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IDENTIFICATION OF GENETIC BIOMARKERS IN URINE FOR EARLY DETECTION OF PROSTATE CANCER

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THESIS APPROVAL

Eyyup Kavalcı's thesis titled "Identification of Genetic Biomarkers in Urine for Early Detection of Prostate Cancer" was accepted by our jury on July 17, 2020 as a doctoral dissertation in the Department of Medical Biology and Genetics of the Faculty of Medicine.

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APPROVAL

This doctoral thesis exam has been made online via Google Meet and the jury members have declared their acceptance orally.

STATEMENT (DECLARATION)

Hereby I declare that this thesis study is my own study, I had no unethical behavior in all stages from planning of the thesis until writing thereof, I obtained all the information in this thesis in academic and ethical rules, I provided reference to all of the information and comments which could not be obtained by this thesis study and took these references into the reference list and had no behavior of breeching patent rights and copyright infringement during the study and writing of this thesis.

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LIST OF ABBREVIATIONS

ACS: The American Cancer Society

AUA: The American Urological Association

BPH: Benign Prostatic Hyperplasia

BPH: Benign Prostatic Hypertrophy

DNA: Deoxyribonucleic Acid

DRE: Digital Rectal Examination

EAU: The European Association of Urology

ED: Erectile Dysfunction

FDA: The Food and Drug Administration

MRI: Magnetic Resonance Imaging

PB: Prostate Biopsy

PCA3: Prostate Cancer Antigen 3

PET: Positron Emission Tomography

PHI: Prostate Health Index

PIN: Prostatic Intraepithelial Neoplasia

PSA: Prostate-Specific Antigen

PSMA: Prostate-Specific Membrane Antigen

RP: Radical Prostatectomy

RT: Radiation Therapy

TRUS: Trans-rectal Ultrasonography

WHO: World Health Organization

ABSTRACT

Prostate cancer screening is a challenging and vital issue in the aspects of the current tests and risk assessments. Prostate cancer risk assessments are currently carried out by using blood, urine and tissue biomarkers with radiological imaging methods. Here, we introduce a novel non-invasive screening tool for a further in-depth selection of eligible cases for prostate biopsies which is based on sequencing somatic and hereditary HOXB13 mutations in urine samples. This approach provides diagnostic information to the physician about the presence of prostate cancer while aiming to screen for specific prostate biopsies and save biopsies potentially when there are no mutations related to prostate cancer. Findings suggest that this method is reliable, cost-effective and has a promising potential in prostate cancer screening.

Keywords: Prostate Cancer, Prostate Cancer Screening, Urine, Biomarkers, HOXB13

ÖZET

Prostat kanseri taraması, mevcut testler ve risk değerlendirmeleri açısından zorlu ve hayati bir konudur. Prostat kanseri risk değerlendirmeleri, radyolojik görüntüleme yöntemleri ile kan, idrar ve doku biyobelirteçleri kullanılarak yapılmaktadır. Burada, prostat biyopsileri ve uygun vakaların daha ayrıntılı seçimi için idrar örneklerinde somatik ve kalıtsal HOXB13 mutasyonlarının dizilenmesine dayanan yeni bir invaziv olmayan tarama aracı sunuyoruz. Bu yaklaşım, prostat kanseri ile ilgili herhangi bir mutasyon olmadığında, doktora prostat kanseri varlığı hakkında teşhis bilgileri verirken, prostat biyopsilerini potansiyel olarak azaltmayı amaçlamaktadır. Bulgular, bu yöntemin güvenilir, uygun maliyetli ve prostat kanseri taramasında umut verici bir potansiyele sahip olduğunu göstermektedir.

Anahtar Kelimeler: Prostat Kanseri, Prostat Kanseri Taraması, İdrar, Biyobelirteçler, HOXB13

CHAPTER I

1. INTRODUCTION

1.1.Definition of Cancer

Cancer is the occurrence of uncontrolled cell division and spread into the body where it represents a group of diseases. In the human body, it is formed by trillions of cells and it can start almost anywhere. In other words, cancer is a genetic disease which occurs when there are changes to genes which are responsible for regular cell functions (https://www.cancer.gov/about-cancer/understanding/what-is-cancer, Accession date: 03 June 2020).

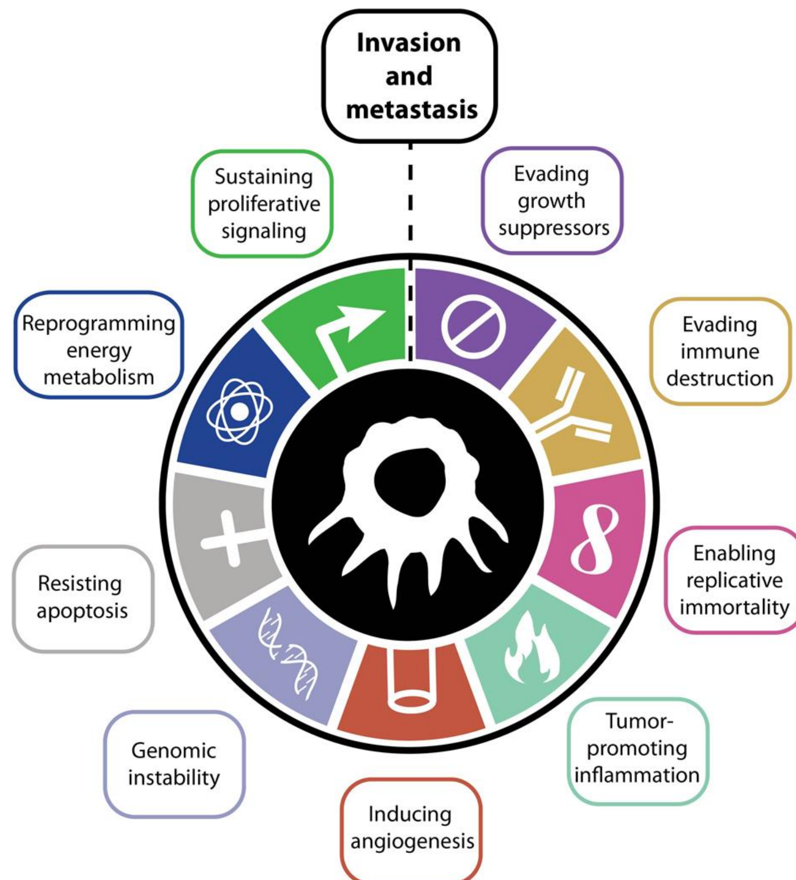


Figure 1.1. Hallmarks of cancer with invasion and metastasis. The figure was adopted from (Meirson et al., 2020).

1.2.Carcinogenesis and Tumorigenesis

Tumorigenesis is the formation of a tumour caused by a large number of cells where it can be benign or malignant; however, carcinogenesis or oncogenesis is specifically the formation of cancer by transformed cells. When genes that are responsible for the regulation of cell growth and differentiation are altered, a normal cell transforms into a cancer cell which leads to disease (Croce, 2008).

When genes are affected they are classified into two categories. Oncogenes are responsible for cell growth and reproduction. Tumour suppressor genes prevent cell division and survival. Tumours become malignant when tumour suppressor genes are disabled or under-expressed, or novel oncogenes are formed, or inappropriately over-expressed (Knudson, 2001).

Cancer cells transfer to the parts of a body through the blood and/or lymphatic system, especially in the late stages of cancer and this is called metastasis. The original tumour is called primary where the spread ones called metastatic tumours. Most of the late stages of metastasis lead to cancer death. Cancers have tendencies to metastasize to different organs in the body; however, most of the metastases occur in brain, lungs, bones and liver (S. Kim, 2015).

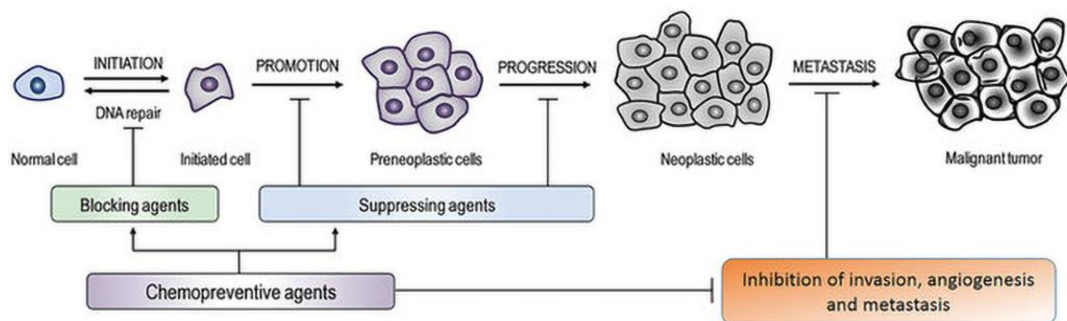


Figure 1.2. Phases of carcinogenesis as initiation, promotion, progression and metastasis. The figure was adopted from (Siddiqui et al., 2015).

1.3.Causes of Cancer Development

The 5-10% of cancer developments occur by inherited mutations where 90-95% of them are caused by somatic mutations which are caused by environmental and lifestyle factors. Besides, errors in DNA replication can cause mutations which promote cancer formation (Anand et al., 2008).

Lifestyle, diet, behavioural and economic factors refer to environmental factors which are not inherited genetically. Obesity, smoking, infections, alcohol consumption, radiation, pollution and lack of physical activity are major environmental factors that cause and/or promote cancer formation and death (Islami et al., 2018).

1.4.Types of Cancer

Classification of cancers is done by the type of tumour cells where there are many cancer types. Generally, types of cancer are named according to the place of cancer formation for the tissues or organs and it is assumed to be the origin of the tumour. Carcinoma, sarcoma, leukaemia, lymphoma, multiple myeloma, melanoma, brain and spinal cord tumours, germ cell tumours, neuroendocrine tumours and carcinoid tumours are some of the categories that are formed due to specific types of cells (https://www.cancer.gov/about-cancer/understanding/what-is-cancer#types, Accession date: 03 June 2020).

1.5.Diagnosis and Treatment

There are different types of diagnosis and treatment programmes available for cancer patients to cure or prolong their lives. Besides, for cancer survivors, the goal is to provide the best quality of life for them. Diagnosis is the crucial step in the management of cancer in the basis of pathological examination through a sample of biopsy or aspiration which can be an endoscopy or image-guided procedure.

Laboratory and pathology services are important during these processes because staging is the most critical part in order to specify the degree of tumour spread to determine the treatment options.

Surgery, radiotherapy and systemic therapy are the major therapeutic modalities in cancer treatment and this process needs special care and consideration with a collaboration of well-coordinated multi-disciplinary cancer team to get the best effective treatment for the patient (https://www.who.int/cancer/treatment/en/, Accession date: 03 June 2020).

1.6.Prostate Cancer

Prostate cancer is the most common type of cancer which develops in the prostate gland. Normally, prostate cancers grow slowly; however, in some cases, it grows relatively quickly. It may not cause any symptoms at the beginning of the disease, but patients may have pain in the pelvis, difficulty when urinating or blood in the urine in later stages. In addition, metastasis may occur from the prostate gland to other parts of the body, such as bones and lymph nodes.

1.6.1. Anatomy of the human prostate gland

The prostate is an exocrine and the largest gland of the male reproductive system. It is located in front of the rectum and under the bladder. Figure 1.3 shows the four anatomic zones of the prostate which are peripheral, central, transition and fibromuscular stroma. Peripheral zone is the largest part which covers about 70% of the gland and encompasses the distal urethra (Bhavsar & Verma, 2014).

The central and transition zones cover about 25% and 5% of the gland respectively, where the central zone encompasses the ejaculatory ducts and the transition zone encompasses the proximal urethra with glandular tissue. The fibromuscular stroma does not have glandular tissue, but it is made up of fibrous and smooth muscle. The prostate secretes a prostatic fluid which is white, milky and slightly alkaline and contributes to the volume of the semen.

The vaginal tract has acidity medium and the alkalinity of the semen neutralizes it in order to prolong the lifespan of sperm. The prostatic fluid squirts just at the beginning of the ejaculation with the sperm (Bhavsar & Verma, 2014; Mawhinney & Mariotti, 2013).

