room temperature until used for DNA and RNA isolations. RNA was isolated with the Quick-RNA FFPE Kit (Zymo Research) following the protocol suggested by the manufacturer with some modifications.1 ml of xylene was added to the samples and vortexed vigorously for 30 seconds. Then the samples left in Shaker for 1 hour at 24 °C and centrifuged at 10,000 x g for 2 minutes. Xylene was removed and discarded. The samples were washed twice with 1 ml ethanol (100 %), 1 ml ethanol (75 %), and1 ml ddIH2O, respectively and each time incubated for 5 minutes with gentle rocking.Then supernatant was removed and 400µl of Deparaffinization Solution was added to the samples, vortexed and incubated for 3 minutes at 55°C. Then centrifuged at 10,000 x g for 1 minute. To the deparaffinized tissue samples were added 95 µl of DNAse/RNAse free water, 95 µl of 2X Digestion Buffer and 10 µl of Proteinase Kand incubated at 55°C for 1 hour. After digestion, the samples were incubated at 65°C for 15 minutes in order to be de-crosslinked. 600 µl of RNA Lysis Buffer was added to the tubes and centrifuged at16,000 x g for 2 minutes. The supernatant was transferred to an RNase-free tube and mixed with 1 volume of ethanol (96 %). Then mixture was transferred into a Zymo-Spin™ IIC Column2 in a Collection Tube and centrifuged at 12,000 x g for 20 seconds and the flow-through was discarded. 400 µl of RNA Wash Buffer was added to the columns, centrifuged at 12,000 x g for 20. Then 5 µl of DNAse and 75 µl of Digestion Buffer were added and incubated at room temperature for 15 minutes. After incubation 400 µl of RNA Prep Buffer was added to the columns, centrifugedat 12,000 x g for 20 seconds and the flow-through was discarded. 700 µl of RNA Wash Buffer was added to the columns, centrifuged at 16,000 x g for 2 minutesto make sure of complete removal of the wash buffer. The columns were transferred carefully into an RNAse-free tubes (1.5 ml). 50 µl of

DNase/RNase-Free Water was added directly to the column matrix and centrifuged to elute RNA.

3.4.1.Determination of the concentration and quality of the isolated RNAs

3 µl from all isolated RNA samples were taken and their concentrations and quality assays were measured using a Spectrophotometer at 260 nm and 280 nm wavelengths.

3.5.cDNA synthesis

CDNA synthesis from total RNA was performed using commercial MiScript Reverse Transcription Kit (Qiagen) in accordance with the manufacturer's instructions. The mixture was prepared as in the Table 3.2. and Table 3.3. for each sample. The synthesis protocol is as follows;

Table 3.2. Mix amounts of cDNA synthesis. Step 1.

Mix	Amount
Poly A tailing buffer	1 µl
Poly A Polymerase	0,5 µl
miRNA	3,5 µl
Total Volume	5 µl

Table 3.3. Mix amounts of cDNA synthesis. Step 2.

Mix	Amount
Poly A tailing reaction mix	5 µl
Hsa-mir-NA cDNA reaction mix	4,5 µl
Reverse Transcriptase	0,5 µl
Total Volume	10 µl

All mixture operations were carried out on ice.5 µl of mixture from the step 1 was incubated in the PCR machineat 37°C for 20 minutes andat 70°C for 5 minutes respectively.10 µl of mixture from the step 2 was incubated in the PCR machineat 42°C for 20 minutes andat 85°C for 5 minutes.The obtained cDNAs were stored at -20°C for later use.

3.6. Real-Time PCR

SensiFast SYBR Lo-ROX Kit (Bioline) was used in the MicroRNA Real Time PCR experiments and manufacturer's instructions were applied as protocol. Amounts of mixture prepared for PCR are shown in the Table 3.4.Reference genes RNU6 and hsa-mir-192 and also hsa-mir-572, hsa-mir-512-5p, hsa-mir-513a-2 were purchased from Sentegen.The Real-Time PCR reaction steps are shown in the Table 3.5.Detailed information about primers used in RT-PCR analysis are shown in Table 3.6.

Table 3.4. Real-Time PCR mixture quantities

Mix	Amount
SensiFast SYBR Lo-ROX	10 µl
RNase-free water	5µl
Template cDNA	3µl
10x miScript Universal Primer	1µl
10x Primer	1 µl
Total Volume	20 µl

Table 3.5. Real-Time PCR reaction conditions

Mix	Amount
Pre-denaturation	95°C for 2 min
Denaturation	95°C for 10 seconds
Annealing	60°C for 10 seconds
Extension	72°C for 10 seconds
Melte Curve	60°C to 95°C
Number of Cycles	39 Cycles

Table 3.6. Primer sequences used in RT-PCR

Name	**Sequence**	**Base Number**
Hsa-mir-192 F	5’ CTG CCA ATT CCA TAG GTC ACA G 3’	22 bp
Hsa-mir-512-5p F	5’ CAC TCA GCC TTG AGG GCA CTT TC 3’	23 bp
Hsa-mir-513a-2 F	5’ TAA ATT TCA CCT TTC TGA GAA GG 3’	23 bp
Hsa-mir-572 F	5’ GTC CGC TCG GCG GTG GCC CA 3’	20 bp

Universal primer was used as reverse primer. The binding temperatures were determined as 60°C for reference gene RNU6, 60°C for hsa-mir- 513a-2 and hsa-mir- 572, 63°C for hsa-mir-192 and 65 for hsa-mir- 512-5p.

3.7.DNA Isolation

DNA was isolated with the Quick-DNA Miniprep Plus Kit(Zymo Research) following the protocol suggested by the manufacturer with some modifications. 800 µl of xylene wasadded to the samples and vortexed vigorously for 30 seconds. Then the samples left in Shaker for 10 minutes at 24°C and centrifuged at 16,000 x g for 3 minutes. Xylene was removed and discarded. The samples were washed with 800 µlethanol (100 %),800 µl ethanol (75 %) and 800 µlof ddIH2O, then vortexed and centrifuged at 16,000 x g for 3 minutes. Then supernatant was removed and 45 µl of DNAse/RNAse free water, 45 µl of Solid Tissue Buffer and 10 µl of Proteinase Kwere added to the deparaffinized tissue samples. Then samples were vortexed and incubated at 55°C for 14-16 hours, at 94°C for 20 minutes and centrifuged at 16,000 x g for 2 minutes. The supernatant with DNA was transferred to new tubes. 500 µl of Genomic Binding Buffer was added to the tubes and vortexed gently. The supernatant was transferred to aZymo-Spin™ IIC-XL Column in a Collection Tubeand centrifuged at 12,000 x g for 2 minutes and the collection tube with flow-through was discarded. 400 µl of DNA Pre-Wash Buffer, 700 µl of g-DNA Wash Bufferand again 200 µl g-DNA Wash Bufferwere added to the spin column in a new Collection Tube, respectively, each time centrifuged at 12,000 x g for 1 minuteand the flow-through was discarded. Each column was transferred to a clean microcentrifuge tube. 40 µl of DNA Elution Buffer was added directly to the column matrix and centrifuged at 16,000 x g for 2 minutes to elute DNA.

3.7.1.Determination of the concentration and quality of the isolated DNAs

The concentrations of the DNA samples were determined by the absorbance value at 260 nm in the NanoDrop Spectrophotometer and the purity was determined by the ratio of the absorbance at 260 nm to 280 nm.

3.8.Bisulfite DNAmodification

In methylation analysis, the separation of methylated cytosines from unmethylated cytosines is essential. For this, unmethylated cytosines after the bisulfite modification process are transformed into Uracil, while cytosines bearing the methyl group remain unchanged. The process of bisulfite modification is shown in Figure 3.1. The bisulfite modification of the DNAs obtained for the methylation analyzes was carried out using the EZ Methylation-Gold Kit. For this, 20 µl of 200-500 ng / µl DNA was used. 900 µl of water, 300 µl of Dilution Buffer and 50 µl of M-Dissolving Buffer were added to a tube ofCT Conversion Reagent and mixed with frequent vortexing for 10 minutes. 130 µl of

CT Conversion Reagent was added to 20µl of DNA sample in a PCR tube. Then samples were placed in a thermal cycler, performing the following steps:

- 98°C for 10 minutes
- 64°C for 2.5 hours
- 4°C for 20 hours

Then 600 µl of M-Binding Buffer was added to a Zymo-Spin™ IC Column and placed into the provided Collection Tube. The samples were loaded into the Zymo-Spin™ IC Column containing the M-Binding Buffer and mixed by inverting several times. Then centrifuged at 16,000 x g for 30 seconds and f

low-through was discarded. 100 µl of M-Wash Buffer was added to the column and centrifuged at 16,000 x g for 30 seconds. 200 µl of M-Desulphonation Buffer was added to the column and stood at room temperature for 15-20 minutes and then centrifuged at 16,000 x g for 30 seconds. 200 µl of M-Wash Buffer was added to the column and centrifuged at 16,000 x g for 30 seconds. The columns were placed into a 1.5 ml microcentrifuge tubes and 10 µl of M-Elution Buffer was added directly to the column matrix.Then centrifuged at 16,000 x g for 1 minute to elute the DNA.

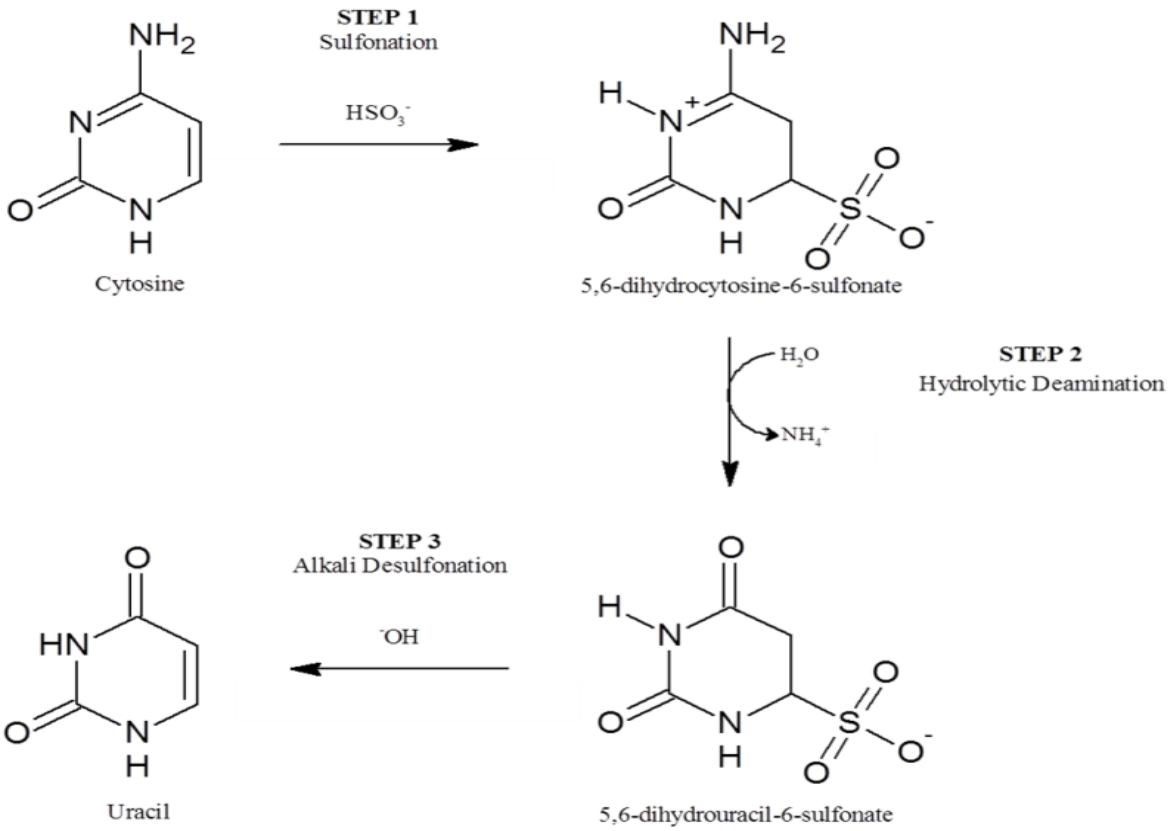

Figure 3.1. Transformation of cytosine to urasil with bisulphite modification. In this image bisulfite conversion mechanism was shown. The mechanism begins with the nucleophilic addition of bisulfite to the C-6 position of cytosine, which allows the rapid deamination of cytosine into 5,6-dihydrouracil-6-sulfonate. Subsequent treatment with an alkaline solution swiftly eliminates the sulfonate group and regenerates the double bond, yielding uracil.

3.9.Methylation-specific PCR

MSP is a useful method for qualitative DNA methylation analysis with many advantages, including ease of design and application, sensitivity to detect very small amounts of methylated DNA, and the ability to quickly scan a large majority of samples without the need to purchase expensive laboratory equipment (Barekati *et al*. 2012). MSP is a qualitative technique used to detect the presence of methylation in bisulfite-modified DNA.The primer to detect the methylated DNA (M primer) was designed under the assumption that the target region was completely methylated, and this region contains cytosines in CG dinucleotide sequences. Conversely, the primer to detect unmethylated DNA (U primer) was designed on the assumption that the target region was not completely methylated, and therefore contains thymines instead of cytosines in CG dinucleotides.MethPrimer is a primer design tool, especially for methylation studies. This software also offers suggestions for primer design for MSP and bisulphite sequencing. The selected genomic sequence simply copied and pasted; this software then performed a silico bisulfite transformation and provided suggestions for primer positions and sequences. The parameter settings included the target size, except for the regions, the number of product primer pairs, the primer Tm, the number of CGs in the primer, the non-CG number in the primer as well as the max difference for MSP (Barekati *et al*. 2012).

For bisulfite treated genomic DNA samples qPCR was applied with methylated and unmethylated primers, respectively.Amounts of mixture prepared for qPCR are shown in the Table 3.7.The qPCR reaction steps are shown in the Table 3.8.

Table 3.7. qPCR mixture quantities

Mix	**Amount**
SensiFast SYBR Lo-ROX	10 µl
ddH2O	7µl
Template DNA	1µl
Primer Forward	1µl
Primer Reverse	1 µl
Total Volume	20 µl

Table 3.8. qPCR reaction conditions

Mix	**Amount**
Pre-denaturation	95°C for 2 min
Denaturation	95°C for 10 seconds
Annealing	10 seconds
Extension	72°C for 10 seconds
Melte Curve	60°C to 95°C
Number of Cycles	41cycles

3.8.1.Primers used in methylation analysis

Table 3.9. Sequences and annealing temperatures for hsa-mir-192

Name	**Sequence**	**Annealing temperature**
Hsa-mir--192 MF(M primer Forward)	5’ TAT GAG TAG AAG GGG TTG ACG GGC 3’	64.4
Hsa-mir-192 MR(M primer Reverse)	5’ CCC GAA CAA ACT AAA CGT AAC CTC C 3’	63.0
Hsa-mir-192 UF(U primer Forward)	5’ GTT ATG AGT AGA AGG GGT TGA TGG GTG 3’	65.0
Hsa-mir-192 UR(U primer Reverse)	5’CCC CCA AAC AAA CTA AAC ATA ACC TCC 3’	63.4

Table 3.10. Sequences and annealing temperatures for hsa-mir-512-5p

Name	**Sequence**	**Annealing temperature**
Hsa- mir-512-5p MF	5’ TTT AGT TTG GGT GAT AGA GCG AGA 3’	59.3
Hsa-mir-512-5p MR	5’ AAA CTA ATC TTA AAT TCC TAA ACT CAA ACG 3’	58.6
Hsa-mir-512-5p UF	5’ TTA GTT TGG GTG ATA GAG TGA GA 3’	57.1
Hsa-mir-512-5p UR	5’ AAT CTT AAA TTC CTA AAC TCA AAC AAT3’	54.3