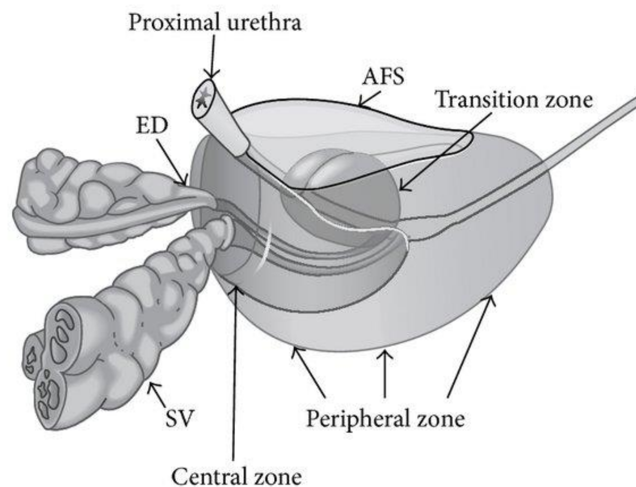


Figure 1.3. Zonal anatomy of the prostate gland. ED – ejaculatory ducts; SV – seminal vesicles; AFS – anterior fibromuscular stroma. The figure was adopted from (Bhavsar & Verma, 2014).

The prostate gland starts to develop in the embryo and continues until the person becomes an adult. Testosterone and other male hormones regulate the embryology and development of the prostate gland and any problems in the hormonal system can cause abnormalities in sexual development, benign prostatic hypertrophy (BPH) or prostate cancer depending on the level of prostate development (Feldman & Feldman, 2001). Normally, BPH evolves in the transition zone and most of the prostatic adenocarcinomas develop in the peripheral zone (Applewhite et al., 2001; Mawhinney & Mariotti, 2013).

1.6.2. Signs and symptoms

If a patient has prostate cancer in early stages, it may not have any symptoms. Usually, it has the same symptoms such as benign prostatic hyperplasia (BPH). Dysuria (painful urination), frequent urination, difficulty in a steady stream of urine, nocturia (increased urination at night) and hematuria (blood in the urine) are the most common symptoms of prostate cancer. The prostatic urethra is encompassed by the prostate gland and therefore, a patient who has prostate cancer has urinary dysfunction. Thus, the urinary function is directly affected by the changes within the gland. In addition, prostate cancer patients may have difficulties in achieving an erection or painful ejaculation because of the structure of the prostate gland. Bone pain, compressing in the spinal cord, urinary and faecal incontinence and leg weakness are some of the additional symptoms of prostate cancer when metastatic cancer cells spread to the other parts of the body (Miller et al., 2003).

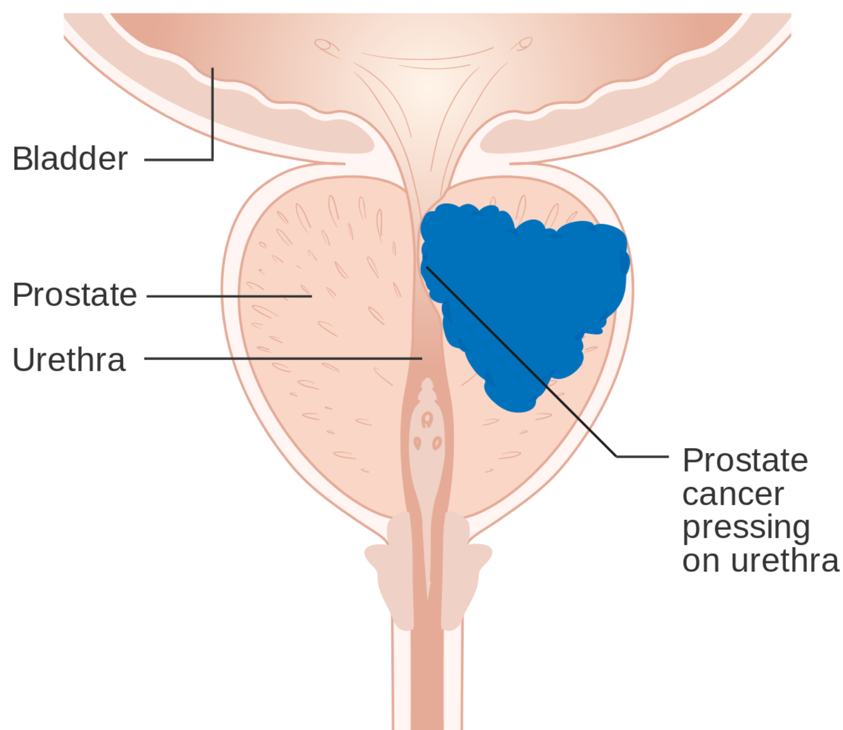


Figure 1.4. A diagram of prostate cancer touching to the urethra (Cancer Research UK, 2020).

1.6.3. Risk factors

Prostate cancer is generally diagnosed after the age of 45 and it is more common in older ages. 70 years of age is the average time of diagnosis (Bell et al., 2015). Age, heredity, race, obesity, inflammation, hormones, metabolic syndromes, vitamins and fatty acids, smoking, excess alcohol consumption and lack of exercise are the most common risk factors of prostate cancer. There is a double risk of having prostate cancer if a man has first-degree family members that have prostate cancer. Besides, there is a greater risk for a man that has an affected brother compared to an affected father. High blood pressure and lack of exercise also increase the risk of having prostate cancer (Aslam N, Nadeem K, Noreen R, 2015; Zeegers et al., 2003).

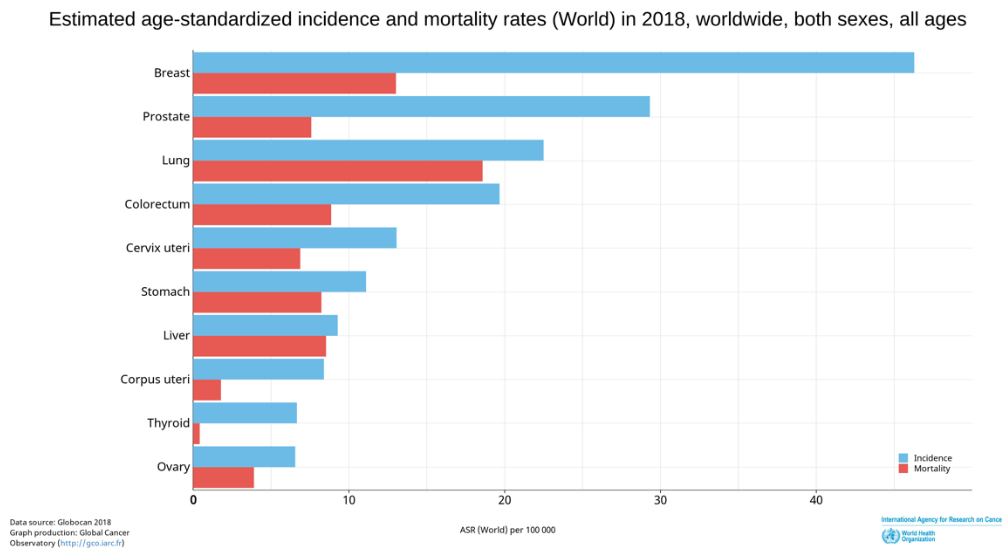


Figure 1.5. Estimated age-standardized incidence and mortality rates in 2018 (WHO, 2018).

1.6.3.1.Genetics factors

Genetic factors are important with the association of race, family and specific gene variants in developing prostate cancer. Family history plays a critical role when first-degree relatives have prostate cancer and increase the risk of having it.

In the USA (United States of America) black men are more affected than white and Hispanic men and also mortality rates are greater in black men. Studies have shown that approximately 10% of prostate cancer cases are developed by inherited factors (Attard et al., 2016).

BRCA1, BRCA2, CHEK2, PALB2 are some of the genes that have found in studies that are associated with prostate cancer; however, the homeobox gene HOXB13 is the only one that has identified in genetic linkage studies in multiple-case families as definite prostate cancer predisposition gene (Ewing et al., 2012; Struewing et al., 1997).

HOX genes or homeobox genes are a set of related genes that are responsible for the formation of the body plan of an embryo. In addition, HOX genes are the main transcriptional regulators and play important roles in embryo and carcinogenesis.
There are 39 HOX genes which are clustered on four different chromosomes in humans and these clusters are known as the four HOX families: HOXA, HOXB, HOXC and HOXD. These HOX genes have important roles during stem cell differentiation in the entire development period and it is found that HOX mutations can cause human disorders with different variation (Bhatlekar et al., 2018).

Prostate cancer has the highest heritability reported compared to other major cancers. Many studies have done on the family-based linkage of prostate cancer on European descent to identify the responsible genes for prostate cancer. HPC1 (Berry, Schaid, et al., 2000; B. S. Carter et al., 1992; Cooney et al., 1997), PCAP (Berry, Schaid, et al., 2000; Neuhausen et al., 1999; J. Xu et al., 2001), HPCX (Schleutker et al., 2000), CAPB (Berry, Schaid, et al., 2000; J. Xu et al., 2001), HPC20 (Berry, Schroeder, et al., 2000) and HOXB13 (Breyer et al., 2012; Jianfeng Xu et al., 2013) are the major found genes.

1.6.3.2.Dietary and other factors

Studies have shown that consumption of vegetables and fruits reduces prostate cancer risk. Red meat and processed products seem to have little effect on daily consumption (Venkateswaran & Klotz, 2010). Also, obesity (Calle et al., 2003), lower blood levels of vitamin D (Wigle et al., 2008) and higher blood levels of testosterone (Gann et al., 1996) have potential to increase the risk for prostate cancer.

Prostatitis is the infection or inflammation of the prostate gland and it may cause to have prostate cancer. Sexually transmitted infections, syphilis, gonorrhoea or chlamydia also increase the risk (Caini et al., 2014; Dennis et al., 2002).

1.6.4. Prevention

In recent studies, it was found that there is a relationship between diet and prostate cancer but the data is not sufficient to prove that. In this context, the risk rate of prostate cancer depends on the consumption of the Western diet. In addition, the results showed that a vegetarian diet reduces the risk of prostate cancer. Therefore, consuming vegetables, beans, soy and other legumes with regular exercises may prevent prostate cancer. Some reports support lycopene and selenium (Masko et al., 2013; Rowles et al., 2018).

1.6.5. Screening and early detection

Prostate cancer screening aims to detect prostate cancers in early stages before it metastasized especially when there are no symptoms to reduce the mortality rates. The gold standard of prostate cancer screening is measuring the prostate-specific antigen (PSA) level in the blood with the digital rectal examination (DRE) followed by a prostate biopsy.

It is known that prostate cancer screening is vital for the early detection of a tumour with a well-managed treatment in order to prevent the progression and spread of cancerous cells to the body. A study between different races was done in England between 2008 and 2010 and the lifetime risk of patients was calculated.

The results showed that prostate cancer diagnosis and death risks of white men were 1 in 8 and 1 in 24 respectively where it was 1 in 13 and 1 in 44 respectively for Asian men. Therefore, white men are at risk of diagnosis as 1.6 times higher than Asian men and this also shows the ratio of death is 1.8 among them (Bokhorst & Roobol, 2015; Lloyd et al., 2015).

A significant number of men can be saved from metastatic prostate cancer if PSA-based screening starts at age 45-49 compared to 51-55 years which is indicated by the study of The Malmo Preventive Project. According to this observation, it is suggested to start prostate cancer screening in early ages by several guidelines. To find out the risks of prostate cancer, PSA testing should start at 40s ages for men which are recommended by the European Association of Urology (EAU) (Heidenreich et al., 2014; A. J. Vickers et al., 2013).

The limitations of the current screening methods have led to the need for more reliable and specific biomarkers for prostate cancer in clinical applications. Prostate cancer antigen 3 (PCA3) (Auprich et al., 2011), Transmembrane protease serine-2: ERG (TMPRSS2: ERG) gene fusion (Tomlins et al., 2005), 4KScore (A. Vickers et al., 2010), MiProstate Score (Tomlins et al., 2016), SelectMDx (Leyten et al., 2015), ConfirmMDx (Stewart et al., 2013), ExoDx (McKiernan et al., 2016) and Prostate Health Index (PHI) (Le et al., 2010) are the most known and commonly used biomarkers and tests. However, none of these biomarkers and tests is sufficient to replace PSA and DRE for prostate cancer screening.