Table 3.11. Sequences and annealing temperatures for hsa-mir-513a-2

Name	Sequence	Annealing temperature
Hsa- mir-513a-2 MF	5’ GGA GAA TAT TGG TAG GGT GGT C 3’	60.3
Hsa-mir-513a-2 MR	5’ TCT TTA AAT AAA TCC CTA ATC CCG 3’	55.9
Hsa- mir-513a-2 UF	5’ GGA GAA TAT TGG TAG GGT GGT T 3’	58.4
Hsa- mir-513a-2 UR	5’ TCT TTA AAT AAA TCC CTA ATC CCA3’	54.2

Table 3.12. Sequences and annealing temperatures for hsa-mir-572

Name	Sequence	Annealing temperature
Hsa- mir-572 MF	5’ GGG TTG TTC GGT AGG ACG TA 3’	59.4
Hsa- mir-572 MR	5’ GAA CGA ACG AAA CAC AAA CG 3’	55.3
Hsa-mir-572 UF	5’ GGG TTG TTT GGT AGG ATG TA 3’	55.3
Hsa-mir-572 UR	5’ AAA CAA ACA AAA CAC AAA CAA C 3’	52.8

3.9.1.Agarose Gel Electrophoresis

Following the PCR, qualitative detection of bands was visualized under UVlight after Ethidium Bromide (EtBr) staining to determine the presence or absence of methylation in the template DNA. Since the MSP can not be quantitative as described above, the results here gave information about the methylation in the form of "present or absent".

3.9.1.1.Preparation of 10X tris-acetate-EDTA (TAE)

48.4 g of Tris base, 11.4 mL of acetic acid, 3.7 g of 0.02 M EDTA, 800 mL of ddH2O were weighed and added in a bottle and then dissolved in a magnetic stirrer to prepare 10X. tris-acetate-EDTA (TAE)

3.9.1.2.Preparation of 2% agarose gel

The prepared 10X TAE buffer was diluted to 1X. 1.6 gr of agarose was weighed, taken out of a beaker and dissolved in 80 ml of 1X TAE. The solution was heated in a microwave for

1 minute and boiled. 4µl of EtBr was added to the prepared gel bed. The spattering solution was placed on the comb to freeze to form the loading wells. The frozen jelly comb was carefully removed and placed in the loading tank containing the gel 1X TBE.The poured solution was placed on the comb to freeze to form the loading wells. The comb was carefully removed from thefrozen gel and placed into the loading tank containing the 1X TAE.

3.9.1.3.Loading samples into agarose gel

15 µl of the qPCR product and 3 µl of the loading buffer were mixed and the samples were loaded to gel respectively. The electrophoresis tank lid was closed and connected to a power source set to 150 mA at 134 V and run for 20 minutes. For ease of evaluation, two PCR products that made with the same patient's methylated and unmethylated primers were loaded side-by-side. The agarose run was examined under UV light in the imaging device.

3.10.Statistics

3.10.1.RT-PCR

Statistical analyzes for RT-PCR were performed on the log-transformed data using "two-sided Student's t test" .ΔCt was calculated by subtracting the Cq averageof RNU6 results from the Cq average of Benign and Malign miRNAsresults respectively.ΔΔCt was calculated by subtracting the ΔCtaverage of Benign samples from ΔCtaverage of Malign ones. Fold changes were calculated with formula: 2^ΔΔCt.P-value <0.05 was accepted statistically significant.

3.10.2.qPCR

Statistical analyzes for qPCR were performed on the log-transformed data using "two-sided Student's t test" .ΔCt was calculated by subtracting the melte peak curve average of Benign Unmethylated DNA results from the melte peak curve average of Benign Methylated DNA results.Then ΔCt was calculated for Malign Methylated and Unmethylated DNAs.ΔΔCt was calculated by subtracting the ΔCtaverage of Benign samples from ΔCtaverage of Malign ones. Fold changes were caculated with formula: 2^ΔΔCt.P-value <0.05 was accepted statistically significant.

4.RESULTS

In this study 90 patients were included. 50 of them are patientsof Adenomyomatous Hyperplasia (benign protate tissues) and 30 of them are patients of Prostatic Aciner Adenocarcinoma (malign prostate tissues).Prognostic factors such as age, tumor types, gleason grades of the cases were obtained from patient files and shown in Table 3.1.

4.1.RNA isolation

Table 4.1. Concentration and purity values of RNA samples

Number	Benign/Malign	Concentration ng/µl
1	Benign	597,1
2	Benign	301,5
3	Benign	330,9
4	Benign	1119
5	Benign	534,2
6	Benign	553,0
7	Benign	461,0
8	Benign	170,2
9	Benign	203,4
10	Benign	384,9
11	Benign	438,2
12	Benign	495,2
13	Benign	646,0
14	Benign	366,2
15	Benign	300,2
16	Benign	612,0
17	Benign	1099
18	Benign	419,5
19	Benign	499,5
20	Benign	573,4
21	Benign	401,2
22	Benign	447,7
23	Benign	738,5
24	Benign	598,5
25	Benign	158,2
26	Benign	427,9
27	Benign	738,7
28	Benign	690,9
29	Benign	467,8
30	Benign	501,5
31	Benign	611,2
32	Benign	781,3
33	Benign	620,6
34	Benign	449,8
35	Benign	468,0
36	Benign	390,2
37	Benign	388,5

38	Benign	231,5,
39	Benign	272,3
40	Benign	388,4
41	Benign	534,7
42	Benign	238,9
43	Benign	251,2
44	Benign	157,4
45	Benign	589,7
46	Benign	342,8
47	Benign	706,5
48	Benign	1110
49	Benign	566,0
50	Benign	430,0
51	Malign	299,1
52	Malign	983,9
53	Malign	342,5
54	Malign	336,4
55	Malign	452,1
56	Malign	958,2
57	Malign	1173
58	Malign	308,2
59	Malign	1883
60	Malign	2606
61	Malign	1018
62	Malign	2140
63	Malign	1478
64	Malign	2569
65	Malign	1965
66	Malign	2120
67	Malign	1781
68	Malign	1388
69	Malign	1401
70	Malign	1494
71	Malign	1112
72	Malign	2354
73	Malign	656,6
74	Malign	1112
75	Malign	678,2
76	Malign	2161
77	Malign	1031
78	Malign	792,8
79	Malign	594,3
80	Malign	980,2

4.2.DNA isolation

Table 4.2. Concentration and purity values of DNA samples

Number	Benign/Malign	Concentration ng/µl
1	Benign	26,25
2	Benign	30,19
3	Benign	50,48
4	Benign	53,46
5	Benign	104,4
6	Benign	72,21
7	Benign	22,15
8	Benign	45,96
9	Benign	52,98
10	Benign	192,8
11	Benign	83,37
12	Benign	51,44
13	Benign	72,40
14	Benign	70,38
15	Benign	46,46
16	Benign	24,22
17	Benign	28,75
18	Benign	15,48
19	Benign	47,69
20	Benign	160,5
21	Benign	18,46
22	Benign	18,37
23	Benign	15,77
24	Benign	14,04
25	Benign	25,38
26	Benign	13,46
27	Benign	25,87
28	Benign	15,00
29	Benign	17,98
30	Benign	33,65
31	Benign	24,04
32	Benign	40,77
33	Benign	11,44
34	Benign	21,54
35	Benign	16,06
36	Benign	21,15
37	Benign	39,62
38	Benign	37,40
39	Benign	27,60
40	Benign	37,73
41	Benign	19,33
42	Benign	208,8
43	Benign	122,2
44	Benign	178,6

45	Benign	117,1
46	Benign	99,13
47	Benign	58,37
48	Benign	92,50
49	Benign	82,69
50	Benign	74,13
51	Malign	39,71
52	Malign	37,50
53	Malign	15,10
54	Malign	31,63
55	Malign	55,48
56	Malign	50,19
57	Malign	76,06
58	Malign	61,35
59	Malign	86,15
60	Malign	160,2
61	Malign	294,8
62	Malign	174,9
63	Malign	434,2
64	Malign	255,9
65	Malign	44,04
66	Malign	234,4
67	Malign	175,7
68	Malign	160,3
69	Malign	245,3
70	Malign	242,9
71	Malign	225,8
72	Malign	255,9
73	Malign	89,04
74	Malign	202,6
75	Malign	37,69
76	Malign	179,3
77	Malign	205,0
78	Malign	170,3
79	Malign	28,46
80	Malign	233

4.3.RT-PCR

The relative gene expression results of microRNAs from all malign and benign patient tissues are shown in Figure 4.1. hsa-mir-192 and hsa-mir-512-5p were statistically significantly downregulated while hsa-mir-572 and hsa- mir-513a-2 showed statistically significant upregulation. As shown in Table 4.2, p value0,05 for hsa-mir-192 real-time PCR analysis, p value0,02 for hsa-mir-512-5p real-time PCR analysis, p value2E-08 for hsa- mir-513a-2 real-time PCR analysis, p value0,01 for hsa-mir-572 real-time PCR

analysis result and tissue samples using reference gene RNU6 was found to be statistically significant (Table 4.3).

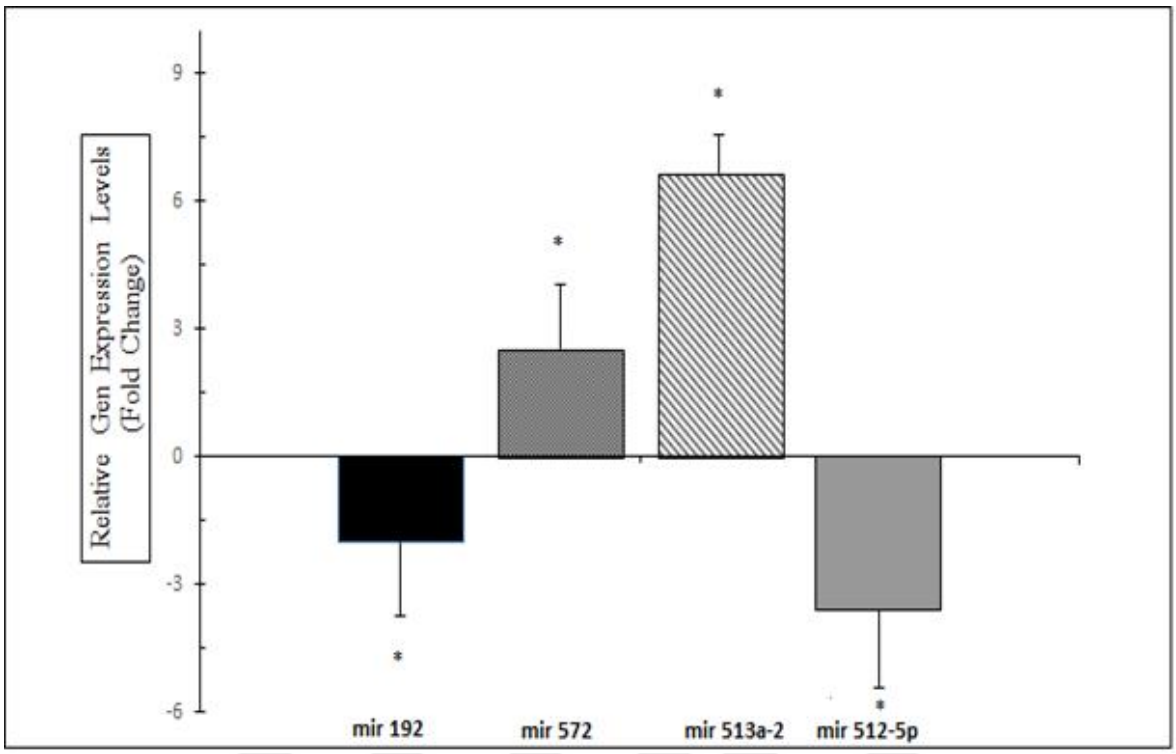

Figure 4.1. Gen Expression Levels of MicroRNAs: Figure shows the comparison of hsa-miRNA expression levels in benign and malign tissues.hsa-mir-192 and hsa-mir-512-5p were significantly downregulated, hsa-mir-572 and hsa- mir-513a-2 were significantly upregulated. This figureshows that the expression wassuccessful. * is a sign of significance.

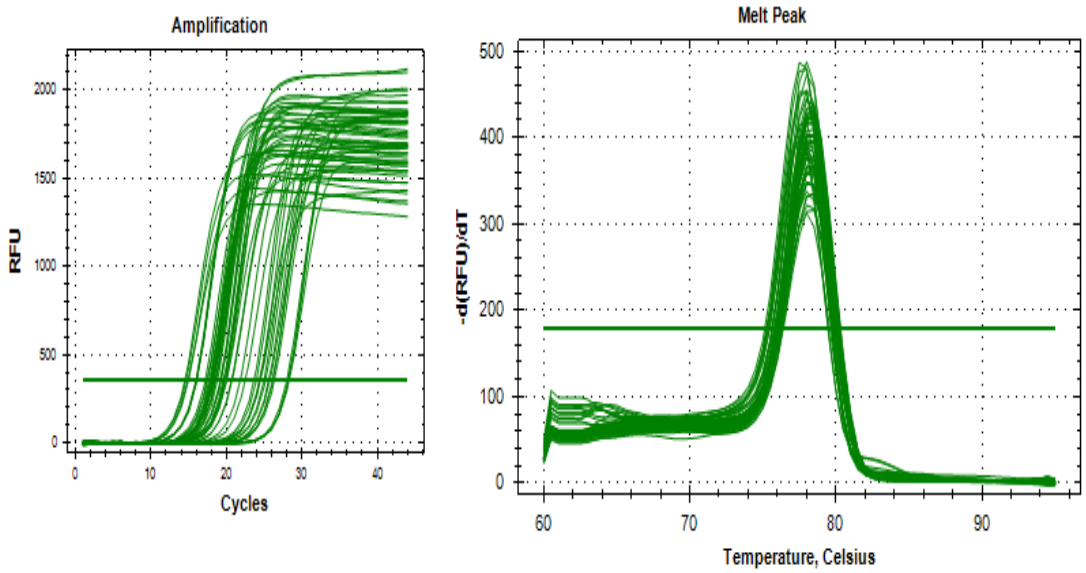

Figure 4.2. Rt-PCR Amplification and Melt Peak results. Figure on the left shows samples amplification curve. Figure on the right shows melting curve analysis. Thesepeaks

represent a normal distribution. The normal distribution is symmetric and has a bell-shaped curve with a single peak. This figure shows that the expression has been successful.

Table 4.3. RT-PCR Cq results

microRNA	ΔCt average benign/ malign	Standart deviation benign/malig n	ΔΔCt	Fold Change	T-Test P value	Up/Down regulation
Hsa-mir-192	10,71/11,72	1,02/1,75	1,01	2,01	P ≤ 0,05	Down regulated
Hsa-mir-512-5p	7,28 / 9,14	2,04 / 1,81	1,85	3,61	P ≤ 0,05	Down regulated
Hsa-mir-513a-2	10,13/7,40	0,49/0,92	-2,73	6,63	P ≤ 0,05	Upregulated
Hsa-mir-572	0,59 / -0,76	0,86/1,60	-1,34	2,54	P ≤ 0,05	Upregulated

4.4.Methylation

In this study, the methylation status of four oncomir microRNAs (hsa-mir-192, hsa-mir-512-5p, hsa-mir-513a-2 and hsa-mir-572), which were normally unmethylated, has been investigated in 50 benign and 30 malign tissues. The methylation profile of the hsa-mir-192, hsa-mir-512-5p, hsa-mir-513a-2 and hsa-mir-572 genes was evaluated by the qPCR method. Only hsa-mir-572 results were found as significant. Other 3 miRNAs remained as unchanged. It is possible to see in the results of the students t-test that the Malign samples are more methylated than the Benignsamples in hsa-mir-572 (Table 4.6). As shown in Figure 4.3, values below 5% were unmethylated, values between 5-25% were low, values between 25-75% were moderate, and values above 75% were considered as high-level of methylation (Hoque MO *et al.* 2004).

Table 4.4. Melt peak results of Benign Tissue Samples.