1.6.5.1.Prostate-specific antigen (PSA) test

PSA is a kallikrein-related peptidase 3; KLK3 which liquidizes the seminal coagulum. PSA is produced by both cancerous and noncancerous epithelial cells and it is highly organ-specific but not tumour specific. Different causes may increase the level of PSA such as prostatitis and BPH. PSA level may also increase after the DRE by up to ten weeks. PSA has a complex structure with the protease inhibitor α_1-antichymotrypsin and it circulates inactively within the blood.

Elimination of PSA depends on protease inhibitors and it progresses slowly (approximately 1-2weeks) and kidneys clear the most of it. After radical prostatectomy (complete removal of the prostate) PSA levels become undetectable in 6 weeks (Eastham, 2017).

In most cases, men with (about 70%) elevated PSA levels do not show any evidence for prostate cancer until obtaining the biopsy results. Thus, about 15% of men may have prostate cancer when the PSA levels are normal. PSA test is not able to distinguish the clinical significance or insignificance of prostate cancer during the man's lifetime. Although the PSA test has some limitations, it is the most widely used test for screening the early stages of prostate cancer (Thompson et al., 2004, 2005).

The United States Food and Drug Administration (FDA) approved PSA testing as an early detection test for prostate cancer in 1994. After the use of PSA testing in clinical applications, over %80 of new cases was diagnosed with prostate cancer in early-stage. Deaths due to prostate cancer generally occur when PSA levels are >2 ng/ml. Although there are reports from large randomized studies that PSA test reduces mortality rates, there are contradictions regarding the routine use of this test (Carlsson et al., 2014).

The United States Preventive Services Task Force updated the guidelines for prostate cancer screening in 2017. According to these guidelines, men who are 55-69 years old is recommended as "C" which means benefits and harms of prostate cancer screening must be informed to these patients and offered to choose PSA testing by themselves. "D" or "do not screen" recommendation was done for men who are ≥70 years' old which means "there is moderate or high certainty that this service has no net benefit or that the harms outweigh the benefits." Men who are 55 to 69 years old are recommended for shared decision-making about the harms and benefits of PSA-based screening by The American Urological Association. Men who are not in this age range are not recommended for the routine of PSA-based screening (Eastham, 2017; Grossman et al., 2018).

The risk of over-diagnosis is the negative side of prostate cancer screening especially in Western countries (Draisma et al., 2009; Pashayan et al., 2009). The results showed that there were no benefits for prostate cancer screening in the case of having medical complications while other studies claimed that the mortality rates were reduced significantly by screening programmes (Bokhorst et al., 2014; Djulbegovic et al., 2010).

There is a contradiction between the guidelines for prostate cancer screening in the USA. Screening is recommended at the age of 40-45 years for high-risk men and 50 years of age for average-risk men by the American Cancer Society where it is only recommended in every two years routinely for average-risk men between 55-69 years of age and individualised for high-risk men by the American Urological Association (H. B. Carter et al., 2013; Wolf et al., 2010). These guidelines are opposed to the guidelines of the United States Preventive Services Task Force where no screening at any age is recommended. Besides, prostate cancer screening is offered by physicians or requested by patients if they have anything related to their prostate health (Moyer, 2012).

1.6.5.2.Digital rectal examination (DRE)

DRE is the procedure of observing the face of prostate gland from the rectum side by inserting the index finger into the rectum whether it has lumps, spots or any atypical characteristic. Posterior and lateral aspects are the only parts of the prostate gland that can be accessed through the rectum. Therefore, tumours can only be detected in the peripheral zone by the DRE where 70% of prostate cancers develop in that part (Applewhite et al., 2001; Bhavsar & Verma, 2014).

DRE is not a sufficient procedure by itself because 40-50% of men with atypical findings did not have any pathological finding related to prostate cancer, where 40% of men with normal DRE had prostate cancer. Therefore, the usefulness of DRE was questioned and later recommended to use with PSA test in order to detect cancer and determine the degree of cancerous mass (Philip et al., 2005).

1.6.5.3.Follow-up tests

After an elevated PSA level and/or abnormal DRE finding, a prostate biopsy is a must for the detection of prostate cancer and other diseases related prostate as well as staging the tumour if exists. Although prostate biopsy is the gold standard in the detection, it may cause medical complications, such as infection or bleeding. Biopsies may be taken via rectum or penis.

Besides, magnetic resonance imaging (MRI) guided biopsies have improved the quality and diagnostic accuracy of the method (Bennett et al., 2016; Borghesi et al., 2017; Loeb et al., 2013).

A better alternative to MRI technique is the transrectal ultrasonography (TRUS) where it is fast and minimally invasive for the evaluation of superficial tumours. The layers of the rectum wall can be seen accurately and primary rectal cancer can also be staged. For the staging of perirectal lymph nodes, locally advanced and stenosing cancers both techniques can be used where MRI is better in visualization (M. J. Kim, 2015).

When there is a suspicious malignancy, MRI scanning technique is used to observe the tumour better in order to eliminate unnecessary prostate biopsies and increase the biopsy yield (Sarkar & Das, 2016). Studies showed that MRI scans cost much more than other techniques; however, when it is compared to PSA and TRUS biopsy-based standard of care, in the long run, it has been found as more cost-effective. Although MRI-targeted biopsy technique has advantages, a consensus should be determined when to use this technique (Giganti & Moore, 2017; Turkbey & Choyke, 2018).

In recent years Ga^{68}-PSMA (Prostate-specific membrane antigen) PET/CT (Positron emission tomography/computed tomography) became the gold standard for restaging recurrent prostate cancers within a relatively less time (Lenzo et al., 2018). Intermediate-to-high risk primary prostate cancers can be staged with this modality.

The appropriate use of Ga68-PSMA PET/CT or PET/MR (Positron emission tomography/Magnetic resonance) is with the combination of mpMRI (multi-parametric magnetic resonance imaging) to locate cancer for primary prostate cancer where mpMRI helps to evaluate the recurrence of cancer (Bouchelouche & Choyke, 2018; Gaur & Turkbey, 2018; Virgolini et al., 2018).

1.6.5.4.Biomarkers for prostate cancer

Biomarkers for prostate cancer screening are the tools which are used to reduce unnecessary prostate biopsies, predict the probability of the first positive biopsy, distinguish low, intermediate and high-risk tumours, classify the degree of the disease, and predict the response to the treatment (Cucchiara et al., 2018; Kretschmer & Tilki, 2017).

The Prostate Health Index (PHI, Beckman Coulter Inc., CA, USA) is a mathematical score which consists of three PSA forms that are tPSA, %fPSA and [-2] proPSA (p2PSA). This score supports more information which is used to distinguish benign and suspected prostate cancer cases. Studies showed that PHI had greater specificity (AUC 0.73) compared to PSA or combination pro PSAs. In addition, it was found that increased PHI scores were associated with significant prostate cancers with Gleason Grade 7 or higher (Le et al., 2010; Wei et al., 2014). PHI score is recommended for early detection of prostate cancer and risk assessment with PSA level between 2.0 and 10.0 ng/ml by the European Association of Urology (EAU) and National Comprehensive Cancer Network (NCCN) guidelines (Mottet et al., 2017).

Prostate Cancer Antigen 3 (PCA3) also referred to as DD3 is a gene that expresses a non-coding RNA. It is a prostate-specific gene and it highly expresses in prostate cancer. Therefore, it is used for the early detection of prostate cancer especially after an initial negative biopsy. PCA3 had an FDA approval in 2012 as the Progensa PCA3 test (Hologic, Marlborough, MA, USA) where it has promising results on repeat biopsies (AUC= 0.71-0.75 with the combination of clinical findings and PSA). Studies showed that PCA3 correlates with the aggressiveness of prostate cancer.

If a patient has at least one negative initial biopsy and at risk of prostate cancer, the PCA3 test is recommended by the National Comprehensive Cancer Network (NCCN) guidelines. However, the use of this test on repeat biopsies can be problematic when compared with clinical findings or PSA alone (Auprich et al., 2011; Wei et al., 2014).

TMPRSS2: ERG is the gene fusion of TMPRSS2 gene and E26 transformation specific (ETS) oncogene which is highly associated with prostate cancer (Tomlins et al., 2005).

TMPRSS2: ERG is considered to have an important role in tumorigenesis and Laxman et al. found that this fusion transcript can be obtained in patients' urine samples. However, this fusion has high specificity (86%) with poor sensitivity (45%). Thus, when this fusion combined with PCA3, 73% of sensitivity was obtained (Hessels et al., 2007; Laxman et al., 2006; Prensner & Chinnaiyan, 2009; Sanguedolce et al., 2016).

Mi-Prostate Score (MiPS) is a urine test which has been introduced by the University of Michigan, USA. This score is obtained with the combination of PSA, TMPRSS2: ERG and PCA3. Statistical models showed that MiPS had greater AUC (0.751) than PSA plus PCA. Therefore, it is listed as an investigational biomarker by NCCN guidelines; however, more evidence was needed (Mottet et al., 2017; Tomlins et al., 2016).

4KScore (OPKO Lab, Miami, USA) test works similar to PHI which is based on serum levels of total PSA, free PSA, intact PSA, and human kallikrein 2. The score is determined with clinical findings such as age, DRE, etc. All these variables put in an algorithm and a specific percentage risk is calculated for the patient. It has been reported that 4KScore reduced the number of prostate biopsies and it may help to catch high-risk prostate cancers. However, in a study of 12 out of 100 high-grade cancers were missed. Therefore, it is mentioned as a potential marker test by EAU guidelines in particular for patients with PSA levels between 2.0 and 10.0 ng/ml for further risk assessments. It is also mentioned as a potential tool for pre-biopsy and post-negative-biopsy cases by NCCN where highlighted that there are no validated cut-off values (Mottet et al., 2017; A. Vickers et al., 2010).

SelectMDx (MDxHealth, Irvine, USA) is an assay for the selection of patients for an initial biopsy which works with post-DRE urine samples. It is a three-gene panel (TDRD1, HOXC6, and DLX1) which has higher accuracy (AUC= 0.77) compared to PSA (AUC= 0.72) and the Progensa PCA3 test (AUC= 0.68) for the detection of clinically significant prostate cancers (Leyten et al., 2015). Clinical findings such as age, tPSA level, etc. are combined with the test results for the final decision. Studies showed that properly use of SelectMDx test for the patients who have PSA levels of >3 ng/ml can reduce the overdiagnosis and overtreatment as well as the cost while increasing the quality of life. Patients who also had suspicious lesions in mpMRI found that they had high SelectMDx scores. SelectMDx is indicated as an investigational biomarker in current NCCN guidelines. However, the panel needs more evidence (Dijkstra et al., 2017; Hendriks et al., 2017; Van Neste et al., 2016).

ExoDx prostate intelliscore (EPI, exosome diagnostics, Boston, USA) is an exosome-based gene signature which is obtained from urinary mRNA expression. This test does not require pre-catch DRE or post-catch handling and can easily be used in clinical applications. Studies show that this test increases the diagnostic performance of standard care for high-grade prostate cancers. EPI is indicated as an investigational biomarker by current NCCN guidelines; however, more evidence is needed (Donovan et al., 2015; McKiernan et al., 2016; Mottet et al., 2017).

The ConfirmMDx (MDxHealth)is a tissue-based methylation marker test which is used to find the epigenetic changes around the tumour lesions (halo effect) to reduce the number of unnecessary prostate biopsies. GSTP1, APC, and RASSF are the basic genes of this test and after a negative biopsy, this test is used to identify the hypermethylation pattern of CpG island promoter regions of these genes. Studies showed that the ConfirmMDx test has %68 of sensitivity and 64% of specificity and in a multivariate analysis it was confirmed that this biomarker could be used as an independent predictor for any prostate cancer biopsy. ConfirmMDx test is indicated by current EAU Guidelines to gain additional information in re-biopsy cases; however, due to limited available data, this test has no recommendation in routine applications. Conversely, the NCCN guidelines indicated that the ConfirmMDx test can be recommended to men before repeat biopsy after a negative first biopsy (Leapman & Carroll, 2016; Stewart et al., 2013).

OncotypeDX genomic prostate score (Genomic Health, Redwood City, USA) assay consists of 17 genes where 12 genes are related to proliferation, androgen metabolism, stromal response, cellular organization, and 5 reference genes. Combining different biological pathways is considered to increase accuracy. This essay aims to select the candidates for the active surveillance and give results especially for the small size of tumours by predicting the adverse pathologic effects for the radical prostatectomy (Klein et al., 2014). When active surveillance is considered OncotypeDX assay can be used (Davis, 2015; Ross et al., 2016).

EAU guidelines indicated overall results from multi-variate studies in order to make the final recommendation. OncotypeDX assay is recommended for low-risk and very-low-risk prostate cancer patients in the post-biopsy cases if a 10 years or more life expectancy is present by the NCCN guidelines. AUA guidelines stated that this assay needed to be proven for the selection of active surveillance patients (Mottet et al., 2017; Sanda et al., 2018).

Prolaris (Myriad Genetics Inc., Salt Lake City, USA) test is based on the study of Cuzick and colleagues where the test includes a consecutive score and a gene signature. A gene signature was developed which includes 31 cell cycle genes by evaluating 126 genes that are related to cell cycle regulation (Cuzick et al., 2011). Results of the studies made for the Prolaris test showed that it might be used in the improved pre-therapy classification of disease risk in the future to reduce the number of unnecessary prostate biopsies. However, expert opinions indicated that this test was not safe enough to be used in clinical applications and EAU guidelines stated the need of more trials for the final recommendation. Similar to OncotypeDX assay Prolaris is also recommended for low-risk and very-low-risk prostate cancer patients in the post-biopsy cases if a 10 years or more life expectancy is present by the NCCN guidelines. AUA guidelines also stated that this assay needed to be proven for the selection of active surveillance patients (Mottet et al., 2017) (Sanda et al., 2018).

Decipher (GenomeDX, Vancouver, Canada) gene signature consists of a 22-gene panel and it was developed to predict the comprehensive progression after the treatment where the panel has different biological pathways such as cell structure, androgen signalling, cell proliferation, cell cycle progression and immune system modulation.

The range of scores is from 0 to 1 where >0.6 are count as high-risk for progression (Nakagawa et al., 2008). Expert opinions suggested that the Decipher test can be used if adjuvant radiotherapy is referred for a high-risk patient in the clinical application for the risk stratification. AUA guidelines indicated that the Decipher test has not had a major role in the selection of active surveillance patients. It is also mentioned in NCCN guidelines as a potential tool for patients who are referred to as radical prostatectomy (Sanda et al., 2018) (Ross et al., 2016).

1.6.6. Diagnosis of Prostate Cancer

Diagnosis of prostate cancer starts with the results of PSA and DRE findings. If a suspicious lump is detected in DRE and TRUS-guided prostate biopsy is referred to the patient to identify the presence of prostate cancer and its stage of tumour development. During the biopsy procedure, samples are obtained from different sides of the prostate gland especially from the peripheral zone. Later, pathological analysis is done to find out the presence of prostate cancer and its stage. Approximately 20-30% of tumours may be missed due to under-sampling during the biopsy procedure due to the small number of samples and/or newly developed tumours. If a patient has elevated PSA levels and a negative initial biopsy, a re-biopsy with more core samples is offered in the follow-up process. After a positive biopsy, further analysis is done to investigate the degree of prostate cancer (Castillejos-Molina & Gabilondo-Navarro, 2016; Klein et al., 2014).

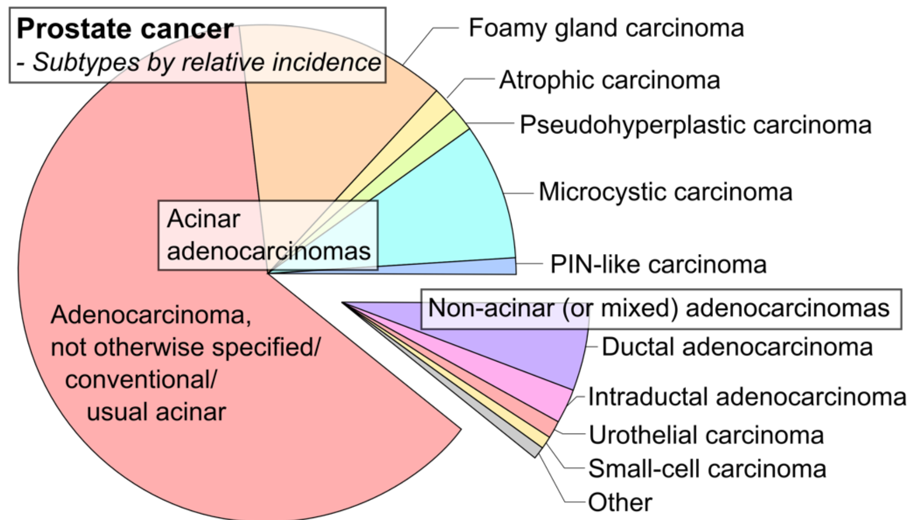


Figure 1.6. Pie chart of histopathological sub-diagnoses of prostate cancer. The chart was adopted from (Haggström, 2020).

Prostatic intraepithelial neoplasia (PIN) is a form of adenocarcinoma (development of cancer in gland cells) and it is diagnosed about 5-16% of patients who referred to prostate biopsies. It is the condition when epithelial cells and ducts in the prostate gland grow abnormally compared to normal cells. The PIN can be low-grade (LG-PIN) or high-grade (HG-PIN) and there is a risk when it is high-grade. Therefore, HG-PIN should be followed-up properly (Bishara et al., 2004; Brawer, 2005; Montironi et al., 2011).

1.6.6.1.Gleason grading system

The Gleason Grading System is a tool which is used to find out the degree of tumour aggressiveness and differentiation when a tumour is detected in prostate biopsies. Besides, it is used to predict the prognosis of cancer and guide the therapy together with other clinical parameters. In this grading system, there is a grade range from 1 (well-differentiated) to 5 (poorly differentiated) which is given due to the glandular differentiation of cancer on the microscope. Higher Gleason score means the cancer is aggressive and its prognosis is not good.

Meanwhile, the range of pathological scores is from 2 to 10 which indicates high risk and mortality as the score increases. The appearance of cells under the microscope determines the total score where the first half of it is calculated by the dominant or most common cell morphology (scored from 1 to 5) and the second half by the non-dominant cell pattern with the highest grade (scored from 1 to 5). Finally, these two scores are combined to generate the final score for cancer (Epstein et al., 2005; Gleason, 1977, 1992).

In other words, an overall Gleason score is the sum of the most common Gleason grade in all of the samples and the highest grade found in the rest of the samples when there is more than one grade of cancer in biopsy samples. A Gleason grade of 5+2=7 can be explained as there are plenty of grade 5 tumours where there are fewer cases of grade 2 tumours. If a tumour has a Gleason score of 6 or less, it is accepted as a low risk where a Gleason score of 7 tumours is accepted as intermediate risk. If there is a score between 8 and 10, it is accepted as high-risk tumours and maybe more aggressive (Pan et al., 2000; Pierorazio et al., 2013).

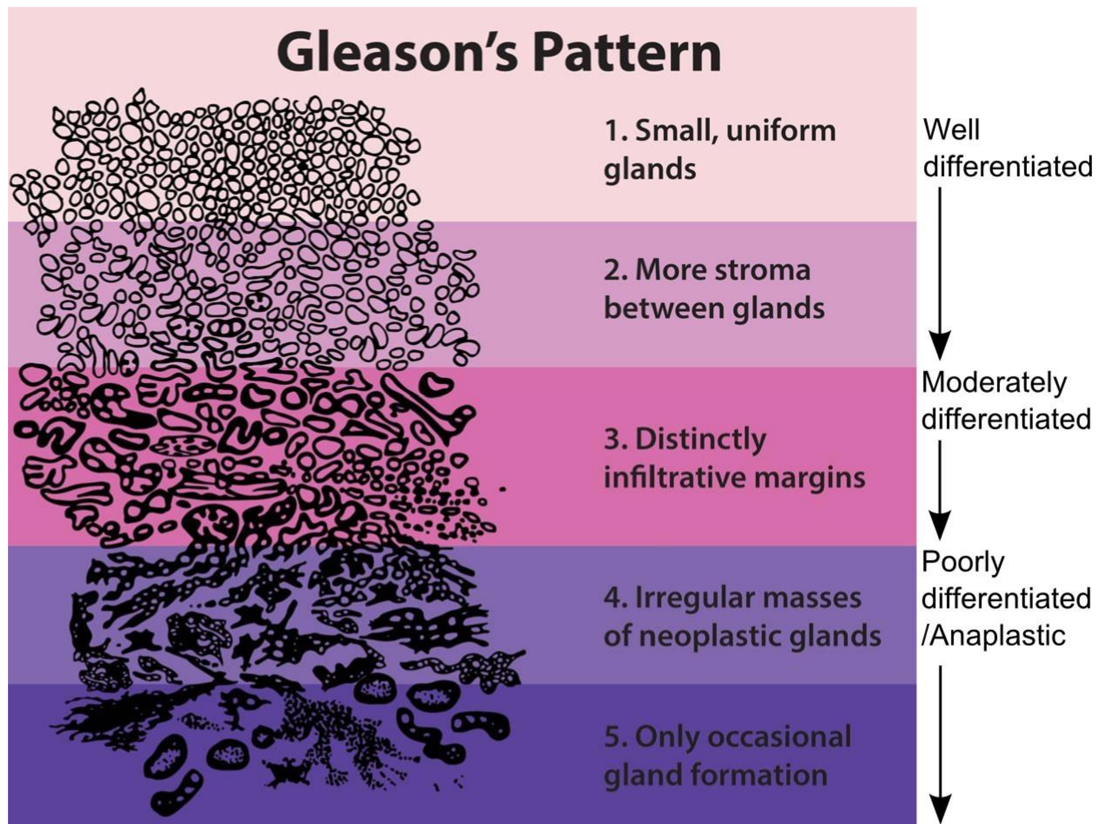


Figure 1.7. Gleason's pattern of Gleason grading system (Morphology & Grade, ICD-O-3 Morphology codes, National Institutes of Health; Accession date: 04 June 2020).

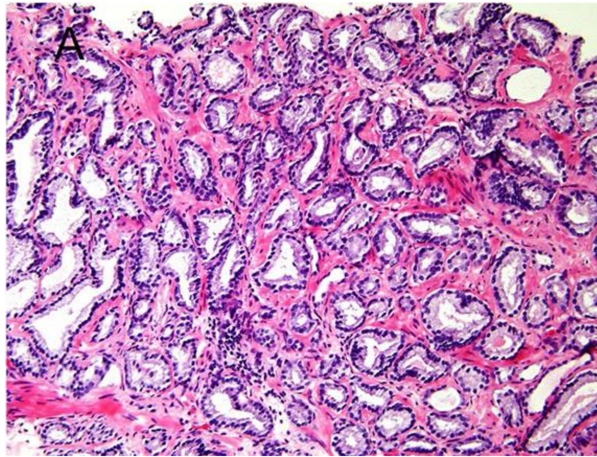
Gleason 1 is the most well-differentiated and well-defined tumour pattern which has a back-to-back, closely or densely packed, single or separate gland pattern that does not invade to the healthy prostatic tissue. The glands are large and round to oval-shaped and nearly equal in size and shape comparing to Gleason pattern 3 tumours. ***Gleason 2*** is quite restrained nodules of separate of single glands, but they are not uniform compared to patter 1. A small amount of invasion may be seen by neoplastic glands and they are larger and round to oval in shape similar to Gleason 1 when compared to Gleason 3. Minimal invasion and density of packing are the main difference between 1 and 2. ***Gleason 3*** glands have alterations in shape and size with a clear invasion into from neoplasm to adjacent healthy prostate tissue. Their glandular structure is small or micro compared to 1 and 2 grades. Sometimes, they may be medium to large in size. Glandular units of 3 and 4 are different from each other. ***Gleason 4*** glands are not single or separated like pattern 1 to 3. They are blended and it is difficult to differentiate them because of their lumen formation.

Occasional stroma gives an appearance of partial separation to blended glands. In addition, sometimes edges of the glands can be seen as scalloped because of the partial separation. ***Gleason 5*** is the pattern that neoplasms do not have glandular differentiation. In other words, they no longer look like normal prostate tissue. They are a combination of solid cords, sheets, and individual cells. No round glands with luminal spaces can be seen compared to other patterns (Epstein et al., 2005; Pierorazio et al., 2013).