Benign methylated	Benign unmethylated	Metylation percentages
81,50	73,50	25,00
78,50	74,00	0,00
87,50	73,50	100,00
87,00	72,50	100,00
79,50	73,00	5,00
80,00	73,50	5,00
86,50	73,00	100,00
85,00	73,00	75,00
78,50	73,50	0,00
86,50	72,00	100,00
86,50	73,00	100,00
86,50	79,00	100,00
86,00	73,00	75,00
86,50	72,50	100,00
85,50	72,50	75,00
87,00	72,50	100,00
86,00	72,00	75,00
77,00	73,00	0,00
86,50	73,00	100,00
85,50	72,50	75,00
85,00	72,50	75,00
86,00	72,50	75,00
85,50	73,00	75,00
86,00	72,00	75,00
86,50	72,00	100,00
86,50	73,00	100,00
87,00	73,00	100,00
84,50	72,50	50,00
85,50	71,50	75,00
85,50	72,50	75,00
86,00	72,50	75,00
87,00	73,00	100,00
86,50	73,00	100,00
84,00	73,00	50,00
86,00	73,00	75,00
86,50	73,50	100,00
86,00	73,50	75,00
85,50	72,50	75,00
84,00	72,50	50,00
81,00	73,00	25,00
86,50	72,00	100,00
86,50	81,50	100,00
86,00	80,50	75,00

86,50	81,50	100,00
87,50	82,00	100,00
84,50	80,00	50,00
82,50	81,00	25,00
80,50	79,00	5,00
86,00	80,00	75,00

Table 4.5. Melt peak results of Malign Tissue Samples.

Malign metylated	Malign unmethylated	Metylation percentages
86,50	80,50	100,00
85,50	72,50	50,00
82,00	72,50	5,00
86,50	72,50	100,00
81,00	72,00	0,00
86,50	72,50	100,00
86,00	72,50	75,00
81,50	82,00	5,00
85,00	80,50	50,00
86,00	81,50	75,00
86,00	81,50	75,00
85,50	72,50	50,00
86,00	80,50	75,00
86,50	80,50	100,00
85,00	71,00	50,00
86,50	81,00	100,00
84,00	80,50	25,00
86,50	80,50	100,00
87,00	80,50	100,00
86,50	72,50	100,00
85,50	80,50	50,00
86,00	72,50	75,00
87,00	81,50	100,00
86,50	81,50	100,00
87,00	82,00	100,00
87,00	73,00	100,00

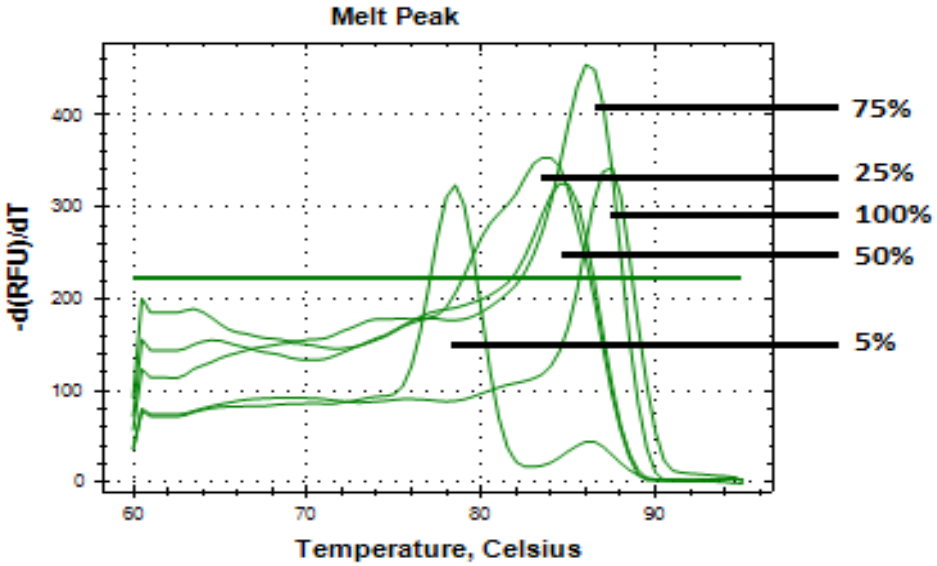

Figure 4.3. Methylation percentages. This figure shows different methylation ratio of benign and malign samples, which were determined by qPCR melt curve peak results.

Table 4.6. qPCR Melt curve peak results. Comparison of melt peak results in malign and bening tissue samples. * hsa-mir-572 qPCR results are significant.

miRNAs	ΔCt average benign/malign	Standart deviation benign/malign	ΔΔCt	Fold Change	T-Test P value
hsa-mir-192	1,87 / 1,95	0,55 / 0,91	0,08	1,06	P ≥0,34
hsa-mir-512-5p	0,41 / 0,38	1,59 / 0,47	-0,03	1,03	P ≥0,44
hsa-mir-513a-2	0,63 / 0,67	0,27 / 0,58	0,04	1,03	P ≥0,37
hsa-mir-572	12,37 / 9,61	1,99 / 3,88	-2,76	6,78	P≤ 0,003*

Table 4.7. Methylation. Number of patients with 5 %, 25 %, 50 %, 75 % and 100 % of methylationin malign and benign tissue samples.

Percentage (%)	Hsa-mir-572 Benign n=50	Hsa-mir-572 Malign n=30
0 %	4	4
5 %	3	2
25 %	3	1
50 %	4	5
75 %	17	5
100 %	19	13

Table 4.4 results showed that the methylation profile of hsa-mir-572 oncomir gene region wasobserved. When the difference of expression value of methylatedand non-methylated malign tissues primers were compared with the benign tissues, interestingly the gene region was methylated at a statistically significant level (p = 0,0003) 6,78 times.Table 4.5 shows that the total number of patients in malign group is 30 andnumber of patients with 100% methylase profile in hsa-mir-572 oncomir gene is 13, number of patients with 75% methylase profile is 5, number of patients with 50% methylase profile is 5, number of patients with 25% methylase profile is 1, number of patients with5%methylase profile is 2 and finally number of patients with unmethylated samples is 4.In a benign patient group with a total number of 50 patientsnumber of patients with 100% methylase profile is 19, number of patients with 75% methylase profile is 17, number of patients with 50% methylase profile is 4, number of patients with 25% methylase profile is 3, number of patients with 5% methylase profile is 3 and finally number of patients with unmethylated samples is 4. In Table 4.6, when looking at the percentages of benign and malign patients, 87% of malign patients and 92% of benign patients were found to be methylated.

Table 4.8. The methylation profile of hsa-mir-572. The methylation profile of hsa-mir-572 oncomir on the benign and malign tissue samples of prostate cancer patients

Hsa-mir-572 Methylation status	Hsa-mir-572 Benign patient n=50 amount (%)	Hsa-mir- 572 Malign patient n=30 amount (%)
Methylated	92	87
Unmethylated	8	13

When the methylation profile of hsa-mir-192 oncomir miRNA in malign and benign patient groups was compared, there was no statistically significant difference between them (p = 0,34) (Table 4.4). As seen in Figure 4.3, there is no difference in the gene expression and the melting curve peak results of methylated and unmethylated primers of malign tissue samples and methylated and unmethylated primers of benign tissue sapmles. There was no statistically significant difference between the two groups.