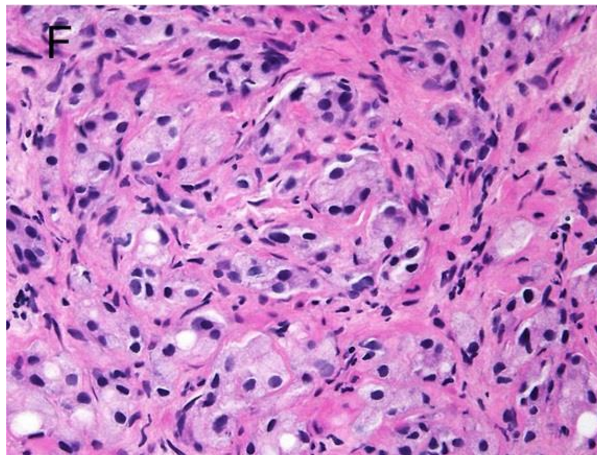
1.6.6.2.Staging

Cancer staging is a procedure which is used to determine the degree of developed cancer by growing and spreading. In clinical applications, a number from I to IV is used to determine the extent of cancer where I is an isolated cancer and IV is the spread to the limit. For the therapeutic and prognostic decisions, the stage of cancer plays a critical role. TNM (Tumour, Node, and Metastasis) scale is used to classify pathological and clinical stages in contemporary practice. Besides, there are other validated scales such as D'Amico scale to classify patients according to their risk of recurrence and mortality (Castillejos-Molina & Gabilondo-Navarro, 2016).

TNM system was developed by the Union for International Cancer Control (UICC) (It is also used by the American Joint Committee on Cancer (AJCC) and the International Federation of Gynecology and Obstetrics (FIGO) for the staging of developed cancer. The degree of the primary tumour (T), participation of the regional lymph nodes (N) and the existence of distant metastasis (M) is expressed with this system. Besides, cancer staging consists of two parts as pathological and clinical stages. The letter "c" or "p" is used before the stage in the TNM system to indicate pathological and clinical stage (e.g., cT3N1M0 or pT2N0). Clinical findings such as PSA levels, DRE, biopsy results together with imaging form clinical staging which includes microscopic examination and gross after radical prostatectomy form pathological staging. When T, N and M are defined for the disease, a stage from I to IV is given where I means the least and IV the most advanced form of prostate cancer. This system is also common for most forms of cancer except haematological malignancies and brain tumours (Falzarano & Magi-Galluzzi, 2010).



(a)



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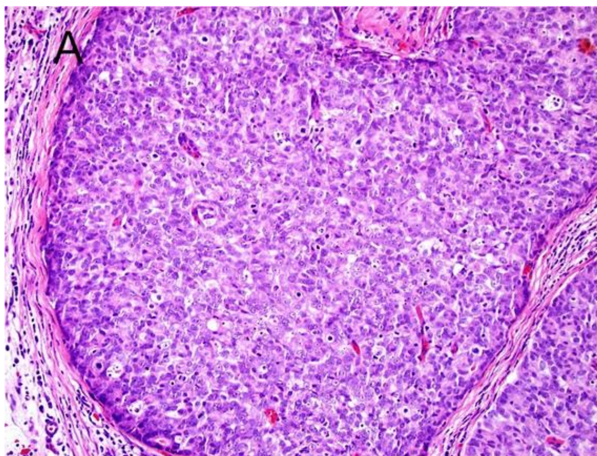


(c)

Figure 1.8. Gleason grading of prostatic adenocarcinoma. Figure a) Gleason score 6 (3+3), b) Gleason score 8 (4+4) and c) Gleason score 10 (5+5). Patterns were adopted from (Gordetsky & Epstein, 2016).

Table 1.1. TNM stages of prostate cancer. All definitions are based on AJCC 8th edition. Table was taken from AJCC Cancer Staging Manual (Gress et al., 2017).

Primary tumour (T)	
Category	Criteria
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Clinically inapparent tumour that is not palpable
T1a	Tumour incidental histologic finding in ≤5% of tissue resected
T1b	Tumour incidental histologic finding in >5% of tissue resected
T1c	Tumour identified by needle biopsy found in one or both sides, but not palpable
T2	Tumour is palpable and confined within prostate
T2a	Tumour involves one-half of one side or less
T2b	Tumour involves more than one-half of one side but not both sides
T2c	Tumour involves both sides
T3	Extra-prostatic tumour that is not fixed or does not invade adjacent structures
T3a	Extra-prostatic extension (unilateral or bilateral)
T3b	Tumour invades seminal vesicle(s)
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
Regional lymph nodes (N)	
Nx	Regional lymph nodes cannot be assessed
N0	No positive regional nodes
N1	Metastasis in regional lymph node(s)
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

Table 1.2. Staging of prostate cancer. All classifications are based on AJCC 8th edition. X means not assessable and table was taken from AJCC Cancer Staging Manual (Gress et al., 2017).

Group	T	N	M	PSA	Gleason Score (GS)
I	T1a-c	N0	M0	PSA<10	GS≤6
	T2a	N0	M0	PSA<10	GS≤6
	T1-2a	N0	M0	PSA x	GS x
IIA	T1a-c	N0	M0	PSA<20	GS 7
	T1a-c	N0	M0	PSA≥10<20	GS≤6
	T2a	N0	M0	PSA≥10<20	GS≤6
	T2a	N0	M0	PSA<20	GS 7
	T2b	N0	M0	PSA<20	GS≤7
	T2b	N0	M0	PSA x	GS x
IIB	T2c	N0	M0	Any PSA	Any GS
	T1-2	N0	M0	PSA≥20	Any GS
	T1-2	N0	M0	Any PSA	GS≥8
III	T3a-b	N0	M0	Any PSA	Any GS
IV	T4	N0	M0	Any PSA	Any GS
	Any T	N1	M0	Any PSA	Any GS
	Any T	Any N	M1	Any PSA	Any GS

1.6.7. Management and treatment

Surgery, chemotherapy, radiation therapy and active surveillance are the treatment options of prostate cancer which include external-beam radiation therapy, prostate brachytherapy, proton therapy, hormonal therapy, cryosurgery, high-intensity focused ultrasound (HIFU), and some combinations of these methods. Treatments may include some interventions which are based on survivorship.

Surveillance, care coordination, psychological symptoms, physical symptoms and health promotion are the five domains which are focused by survivorship based interventions. However, there are only reviews on health promotion, psychological and physical symptom management (Crawford-Williams et al., 2018; Resnick et al., 2015).

Patients that have prostate cancer may die from other causes due to the age of diagnosis, such as stroke, cardiovascular diseases, diabetes among others because of the slow progression of prostate cancer. Therefore, patient-based assessments should be applied in order to choose the best therapeutic modalities. Besides, PSA level, the Gleason score, the stage of the disease and other factors like patient's general health status, age, feelings about potential treatments and their possible side-effects must be taken into consideration. Urinary incontinence and erectile dysfunction are the most significant side-effects, therefore treatment options should balance the goals and risk of lifestyle alterations (Attard et al., 2016; Castillejos-Molina & Gabilondo-Navarro, 2016).

1.6.7.1.Active surveillance

Active surveillance is the process of monitoring and observing the progression of the disease with PSA levels and prostate biopsies. In other words, it is the monitoring of the disease without invasive treatment. Generally, it is used to observe a slow-growing, early-stage prostate cancer when it is suspected. However, watchful waiting may also be offered when hormonal therapy, radiation therapy or surgery have high risks than their benefits. If there are signs that cancer accelerates, then other treatments should be applied concerning the current health status of the patient (Klotz, 2015).

To predict the development of a tumour, some statistical models have been formed. The Epstein criteria are one of the most commonly used one for "insignificant cancer" (Gleason<7, organ-confined and tumour volume<0.2cc). Besides, there is no tumour marker to identify prostate cancer as indolent or insignificant (Wilt et al., 2012).

Studies showed that nearly one-third of men under active surveillance showed signs of tumour progression and they needed treatment within 3 years. Active surveillance is an option for men who are at low risk and have other diseases that do not allow to have a ten-year life expectancy. However, men who have active surveillance has a risk of metastasis but, at the same time avoiding the risk of radiation, surgery and, other treatments. However, if the surveillance program is followed exactly, the metastasis risk is very small in comparison (Bill-Axelson et al., 2011; Wu et al., 2004).

1.6.7.2.Surgery

When radiation therapy fails or cancer localizes to the prostate gland, then it is removed surgically which is called prostatectomy operation. This procedure is done in several ways but, radical retropubic prostatectomy and radical perineal prostatectomy are the most commonly used ones. This surgical operation can also be performed laparoscopically with or without the assistance of a surgical robot. Radical prostatectomy is very effective when there is no metastasis, but of course PSA level and Gleason grade affect the cure rates. Although it is an effective procedure, it may cause some medical complications which lower the quality of life of the patient. Urinary incontinence (5-20%) and erectile dysfunction (40 and 80%) are the most common complications of radical prostatectomy (Briganti et al., 2013; Ko et al., 2013).

In the last decade, new technology approaches to surgical operations such as laparoscopic and robotic techniques gained a demand because of their less medical complications compared to traditional methods. They are not better in cancer control, but they have better results for urinary incontinence and erectile dysfunction. Therefore, the risks and benefits should be explained well to find the best option for the patient (Castillejos-Molina & Gabilondo-Navarro, 2016).

1.6.7.3.Radiotherapy

Radiation therapy or radiotherapy is a procedure which is used as External beam radiation therapy and Brachytherapy to treat the prostate gland in all stages of prostate cancer by applying a dose of radiation to it.

Ionizing radiation is used to kill prostate cancer cells and it can be used after the surgery if it is not successful at curing cancer. This procedure can be used for localised and advanced diseases; however, it is not very acceptable in popularity. Although it has better oncological control compared to radical prostatectomy, it has similar side effects in the medium and long term. Recurrent rate is also comparable to radical prostatectomy and after the treatment, the follow-up must be applied for up to 15 years (Critz et al., 2013; Gray et al., 2014; Maggio et al., 2012)

1.6.7.4.Other modalities

High intensity focused ultrasound and cryotherapy have been referred for localised prostate cancer. Tissue necrosis is the main objective of these methods by freezing or ultrasonic waves. For some cases, control rates can be comparable with radical prostatectomy and radiotherapy. However, there is still no exact evidence on erectile dysfunction and urinary incontinence (Castillejos-Molina & Gabilondo-Navarro, 2016).

Androgen deprivation therapy is another option with other surgical procedures. It is found that high levels of androgen are associated with the formation of prostate cancer. Therefore, lowering androgen stimulation may potentially reduce the progression of the disease. Degarelix and Bicalutamide do not allow the synthesis of testosterone or penetrating the cancer cells. If there is a possibility of recurrence due to the degree of the disease, then androgen deprivation therapy is recommended as a supportive therapy. If cancer responds to this therapy, it is called androgen-dependent and vice-versa. This therapy has significant benefits as well as side effects but, does not have any survival advantage (Bolla et al., 2010; Kumar et al., 2006).

Chemotherapy is another treatment method which uses powerful drugs to kill cancerous cells in metastasis stage. Chemotherapy is a systemic treatment where drugs circulate through the body and this makes it different from other treatments such as radiotherapy and surgery. The goals of this treatment are cure, control and palliation. Chemotherapy may reduce the speed of development of cancer and symptoms while increasing life expectancy a few more months. However, it is not very successful in prostate cancer.

In a randomised clinical trial it was found that combination of androgen deprivation therapy with chemotherapy (docetaxel) added 17 more months in the survival compared to androgen deprivation therapy only (Sweeney et al., 2015).

Prostate-specific membrane antigen (PSMA) targeted therapies gained importance in the last decade. Highly specific and quality PET imaging has been obtained by the development of small-molecule peptides for PSMA with highly binding features for prostate cancer in the field of targeted radionuclide therapy. Lutetium 177 (^{177}Lu) labelled PSMA peptides are the most commonly used peptides for prostate cancer. Although it has valuable results for metastatic patients who have failed in the previous treatments, it may not be successful for all men with metastatic castration-resistance prostate cancer where 30% of men do not respond to ^{177}Lu therapy. Some factors such as heterogeneity of PSMA receptors, uniform expression of high density PSMA receptors, and high PSA levels may affect the treatment impact of this therapy (Emmett et al., 2017).

CHAPTER II

2. AIM OF THE STUDY

Prostate-Specific Antigen (PSA) test is the most commonly used biomarker for prostate cancer (PCa) screening as well as for the clinical diagnosis of other diseases related to prostate such as infection and inflammation (Carroll et al., 2014). A PSA test is inexpensive, quick and easy to apply; however, it is not a tumour-specific biomarker and non-malignant diseases such as benign prostatic hyperplasia (BPH) or prostatitis can increase PSA level (Dijkstra et al., 2014). In clinical applications, if a patient has elevated PSA levels and/or atypical prostate finding in digital rectal examination (DRE), transrectal ultrasound (TRUS) guided prostate biopsy (PB) is a must according to the guidelines for the detection of PCa and other diseases. However, this procedure is painful for the patient and may cause medical complications and mostly has negative results for PCa due to false-positivity of PSA tests (Alberts et al., 2015).

Lack of diagnostic precision of the PSA results in PCa screening causes overdiagnosis and overtreatment of prostate cancer including unnecessary biopsies. To overcome this problem urine, blood and tissue biomarkers have been developed (Tan et al., 2019). Prostate Cancer Antigen 3 (PCA3), Transmembrane Protease Serine-2 - ERG (TMPRSS2: ERG) fusion, 4KScore, MiProstate Score, SelectMDx, ConfirmMDx, ExoDx and Prostate Health Index (PHI) are the most commonly used biomarkers and tests. However, there is still no straight-forward test or method to diagnose PCa from specimen collection to the final result. Some of them have promising results when combined while most have sensitivity and/or specificity problems with confusing cut-off values. Therefore, physicians should make the risk stratification very carefully considering the cost and harms of tests to their patients (Borghesi et al., 2017).

HOX genes are the main transcriptional regulators and play important roles in embryo and carcinogenesis. There are 39 HOX genes which are clustered on four different chromosomes in humans and these clusters are known as the four HOX families: HOXA, HOXB, HOXC and HOXD.

These HOX genes have important roles during stem cell differentiation in the entire development period and it is found that HOX mutations can cause human disorders with different variation (Bhatlekar et al., 2018). G84E (Ewing et al., 2012), G135E (Lin et al., 2013), A128D and F240L (Attard et al., 2016), F127C and G132E (Hayano et al., 2016) are the best-characterized genetic variants of HOXB13 that are associated with PCa and have further been described in different populations. Therefore, this study aims to propose a novel approach that gives early diagnostic information to the physician about the possible presence of PCa by sequencing and analysing the hereditary and somatic HOXB13 mutations through a small sample of patient's urine which is taken right after the DRE. Besides, to contribute to the risk assessment of PCa as a non-invasive screening tool together with clinical findings for the selection of eligible cases for PB simply and cost-effectively based on the patient's PCa mutation profile.

CHAPTER III

3. MATERIALS AND METHODS

3.1.Sample Collection and Ethical Approval

Approval of this study was obtained from the Near East University Scientific Research Assessment Ethics Committee (YDU/2017/52-479) following all medical ethical requirements. Urine samples were collected from ten patients at the Near East University Hospital-Urology Department (North Cyprus) who were referred for prostate needle core biopsy due to high PSA levels and/or abnormal DRE during the period January to May in 2018.

The mean age of the patients was 62,8 at the time of diagnosis (range, 51-74 years). Written informed consent was obtained from all patients before their biopsy procedure. Firm pressure was applied to the prostate from base to apex and lateral to the medial side (Groskopf et al., 2006) during the DRE and 20-30ml of urine samples were collected into sterile cups right after the DRE and stored at -20 °C for later processing.

3.2.mRNA Isolation and cDNA Synthesis

Messenger RNAs (mRNAs) were extracted from the urinary sediments using GeneAll Ribospin vRD II (Cambio, Cat. No: 322-150) and complementary DNA (cDNA) was synthesized using HelixCript 1st strand cDNA Synthesis Kit (Nanohelix Ltd, Cat. No: CDNA-100) according to the manufacturers' protocols. Isolated mRNAs were stored at -80 °C for later processing.

3.3.Polymerase Chain Reaction (PCR) Procedures

Beta-actin ACTB; (NM_001101.5) was used as a housekeeping gene and Human Prostate Specific Gene-1 (HPG-1, NAALADL2; NM_207015.3) was used to check the existence of prostate cells in urine samples (Herness & Naz, 2003).

Amplification reactions of Beta-actin PCR were performed in a 50 µL volume containing: 5 µL of (10x) Taq buffer (Thermo Scientific), 3 µL of (10mM) MgCl2 (Thermo Scientific), 5 µL of (2mM) dNTP (Thermo Scientific), 0.4 µL (20µM) of forward primer (Oligomer), 0.4 µL (20µM) of reverse primer (Oligomer), 0.3 µL of (5µ/µL) Taq polymerase (Thermo Scientific), 30.9 µL of DEPC-Treated H2O and 5 µL (0.8ng/µL) of cDNA template were used with the following cycling conditions: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 59 °C for 45 sec and 72 °C for 45 sec, then, 72 °C for 7 min. Following the PCR amplification, 5 µL of each PCR product was run on a 1.5% agarose gel in 1X Tris-Borat EDTA buffer and visualized by staining with ethidium bromide using 1 kb DNA ladder (Nanohelix) as a molecular marker. The electrophoresis (Biorad) was conducted at 130 V for 20 minutes. Separated products were visualized under an ultraviolet transilluminator (UV Star) and the product bands were evaluated.

Amplification reactions of HPG-1 PCR were performed in 25 µL volume containing: 2.5 µL of (10x) Taq buffer (Thermo Scientific), 1.5 µL (10mM) of MgCl2 (Thermo Scientific), 0.5 µL of (2mM) dNTP (Thermo Scientific), 0.8 µL of (20µM) forward primer (Oligomer), 0.8 µL of (20µM) reverse primer (Oligomer), 0.3 µL of (5µ/µL) Taq polymerase (Thermo Scientific), 13.1 µL of DEPC-Treated H2O and 5 µL of (0.8ng/µL) cDNA template were used with the following reaction parameters: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 58 °C for 120 sec and 72 °C for 60 sec, then, 72 °C for 5 min. Following the amplification of cDNA samples, 5 µL of the PCR products were run on a 2% agarose gel in 1X Tris-Borat EDTA buffer and visualized by staining with ethidium bromide by using 1 kb DNA ladder (Nanohelix) as a molecular marker. The electrophoresis was conducted at 100 V for 30 minutes and separated products were visualized under the ultraviolet transilluminator and the product bands were evaluated at 700 bp.

PCR primers that were used for Beta-actin and HPG-1 are given in Table 3.1 and all PCR experiments were performed on the Applied Biosystems Veriti instrument.

Table 3.1. PCR primers for Beta-actin and HPG-1

Gene	Primer	Sequence	Position, (nt)	Product, (bp)
Beta-actin	NT Forward	5' CTG TGC TAT CCC TGT ACG CC 3'	c.589-608	196
	NT Reverse	5' GTG GTG GTG AAG CTG TAG CC 3'	c.414-432	
HPG-1	NT Forward	5' TGG AAC AAG CCA AGA ATA CCA CCT GTC A 3'	c.437-464	718
	NT Reverse	5' GTT TTT ATG CCA ATT CCA TGC TGC TTT G 3'	c.1100-1127	

3.4.Point Mutation and Sanger Sequencing Analysis

To sequence the specific (G84E, F127C, A128D, G132E and G135E) and somatic HOXB13 mutations, Sanger sequencing analysis was performed. The Primer-BLAST design tool of the National Center for Biotechnology Information (NCBI) was used for designing specific primer pairs which are listed in Table 3.2.

Table 3.2. Specific primers of G84E, F127C, A128D, G132E, G135E and F240L

Mutations	Primer	Sequence
G84E, F127C,	NT Forward	5'-CAT GGA GCC CGG CAA TTA TG-3'
A128D, G132E	NT Reverse	5'-AGT AGT ACC CGC CTC CAA AG-3'
G135E	NT Forward	5'- TTA CTT TGG AGG CGG GTA CT-3'
	NT Reverse	5'-AAG GGG ACC CAG GGT AAT AG-3'
F240L	NT Forward	5'-TTG CCT GTG GAC AGT TAC CA-3'
	NT Reverse	5'-AGG GGA CCC AGG GTA ATA GA-3'

Before Sanger sequencing analysis, a PCR procedure was performed: 2.5 µL of (10x) Taq buffer, 1.5 µL of (10mM) $MgCl_2$, 0.5 µL of (2mM) dNTP, 0.8 µL of (20µM) forward primer, 0.8 µL of (20µM) reverse primer, 0.3 µL of (5µ/µL) Taq polymerase, 12.3 µL of DEPC-Treated H_2O and 5 µL of (0.8ng/µL) cDNA template were used with the following reaction for the PCR protocol: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 58 °C for 120 sec and 72 °C for 60 sec, then, 72 °C for 5 min. Amplicons were used for Sanger sequencing analysis with the specific primer pairs for mutation screening.

Sanger Sequencing is a DNA sequencing method based on chain-termination of dideoxynucleotides by DNA polymerase in the process of in vitro DNA replication. It was developed by Frederick Sanger and colleagues in 1977 and then, it was commercialized by Applied biosystems in 1986 and it became the most commonly used sequencing method (Sanger et al., 1977; Sanger & Coulson, 1975).

BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for Sanger sequencing according to the manufacturer's instructions. 3500 Genetic Analyzer (Applied Biosystems) was used to run the products and all sequences were compared and analysed with the HOXB13 NCBI reference sequence (NM_006361.5) by using 4Peaks (Nucleobytes, The Netherlands) software. Hereditary and somatic mutations of HOXB13 were sequenced from region 17:48728004 to 17:48728588, GRCh38. To predict the possible effects of the mutations found, the bioinformatics tools PolyPhen 2.0 (RRID: SCR_013189) (Adzhubei, I. A. et al., 2010), Provean (RRID: SCR_002182) (Choi et al., 2012), SIFT (RRID: SCR_012813) (Ng & Henikoff, 2001), MutationTaster (RRID: SCR_010777) (Schwarz et al., 2014) and CADD Score (RRID: SCR_018393) (Rentzsch et al., 2019) were used.

PolyPhen 2.0 (http://genetics.bwh.harward.edu/pph2/) is a protein based polymorphism and phenotyping server which is used to predict the effects of nsSNPs (non-synonymous single nucleotide polymorphisms). In this server, nsSNPs are classified as "probably damaging", "possibly damaging" or "benign" based on the specific protein sequence. "Damaging" means that the mutation affects protein structure and no loss or gain of function. 0.50 is the cut-off score and if a mutation scores over 0.50, it is predicted as pathogenic by the server.

Provean (The Protein Variant Effect Analyzer) is used to predict the functional impact of an amino acid substitution over a protein function. -2.5 is the cut-off score where less than -2.5 is considered as deleterious while greater of it is accepted as neutral variant. The input is in the FASTA format of a protein sequence. It can be accessed at http://provean.jcvi.org/genome_submit_2.php?species=human.

SIFT (The Sorting Intolerant from Tolerant) server is based on amino acid sequences of various species and it makes orthologous and paralogous alignment due to given protein sequence. 0.05 is the default intolerance threshold and a score over 0.05 is considered as tolerated by the protein. It can be accessed at http://sift.bii.a-star.edu.sg/www/Extended_SIFT_chr_coords_submit.html.