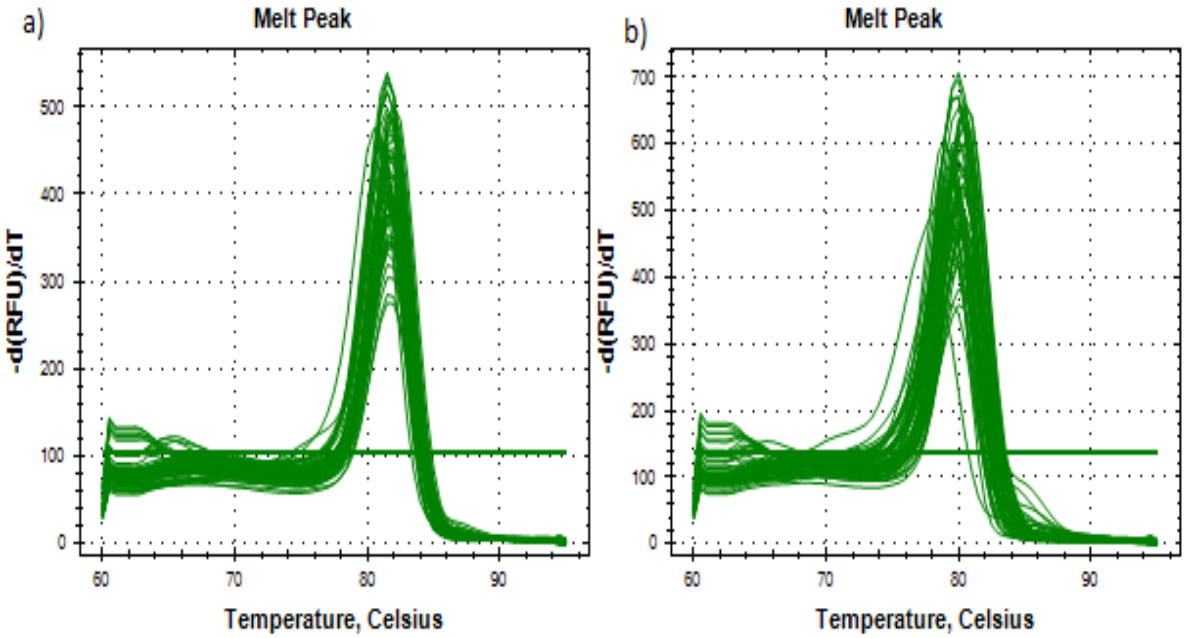

Figure 4.3. qPCR results of hsa-mir-192. Both Figures shows that expression was successful, but there is no difference between methylated and unmethylated, benign and malign samples. These peaks represent a normal distribution. The normal distribution is symmetric and has a bell-shaped curve with a single peak. a)methylated, b)unmethylated

When the methylation profile of Hsa-mir-512-5p oncomir miRNA in malign and benign patient groups were observed, there was no statistically significant difference (p = 0,44) in Table 4.4. As seen in Figure 4.4, there is no difference in the gene expression and the melting curve peak results of methylated and unmethylated primers of malign tissue samples and methylated and unmethylated primers of benign tissue sapmles. There was no statistically significant difference between the two groups.

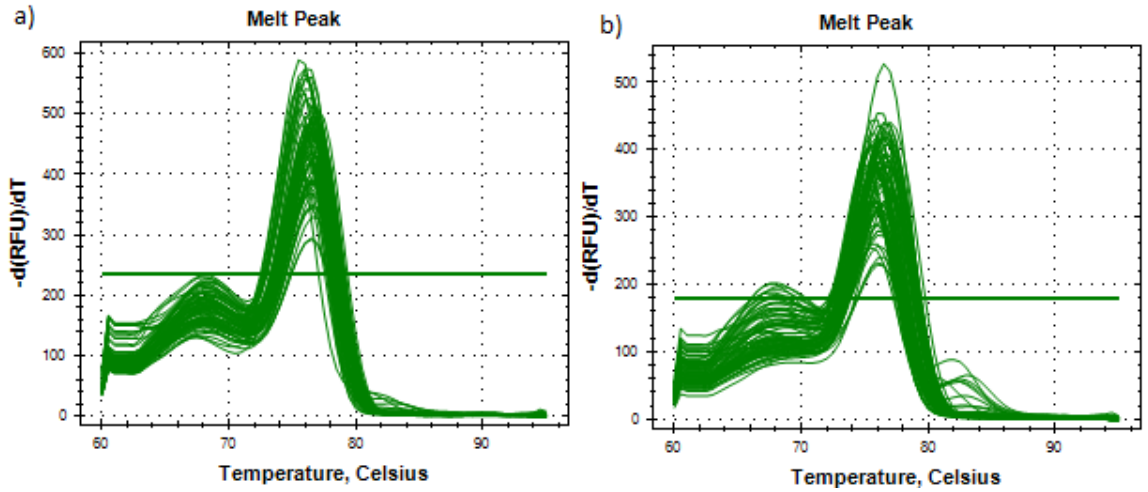

Figure 4.4. qPCR results of hsa-mir-512-5p. Both Figures shows that expression was successful, but there is no difference between methylated and unmethylated, benign and malign samples. These peaks represent a normal distribution. The normal distribution is symmetric and has a bell-shaped curve with a single peak.a)methylated, b)unmethylated

When methylation profile of Hsa-mir-513a-2 oncomir miRNA in malign and benign patient groups were examined, there was no statistically significant difference (p = 0,37) (Table 4.4). As seen in Figure 4.5, there is no difference in the gene expression and the melting curve peak results of methylated and unmethylated primers of malign tissue samples and methylated and unmethylated primers of benign tissue sapmles.There was no statistically significant difference between the two groups.

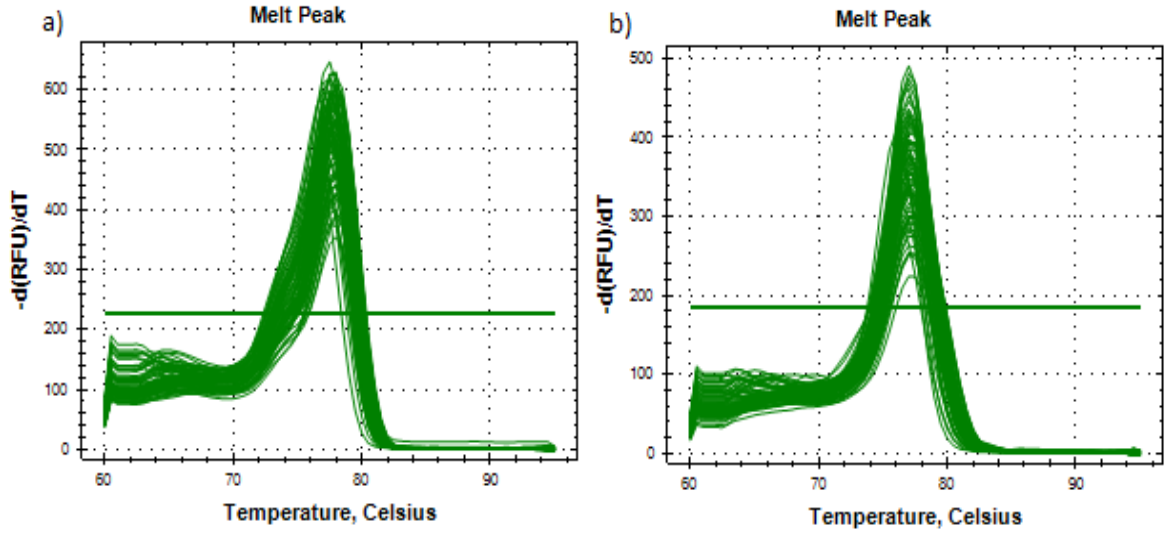

Figure 4.5. qPCR results of Hsa-mir-513a-2. Both Figures shows that expression was successful, but there is no difference between methylated and unmethylated, benign and malign samples. These peaks represent a normal distribution. The normal distribution is symmetric and has a bell-shaped curve with a single peak.a)methylated, b)unmethylated

4.4.1. Gel screening results

The modified DNAs were amplified by methylation-specific primers and then run on a 2% agarose gel and amplified fragment-specific bands were analyzed on a gel documentation system. Gel imaging results from the analyzes are performed below in Figure 4.6.

Figure 4.6. A gel image of the methylation analysis of Hsa-mir--572 in benign (a) and malign (b) tissues samples.M: methylated, U: unmethylated, O'GeneRuler DNA ladder Mix was used as DNA marker.

5.DISCUSSION

At Bezmialem Vakif University, Faculty of Medicine, Department of Medical Pathology,30 patients histopathologically diagnosedwith Aciner Adenocarcinoma and 50 patients with Adenomyomatous Hyperplasia were included and studied in this thesis. Tissue samples were used which embedded with paraffin blocks fixed with 10% formaldehyde solution. From all cases, the best tumor tissue reflecting cases with the least necrosis, hemorrhage, inflammatory cells and stromal elements containing areas's sections have been identified. Paraffin blocks from these sections were taken: 40 microns for genomic DNA isolation and 60 microns for RNA isolation.Haematoxylin-eosin stained archive preparations were graded again according to WHO / ISUP 2004 classification criteria and staged according to 2004 TNM classification. The ages, gender, biopsy pattern, follow-up intervals, disease pathology reports of the cases were obtained from patient files.cDNA was obtained from the obtained RNA samples and gene expressionof hsa-mir-192, hsa-mir-512-5p, hsa-mir-513a-2, hsa-mir-572 was performed by RT-PCR.After RT-PCR,significant miRNAs were investigated for the methylation patterns. Methylation-specific PCR was performed with isolated DNA samples from the tissues and the methylation profiles of hsa-mir-192, hsa-mir-512-5p, hsa-mir-513a-2, hsa-mir-572 were investigated.

Although cancer appears to be uncontrolled cell proliferation only, when looking at reasons it is also an oncogene activation, tumor suppressor genes's activation and an out of control cell division process which is appear by DNA repair mechanism disruption (Alberts *et al*, 2002) . That is, it is a multi-step disease where several conditions come together to form it. The changes that occur in these genes are directly related to the formation of cancer as they ultimately affect the basic step of cell proliferation.Modification of the genes may be genetic like mutations, or may be epigenetic, like methylation or histone modifications. Ongogenes that play role in cell division, are accelerating cell division process, while in contrasttumor suppressor genes under normal conditions suppress the cell proliferation. However, any change that may occurwill also remove their control upon cell cycle and cell division affecting function of these genes.

Changes in tumor suppressor genes that may occur and interfere their function may be collected in 2 subclasses, genetic and epigenetic changes. Genetic changes are features that able to be inherited andappear by degradation of gene structure such as mutation, deletion.

It affects the gene structure as well as its function.But epigenetic changes because of not having structurally inheritance properties can only affect the gene functioning. Since these changes can not be identified by DNA sequence alterations and can be reversible, they are thought to be an important step that can be used for the treatment of cancer after prognosis (Riya *et al*, 2014)

Many recent cancer studies have been conducted, and it is aimed to be able to have more information about the cancer mechanism and recycling of the methylated genes in this study and to be able to create a new step for treatment in this way.

Epigenetic mechanisms can be divided into 2 classes:

1. DNA methylation
2. Histon Modifications

DNA methylation is the addition of a methyl (CH_3) group to DNA, thereby often modifying the function of the genes and affecting gene expression. It plays an important role in the regulation of transcription in the genomes of higher eukaryotes (Bogdanovic and Veenstra, 2009, Chen *et al* 2011).

DNA methylation makes some modifications on DNA structure and also makes CpG regions for binding.These regions include a number of transcription repressors linked by the methyl-CpG binding domain (MBD) which shows the region of methylation during the transcription repression

In this study, from 80 PCa patients isolated DNAsamples investigated by qPCR technique and oncomir tumor suppressor gene regions mutation level was measured. Initial number of patients was 100, but according to quality of DNA and microRNA some of the samples were discarded.

Recently, Molecular Biology techniques inform us like transcriptional repressor dependent promoter regions, DNA methylation and epigenetic changes etc. Genes hypermethylation pathways in promoter region was thought to be possible target for the prostate cancer.According to the Mihi Yang ve Jong Y. Park 2013, development and growth duration of Prostate cancer linked over 40 methylated genes.These genes are very important pathways like repair, invasion and metastasis. New findings provide us a personel cancer treatment and information about its pathogenesis.Nowadays, personel

treatment becomes extremely important to improve early diagnosis and treatment to reduce cancer.

Genetic and epigenetic modification cause cancer and can be related with tumor supressor genes and DNA repair mechanisms. DNA metyhlation is a very important mechanism and histone modifications, and noncoding RNA could also potentially provide new tools for prognosis of prostate cancer, affecting clinical management of patients (Matteo F. *et al*. 2017).

Gonzales and colleagues (2011) investigated the role of miR-141 as a biomarker in advanced prostate cancer by looking at a retrospective cohort of 21 patients with metastatic prostate cancer. They analysed miR-141 levels using qRT-PCR along with lactate dehydrogenase (LDH), prostate specific antigen (PSA), and circulating tumour cell count (CTC) in a number of stored blood samples at taken at varying intervals during the patient's clinical course. Increasing miR-141 levels demonstrated a significant ability to predict clinical progression via univariate regression modelling, with an odds ratio of at least 8. miR-141 levels also correlated with changes in the other biomarkers under study. The authors suggested that miR-141 may therefore be a suitable biomarker for progression in metastatic prostate cancer, but accepted that larger, prospective studies would be required to validate that contention (Gonzales *et al*. 2011)

Shahana Majid and colleagues (2012) have identified that miR-23b is a methylation-silenced tumor suppressor in prostate cancer (PCa). They demonstrated that miR-23b expression is controlled by promoter methylation and has great promise as a diagnostic and prognostic biomarker in PCa. High levels of miR-23b expression are positively correlated with higher overall and recurrence-free survival in PCa patients. Further they elucidated the tumor suppressor role of miR-23b using in vitro and in vivo models. They demonstrated that proto-oncogene Src kinase and Akt are direct targets of miR-23b. Increased expression of miR-23b inhibited proliferation, colony formation, migration/invasion and triggered G0/G1 cell cycle arrest and apoptosis in PCa. Over-expression of miR-23b inhibited epithelial to mesenchymal transition (EMT) causing a decline in mesenchymal markers Vimentin and Snail and increasing the epithelial marker, E-cadherin. Depletion of Src by RNA interference conferred similar functional effects as that of miR-23b reconstitution. miR-23b expression caused a dramatic decrease in tumor growth in nude mice and attenuated Src expression in excised tumors compared to a control miR. These findings suggest that miR-23b is a methylation-silenced tumor

suppressor that may be useful biomarker in PCa. Loss of miR-23b may confer proliferative advantage and promote PCa migration and invasion and re-expression of miR-23b may contribute to the epigenetic therapy for PCa (Majid *et al.* 2012).

Mingliang Chu and colleagues (2015)showed that microRNA miR-124 exerts a tumor suppressive function in prostate cancer. They found a negative feedback loop between AR and miR-124 expression. On one hand, miR-124 was a positively regulated target gene of the AR, on the other hand, overexpression of miR-124 inhibited the expression of AR. In addition, they found that miR-124-2 and miR-124-3 promoters were hypermethylated in AR-negative PCa cells. Furthermore, overexpression of miR-124 inhibited proliferation rates and invasiveness capacity of PCa cells in vitro, and suppressed xenograft tumor growth in vivo. Taken together, their results support a negative feedback loop between AR and miR-124 expression. Methylation of miR-124-2 and miR-124-3 may serve as a biomarker for AR-negative PCa cells, and overexpression of miR-124 might be of potential therapeutic value for the treatment of PCa (Chu et al. 2015).

Again Mingliang Chu and colleagues (2014)found that AR-positive prostate cancer (PCa) cells showed high expression levels and hypomethylation of the miR-375. In contrast, AR-negative PCa cells displayed low levels and hypermethylation of the miR-375. Addition of 5-Aza-2'-deoxycytidine, a specific inhibitor of DNA methylation, into the culture medium reversed the low expression levels of miR-375 in the AR negative PCa cells. In addition, the total activity levels of DNA methyltransferases (DNMTs) were high in AR-negative PCa cells, in which hypermethylation of miR-375 promoter and low expression levels of miR-375 were observed. Taken together, these findings indicate that the negative correlation between AR and total DNMT activity is one of mechanisms to influence the methylation status of miR-375 promoter, which in turn regulates the expression of miR-375 (Chu *et al.* 2014)

Helle Kristensen and colleagues (2014) have found that GABRE_miR-452_miR-224 was significantly downregulated in prostate cancer compared with nonmalign prostate tissue and had highly cancer-specific aberrant promoter hypermethylation (AUC ¼0.98). Their functional studies and GSEA suggested that miR-224 and miR-452 inhibit proliferation, migration, and invasion of PC3 and DU145 cells by direct/indirect regulation of pathways related to the cell cycle and cellular adhesion and motility. Finally, in uni- and multivariate analyses, high GABRE_miR-452_miR-224 promoter methylation was significantly associated with biochemical recurrence in RP cohort 1, which was successfully validated

in RP cohort 2. The GABRE_miR-452_miR-224 locus is downregulated and hypermethylated in prostate cancer and is a new promising epigenetic candidate biomarker for prostate cancer diagnosis and prognosis. Tumor-suppressive functions of the intronic miR-224 and miR-452 were demonstrated in two prostate cancer cell lines, suggesting that epigenetic silencing of GABRE_miR-452_miR-224 may be selected for in prostate cancer (Kristensen *et al*, 2014).

Based on the results of our study, mir-572 have been proven to be tumor-suppressing activity. This miRNA has been found to significantly reduce cell proliferation, migration, invasion and colony formation from their natural ability of tumor cells. We have proven that our hypothesis is correct at the the end of our work. We have determined that mir-192, mir-512-5p and mir-513a-2 have no effect on Adenomyomatous Hyperplasia and Aciner Adenocarcinoma. It is understood that mir-572 can easily be used as candidate for diagnostic biomarkers according to the data obtained in this study. This study will lead the work to be done in cancerology. It will provide a step for the progress of the disease in the diagnosis treatment. This study might shed a light further experiments in order to understand this cancer mechanisim.

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7.ADDITIONS

7.1.Resume

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State of education:MSc

Degree	School Name and Department	Year of graduation
Bachelor	**Baku State University, Biology**	**2015**
Master	**Biruni University, Molecular Biology and Genetics**	**-**

Publication

Awards

7.2.Plagiarism Report

INVESTIGATION OF SPECIFIC ONCOMIR MICRORNAS THAT MODIFY METHYLATION PATTERNS ONTO EFFECTION OF PROSTATE CANCER

ORIJINALLIK RAPORU

%26	%18	%21	%9
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7.3.Ethics Committee Approval

Evrak Tarih ve Sayısı: 02/03/2018-3417

T.C.
BEZMİALEM VAKIF ÜNİVERSİTESİ REKTÖRLÜĞÜ
Girişimsel Olmayan Araştırmalar Etik Kurulu

Sayı : 54022451-050.05.04-
Konu : Etik Kurul Kararı

Sayın Uz. Dr. Ganime ÇOBAN

27.02.2018 tarihinde yapılan Girişimsel Olmayan Araştırmalar Etik Kurulu toplantısında "Metilasyon Paternleri Değişen Belirli Onkomir MikroRNA'ların Prostat Kanseri Üzerindeki Etkisinin Araştırılması" başlıklı başvurunuz değerlendirilmiş olup karar yazısı ektedir.

Bilgilerinize.

e-imzalıdır
Prof.Dr. İsmail MERAL
Başkan

Ek: Karar Yazısı (2 sayfa)

02/03/2018 Sek. Elif Gamze ASLAN

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KARAR FORMU

ARAŞTIRMANIN AÇIK ADI	Metilasyon Paternleri Değişen Belirli Onkomir MikroRNA'ların Prostat Kanseri Üzerindeki Etkisinin Araştırılması

27.02.2018

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	KOORDİNATÖR/SORUMLU ARAŞTIRMACININ UZMANLIK ALANI	Patoloji			
	ARAŞTIRMAYA KATILAN MERKEZLER	TEK MERKEZ ☒	ÇOK MERKEZLİ ☐	ULUSAL ☐	ULUSLARARASI ☐

	Belge Adı	Tarihi	Versiyon Numarası	
DEĞERLENDİRİLEN BELGELER	ARAŞTIRMA PROTOKOLÜ	-	-	Gerekli Değil ☐ Var ☒
	BİLGİLENDİRİLMİŞ GÖNÜLLÜ OLUR FORMU	-	-	Gerekli Değil ☒ Var ☐
KARAR BİLGİLERİ	**Karar No:5/36**	**Tarih: 27.02.2018**		
	Yürütücülüğünü Uzm. Dr. Ganime ÇOBAN 'ın yaptığı "Metilasyon Paternleri Değişen Belirli Onkomir MikroRNA'ların Prostat Kanseri Üzerindeki Etkisinin Araştırılması" Girişimsel Olmayan Araştırmalar Etik Kurulu tarafından değerlendirilmiş ve etik açıdan uygun bulunmuştur.			

Etik Kurul Başkanı
Prof. Dr. İsmail MERAL

BEZMİALEM VAKIF ÜNİVERSİTESİ GİRİŞİMSEL OLMAYAN KLİNİK ARAŞTIRMALAR ETİK KURULU (2011-KAEK-42)
KARAR FORMU

ARAŞTIRMANIN AÇIK ADI	Metilasyon Paternleri Değişen Belirli Onkomir MikroRNA'ların Prostat Kanseri Üzerindeki Etkisinin Araştırılması

BEZMİALEM VAKIF ÜNİVERSİTESİ GİRİŞİMSEL OLMAYAN KLİNİK ARAŞTIRMALAR ETİK KURULU	
ETİK KURULUN ÇALIŞMA ESASI	İlaç ve Biyolojik Ürünlerin Klinik Araştırmaları Hakkında Yönetmelik, İyi Klinik Uygulamaları Kılavuzu
BAŞKANIN UNVANI / ADI / SOYADI:	Prof. Dr. İsmail MERAL

Unvanı/Adı/Soyadı	Uzmanlık Alanı	Kurumu	Araştırma ile ilişki		Katılım *		İmza
Prof. Dr. İsmail MERAL	Fizyoloji	Bezmialem Vakıf Üniversitesi Tıp Fakültesi	E ☐	H ☒	E ☑	H ☐	
Prof. Dr. Ömer SOYSAL	Göğüs Cerrahisi	Bezmialem Vakıf Üniversitesi Tıp Fakültesi	E ☐	H ☒	E ☑	H ☐	
Prof. Dr. Nuran YILDIRIM	Tıp Tarihi ve Etik	Bezmialem Vakıf Üniversitesi	E ☐	H ☒	E ☑	H ☐	
Prof. Dr. Türkinaz AŞTI	Hemşirelik Bölümü	Bezmialem Vakıf Üniversitesi Sağlık Bilimleri Fakültesi	E ☐	H ☒	E ☑	H ☐	
Prof. Dr. Semra ÖZÇELİK	Tıp Eğitimi ve Bilişimi	Bezmialem Vakıf Üniversitesi Tıp Fakültesi	E ☐	H ☒	E ☑	H ☐	
Prof. Dr. Teoman AYDIN	Fiziksel Tıp ve Rehabilitasyon	Bezmialem Vakıf Üniversitesi Tıp Fakültesi	E ☐	H ☒	E ☑	H ☐	
Doç. Dr. Fahri AKBAŞ	Tıbbi Biyoloji	Bezmialem Vakıf Üniversitesi Tıp Fakültesi	E ☒	H ☐	E ☐	H ☒	KATILMADI
Doç. Dr. Binnur AYDOĞAN TEMEL	Eczacılık	Bezmialem Vakıf Üniversitesi Eczacılık Fakültesi	E ☐	H ☒	E ☑	H ☐	
Doç. Dr. Aclan ÖZDER	Aile Hekimliği	Bezmialem Vakıf Üniversitesi Tıp Fakültesi	E ☐	H ☒	E ☐	H ☑	KATILMADI
Doç. Dr. Mustafa TUNALI	Periodontoloji	Bezmialem Vakıf Üniversitesi Diş Hekimliği Fakültesi	E ☐	H ☒	E ☑	H ☐	
Yrd. Doç. Dr. Nur BÜYÜKPINARBAŞILI	Tıbbi Patoloji	Bezmialem Vakıf Üniversitesi Tıp Fakültesi	E ☐	H ☒	E ☐	H ☑	KATILMADI
Av. Mustafa Fırat ALKAYA	Hukuk	Bezmialem Vakıf Üniversitesi	E ☐	H ☒	E ☑	H ☐	
Eda BAYRAKTAR	Sivil Üye	Bezmialem Vakıf Üniversitesi	E ☐	H ☒	E ☑	H ☐	

* :Toplantıda Bulunma

Karar: ☒ Onaylandı ☐ Reddedildi

Etik Kurul Başkanı
Prof. Dr. İsmail MERAL