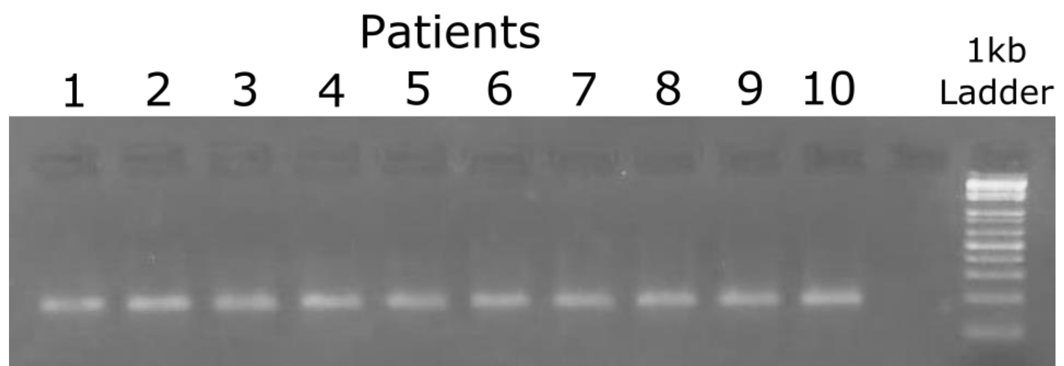
MutationTaster is a server which is used to evaluate pathogenic impact of DNA sequence changes. Functional impacts of amino acid substitutions are predicted by the server and indicated as “Disease causing” or “Polymorphism” due to obtained score. Short insertion and/or deletion, synonymous and intronic changes are also predicted. It can be accessed at http://mutationtaster.org.

CADD (Combined Annotation Dependent Depletion) is a tool which predicts the damaging effect of a single nucleotide variant, plus the insertion or deletion variant. The score is generated due to a specific gene and genomic position with entering the alterations where 20 is the recommended cut-off score. It can be accessed at http://cadd.gs.washington.edu/snv.

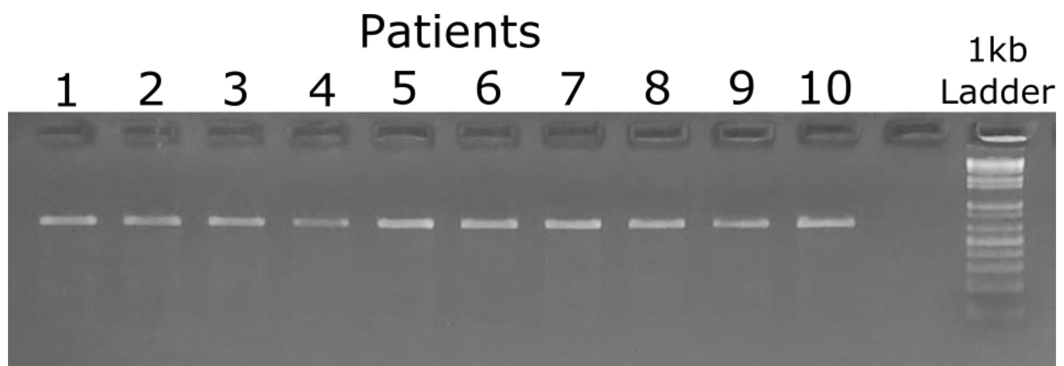
CHAPTER IV

4. RESULTS

Beta-actin and HPG-1 were found in all samples (Figure 4.1 a & b). The HOXB13 G84E, F127C, A128D, G132E and G135E mutations were not found in any of the ten patients. However, nine patients (P1 - P8 and P10) carried different heterozygous and/or homozygous variants of which thirteen has not been described in any of the public databases 1000 Genomes Project or the Exome Variant Server (Table 4.1).



a)



b)

Figure 4.1. Gel electrophoresis images of a) Beta-actin as expression control and b) HPG-1 to demonstrate that prostate cells thus, DNA was detected.

Table 4.1. Germline variants detected in patients.

IDs	Variant GRCh38 position	rs ID	cDNA change	Genotype	Protein change	ClinVar	1000G* MAF%	Exome Variant Server MAF%
P5	17:48728081	rs9900627	c.513T>C	Het/Hom	p.Ser171=	Benign	EUR: 11.90% (107/899); EAS: 22.48% (185/823); ALL: 13.79% (607/4401).	EA: 9.60% (826/7774); AA:10.74% (473/3933); All: 9.99% (1299/11707).
P5	17:48728124	N/A	c.470A>C	Het	p.Glu157Ala	N/A	Not reported	Not reported
P1	17:48728142	rs1555558604	c.452C>A	Het	p.Thr151Asn	N/A	Not reported	Not reported
P1	17:48728145	N/A	c.449A>C	Het	p.Gln150Pro	N/A	Not reported	Not reported
P1	17:48728151	N/A	c.443T>A	Het	p.Val148Glu	N/A	Not reported	Not reported
P10	17:48728157	N/A	c.437T>A	Het	p.Val146Glu	N/A	Not reported	Not reported
P10	17:48728168	N/A	c.426T>A	Het	p.Ser142Arg	N/A	Not reported	Not reported
P10	17:48728203	N/A	c.391C>T	Het	p.Pro131Ser	N/A	Not reported	Not reported
P2, P6	17:48728226	rs201428095	c.368G>C	Het	p.Arg123Pro	Uncertain significance	Not reported	Not reported
P1, P2, P3, P4	17:48728228	rs8556	c.366C>T	Het/Hom	p.Ser122=	Benign	EUR: 14.44% (127/879); EAS: 3.38% (33/975); ALL: 21.29% (879/4129)	EA: 13.21% (1136/7464); AA: 26.24% (1156/3250); All: 17.62% (2292/10714)
P10	17:48728233	rs766909225	c.361C>A	Het	p.Pro121Thr	Uncertain significance	Not reported	Not reported
P7	17:48728297	N/A	c.297C>A	Het	p.Pro99=	N/A	Not reported	Not reported
P8	17:48728382	N/A	c.212A>C	Het	p.Gln71Pro	Uncertain significance	Not reported	Not reported
P4	17:48728481	rs587780160	c.113C>G	Het	p.Ala38Gly	Uncertain significance	EUR: -; EAS: 100%; ALL: 100%	Not reported
P2	17:48728499	N/A	c.95C>G	Het	p.Pro32Arg	N/A	Not reported	Not reported
P7	17:48728559	N/A	c.35C>A	Het	p.Ala12Asp	Uncertain significance	Not reported	Not reported

*1000 Genomes Project phase 3; AA–African-American; ALL-All Population; EA–European-American; EAS-East Asian Population; EUR-European Population; Het–Heterozygous; Hom–Homozygous; MAF–Minor allele frequency; =- protein analysis has not been done, but no change is expected; N/A–Not applicable.

Five patients (P1 - P4 and P5) carry the known benign heterozygous/homozygous variants c.366C>T and c.513T>C, respectively, which were found by Maia et al. (Maia et al., 2015). c.368G>C (P2 and P6), c.361C>A (P10), c.212A>C (P8), c.113C>G (P4) and c.35C>A (P7) variants were detected in different patients where they are characterized as of uncertain significance in ClinVar - NCBI. The c.470A>C (P5), c.452C>A (P1), c.449A>C (P1), c.443T>A (P1), c.437T>A (P10), c.426T>A (P10), c.391C>T (P10), c.297C>A (P7) and c.95C>G (P2) variants were not found in any published study.

Table 4.2 shows the pathogenicity prediction of the coding HOXB13 variants. The scores of PolyPhen 2.0, Provean, SIFT, MutationTaster and CADD tools showed that c.470A>C, c.443T>A, c.437T>A, c.426T>A and c.368G>C variants have potential to be deleterious and highly associated with PCa. c.452C>A, c.391C>T, c.212A>C, c.95C>G and c.35C>A can also be damaging and associated with PCa based on the average of the scores.

Table 4.2. Pathogenicity prediction of the coding HOXB13 variants.

cDNA change, Protein change	Polyphen2 (Cut-off=0.50)	Provean (Cut-off=-2.5)	SIFT (Cut-off=0.05)	Mutation Taster (Probability values)	CADD Score (Cut-off=20.0)
c.513T>C, p.Ser171=	N/A	Neutral (0.00)	Tolerated (0.373)	Polymorphism (No AA changes)	13.64
c.470A>C, p.Glu157Ala	Probably Damaging (0.997)	Deleterious (-5.32)	Damaging (0.001)	Disease causing (AA score:107)	25.7
c.452C>A, p.Thr151Asn	Probably Damaging (0.979)	Neutral (-1.11)	Tolerated (0.055)	Disease causing (AA score:65)	23.6
c.449A>C, p.Gln150Pro	Benign (0.067)	Neutral (2.85)	Tolerated (1.000)	Disease causing (AA score:76)	18.27
c.443T>A, p.Val148Glu	Probably Damaging (0.999)	Deleterious (-4.10)	Damaging (0.000)	Disease causing (AA score:121)	28.8
c.437T>A, p.Val146Glu	Probably Damaging (0.999)	Deleterious (-3.50)	Damaging (0.001)	Disease causing (AA score:121)	32
c.426T>A, p.Ser142Arg	Probably Damaging (0.974)	Deleterious (-2.73)	Damaging (0.001)	Disease causing (AA score:110)	24.7
c.391C>T, p.Pro131Ser	Probably Damaging (0.976)	Neutral (-1.90)	Tolerated (0.115)	Disease causing (AA score:74)	24.7
c.368G>C, p.Arg123Pro	Probably Damaging (1.000)	Deleterious (-5.97)	Damaging (0.001)	Disease causing (AA score:103)	27.4
c.366C>T, p.Ser122=	N/A	Neutral (0.00)	Tolerated (1.000)	Polymorphism (No AA changes)	17.85
c.361C>A, p.Pro121Thr	Benign (0.297)	Neutral (-0.75)	Tolerated (0.432)	Disease causing (AA score:38)	15.38
c.297C>A, p.Pro99=	N/A	Neutral (0.00)	Tolerated (0.619)	Disease causing (No AA changes)	15.24
c.212A>C, p.Gln71Pro	Probably Damaging (0.995)	Neutral (-1.16)	Tolerated (0.052)	Disease causing (AA score:76)	24.6
c.113C>G, p.Ala38Gly	Benign (0.000)	Neutral (0.16)	Tolerated (0.635)	Disease causing (AA score:60)	22.2
c.95C>G, p.Pro32Arg	Benign (0.278)	Neutral (-0.45)	Damaging (0.013)	Disease causing (AA score:103)	26
c.35C>A, p.Ala12Asp	Benign (0.077)	Neutral (-0.37)	Damaging (0.042)	Disease causing (AA score:126)	22

*=-protein analysis has not been done, but no change is expected; N/A–Not applicable.

Table 4.3. Risk summary of the found mutations according to the scores of the tools compared with the pathology report and final clinical diagnosis.

Patient ID	Classification of the Found Mutations			Pathology Report			Final Diagnosis
	Deleterious	Probably Damaging	Benign	PB Result	Diagnosis	Stage	
P1	c.443T>A	c.452C>A	c.449A>C, c.366C>T	+	PCa	Early	RP
P2	c.368G>C	c.95C>G	c.366C>T	-	BPH	N/A	TURP
P3	N/A	N/A	c.366C>T	+	PCa	Early	TURP
P4	N/A	N/A	c.366C>T, c.113C>G	-	BPH	N/A	TURP
P5	c.470A>C	N/A	c.513T>C	-	BPH	N/A	TURP
P6	c.368G>C	N/A	N/A	+	PCa	Late	RT+RP
P7	N/A	c.35C>A	c.297C>A	-	BPH	N/A	TURP
P8	N/A	c.212A>C	N/A	+	PCa	Late	RT
P9	N/A	N/A	N/A	+	PCa	Late	RT
P10	c.437T>A, c.426T>A	c.391C>T	c.361C>A	-	BPH	N/A	TURP

*PB-Prostate Biopsy; PCa-Prostate Cancer; BPH-Benign Prostatic Hyperplasia; RP-Radical Prostatectomy; TURP-Transurethral Resection of the prostate; RT-Radiation Therapy; N/A–Not applicable.

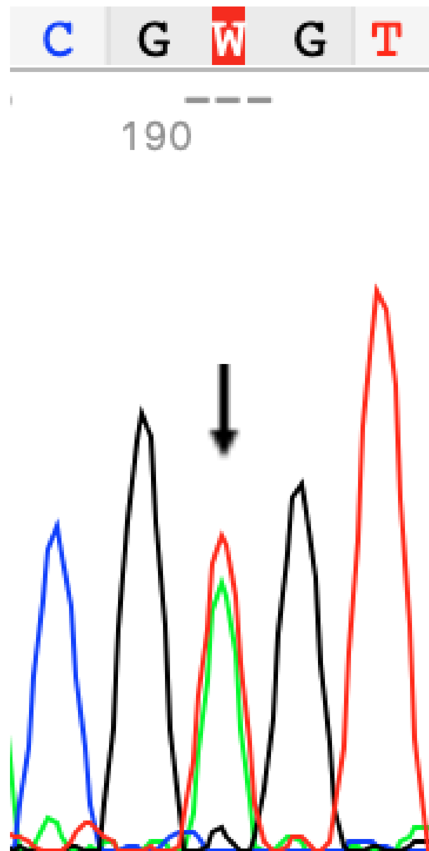


Figure 4.2. Electropherogram of P10 having the HOXB13 c.437T>A, p. (Val146Glu) variant in heterozygosity. The arrow indicates the mutation.

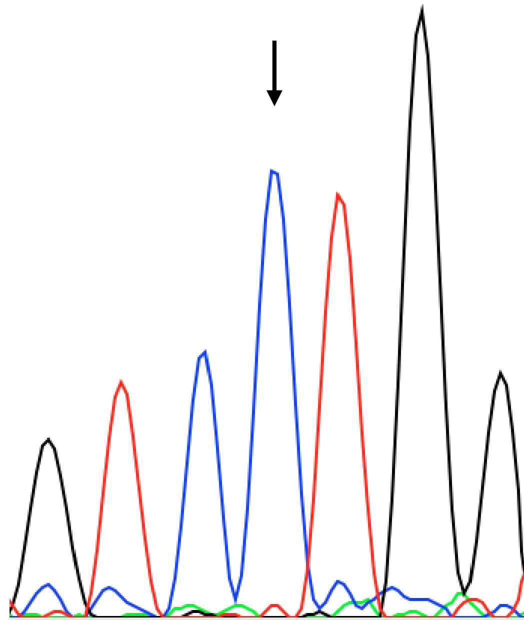


Figure 4.3. Electropherogram of P5 having the HOXB13 c.513T>C, p. (Ser171=) variant in heterozygosity/homozygosity. The arrow indicates the mutation.

CHAPTER V

5. DISCUSSION

PCa is the second main cause of cancer-related death among men with 1.2 million new cases in the world according to the statistics of the World Health Organization (WHO) in 2018 (Bray et al., 2018). There are high morbidity rates in Western countries compared to Eastern countries caused by lifestyle and diet (Li et al., 2018).

Insufficiency of PSA test has raised the need for identifications of new biomarkers to screen and diagnose PCa in a more efficient, sensitive and specific manner. In this perspective, we developed a novel method to catch PCa with a small sample of patient's post-DRE urine by sequencing the HOXB13 gene to find the known and unknown somatic and hereditary mutations to help the physician to make a more precise risk assessment and reduce the unnecessary PBs.

In this study, we found deleterious, most-likely damaging and benign mutations for all patients (except P9) according to the scores of PolyPhen 2.0, Provean, SIFT, MutationTaster and CADD prediction tools as listed in Table 4.2. Unfortunately, we did not detect any of the known deleterious PCa mutations (G84E, F127C, A128D, G132E and G135E) perhaps due to small size study or because of the geographic heterogeneity of the disease.

Risk summary of the mutations is listed in Table 4.3 where five patients (P1, P3, P6, P8 and P9) had positive PBs and we found mutations that are possibly associated with their PCa except for P9. We also detect benign mutations for P4 and no mutations for P9. Further, P2, P5, P7 and P10 had negative PBs, however, we found mutations that could be deleterious or probably damaging and associated with PCa. Since PB is still the gold standard for cancer diagnosis, this situation indeed elucidates the fact that tumours are heterogeneous and undersampling can occur due to insufficient biopsy specimens or size of the tumour (Klein et al., 2014). Therefore, these results can play a critical role in the risk assessment of PB before and after.

PCA3, HOXC6/DLX1 (Leyten et al., 2015), MiProstate Score, SelectMDx and ExoDx are the tests for PCa screening that works with post DRE urine samples. Every test has different specificity and sensitivity rates for PCa detection where the only PCA3 has an FDA (Food and Drug Administration) approval since 2012. Since our method has a working flow of PCR procedures and the Sanger sequencing analysis, it is easy to perform in almost any genetic laboratory. The results of the Sanger sequencing analysis are easy to perform. All tools are easy to use to calculate the possible risk scores of the mutations found and hence, to obtain the exact results. Additionally, compared to the above-mentioned approaches, this method has an ability to find known and unknown mutations easily with a reasonable price, time, sensitivity and specificity with no confusing cut-off values.

Since this is a proof-of-principle study, we obtained remarkable results strongly suggesting that this is a potentially promising tool in prostate cancer screening. Thus, the success and the sensitivity of the method yet to be improved with a large cohort study by sequencing the entire HOXB13 gene with a Next-Generation Sequencing (NGS) method including the other known genes (BRCA1/2, etc.) that are associated with prostate cancer.

CHAPTER VI

6. CONCLUSION

Findings suggest that the proposed method has a promising and reliable tool for prostate cancer screening in clinical applications cost-effectively to help physicians for making the risk stratification of more specific biopsy decision without any confusing details.

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APPENDIX 1

CURRICULUM VITAE

Name	Eyyup	**Surname**	KAVALCI
Place of Birth	Gazi Mağusa	**Date of Birth**	03.09.1988
Nationality	TRNC	**Tel**	0 533 821 07 27
E-mail	eyup.kavalci@neu.edu.tr		

Education Level

	Name of the Institution where he/she was graduated	**Graduation Year**
Postgraduate/Specialization	Near East University-Medical Biology and Genetics	2020
Masters	Near East University-Biomedical Engineering	2013
Undergraduate	Near East University-Electric & Electrical Engineering	2010
High School	Akdoğan Polat Paşa Lisesi	2005

Job Experience

Duty	**Institution**	**Duration (Year-Year)**
Purchasing Manager	Near East University Hospital	2010 - Present

Foreign Languages	**Reading comprehension**	**Speaking**	**Writing**
English	Good	Good	Good

Foreign Language Examination Grade								
YDS	**ÜDS**	**IELTS**	**TOEFL IBT**	**TOEFL PBT**	**TOEFL CBT**	**FCE**	**CAE**	**CPE**
Good								

Computer Knowledge

Program	**Use proficiency**
MS Office	Good
Adobe Photoshop	Good
Adobe Premiere Pro	Good
Corel Draw	Good

APPENDIX 2

ETHICS COMMITTEE APPROVAL FORM

APPENDIX 3

ARAŞTIRMA AMAÇLI ÇALIŞMA İÇİN AYDINLATILMIŞ ONAM FORMU

(Araştırmacının Açıklaması)
Prostat Kanseri ile ilgili yeni bir araştırma yapmaktayız. Araştırmanın ismi "Prostat Kanserinin Erken Tespiti İçin İdrarda Genetik Biyobelirteçlerin Tanımlanması"dır.

Sizin de bu araştırmaya katılmanızı öneriyoruz. Bu araştırmaya katılıp katılmamakta serbestsiniz. Çalışmaya katılım gönüllülük esasına dayalıdır. Kararınızdan önce araştırma hakkında sizi bilgilendirmek istiyoruz. Bu bilgileri okuyup anladıktan sonra araştırmaya katılmak isterseniz formu imzalayınız.

Bu araştırmayı yapmak istememizin nedeni, prostat kanserinin erken teşhisidir. Yakın Doğu Üniversitesi Tıp Fakültesi Tıbbi Biyoloji ve Genetik ile Üroloji Anabilim Dalları'nın ortak katılımı ile gerçekleştirilecek bu çalışmaya katılımınız araştırmanın başarısı için önemlidir.

Eğer araştırmaya katılmayı kabul ederseniz, kan numuneniz ve size yapılacak prostat muayenesi öncesinde ve sonrasında idrar örneğiniz alınacaktır. Alınan idrar örneklerinde, prostat kanserine neden olan tümör hücreleri araştırılacaktır ve elde edilen bulgular kan örneği ile de kıyaslanacaktır. Bu çalışmaya katılmanız için sizden herhangi bir ücret istenmeyecektir. Çalışmaya katıldığınız için size ek bir ödeme de yapılmayacaktır.

Sizinle ilgili tıbbi bilgiler gizli tutulacak, ancak çalışmanın kalitesini denetleyen görevliler, etik kurullar ya da resmi makamlarca gereği halinde incelenebilecektir.

Bu çalışmaya katılmayı reddedebilirsiniz. Bu araştırmaya katılmak tamamen isteğe bağlıdır ve reddettiğiniz takdirde size uygulanan tedavide herhangi bir değişiklik olmayacaktır. Yine çalışmanın herhangi bir aşamasında onayınızı çekmek hakkına da sahipsiniz.

Katılımcı	Görüşme tanığı	Araştırmacı
Adı, soyadı:	Adı, soyadı:	Adı soyadı, unvanı:
Adres:	Adres:	Adres:
Tel.:	Tel.:	Tel:
İmza:	İmza:	İmza:

ARAŞTIRMA AMAÇLI ÇALIŞMA İÇİN AYDINLATILMIŞ ONAM FORMU

(Katılımcının / Hastanın Beyanı)

Sayın Eyyup Kavalcı tarafından Tıbbi Biyoloji ve Genetik ile Üroloji Anabilim Dalları'nda Prostat Kanserinin Erken Tespiti İçin İdrarda Genetik Biyobelirteçlerin Tanımlanması konusunda bir araştırma yapılacağı belirtilerek bu araştırma ile ilgili yukarıdaki bilgiler bana aktarıldı. Bu bilgilerden sonra böyle bir araştırmaya "katılımcı" olarak davet edildim.
Eğer bu araştırmaya katılırsam araştırmacı ile aramda kalması gereken bana ait bilgilerin gizliliğine bu araştırma sırasında da büyük özen ve saygı ile yaklaşılacağına inanıyorum. Araştırma sonuçlarının eğitim ve bilimsel amaçlarla kullanımı sırasında kişisel bilgilerimin ihtimamla korunacağı konusunda bana yeterli güvence verildi.
Projenin yürütülmesi sırasında herhangi bir sebep göstermeden araştırmadan çekilebilirim. (Ancak araştırmacıları zor durumda bırakmamak için araştırmadan çekileceğimi önceden bildirmemim uygun olacağının bilincindeyim) Ayrıca tıbbi durumuma herhangi bir zarar verilmemesi koşuluyla araştırmacı tarafından araştırma dışı tutulabilirim.
Araştırma için yapılacak harcamalarla ilgili herhangi bir parasal sorumluluk altına girmiyorum. Bana da bir ödeme yapılmayacaktır.
İster doğrudan, ister dolaylı olsun araştırma uygulamasından kaynaklanan nedenlerle meydana gelebilecek herhangi bir sağlık sorunumun ortaya çıkması halinde, her türlü tıbbi müdahalenin sağlanacağı konusunda gerekli güvence verildi. (Bu tıbbi müdahalelerle ilgili olarak da parasal bir yük altına girmeyeceğim).
Araştırma sırasında bir sağlık sorunu ile karşılaştığımda; herhangi bir saatte, Eyyup Kavalcı'yı 0 392 444 0 535 - 1017 (iş) veya 0 542 880 29 38 (cep) no'lu telefonlardan ve Yakın Doğu Üniversitesi Hastanesi adresinden arayabileceğimi biliyorum. Bu araştırmaya katılmak zorunda değilim ve katılmayabilirim. Araştırmaya katılmam konusunda zorlayıcı bir davranışla karşılaşmış değilim. Eğer katılmayı reddedersem, bu durumun tıbbi bakımıma ve hekim ile olan ilişkime herhangi bir zarar getirmeyeceğini de biliyorum.
Bana yapılan tüm açıklamaları ayrıntılarıyla anlamış bulunmaktayım. Kendi başıma belli bir düşünme süresi sonunda adı geçen bu araştırma projesinde "katılımcı" olarak yer alma kararını aldım. Bu konuda yapılan daveti kabul ediyorum.
İmzalı bu form kâğıdının bir kopyası bana verilecektir.

Katılımcı	Görüşme tanığı	Araştırmacı
Adı, soyadı:	Adı, soyadı:	Adı soyadı, unvanı:
Adres:	Adres:	Adres:
Tel.:	Tel.:	Tel:
İmza:	İmza:	İmza: