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The Study of TMPRSS-ERG Gene Fusion in Prostate Cancer in Pakistan

by

Samra Manzoor

A thesis submitted in partial fulfillment for the degree of Master of Science

in the

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This thesis is dedicated to my husband Atif Mansoor Khan and my brother Naeem Ahmad who's support, encouragement, and constant love have sustained me throughout my life. I would like to dedicate this thesis to my best friend, Anila Sajjad and my kids (Shanzay and Aniq). Thank you for always believing in me, even when I did not, and cheering me on until the end. Words cannot express how much I love you all and appreciate everything you all have done for me.

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Abstract

TMPRSS-ERG fusion is the most common genomic aberrations in prostate cancer. This fusion occurs at a high frequency in the prostate cancer. In Pakistan there is no reliable markers available except for prostate specific antigen (PSA) for the diagnosis of prostate cancer. Prostate cancer is a heterogeneous disease a combination of marker will be important in prostate cancer diagnosis. In order to characterize the fusion transcripts in Pakistani population, we designed a research, patient tissues samples after prostatectomy was collected from Pakistan Institute of Medical Sciences Islamabad (PIMS). Using RT PCR 11 patient's RNA were analyzed. We found high prevalence of TMPRSS-ERG fusion in 4 patient's with expression of isoform in which exon 1 of TMPRSS fused in frame with exon number 5/6 of ERG with high expression fold while low expression with exon 1/2of ERG. The fusion was characterized as Type I and type IV, these types of fusion are related to aggressive state of disease. This research represents a different approach in clinical management of prostate cancer and to characterize the fusion transcript in Pakistani population. This marker can be included in the diagnosis and prognosis of the prostate cancer to improve the treatment.

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List of Abbreviations

AR	Androgen Receptor
ARE	Androgen Receptor Element
CRPC	Castration Resistant PC
DHT	5-dihydrotestosterone
EMT	Epithelial-to-Mesenchymal Transition
ERG	V-ets Erythroblastosis Virus E26 Oncogene homolog (avian)
ETS	Avian Erythroblastosis Virus E26 Homolog
EZH2	Enhancer of Zeste Homolog 2
GS	Gleason Score
HGPIN	High-Grade Prostatic Intraepithelial Neoplasia
HPC	Hereditary Prostate Cancer
LOH	Loss of Heterozygosity
mRNA	Messenger Ribonucleic Acid
MYC	V-myc Myelocytomatosis Viral Oncogene Homolog (avian)
NKX3.1	NK3 Homeobox 1
PC	Prostate Cancer
P53	Tumor Protein 53
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide-3-Kinase
PIN	Prostatic Intraepithelial Neoplasia
PSA	Prostate Specific Antigen
PTEN	Phosphatase and Tensin Homolog
QRT-PCR	Quantitative Real-time PCR
RNA	Ribonucleic Acid

TMPRSS2 Transmembrane Protease, Serine 2

Chapter 1

Introduction

1.1 Background

In the modern-day world, science has made great advancements for the welfare of mankind but still there are things which are continuously challenging the researchers. The advancements such as stem cell and gene therapy etc. has provided immense benefits in improving our health care systems and revolutionized the methods of disease treatment [1]. But with the evolution of complex diseases whose remedy is challenging for biomedical researchers. Complex diseases are not caused due to a single gene mutation (as in case of simple diseases) rather they are controlled by polygenic (Multiple genes) factors along with some environmental factors, lifestyle and are heritable in nature [2]. In such diseases, genetic factors contribute partially in disease risk and they do not exhibit apparent inheritance patterns. This environment-gene association facilitates in imparting better insights of disease causal and later helps in development of targeted therapy [3]. Most important example of complex disease is cancer which is defined as the uncontrolled/abnormal proliferation of cells due to mutation in certain gene under the control of environmental or inherited factor. Cancer is complex in the sense that it involves a series of interaction of genetic and environmental factors that directly deregulate various mechanisms of human body such as Immune system,

DNA repair mechanism and apoptosis etc. We know that different gene fusions contribute to the disease state. Prostate cancer is most frequent in elderly men especially in developed countries and is the major causes of the death around the world [4]. Prostate cancer is the fourth common cancer in both sexes and second common in men worldwide. It was estimated that 1.1 million men were diagnosed with prostate cancer however the mortality rate diagnosed in 2012 is around 307,000 deaths the molecular reason behind the initial progression of the prostate cancer remains unfolded. [5] Advancement in the field of cytogenetics has given insight into characterizing the reason behind of Cap, which are deletions [6]. One of the early causes of prostate cancer is ERG gene increased expression, which is usually the cause of the half of the prostate cancer cases. ERG is a member of ETS transcription factor family [7]. The over expression of ERG is due to fusion with TMPRSS2, which is androgen responsive gene which lies on the same chromosome very close ERG.ERG overexpression may contribute to condition in tumor. This gene fusion is observed near PIN [8]. TMPRSS2 gene have gained a central importance in the molecular biology of cancer and have raised a great potential as valuable biomarker for the disorder. The discovery of ETS gene fusion in the cancer has been a valuable factor in increasing our knowledge of the molecular and biological mechanism of the the disease. Further research will contribute to the knowledge of the mechanism of the gene fusion. The gene fusion is an important factor for clinical prostate cancer. Till now ERG overexpression is a key tool for the diagnosis and treatment of prostate cancer [10]. Moreover, understanding of the mechanisms of ETS gene expression and function increase provide a target to find new therapeutic targets for early and late stage prostate cancer. The localized cancer can be cured with surgery and radiotherapy but in case of the metalized disease it cannot be cured. The androgen level is very crucial for the disease.The androgen level control the growth and progression of the prostate cancer. The androgen belongs to a family of ligand dependent nuclear receptor transcription factor [11]. Therefore, in order to treat the metasized cancer it is usually important to target the endocrine system to target the androgen receptor and inhibit their function. Paolini identified a novel gene which

was named TMPRSS gene.TMPRSS encodes a protein which possess a serine domain[12]. TMPRSS gene was mapped on 21q 22.3 near ERG by [13]. ETS transcription were first discovered as gag-myb-ets fusion of the transforming virus that cause leukemia in chicken [14]. ETS function as transcription activator's and a repressor's transcription factor play a important role in biological process such as cells pro Proliferation, differentiation, apoptosis, tissue modification, angiogenesis, metastasis and Variation. The ETS gene are expressed in solid tumors. [15]. In addition, high level of ETS are often seen in lung ,breast ,colon, and prostate cancer. ERG is highly expressed in the prostate cancer. The higher expression is in early and late stages. ERG was discovered by Reddy [16] .He isolated a cDNA having the complete coding sequence of oncogene ERG.A protein with two region share 40to 70% homology with the 5 prime and 3 prime region that consist of ETS viral oncogene which concludes that ERG belongs to ETS oncogene family. AR transcriptional enhancement and ERG had tumor suppressor activity which result in PTEN loss that results in oncogenic activity by ERG .ERG upregulation indicate that ERG inhibits the ERF to bind DNA at the site of the both in normal and malignant cancer. Tomlins [20,17] applied the bioinformatics method to identify the candidate oncogenic chromosomal aberration on the basis of gene expression. ERG and ETV 1 are two ETS transcription factor was identified in the [18] identified gene fusion 5 prime untranslated region TMPRSS to ERG in prostate cancer tissues. It was observed that with the simulation of the androgen receptor ligand (DHI) for 60 minutes caused proximately between TMRPSS and ERG loci. The effect depended on the AR. [19] summarized that androgen signaling causes the 5 prime and 3 prime gene hence the probability of the gene fusion increases with DNA double strands breaks. Evidence are available that show androgen receptor induce genomic breaks by recruiting enzyme (TOP2B) or (AID) and ORF2 endonucleases. [20]. The fusion genes play a very important role on molecular level in prostate cancer. It was found through studies that overexpression of the ERG in epithelial prostate cells were the reason behind cell migration and invasion and [21] that limiting the expression of the ERG slowed the invasion.

Experiment demonstrate that androgen receptor association with ERG have important role [22]. ERG activates transcriptional program and induces oncogenesis by upregulation MYC, EZH2 and SOX 9 and repressing NKX3 [23,24]. Whether gene fusion status of the tumor has implications for the timing and the choice of endocrine therapy remain to be clarified.

TABLE 1.1: Therapies Used For Treatment of Prostate Cancer [25].

Androgen deprivation therapy	LHRH agonist Leuprolide,gaserelin,buserelin
	LHRH antagonist Cetrorelix ,ganirelix
	ACTH inhibitors Prednisene,hydrocortisone
	Surgery (orchechtomy)
Androgen biosynthetic enzyme inhibitors	Abiraterone
AR antagonist	Enzalutamide,Bicalutamide,Flutamide
Surgery (prostactomy)	
Radiation	
chemotherapy	

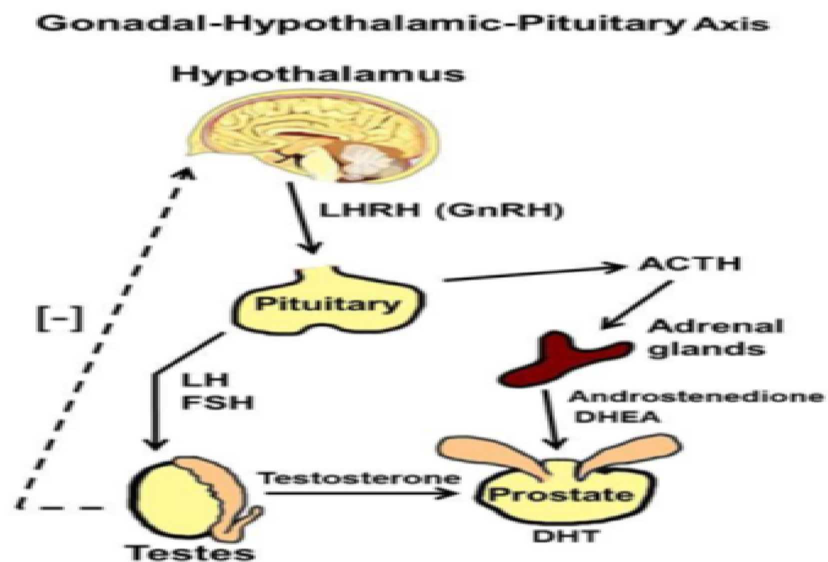

FIGURE 1.1: The signals in prostate cancer [26]

1.2 Problem Statement

Prostate cancer is a serious molecular anomaly that is caused due to many regulators that play a dominant role in disease progression. TMPRSS ERG is a key driver in oncogene transformation of prostate cancer. In Pakistan there are no preexisting accurate and precise assays to evaluate the aggressiveness of the prostate cancer. It seems that the PSA based evaluation of patient do not guarantee proper diagnosis and analysis of the prostate cancer.

1.3 Justification of the Study

A study of Pakistani population was necessary, as the current molecular diagnosis in Pakistan is not accurate in defining the clinical and molecular relation of the fusion in prostate cancer specimen

1.4 Objective of the Study

1.4.1 General Objectives

To investigate the frequency of the fusion genes in Pakistani population and their association with the demographic factors.

1.4.2 Specific Objectives

1. To identify proposed solution on the selection of TMPRSS -ERG fusion based on using as biomarker for prostate cancer.

2. The relevant data on fusion transcript in Pakistan is not available. This study will address the frequency of TMPRSS-ERG transcript.

3. According to the literature it indicates the prostate cancer has miscellany of transcript which are the instrument in determining the aggressiveness of prostate cancer. To identify the transcript type in Pakistani population:

4. To identify the effect of fusion transcript on patient outcome in pakistan

5. These fusion needs to be evaluated to unfold the heterogeneity of the disease and till now in Pakistan there are no reliable marker rather than prostate specific antigen (PSA). The combination of marker will become important in prostate cancer diagnosis.

1.5 Significance of the Study

The information and data yielded in this research will be significant in managing the prostate cancer in Pakistan. The finding and recommendation in the study will be useful in unfolding the complexity and heterogeneity of the disease and provide reliable assessment in Pakistan to diagnose the prostate cancer, which is unavailable.

1.6 Scope and Limitations of the Study

The study focused on providing a reliable marker for the diagnosis and prognosis of the disease in Pakistan. A study was limited to only a period of 10 months. The study faced a lot of challenges and limitation, for instance at the collection of tissue sample from hospitals was time-consuming process in which the problem was encountered at the management and ethical committee of different hospitals The biopsies available for the research work was not available in large amount if there have been large set of sample the correlation with clinicopathological data and fusion would have been much stronger. The study will be helpful in the future as a combination of the marker will be useful in predicting better diagnosis.

Chapter 2

Literature Review

2.1 Oncogene

Oncogene are form of mutated gene which promote cancer from proto -oncogene. These gene are dominant that need only one mutation for transformation. Proto transforming gene also encode protein which are important for transcription factors. When protooncogene become mutated structurally they become oncogene and start to encode oncoproteins [27]. Oncogene is responsible for effecting the function of growth by phosphorylating tyrosine kinases and the receptor which causes ligand -independent receptor activation .One of the example of oncogene is gene fusion BCR-ABL which is found in Philadelphia chromosome in chronic myelogenous leukemia patients . Tyrosine kinases are encoded by BCR- that is active, fast cell propagation throughout cancer development. Cancerous gene can trigger s protein receptors (EGFR) and tube-shaped structure epithelium protein receptor (VEGFR), inflicting stratum growth and oncogenes in cancer [28].

2.2 Tumor Supressor Genes

Tumor repressor gene function as recessive gene which prevents cancer by regulation of DNA damage detection,repair,reponse at cell cycle checkpoints[29].Any

mutation in Tumour repressor can inhibit the cancer suppressive function[30]. As the TSG have recessive allele mutation is needed in each of the alleles so as to silence the sequence. Enhancing the chance of malignant growth., as due to TSGs recessive characteristic, a minimum of pair of change events are needed at every alleles so as to mutate the sequence [31]. The initial change in TSGs generally occurs at the germline level, whereas change of the opposite allele happens in the mitotic level. The second adjustment occasion is named loss of state(LOH), that happens to opening of the body districts preparing to the individual arrangement, and by mitotic recombination or grouping transformation [32]. Haploinsufficient gene are TSGs that need only one allele change for neoplasm development, especially once joined with various changes that adjust the function of oncogenes and TSGs.

2.3 Prostate Cancer

2.3.1 The Anatomy and Physiology of the Prostate

The prostate is found at the anterior of the rectum, and inferior of the bladder with the lower pelvis and is linked by seminal vesicles, forming the reproduction glands. The gland function as a secretory organ and secrete milky, slightly acid which is essential for ejaculation. PSA serum prostate specific antigen is crucial component which is used in screening of prostate cancer patient. The prostate is glandular organ supported by stroma and extracellular matrix (ECM) components including neurovascular tissue, fibroblasts, smooth muscle cells, and lymphocytes. The cell types are of four types in the prostatic epithelium secretory, basal, transient amplifying (intermediate between secretory and basal cells), and neuroendocrine cells [33].

2.3.2 Cellular Compartment

The cell which secrete alkaline are lined in the prostate gland and ducts. This fluid is important for motility and nourishment of sperm cell. Basal cells are connected to the basement membrane that is near to these body fluid cells. They have high proliferative capability and long life. The neuroendocrine cells ar unfold across the prattle secretory organ, the main perform of the cells are the regulation of the cells growth and ductless gland liquid body substance activity [34]. The prostate is split into four anatomical zones: the peripheral, central, transition, and fibromuscular zones. The peripheral zone is that the largest that consist most of glandular cancer arises. Precursor lesions, finest ductless gland intraepithelial pathological process (HGPIN), ductless gland intraepithelial pathological process (PIN), and first prostate malignant neoplastic disease develop within the peripheral zone. Benign ductless gland dysplasia (BPH) sometimes develops within the transition zone [35]. Multifocal glandular cancer arises within the multiple zones.

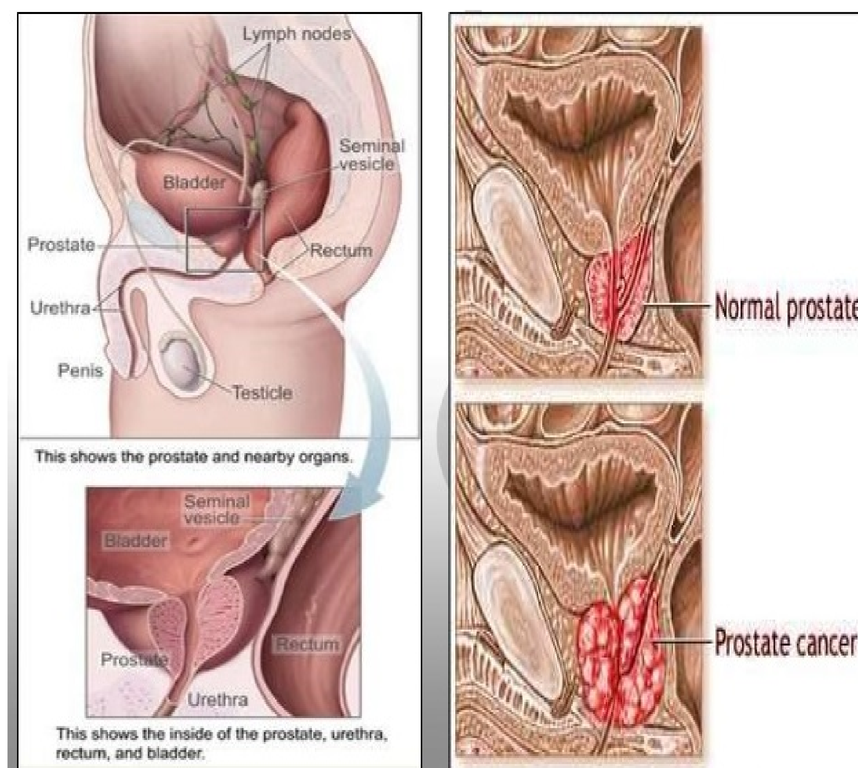

FIGURE 2.1: Pathophysiology of Prostate Cancer [36]

2.4 Incidence and Risk Factor

2.4.1 Incidence

In the recent years, PC rate has redoubled throughout the globe. This could be seen to the raised prostate specific antigen testing and diagnostic test frequency in older population. However, death has reduced [37], PC frequency at the older age within the developing countries is 16% that suggests that on the average, one in each six men in these countries are having PC. Within the developed world, about 650,000 men area unit diagnosed with prostatic adenocarcinoma. The yearly deaths area unit 136,000 round the world.

2.4.2 Risk Factors

The studies have shown the foremost risk factors touching the glandular carcinoma. Progression is a complicated age, ethnicity and family history [38]. The possibility of glandular carcinoma can increase with 3-fold, If initial relative has glandular carcinoma. Hereditary PC is responsible for 5-10% of PC and in similar family having early onset of PC. High penetrance genes can increase the possibility of the PC among hereditary PC.

2.5 The Morphology of Prostate Cancer

The overall morphology of prostate cells changes with aging method that is termed as benign prostatic (BPH). Within the BPH the epithelial willincrease however nuclear look remains traditional. once nuclear compartment in enlarged and nuclei is visible the lesion is thought as precursor lesion to PC [39]. These lesions are best-known a endocrine gland intraepithelial neoplasia or high-grade prostate intra epithelial neoplasia. Multi focal lesions that are characterized by totally different phenotypes at microscopic anatomy level arise perpetually dynamic the small surroundings and cell compartments throughout PC development.

2.6 Epigenetic and Genetic Aberrations

Several genetic and epigenetic anomalies are related to PC which are involved in development of prostate cancer. Which are as follows [40].

TABLE 2.1: Genetic and Epigenetic Aberration [40].

GENETIC ABBERATIONS	*MYC* oncogene overexpression
	loss of *NKX3.1*
	loss of *PTEN*,
	gain-of-function mutations in *EZH2*
	inactivation of *TP53*
EPIGENETIC ABBERATIONS	*GSTP1* (glutathione S-transferase pi 1) promoter hypermethylation
	APC (adenomatous polyposis coli) hypermethylation

2.7 Diagnoses

Prostate specific matter, PSA may be a serine enzyme that belongs to the human Kallikrein family genes. PSA is made in malignant and traditional prostate cell and facilitates the regulation of body fluid liquefication. The PSA is detected in blood. PSA testing is common key within the diagnosing of early glandular cancer, it's additionally a part of the clinical application to live pc progression when prostatectomy therapy and staging [41]. The edge for PSA level for the glandular cancer is 4 ng/ml and men who have PSA above 4 ng/ cc is viable for biopsy. But high PSA will be relating to different disorder too. Like PBH and inflammation PSA testing should be fastidiously understood together with rectal examination, ultrasounds and Gleason scores.

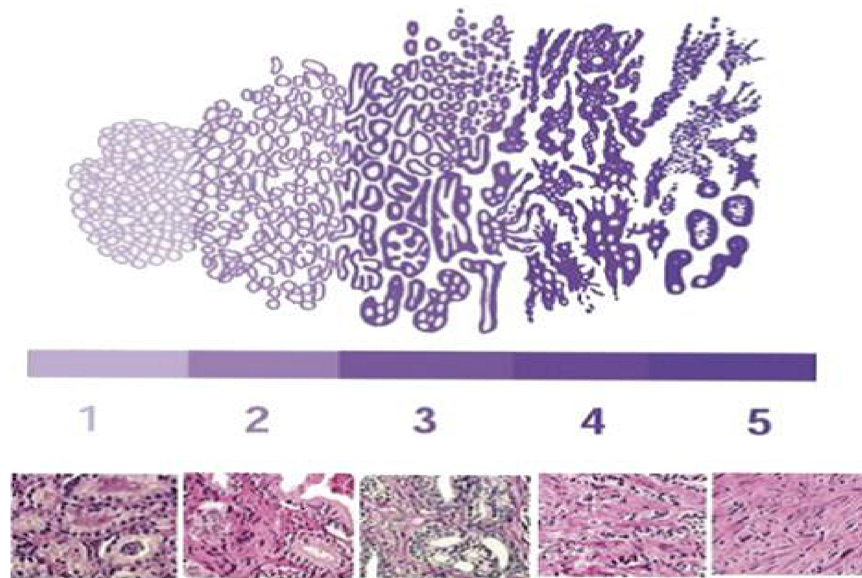

FIGURE 2.2: Prostate Cancer Aggressiveness is Defined According to Five Gleason Patterns [42].

2.8 Tumor Node, Metastasis Staging System

There are two types of TNM staging: Clinical and pathological staging (CT) is the data acquired before medical procedure. Pathological staging is set up based on the data assembled after prostate medical procedure; fundamentally, it depends on histological discoveries prostate tissue test [43]. The PC assessment depends on histological examination while fix is chosen by PSA level, Torganize and pathological findings.TM arranging characterizes the condition of the tumor tissue inside the prostate and other encompassing structures.

TABLE 2.2: TNM Staging Type [45].

Stage I	Stage IIA	Stage IIB	Stage IIc	Stage III	Stage IV
prostate tumor is not visible or palpable	prostate tumor involves ≤ 50% of the lobe	prostate tumor involves ≥ 50% of the lobe	prostate tumor involves both lobes, but is confined within prostate	prostate tumor has spread into seminal vesicles	prostate tumor has spread to lymph nodes and other nearby tissues

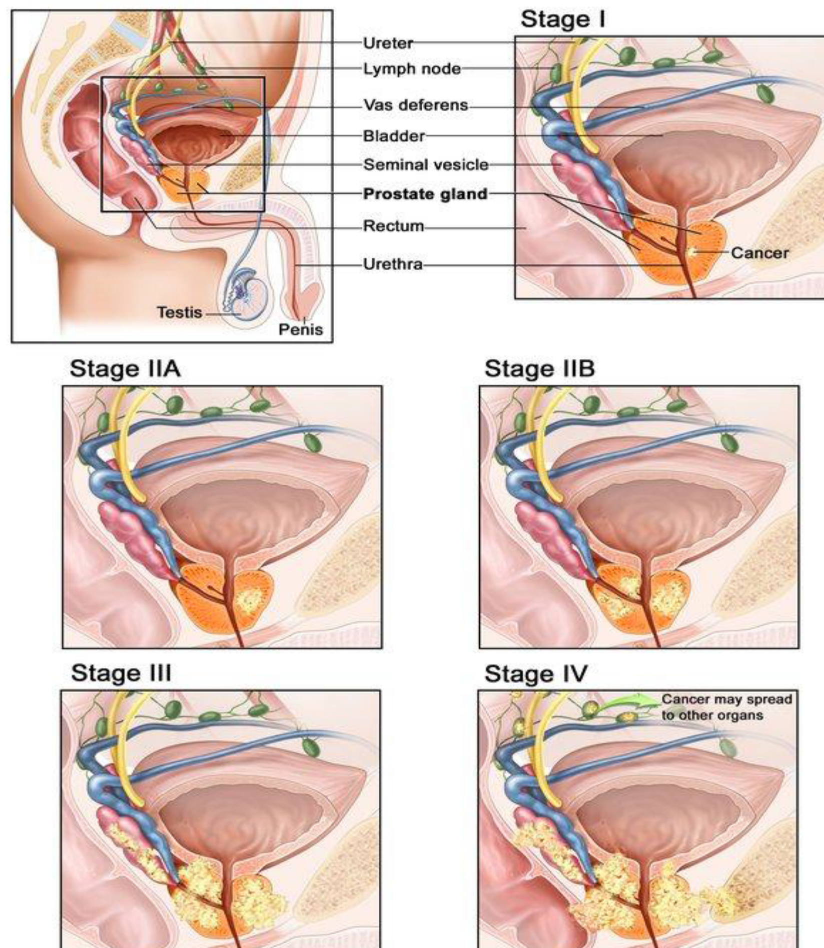

FIGURE 2.3: Tumor, Node, Metastasis (TNM)-staging System [44].

2.9 Types of Cancers

2.9.1 Localized Cancer

PC often becomes inside the prostate organ and which isn't extended through the prostate is known as limited PC. T1-T2 are best clinical stages that the best characterizes restricted PC the patients that have a place with low or normal generally safe accumulations ordinarily have confined PC. With expanding number of restricted moderate developing and well recognize pieces are analyzed to broad increment in the utilization of PSA test testing. The most common how to avoid to keep away from over treatment of patients who have been determined to have limited moderate developing PC[47]. The most suitable solution for persistent who are related to moderate developing PC is dynamic observation and the danger of

TABLE 2.3: The Risk Groups [46]..

Risk Group	PSA ng/ml	Gleason score	Clinical Stage	
Low	< 10	≤ 6	T1a-c, T2a	T1a-c, tumor is not palpable or visible by digital rectal examination or imaging T2, tumor palpable and confined within prostate; T2a, tumor involves $\leq$ 50% of one prostate lobe
Intermediate	$\geq 10 - \leq 20$	7	T2b	T2b, tumor involves $>$ 50% of one lobe
High	> 20	≥ 8	T2c, T3a-c	T2c, tumor involves both prostate lobes; T3a-c, tumor extends through the prostatic capsule and the growth is no longer limited within the prostate lobes.

prostate tumor movement is low sum almost half of patients who are related to restricted moderate developing PC.

2.9.2 Metastatic Prostate Cancer

PC can be metasized restricted to one place or to far away organs. PC is also known nearby progressive if the tumor has ruptured the gland which is usually T2a stage and if it ruptured right outer layer of the two gland to the seminal vessels it is known as T3b stage . When the tumor is in where the seminal vessels for prostate tumor spreads into close organs including bladder, and pelvic cavity[48]. The stages T3 and T4 are referred to restricted progressive PC. The progressive stages of PC clinical stage T4 is referred to distant metastasis are usually found in the bone our lungs. Over 90% of progressive PC tumors are in the bone and 42% to 50% metasized to the lungs and 25 to 30% are metasized to the liver. Still the

molecular mechanism by cross talk between metasized tumor cells and the cells which are associated in the development of the bone is not yet understood.

2.9.3 Castration-Resistant Prostate Cancer

The progression of prostate malignancy depends on the androgen level. The most common cure for metastatic PC is androgen deprivation therapy. It was noted that about 80 to 90% of metastatic PC patients have biological effects are that PSA and serum amount lowers in the blood after castration therapy. Though the cells are used to minimal concentration of androgen which they cope by alternating signal transduction mechanism which includes steroidogenesis reactivation AR and bypass Pathways. CRPC normally create inside 18 to 24 months after the underlying Castration treatment and after CRP recurrence the survival is at least 20 months treatment isn't a cure and is one of the principle clinical test which must be tended to be better treatments against CRPC[49].

2.10 Treatments

2.10.1 Active Surveillance

The cure through active surveillance has exposed to be safe and promising for generally low prostate malignancy patients who have slow and small growing, and well prominent tumors with well examination for the aged patients with life likelihood whose reason of expiry is less to be unconnected to prostate cancer. Through active surveillance the cancer development is followed by PSA data which has to be taken after 3-6 months. Prostate tissue surgeries are examined after 3-5 years. The main reason of active surveillance is to prevent over-treatment with low risked patients [50].

2.10.2 Radical Prostatectomy

The surgical removal of the every or portion of prostate gland and seminal vesicles is known radical prostatectomy. After prostatectomy the following condition should be noted as such PSA should absent from the blood, hence PSA levels are frequently examined by radical prostatectomy. Radical prostatectomy is normally utilized as a cure for minimal or patient at low risk (table 1) identified with clinically with localized cancer. However possible advantages lower after radical prostatectomy amongst high risk PC patients. It has been shown that effects are better after prostatectomy [51]. As the long term and high cancer survival rates.

2.11 TMPRSS Gene

Paolini identified a novel gene which was named TMPRSS gene [52]. TMPRSS encodes a protein which possess a serine domain.This cDNA encodes almost 492 amino acids. There are four domains

1. A serine protease domain (a S1 family which binds to the Arg and Lys residues)
2. SRCR domain (domain involved in the binding to extracellular molecules)
3. LDLRA (domain this serves as binding site for the calcium)
4. A transmembrane domain.

TMPRSS is involved in prostate at high level while low levels are observed in pancreas ,liver lung ,colon and small intestine. TMPRSS gene was mapped on 21q 22.3 near ERG by [53]. TOB2B mediated double strand break[54].

2.11.1 Gene Function

It was shown that androgen regulates the co recruitment of the androgen receptor and TOP2B at the site of TMPRSS-ERG Fusion break point in the genome which activates recombination of TOB2B mediated double strand break [55] .Androgen stimulation causes de novo production of TMPRSS-ERG transcript . TMPRSS have a critical role in different physiological and pathological procedures which includes digestion, blood coagulation, remodeling of tissues, invasion of tumor cells, inflammatory response ,apoptosis. TMPRSS is specified at low levels in liver, kidney, pancreas, lung and Colon. TMPRSS is well expressed in normal prostate and PC cells they have androgen responsive elements and its promoter region in the expression of TMPRSS is redoubled by androgen in PC cells and lowered in androgen independent PC.

2.12 ETS Transcription Factor

ETS transcription were first discovered as gag-myb-ets fusion of the transforming virus that cause leukemia in chicken. This family consist of 27 members which shar a common homology in their C-terminal part of the protein (Figure 1) [56].The structure of the protein consist of 85 amino acids which form helix-turn -helix DNA binding domain which interacts with GGGA/T(the ETS binding site in the promoter region of the target genes [57].Any difference in the binding site in the promoter region effects the overall binding capacity of the ETS factors.PNT is a conserved domain of the ETS factor.

This domains plays a important role in the protein interaction. They can be separated into 11 subfamilies depending on their structural composition and homology [58].ERG family consist of ERG and ETV1 while ETV1,ETV4,ETV4 are belong to the PEA3 family[59].They contain N-terminal acidic transaction Domain(TAD).Many ETS regulate directly or indirectly .ETS function as transcription activator's and a repressor's transcription factor play a important role in

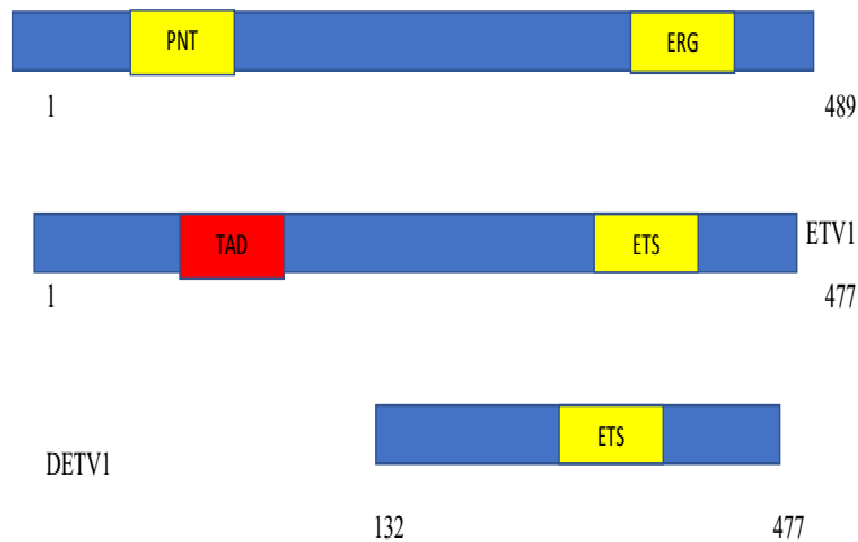

Figure 2.4: ETS Transcription Factors ERG and ETV1.

biological process such as cells Proliferation, differentiation, apoptosis, tissue modification, angiogenesis, metastasis and Variation. The ETS gene are expressed in solid tumors. [60]. In addition, high level of ETS are often seen in lung ,breast ,colon, and prostate cancer. ERG is highly expressed in the prostate cancer. The higher expression is in early and late stages.

2.12.1 ERG Gene

ERG was discovered by Reddy [61]. He isolated a cDNA having the complete coding sequence of oncogene ERG.A protein with two regions share 40to 70% homology with the 5 prime and 3 prime region that consist of ETS viral oncogene which concludes that ERG belongs to ETS oncogene family. With the sequencing of two different ERG transcript were identified ERG1 and ERG2 .Both differ from each other by splicing event that causes frameshift which results in additional amino acid at the N-terminus of the protein .while transcription and translation in vitro which results in two poly peptides which shows that alternative sites of splicing and adenylation with the alternative site of translation initiation causes synthesis of the two Erg poly peptides Erg transcript encodes five proteins which bind on the DNA at the ETs site and act as transcriptional activator.

2.12.2 Gene Function

ERG is a nuclear phosphoprotein that binds specifically to the purine rich sequences [62] .it is considered stable than short lived ETS1 and ETS2 proteins ERG2 is very specific DNA binding protein. ERG also function as regulator of the gene involved in maintenance and differentiation of early hematopoietic cells they are expressed in high level in early myeloid cells than in mature lymphoid cells. It was shown that ERG mutation in prostate cancer cause decreased protein stability and mostly occur in tumors without ERG upregulation .ERF loss sum up the morphological and phenotypic features of the ERG gain in prostate cancer ,including expansion of the androgen receptor[63] .AR transcriptional enhancement and ERG had tumor suppressor activity which result in PTEN loss that results in oncogenic activity by ERG .ERG upregulation indicate that ERG inhibits the ERF to bind DNA at the site of the both in normal and malignant cancer.

2.12.3 Gene Structure

The gene structure of ERG was determined which consist of 17 exons [64]. The 5 prime end contains a major CPG island .The upstream region of exon1 and 11 kb region within intron 1 consist of transcription -binding site.

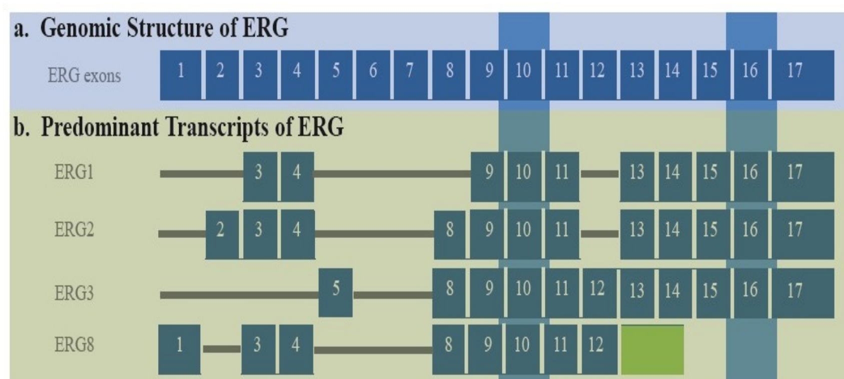

Figure 2.5: The Exon Representation of ERG Gene [65].

2.12.4 Mapping

With somatic cell hybrid analysis ERG gene was mapped on chromosome 21 with family linkage studies to DNA marker translocated along with ETS 2 [66].

2.13 ERG/TMPRSS2 Fusion Gene

2.13.1 The Frequency of TMPRSS-ERG

TMPRSS-ERG is the most dominant fusion which has been detected by distinct fish, array comparative, genomic hybridization and RT PCR essays it has been known that it occurs 42 to 70% of clinical PC. TMPRSS ERG fusion has also been found in 10 to 20% of HGPIN lesion and in 29 to 69% of Advanced PC but they are not exhibit in nonmalignant epithelial cells or stromal cells. This state us that the TMPRSS-ERG combination is an essential event in PC advancement [67].

2.13.2 TMPRSS-ERG Transcripts

Different transcript of TMPRSS-ERG are detected by quick amplification of CDNA ends and RT PCR essays. The most common types of TMPRSS is exon one or two is combined to ERG exon 2,3,4,5. The combination of TMPRSS exon 4, 5 to ERG exon 4 or 5 is less repetitive. There are 19 distinct fusion ranscript of TMPRSS-ERG which are distinguished. The exon of 5 TMPRSS is takes part in the improvement of transcript which is commonly non coding and are converted into protein. The n-terminal truncate structure of ERG protein is the protein which is deciphered by the greater part of the transcripts. There is combination protein transcript variation TMPRSS exon 1:2 ERG exon for which express to combination protein which were the natural connection of n-terminal truncate ERG protein and combination protein encoded by TMPRSS transcript variation in PC improvement stays to be further determined [68].

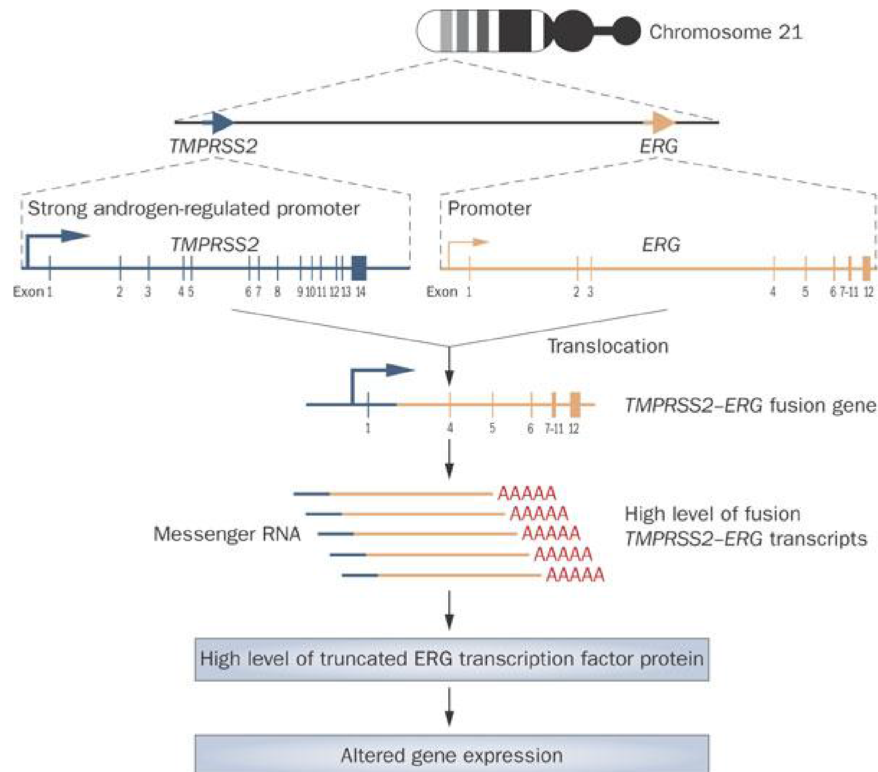

FIGURE 2.6: The Arrangement of TMPRSS2:ERG Fusion Gene by Translocation[69].

2.13.3 ERG-TMPRSS Fusion Gene

Tomlins [70] applied the bioinformatics method to identify the candidate oncogenic chromosomal aberration on the basis of gene expression. ERG and ETV 1 are two ETS transcription factor was identified in the [71] identified gene fusion 5 prime untranslated region TMPRSS to ERG in prostate cancer tissues. Tomblin used FISH to identify that 23 and 29 prostate cancer samples had ERG and ETV 1 rearrangements [72]. Cell line experiment showed that androgen promoter element of the TMPRSS 2 meditate the over impression of ETS family member in prostate cancer. The TMPRSS and ERG are located on chromosomes 21 q 22. The TMPRSS/ERG fusion attached TMPRSS exon one or two to exon two, three, and four which results in activation of ERG transcription factor. This fusion is the reason behind separation of ERG 3 prime centromaric region from 5 times telomeric ends. Deletion can also occur in this region. FISH studies of the TMPRSS -ERG gene in 445 prostate cancer patient showed an alteration called 2+ Edel which was characterized by duplication of TMPRSS 2 / ERG fusion together

with 5 prime ERG sequence deletion. The change was detected in 6.6% of cancers and was linked with poor clinical outcome. The cancers which was characterized by 1 copy of 3 prime of ERG didn't had bad clinical outcome. It proved that over impression of ERG is result of fusion of 5 prime TMPRSS 2 to 3 prime ERG which is cause for cancer development [73].

It was observed that with the simulation of the androgen receptor ligand (DHI) for 60 minutes caused proximately between TMRPSS and ERG loci. The effect depended on the AR. [74] summarized that androgen signaling causes the 5 prime and 3 prime gene hence the probability of the gene fusion increases with DNA double strands breaks.

TMPRSS fusion gene is shown to be present in 50% of PC patients including also the patient with advanced PC. The TMPRSS gene in codes for series transmembrane proteins and hygiene in codes for transcription factor which belongs to ERG belongs family of transcription factors. The TMPRSS gene are located on the same chromosome 21 and are approximately 3Mbs apart. The TMPRSS-ERG Fusion genes are usually formed through 3MB deletion on chromosome 21 which fuses the two genes together. The fusion gene occurs through chromosomal rearrangments and unbalanced chromosomal. The TMPRSS promoter region fuses to ERG coding region. Due to this fusion ERG transcription is under the control of TMPRSS promoter. The coding region of TMPRSS gene is lost during this chromosomal deletion which results in the loss or reduction of TMRSS expression[75].

The promoter region of ERG is lost in chromosomal deletion but ERG negrocoding region on N-terminus is lost which expression of truncated functional protein. These truncated ERG protein does not retain its DNA binding domain so continue to pray transcription factor.There has been a 18 different splice variants of the TMPRSS-ERG which have been identified in PC patient with some multiple splice variants. This file splice variants a result of alternative messenger RNA splicing or chromosomal fusion breakpoints. The most common among variant is T1-T4 variant which is about 75 to 80 6% of ERG variants in PC patients this TMP RSS-ERG fusion shows that this region is an important spot for chromosomal

rearrangement and deletion in PC [77]. The reason behind the cause of TMPRSS-ERG fusion is not exactly known but it is suggested that a signal induces DNA double strand break switch have been a key player in the formation of fusion gene. The Other factor that influences the fusion is from proximity of high levels of AR signaling to TMPRSS energy genes which results in high amount of DNA double strand break [76].

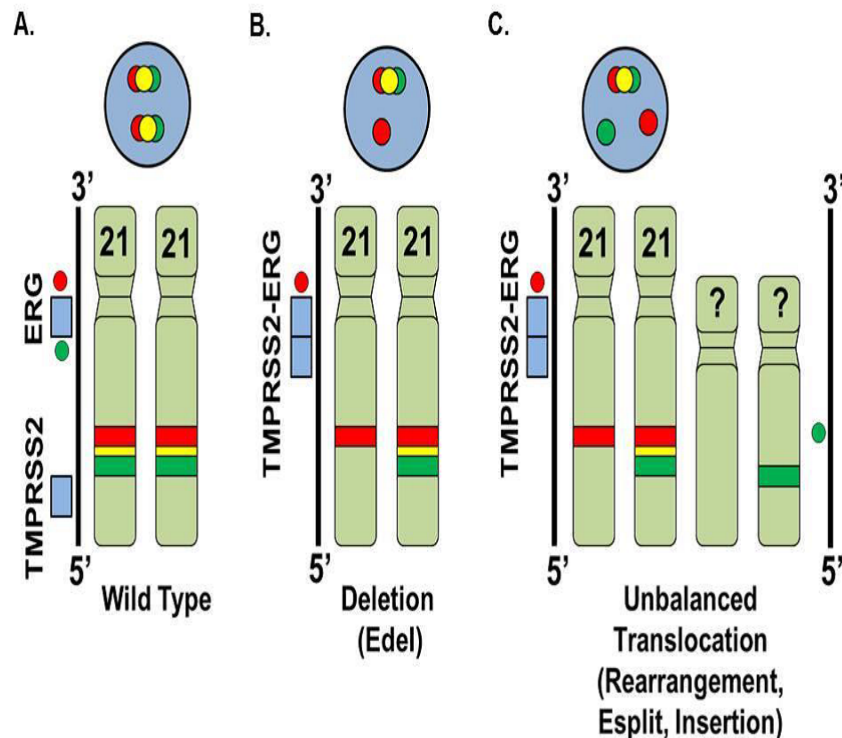

FIGURE 2.7: TMPRSS2-ERG Fusion Gene Formation: 3 Mb Deletion on Chromosome 21[78].

Figure2.10 Illustrating Wild Type ERG and TMPRSS2 Genes Located on Chromosome 21. This represents TMPRSS2-ERG fusion-negative chromosomes. B.) figure Illustrating 3 Mb deletion of chromosome 21 located between TMPRSS2 and ERG, which fuses the two genes together on TMPRSS2-ERG fusion-positive chromosomes. C.) figure Illustrating 3 Mb unbalanced translocation of chromosome 21 located between TMPRSS2 and ERG, which fuses the two genes together on TMPRSS2-ERG fusion-positive chromosomes. The translocated portion of chromosome 21 is inserted into an unknown location in the genome. For A-C, the red and green colors represent ERG specific DNA hybridization probes used in fluorescent in situ hybridization (FISH), a technique normally used for locating

of the TMPRSS-ERG fusion gene. The yellow color appears on TMPRSS2-ERG fusion-negative chromosomes due to overlap of the red and green probes. The blue circles located above chromosome diagrams depict how the FISH results commonly appear. For example, in part B, when the green probe labeled 5' end of RG is lost through deletion, only a single red probe will appear for the affected chromosome [79].

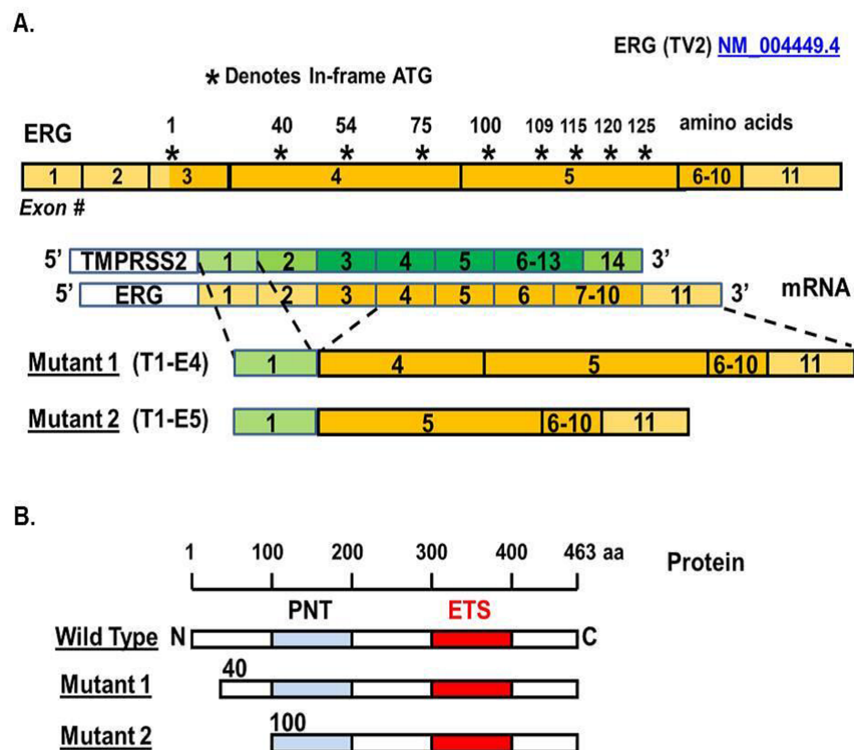

FIGURE 2.8: Formation of the TMPRSS2-ERG Fusion Gene in Prostate Cancer: mRNA [80].

Figure 2.12 Illustrating each of the mRNA splice variants identified for the TMPRSS2-ERG fusion gene in PCa. Full length mRNA sequences for TMPRSS2 and ERG are shown at the top for reference. Numbers on mRNA sequences represent exon numbers. For ERG the light yellow indicates non-coding regions and the dark yellow indicates protein coding regions. For TMPRSS2 the light green indicates non-coding regions and the dark green indicates protein coding regions. Each splice variant is named in the white box according to the TMPRSS2-ERG fusion site. For example, T2-E4 indicates TMPRSS2 exon 2 is fused to ERG exon 4[82].

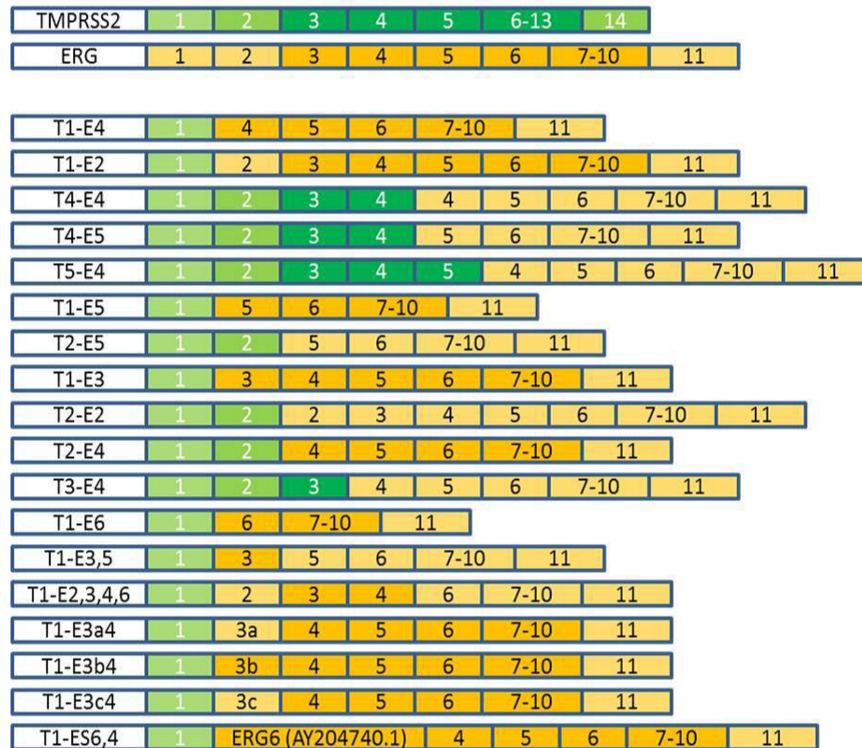

Figure 2.9: Different mRNA Splice Variants of the TMPRSS2-ERG Fusion Gene [81].

The TMPRSS gene promoter contains AR response elements. TMPRSS-ERG fusion positive PC ERG is under the control of AR signaling which results in overexpression of truncated proteins ERG protein belongs to ETS family member transcription factor which contains conserved regions and C-terminal DNA binding domain which is known as ETS domain. ERG regulates transcription of target genes through binding to ETS consensus sequence at the promoter and enhancer of target genes ERG protein is not normally expressed in PC. TMPRSS-ERG fusion ERH regulates expression of target genes which leads to development of PIN lesion and and tumors that have lost the tumor suppressor PTEN [83].

2.13.4 Mechanism of Gene Fusion

The genomic closeness of TMPRSS and ERG is the cause in explaining TMPRSS 2 -ERG fusion as related to other ETS fusion. The other ETS gene are located on different chromosomes, the other ETS fusion may be due to nuclear proximity

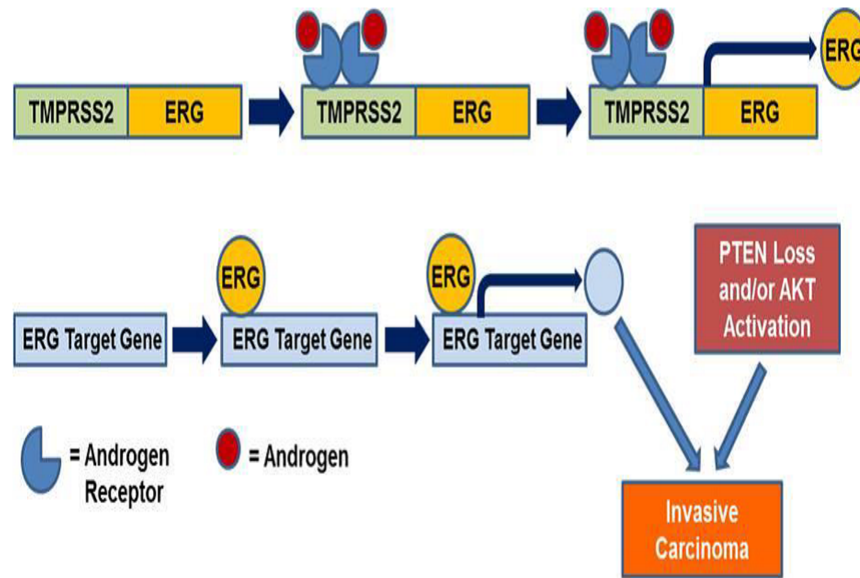

FIGURE 2.10: The TMPRSS2-ERG Fusion Gene in AR Induced Overexpression of ERG [84].

[86]. As stated above, ETS fusion partners are upregulated by androgen and have prostatic specific expression so the mechanism of regulation fusion gene is an important factor in the fusion events [87]. The expression may be also the cause of the nuclear closeness. It is stated that binding of activated androgen receptor to gene which code the fusion partner plays an important role in fusion of genes. Evidence are available that show androgen receptor induce genomic breaks by recruiting enzyme (TOP2B) or (AID) and ORF2 endonucleases [88].

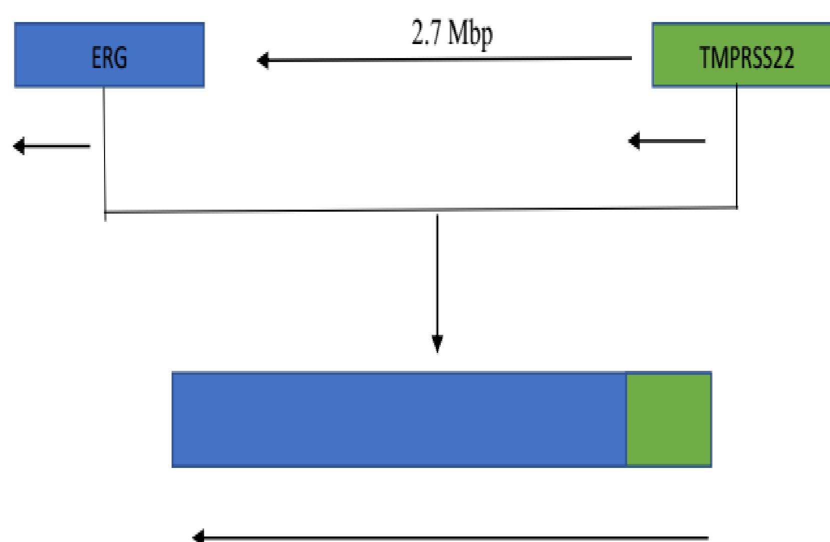

FIGURE 2.11: Mechanism of TMPRSS-FUSION[89].

TABLE 2.4: Fusion Partner Genes[85].

5' fusion partner	Prostate specific	Androgen regulated	ETS partner	Freq
TMPRRS2 (chr21q	+	+	ERG (chr 21q)	<<1%
SLC45A3 (chr 1q)	+/-	+		<1%
NDRG1 (chr 8	+	+		<1%
SLC45A3 (chr 1q)	+	+		<1%
TMPRRS2 (chr21q)	+	+	ETV1 (chr 7p)	<1%
SLC45A3 (chr 1q	+	+	FLI1	<1%
FOXP1 (chr 3p)	ND	ND		<1%
EST14 (chr 14q)	+	+		<1%
HERVK17 (chr 17p)	+	+		<1%
HERV-K_22q11.23	+	+		<1%
C15orf21 (chr 15q	+	+		<1%
HNRPA2B1 (chr 7p	+/-	-		<1%
ACSL3 (chr 2q)	+/-	+		<1%
TMPRRS2 (chr21q)	+	+	ETV4 (chr 17q)	<1%
TMPRRS2 (chr21q)	+	+	ETV5 (chr 3q)	<1%

2.14 Biological and Molecular Functions of ETS Proteins in Prostate Cancer

The role of the ERG gene in prostate tumor is not determined. The ETS high expression is not the only reason sufficient for the progression of the prostate cancer[90]. It was found through studies that overexpression of the ERG in epithelial prostate cells were the reason behind cell migration and invasion and [91] that limiting the expression of the ERG slowed the invasion. The studies showed the suggestion that ERG can collaborate with numerous different oncogenes for the development of the prostate cancer. The expression profile of the has identified genes and pathway associated with ERG over expression [92]. OERG is related to the initiation of the TGFß pathway[93] and WNT pathway[94] .Experiment

demonstrate that androgen receptor association with ERG have important role [95].

2.15 The Role of Signaling by Androgen Receptor

Androgens including testestrone and dihydrsterotestosterone (DHT) are steroid hormones which attaches usually to the ARE's present on the androgen receptor. Both of them regulate and stimulate the formation of male sex organ. The anterior pituitary produces luteinizing hormone (LH) which regulates the production of testosterone. For the LH regulation from the anterior pituitary secretion of the gonadotrophin in the hypothalamus is essential LH secretion causes testosterone production from lydig cells in the testicles. Androgen is important for the event and typical physiological fucntion of AR's are the clarification for the detail of specification of the lineage -specific differentiation and simulation of the prostate cells the ordinary prostate AR maintains the separated form of the prostate epithelial cells[96].PC tumorigenesis the mechanism of air activation is modified most typically CRPC. anti androgen therapy and total steroid hormone blockage are the most necessary treatments used for CRPC patient. The cell has got to get used to a very low level of androgen that they cope up through totally different mechanism. In CRPC AR signal in initiation occurs by many other ways that include amplification that usually happens in one third of CRPC patients. Initiation has been shown to additionally occur by steroid hormones and antiandrogens that include progesterone estrogen this can be due to the mutation in AR sequence. within the literature it's been seen that AR has additionally been shown to be activated by various splicing of AR sequence the result's that altered expression level of AR coactivators and Core processor sometimes cause AR activation[97] .It has been seen that AR activity will increase with increase within the range of gain of operate mutation has been an oversized quantity of proof that recommend that truncated isoform of AR splice variants will cause activation of AR without

a ligand that leads to the event of initiation due to the altered structure of AR splice variants the interaction with coagulated and bindings to AR is could also be ordered AR coregulator like and NCOR 1 2 and 3 regulate histone methylation, acylation thatcauses transcriptional initiation and activation in CRPC patients.

2.16 The Cross Talk Between the Androgen and ERG

The TMPRSS-ERG is regulated by AR but recently it has been suggested that TMPRSS-ERG gene Regulation and AR signaling are associated in PC different data are emerging that supports the hypothesis that ERG corporates with AR negatively and positively regulate AR target genes. It is same as if ERG hijacks the AR and direct target gene expression and regulation in order to facilitate tumor growth. ERG physically interacts with AR and ERG transcriptional regulation often occurs with AR. ERG also has an inhibitory effect on our target genes which are involved in prostrate cell differentiation hence inducing prostate tumor the dedifferentiation.in other studies it has been integrated that ERG activates AR target genes in one of the study it was found that ARG redirected AR to a set of genes including SOX9 and which positively regulated their expression. ERG bound to SOX 9 at an overlapping side and a binding site is dependent on AR.ERG has also shown to regulate the target gene TFF3. ERG negatively regulated TFF3 in a hormone of PC cells and positively regular TFF3 in CRPC cells this regulation is totally dependent Upon AR signaling. ERG regulation of TFF3 CRP cells has shown to be associated with Cellular invasiveness it has been also been reported that AG overexpression alone is not sufficient to induce PIN lesion in prostate and AR overexpression alone did not give rise to any hyperplastic lesions. AR with ERG strongly facilitate PC tumor growth. ERG can activate the AR pathway which includes a study by Chen et al that showed that increase the AR transcriptional output which in turn resulted in PTEN loss and ERG family member are associated with synthesis and also activation energy is not

only associated with androgen synthesis or activation of AR signaling prostate cancer. In the studies that TMPRSS-ERG fusion is present at high frequency in PC patients PTEN negative tumors compared to PTEN positive tumors which suggest that ERG is more associated with PTEN negative environment then PTEN positive environment.

2.17 Androgen Association with TMPRSS-ERG

TMPRSS fusion [100] is a result of the chromosomal rearrangement, with increased expression of the ERG activates transcriptional program and induces oncogenesis by upregulation MYC, EZH2 and SOX 9 and repressing NKX3 [101]. Hence the net result is high levels of ERG expression is prevention of the epithelium that is normally regulated by AR. expression of show a significant correlation with AR expression in tumor [102].The formation of fusion is facilitated by the AR which causes the proximity [103] of genome loci of TMPRSS and ERG. Both are located on the same chromosome 21q22 and fusion occurs due to DNA breaks [104].

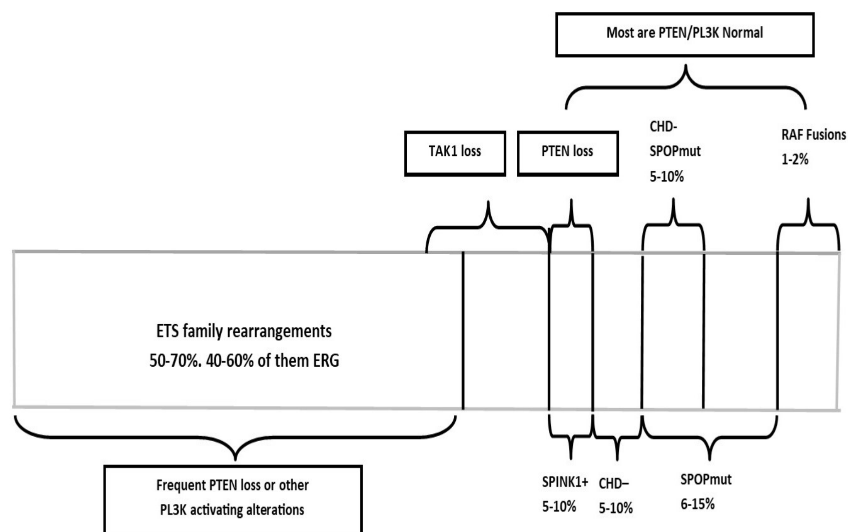

Figure 2.12: Molecular Subtypes of The Prostate Cancer [101].

The ERG rearrangement contains DNA breaks at the AR binding site .How ever DNA breaks in ERG negative cases were at distant from AR binding site [105].The ERG fusion is regulated by androgen signaling .As soon as TMPRSS and ERG at

proximal ,AR induces fusion by recruiting two types of enzymatic activity cytidine deaminase and LINE1 repeat encoded ORF2 endonucleases [106]. These induce double stranded DNA breaks that are ligated by nonhomologous end [107].The prevalence of ETS Fusion is higher in early onset prostate cancer [EO-PCa]. They had higher level AR, 90% had ERG fusion and lack co-repressor NCOR2. The patient with lower level AR have rearrangement such as PTEN,CHDK1,TAK1 which are not androgen dependent [108]. These finding indicate that AR signaling is involved in the rearrangement involving ERG factor and androgen responsive element in TMPRSS.

2.18 Heterogeneity of Prostate Cancer

ETS fusion is common in prostate cancer. Localized prostate cancer is a multifocal disease. Tumors are being tested for the ETS gene identification of different prostate cancer. In almost 50

2.19 ETS Fusion as Diagnostic Tool

As ERG fusion transcript occur in approx. 50% of prostate tumors, it is clear from this that the ERG fusion transcript present in the prostate tissue, urine or overexpression of Erg protein is an important diagnostic marker for prostate cancer. It was found that the patients who had high level of the expression of the Erg showed slow progression than with patient without ERG over expression [111]. with the discovery of the fusion genes (TMPRSS-ERG) were correlated with the poor clinical outcome. It was not determined in later studies [112]. The patient who had gene fusion with intestial deletion between TMPRSS and ERG had poor clinical outcome then patient who had TMPRSS-ERG fusion without genomic deletion between two genes .It was shown that patient who possessed TMPRSS-ERG fusion starting at alternative exon one had good outcome then patient with TMPRSS-ERG exon 1 [113]. The patient who were given testosterone inhibitor

responded well to the therapy [114]. According to the above results it proves that TMPRSS-ERG fusion totally depends on androgen signaling despite CRPCa stage.

2.20 Other Genes Involved in Prostate Cancer

2.20.1 MYC

MYC is a oncogene which is found at 8q chromosomal region transcription factor which regulate cell proliferation, programmed cell death and stem cell renewal [116]. MYC is highly expressed in numerous type of tumors such as lymphomas, leukemias, hematopoietic, colon cancer, breast, prostate cancers. The MYC protein is highly expressed in different stages of PC tumors. MYC lowers the regulation NKX 3.1 and over expresses of EZH2 gene which shows its part in various stages of PC progression [117]. MYC has been detected in 70% of CRP patient which have gain 8 q and one third of this patients have MYC amplification the reason for MYC high expression remains undiscovered but it is evident that inactivation of APC and down regulation of a FOXO3 can be the reason with MYC upregulation [118].

2.20.2 NKX 3.1

NKX 3.1 loss is the most important relation in PC NKX 3.1 8p 21.NKX 3.1 TSG that encodes homeobox containing transcription factor NKX 3.1 and variant of epithelial cells in the prostate so cell proliferation and sell differential increases after the deletion of NKX 3.1 [119]. NKX 3.1 be epigenetically controlled by promoter methylation. The changes in NKX 3.1 as it is located on 8 p21 region and there are no additional mutation observed in the remaining NKX 3.1 expression of NKX 3.1 advanced tumor stages as well as in hormone diseases the expression level of NKX 3.1 is due to the deletion of pretend which leads to the increased activity of AR and tumor progression [120].

2.20.3 PTEN and P13K Pathway

PTEN may be a TSG that is found on chromosome ten it regulates cell pathways by focusing on the proteins that direct cell development and survival [121]. PTEN is the most suppressed in human cancers the practical loss of one PTEN allele disadvantages for pc growth PTEN mutation is determined in four-hundredth of localized PC cases PTEN deletion is way higher regarding 70 to 80% in advanced pc and deletion are considerably related to increased Gleason grade advanced tumor stage and occurrence of ERG gene fusion in CRPC disease and prostate specific antigen repetition. P13k regulate the conversion of phosphate to P1P3 that is concerned in inactivation of amino acid transmembrane macromolecule enzyme activity cell cycle development, macromolecule synthesis, cell growth and cell survival the activity of 13k negatively regulates by PTEN [122]. Enzyme inactivation of PTEN throughout PC by complete loss of PTEN will increase the amount of P1P3 causing increased PC cell growth and increased computer cell survival there's a linkage between AKT, P13K and AR signal inhibition ends up in activation of a AKT signal P13K inhibition activate a signal will the data of any one of signaling pathway activates the opposite pathway resulting in tumour cell survival.

2.20.4 EZH2

EZH2 is a transcription repressor gene is a part of Polycom cluster family is found on 7q36 which regulate cell proliferation in vivo specially in pathological process prostate malignant neoplastic disease typically advance of function [123]. Mutation of EZH2 is one in all the explanation for epigenetics silencing of TSG in pc development that results in poor prognosis of the patient. It had been resolved that TMPRSS-ERG combination is associated with the direction of EZH2 signal it completely was discovered that the epigenetics silencing was regulated by EZH2 is improved with upgraded EZH2 gene expression and and lower EZH2 to target expression of gene by ERG [124].

2.20.5 Tp53

Tp53 is one of the foremost activated genes in human cancer is tumor suppressor. Tp53 growth p53 protien that could be a transcription factor protein [125]. p53 is very important for the regulation of cell cycle and it's concerned in regulating the change from G1 phase to S phase. p53 is triggered and circumstances that are DNA damage like during hypoxia irritation and oncogene initiation or damage follow-on in cell cycle arrest or initiation of caspase-mediated cell death mutation. p53 is detected by immunohistochemistry that is caused because of nuclear accumulation. p53 are observed hormone refractory or pathological process prostate carcinoma [126].

Chapter 3

Material and Methods

3.1 The Area of Study

Main focused area of this research study is frequency of fusion genes in prostate cancer in Pakistani population. Therefore, the participants that were likely for the research were people of major hospitals of Islamabad. More specifically, these participants were taken from Pakistan Institute of Medical Sciences (PIMS) Islamabad Pakistan, for the purpose of narrowing down to research. The research presented here consist of tissue sample collection, RNA extraction, DNA synthesis, RT-PCR, QPCR.

3.2 Tissue Sample Collection

In current study, the subjects with control and affected were selected that were different ages and ethnicity. For participants in research, Clinicopathological information was gathered such as PSA, Chemotherapy, androgen inhibitors were taken. Informed consent was signed from each participant after elucidating purpose of this research. Ethnic committee proved questionnaires were filled by each participant. Tissue sample after proctectomy were drawn from both control and affected participants and immediately transferred to normal saline in a tube. Each

tube was labeled with ID and Tissue sample were stored in refrigerator -80 C until RNA extraction.

3.3 RNA Extraction

Total cellular RNA was extracted from tissue using Trizol RNA extraction protocol [125]. The RNA isolation protocol followed is as follows:

Material

1. DEPC-treated water (AMBION)
2. TRizol Reagent (Invitrogen)
3. Ice cold PBS
4. Cell Scraper
5. 70% ethanol
6. Isopropyl alcohol

The RNA isolation process consisted of the following steps

1. Tissue samples were homogenized in 1ml of Trizol reagent per 50 to 100 mg of tissue using a homogenizer.
2. Volume of TRIZOL Reagent used for the homogenization should be kept at 10%. The monolayer of cell obtained was cleaned with the ice-cold PBS.
3. Cell was lysed using the trizol reagent in culture dish with per 3.5 cm diameter dish and then the cell were scraped using the scraped.
4. The monolayer of the cell obtained were rinsed with the ice-cold PBS once the cell lysate was transferred to culture dish in which per 3.5 cm diameter dish followed by scraping cells with a scraper.

5. The cell lysate was passed through a pipette several times and vortexed carefully. The amount of the trizol relies upon the circumference of the culturing dish and not on the quantity of cells. The unappropriated amount of TRIZOl regent may lead to DNA contamination.

6. Next the cells were spin for 5 min. The cells were removed and the resuspended in ice cold PBS, the pellet was formed by spinning for 5 min. Then the cell lysis was performed with Trizol reagent by pipetting several times.

7. The controlled sample was incubated for 5 min at room temperature so that the nucleoprotein complexes completely dissociates, the cellular debris was removed by the centrifuge.

8. The supernatant was transferred to a new tube. The homogenized sample is incubated at room temperature for 5 min to permit separation of nucleoprotein complexes.

9. For the Phase separation we add 0.2 ml of chloroform in 1 ml of trizol reagent. Then cap the sample tubes tightly. we vortex samples for about 15 seconds and then incubate them at room temperature for 2 to 3 minutes. Then we centrifuged the sample at 12000 for 15 minutes at 2 to 8 degrees centigrade.

10. After centrifugation, the mixture separated into two layers; the lower red, phenol chloroform phase an interphase, and a colorless upper aqueous phase.

11. In the solution RNA left in the aqueous phase. the upper aqueous phase was transferred without disturbing the aqueous phase into new tube.

12. Measure the volume of the actual space which is about 60%. For the RNA separation precipitate it by mixing isopropyl alcohol from aqueous phase.

13. Then add 1 ml of trizol 0.5 ml per isopropyl alcohol for early homogenization. Then leave samples for 10 minutes at fifteen to thirty degree centigrade and then centrifuge at 12000 for approximately 10 minutes at temperature 2 to 4 degree centigrade at this point the RNA precipitates and is of invisible

before centrifugation it will it will form a gel like palette on the side and bottom of the tube.

3.4 cDNA Synthesis

The synthesizes of the cDNA by reverse transcription method. For this process 1 to 5 microgram of RNA was subjected to reverse transcription with random hexamer using first strand cDNA. For this we used 1 ng–5 g of total RNA or 1–500 micro gram of mRNA, then add the following component to nucleus free microcentrifuge tube

Table 3.1: cDNA Synthesis Component.

50–250 ng random primers	1 µl
Total RNA	1 ng to 5 µg total RNA, or 1 ng to 500 ng of mRNA
10 mM dNTP Mix	1 µL
Sterile, distilled water	To 12 µL

We then heat mixture 45 minutes at 65 centigrade and then quick chill it on ice the content of the tube is collected buy brief centrifugation and then we added the following

Table 3.2: cDNA Synthesis Component.

5X First-Strand Buffer	4 µL
0.1 M DTT	2 µL
RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 units/µL)	1 µL

We mixed the content of the tube generally and incubated it at 37 degrees centigrade for 2 minutes. Within added 1 microliter of M-Mlv- RT and mixed it by pipetting it gently up and down then we incubated it at 50 minutes at 37 degrees centigrade. We in activated direction by heating it for 15 min 70 degree

centigrade. The cDNA which was formed was then used as a template for amplification in PCR.

3.5 RT PCR

RT PCR technique utilized for varied gene expression and genetic profiling studies [126]. PCR assay is works on principle reverse transcription of messenger RNA extracted from blood or a tissue the target cDNA is amplified by standard PCR. It is a half quantitative technique numerous RT- PCR technique is being utilized to find and calculate specific expression of the gene. In samples from PC patients, RT-PCR technique which contain PSA prostate specific membrane antigen prostate stem cell antigen, PC specific antigen,mRNA and arrays are detected. RT PCR is carried out for the detection of TMPRSS to ERG transcripts. RT- PCR is carried out using 20 micro litre reaction containing 2 micro liter synthesize DNA in order to carry out the RT-PCR. Master Mix and other frozen regents are thawed so that they can be at room temperature. Mix them thoroughly and spin then and then place it on ice. Then the number of reaction is calculated that is needed to perform and then mix the Master mix and reaction plate on ice or at room temperature. The following percentage of component volumes are used which are as follows .

TABLE 3.3: RT-PCR Component.

Components	50 µl reaction volume	20 µl reaction volume	Final concentration
VeriQuest Probe qPCR Master Mix (2X)	25 µl	10 µl	1X
10 µM Forward Primer	2.5 µl	1.0 µl	500 nM (range 150-900 nM)
10 µM Reverse Primer	2.5 µl	1.0 µl	500 nM (range 150-900 nM)
10 µM TaqMan probe(s)	1.25 µl	0.5 µl	250 nM (range 100-500 nM)
Template DNA	X µl	X µl	see below*
Water, PCR Qualified	up to 50 µl	up to 20 µl	----

For each sample gently, vortex and then spin the content so that there are no bubbles in the sample. Load the plate into the real time PCR instrument and use

TABLE 3.4: Primer List Used in RT-PCR.

Gene		exon	Position	Primer /probe	Sequence 5'-3'
ERG		1	3-22	ERG1-S	CCCGAGGGACATGAGAGAAG
ERG		2	50-69	ERG2-AS	TTCCTCGGGTCTCCAAAGA
ERG		1-2	26-48	ERG12-PR	AGCGGCGCTCAGGTTATTCCAGG
ERG		5	564-583	ERG5-S	CACGAACGAGCGCAGAGTTA
ERG		6	611-630	ERG6-AS	CTGCCGCACATGGTCTGTAC
ERG		5-6	585-609	ERG56-PR	CGTGCCAGCAGATCCTACGCTATGG
TMPRSS2		1	-4-17	TMPRSS2/ERG-S	TAGGCGCGAGCTAAGCAGGAG
ERG		4	252-276	TMPRSS2/ERG-AS	TAGGCGCGAGCTAAGCAGGAG
Tmprss-ERG		-	-	MPRSS2/ERG-PR	GCGCGGCAGGAAGCCTTATCAGTT

the following recommended cycle in condition reaction tube tubes are kept on the ice to prevent any nonspecific amplification then reaction were incubated for 10 minutes at 95 centigrade, at 35 cycles for 1 minute 25 degree centigrade and then for 1 min at 63 C and then 1 min at 72 C. Then final elongation takes place at 10 minutes 72 degree centigrade.

3.6 Quantitative Real Time PCR

Quantitative real time PCR is a technique in which product obtained after PCR is quantitively observed at every PCR cycle [127]. Numerous types of fluorescent dyes bind two different strands DNA can be utilized for the discovery of cDNA. Fluorescent indications are observed by computer based program and concentration of fluorescent linked after each cycle the number of to target PCR product .The quantity of targets rises and a fluorescence signals become noticeable above background the cycle number which is required to hit above a set threshold is known as CT value .Based on the CT values of unidentified samples the concentration can straight be determined from standard curve. Housekeeping genes are used to control the variability in gene expression.

In order to carry out the TMPRSS2-ERG Fusion Transcript Quantification we performed the SYBR Green analysis [132]. Then calculate the number of reaction and then added the following components with the following volume by constantly keeping at room temperature by placing mixing them on ice.

Table 3.5: Quantitative RT-PCR Component.

Components	50 µl reaction volume	20 µl reaction volume	Final concentration
VeriQuest SYBR Green qPCR Master Mix with Fluorescein (2X)	25 µl	10 µl	1X
10 µM Forward Primer	2.5 µl	1.0 µl	500 nM (range 150-900 nM)
10 µM Reverse Primer	2.5 µl	1.0 µl	500 nM (range 150-900 nM)
Template DNA	X µl	X µl	see below*
Water, PCR Qualified	up to 50 µl	up to 20 µl	----

then load the plate into the real time PCR with the following conditions

Table 3.6: Cycle Condition for QPCR.

	UDG treatment	Taq DNA polymerase activation and UDG inactivation	PCR amplification	
			35-45 cycles	
	Hold	Hold	Denature	Anneal/ Extend
Temperature	50°C	95°C	95°C	60°C
Time	2 minutes	10 minutes	15 seconds	30-60 seconds

3.7 DELTA - DELTA CT Method for Fold Expression

The delta - delta CT method was used to calculate the TMPRSS-ERG fusion transcripts The steps are as follows [128].

Step 1:

Calculate the average Ct values for each gene

Step 2:

Calculate the ΔCt (delta Ct) for each sample by using the average Ct values:

ΔCt = Ct (gene of interest) – Ct (housekeeping gene)

Step 3:

$\Delta\Delta$Ct values for every sample (including for each control sample), we first need to average the ΔCt for the 3 control samples

ΔCt Control average = (C1+C1+C1)/3

Step 4:

Then calculate the $\Delta\Delta$Ct relative to the ΔCt Control average.

$\Delta\Delta$Ct = ΔCt (Treated sample) – ΔCt (Control average)

Step 5:

For fold gene expression we need to do 2 to the power of negative $\Delta\Delta$Ct:

Fold gene expression = 2ˆ -($\Delta\Delta$Ct)

Chapter 4

Results and Discussion

4.1 Patient Data

To carry out the current study urology department of different hospital and institute of Islamabad and Rawalpindi were requested for sample collection. PIMS responded to the request, the sample collection was approved from the department of urology ethical committee. The collection of sample and study was approved by ethical committee of Capital University of Science and Technology. In the current study analysis population, the participants were categorized into two groups controls and affected. In this research most of the population belongs to different part of Pakistan, as the sample were collected from Pakistan Institute of Medical Sciences. Demographic factors such as age, gender, ethnicity, height and family history were gathered using a questionnaire. The questionnaire also included clinicopathological information like PSA level, stage, chemotherapy and androgen inhibitors. and informed consent were also obtained from each participant the performer is attached in annexure.

A total of 11 subjects were included in which two were the controls and 9 affected. After Performa filling informed consent taken by all subject participants were requested for tissue sample donation after prostatectomy. The sample were collected within an hour of proctectomy. The tissue sample were labelled and tissue were

collected in normal saline and refrigerated at -80 C at the lab and then RNA isolation was carried out. The information we gathered through the questionnaire are listed in the table

TABLE 4.1: Sample Data Based on the Questionnaire Filled by Target Samples (participant) Listing Age ,Stage ,PSA and Treatments.

Sample Id	AGE	PSA LEVEL	STAGE	CHEMOTHERAPY	ANDROGEN INHIBITOR
PT 001	80	80	II	NON	NON
PT 002	65	110	III	NON	NON
PT003	78	150	II	NON	NON
PT004	42	54	II	NON	NON
PT005	59	110	I	NON	NON
PT006	22	78	NA	NON	NON
PT007	56	89	NA	NON	NON
PT008	40	136	I	NON	NON
PT009	67	67	I	NON	NON
CONTROL 1	74		-	-	-
CONTROL 2	53		-	-	-

4.2 Frequency of TMPRSS2-ERG Fusion

To determine the frequency of the (TMPRSS-ERG) fusion in clinically localized prostate cancer RNA was screened using RT PCR for 11 patient's tissue samples obtained after prostatectomy. There were four positive TMPRSS-ERG fusion messenger RNA positive samples found in our 11 cancer samples which is 37%. The results were similar to those reported by Tomlins et al [70] who discovered the fusion in 15 of 32 sample which is approximately 47% Soller et al [129] have reported expression of TMPRSS-ERG who have reported expression of 14 of 18 cancer. It has been until now known the TMPRSS-ERG is a major reason in the prostate cancer[130]. During the analysis this finding was confirmed using RT-PCR targeting genes with the primers listed. RT-PCR turn out to be the most frequently used method to detect relatively low circulating messenger RNA. However found no expression of TMPRSS diffusion in our control prostrate samples

which confirms the specificity of this fusion is only for cancer tissues. The results of the RT- PCR are listed table

TABLE 4.2: CT Values and Frequency Results for TMPRSS-ERG Fusion Obtained After RT-PCR.

Sample Id	AGE	PSA LEVEL	STAGE	TMPRSS-ERG fusion	CT VALUE
PT 001	78	80	NA	NEGATIVE	-
PT 002	65	110	II	POSITIVE	33.54
PT003	80	150	II	POSITIVE	34.41
PT004	42	54	I	NEGATIVE	-
PT005	59	110	II	POSITIVE	35.05
PT006	22	78	II	NEGATIVE	-
PT007	56	89	ND	NEGATIVE	-
PT008	40	136	III	POSITIVE	41.35
PT009	67	67	I	NEGATIVE	-
CONTROL 1	74		ND	NEGATIVE	
CONTROL 2	53		ND	NEGATIVE	

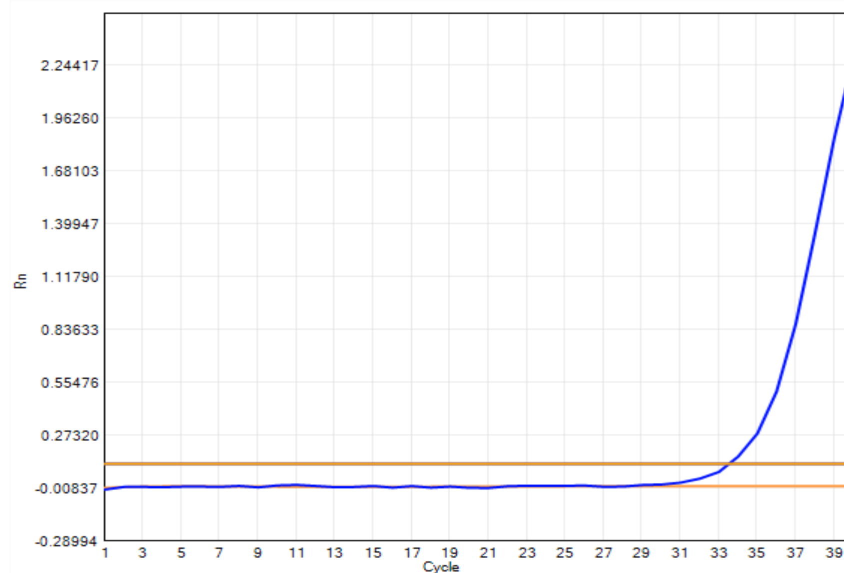

FIGURE 4.1: RT-PCR for Sample PT002.

The sample PT002 showed lowest level of fusion with CT values 33.54.

The figure shows the RT-PCR results for PT003, the CT values for the 34.41.The sample exhibits a second lowest expression of TMPRSS-ERG fusion.

The figure shows result of RT-PCR of sample PT005 with CT values 35.05. The sample shows high expression level of TMPRSS-ERG fusion

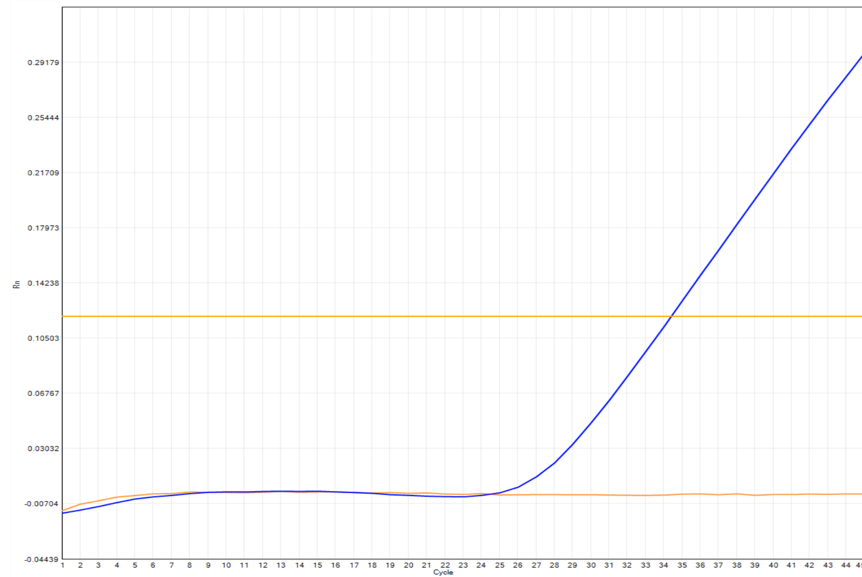

FIGURE 4.2: RT-PCR Results for PT003.

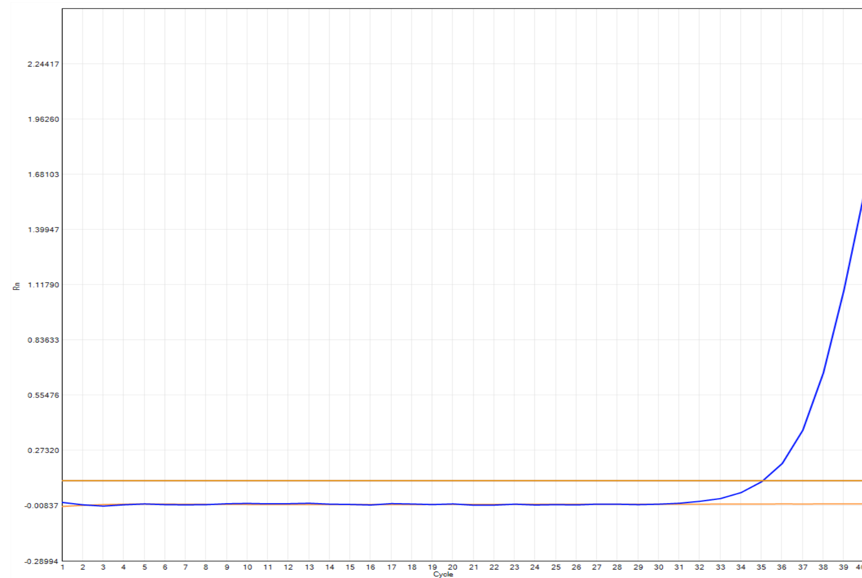

FIGURE 4.3: RT-PCR Results for PT005.

The figure shows result of RT-PCR of sample PT008 with CT values 41.51. The sample shows highest expression level of TMPRSS-ERG fusion. The presence of the TMPRSS-ERG fusion was further compared with other pathologic factors such as prostate serum antigen and stage.

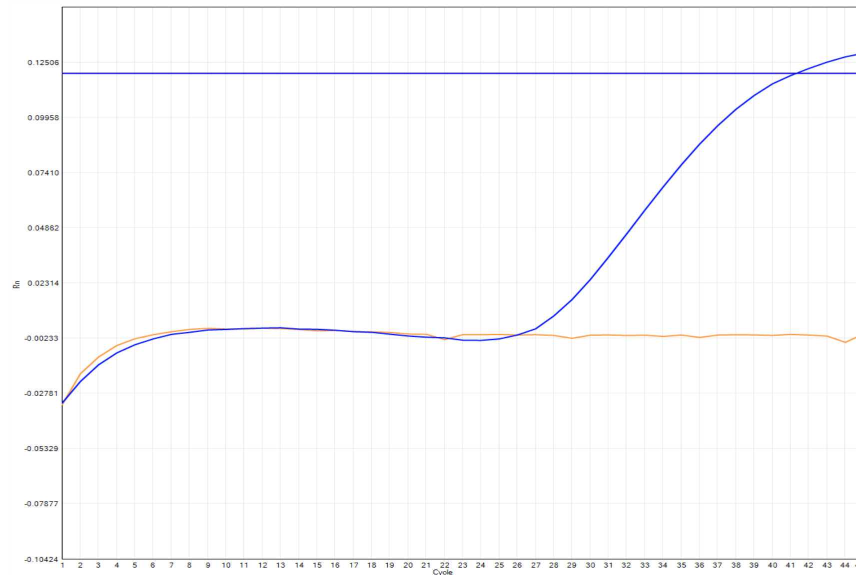

FIGURE 4.4: RT-PCR Results for PT008.

4.3 Relationship Between TMPRSS2-ERG Fusion and ERG Overexpression

To determine the relationship between TMPRSS-ERG detection and over expression different primers for exon 1/2 and exon 5/6 are used with the quantitative RT-PCR using SYBER green of the positive samples. The QPCR results revealed fusion exons of the TMPRSS-ERG. It showed that EXON 5/6 was more prominent in the positive samples. The QPCR results as follows:

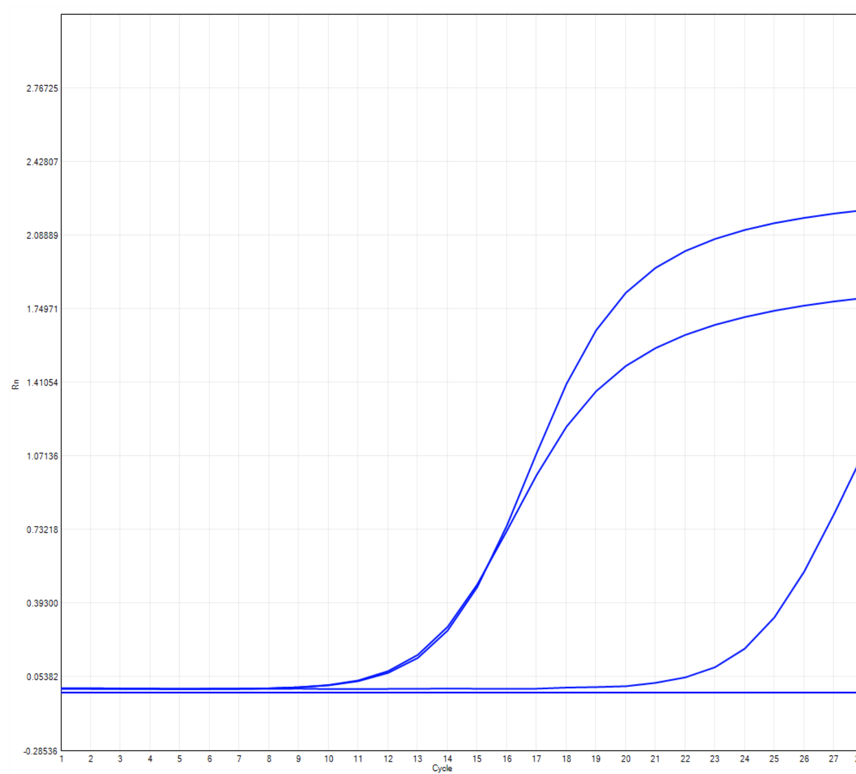

FIGURE 4.5: QPCR Result for 5/6(sample PT003,PT008,Gapdh).

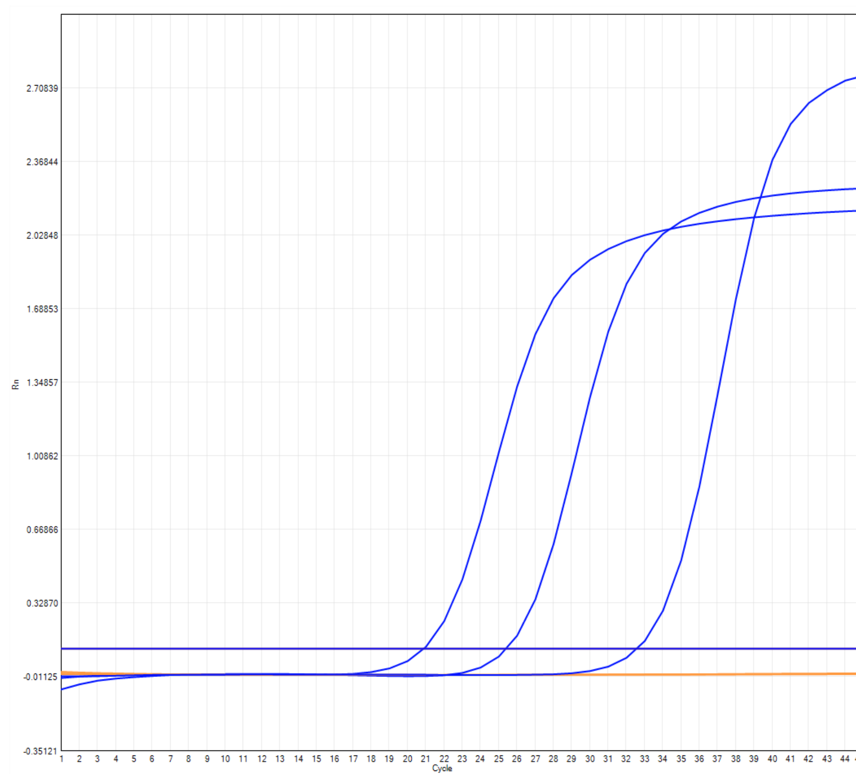

FIGURE 4.6: QPCR Result for 5/6(PT002,PT005,Gapdh).

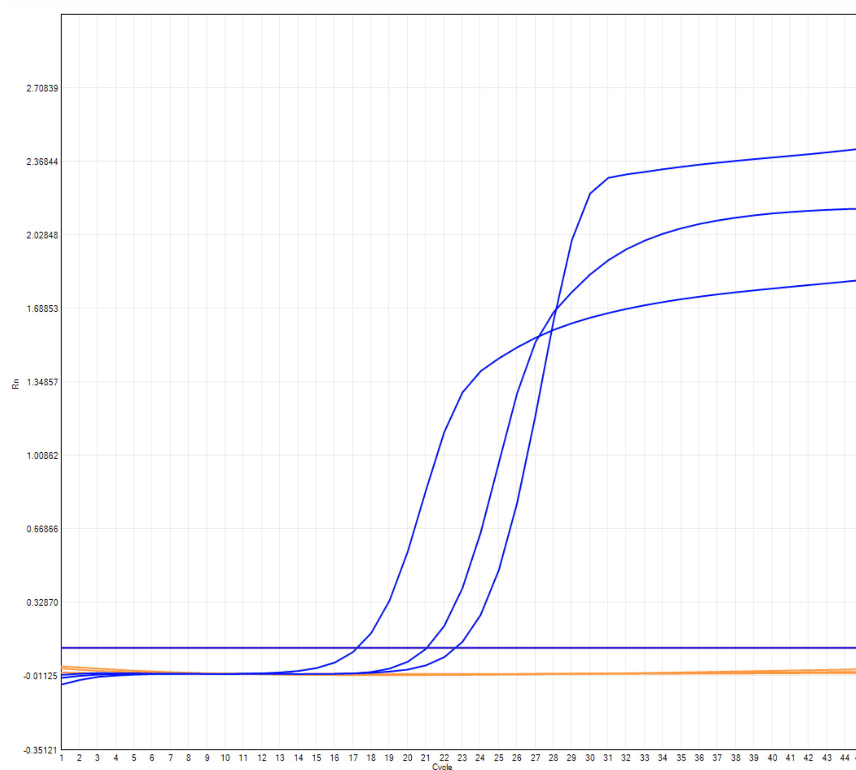

FIGURE 4.7: QPCR RESULT FOR 1/2 (sample PT003, PT008,Gapdh).

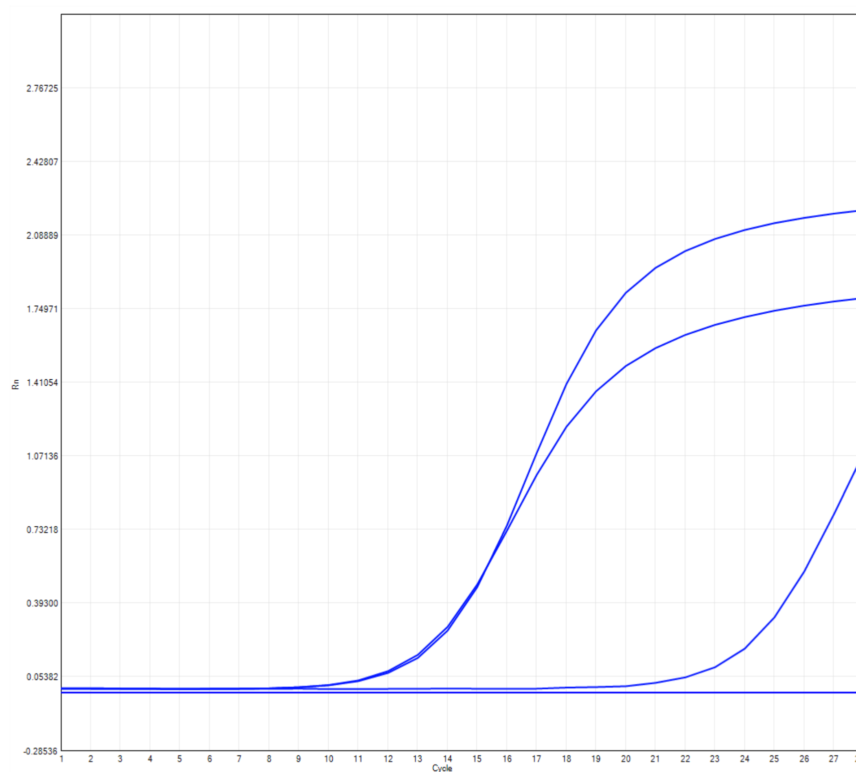

FIGURE 4.8: QPCR Results for 1/2 of (PT002 ,PT005,Gapdh).

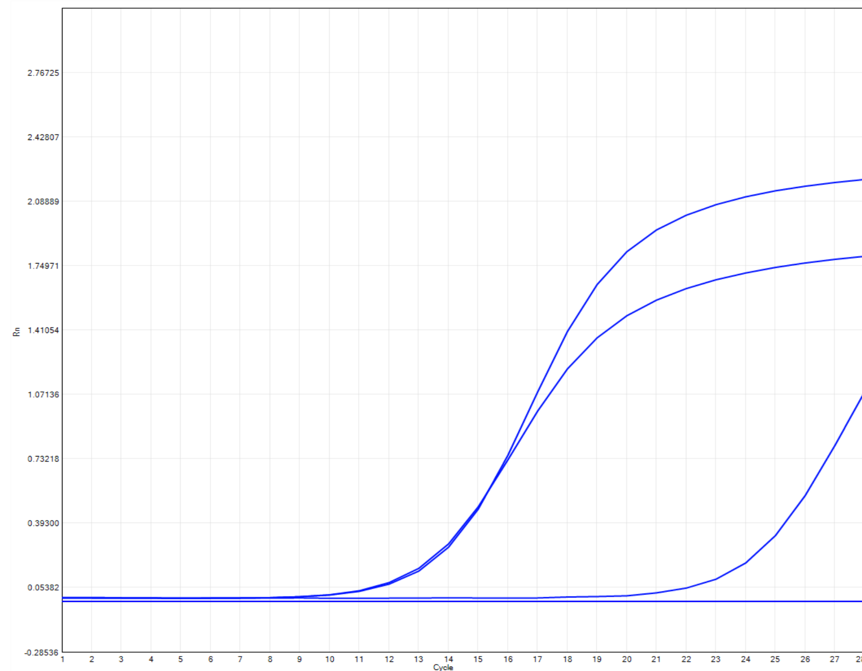

FIGURE 4.9: QPCR Results for 5/6 (GAPDH).

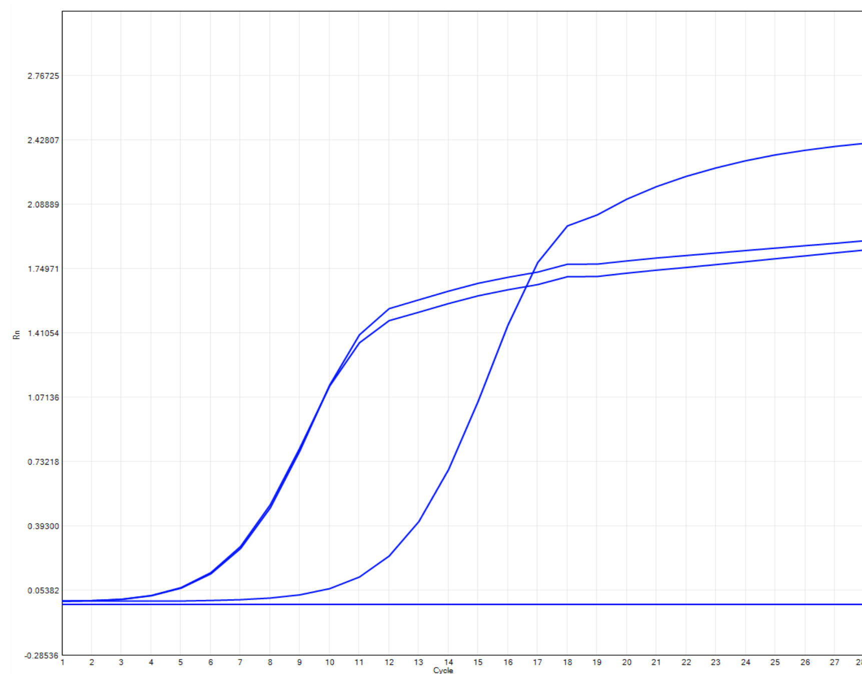

FIGURE 4.10: QPCR Results for 1/2 (GAPDH).

After the quantitative RT PCR, the method below was used to calculate the relative fold gene expression. The formula to calculate relative gene expression used is delta -delta CT which is as follows:

$$2^{-\Delta\Delta Ct}$$ Equation: 1

in which Ct values of gene of interest (TMPRSS-ERG 5/6 and 1/2) and housekeeping gene(GAPDH). These Ct values were obtained from QPCR. The Ct calculated

$$\Delta\ Ct = Ct\ (TMPRSS\text{-}ERG\ 5/6\ and\ 1/2) - Ct\ (housekeeping\ gene(GAPDH))$$

$\Delta\Delta$Ct is calculated as follows

$$\Delta\Delta\ Ct = \Delta\ Ct\ (Treated\ sample) - \Delta\ Ct\ (Control\ average)$$

Finally to get the fold expression of the gene of interest the following is used

$$Fold\ gene\ expression = 2\hat{}\text{-}(\Delta\Delta\ Ct)$$

The results of the fold expression is as follows:

TABLE 4.3: TMPRSS-ERG Expression Fold for Exon 5/6 Fusion.

Ct(GAPDH)				TMPRSS_ERG EXON 5/6					
Sample	Ct1	Ct2	Avg Ct	Ct1	Ct2	Avg Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
PT003	17.22	17.22	17.22	20.87	20.87	20.87	3.65	-0.69	1.61328
PT008	14.51	14.51	14.51	5.62	5.62	5.62	-8.89	-8.89	474.413
PT002	15.15	15.15	15.15	5.69	5.69	5.69	-9.46	-9.46	704.277
PT005	17.03	17.03	17.03	10.89	10.89	10.89	-6.14	-6.14	70.5219
Control	21.04	21.04	21.04	28.43	28.43	28.43	7.39	0	1

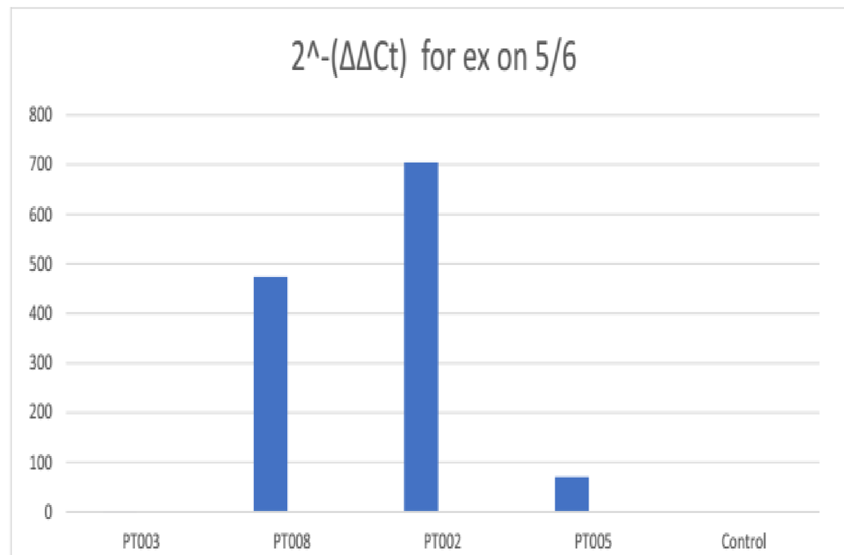

FIGURE 4.11: 2ˆ-(Ct) Exon 5/6 and Fold Expression Level.

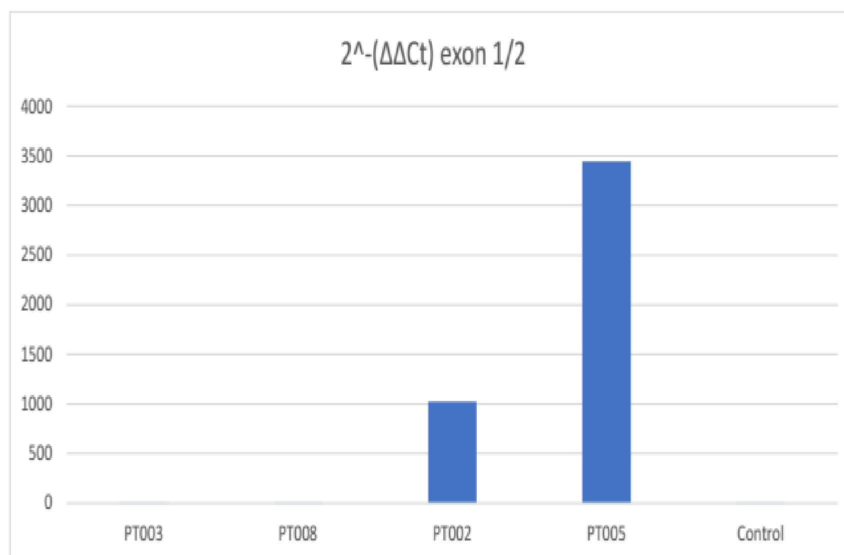

FIGURE 4.12: 2ˆ-(ΔΔCt) Exon 1/2 and fold Expression Level.

4.4 The Heterogeneity of the TMPRSS-ERG Transcript

During the analysis we found significant heterogeneity in the size of fusion transcripts. The transcript that was identified was RT PCR product starting from 444 to 762 and 38-762. The summary of TMPRSS is summarized in table below.

The results show that fusion of the type IV and type I is detected in a study

Table 4.4: Summary of Different Fusion Transcripts [130].

TYPES	TMP	ERG	SIZE	DESCRIPTION
I	12-17	38-762	779	Exon 1 of TMP + beginning of ERG exon 2
II	12-17	140-762	668	Exon 1 of TMP + beginning of ERG exon 3
III	12-17	226-762	591	Exon 1 of TMP + beginning of ERG exon 4
IV	12-17	444-762	373	373 Exon 1 of TMP + beginning of ERG exon 5
V	12-142	38-762	850	Exons 1 and 2 of TMP + beginning of ERG exon 2
VI	12-142	226-762	662	Exons 1 and 2 of TMP + beginning of ERG exon 4
VII	12-142	444-762	444	Exons 1 and 2 of TMP + beginning of ERG exon 5
VIII	12-365	226-762	885	Exons 1-3 of TMP + beginning of ERG exon 4

according to RT PCR results. According to the literature it has been reported that there are 8 fusion transcript types depending upon their size and exon fusion the type ranging from 1 to 4 contain exon 1 on TMPRSS which is fused with exon on 2, 3, 4 and 5 of ERG [130]. The Type 1 and 2 can initiate translation from native ATG code while type 3 and 4 start the translation from an internal ATG as exon 1 is non coding of TMPRSS. Our results were in accordance with Tomlins [62]. The protein translated by type 4 will be a messenger RNA consisting of 39 to 99 amino acid as described in the figure.

This multiple fusion transcript is result of alternative splicing rather than due to multiple genes which are not possible in Cancer [132]. We determine the clinical and pathological variable that correlate with expression of type 4. The type 4 and type I is linked with aggressive disease and the variants of this type of fusion early recurrence early PSA recurrences. Association with seminal vesicle invasion which is one of the variable associated with aggressive disease. However there is a hypothesis that genes are translated more efficiently who have native initiation compared to internal The disease the expression of type 4 and type I is simple association with progression [133]. The table below summarizes the overall results of the research.

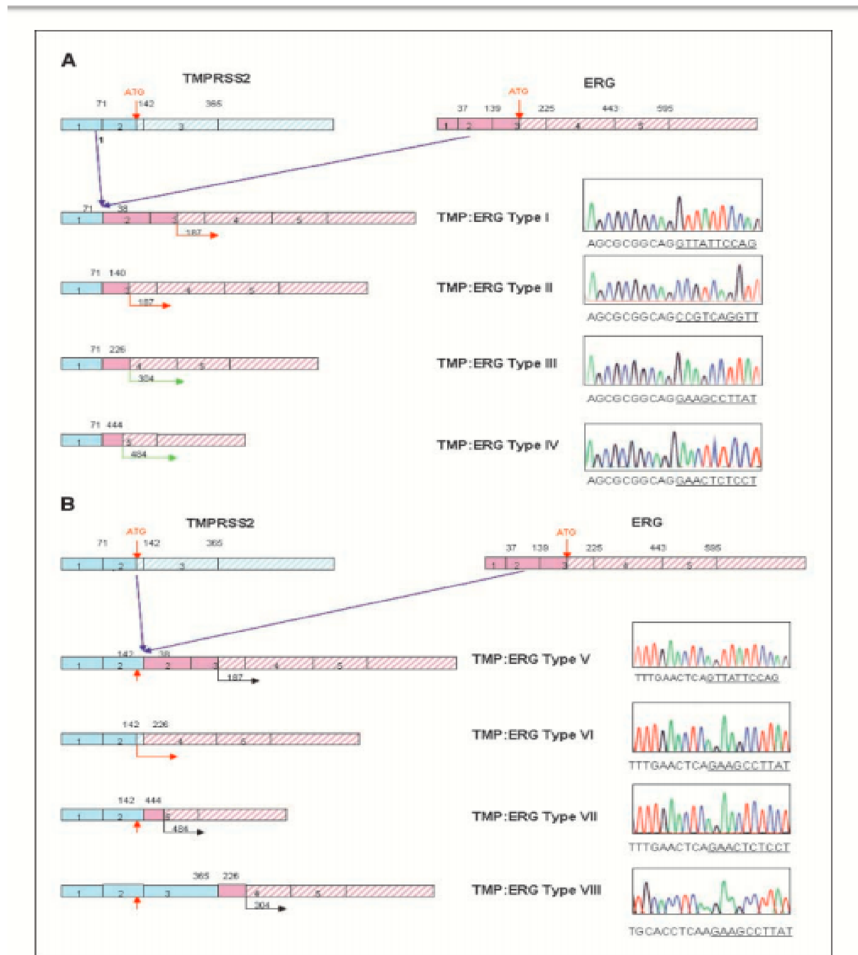

FIGURE 4.13: Identification and Characterization of TMPRSS2/ERG Fusions mRNAs in Prostate Cancer. A, Schematic Structure of TMPRSS2/ERG Fusion mRNAs Types I to IV [131].

4.5 Clinicopathological Data Relation with TMPRSS-ERG Fusion

One of the aim of this study was to find whether or not there was association of PC patients with TMPRSS-ERG fusion. According to literature study the mechanism of TMPRSS-ERG fusion is being studied in vivo and ex vivo but the exact biological function of this Fusion in PC is not yet understood. As it is discussed earlier that TMPRSS-ERG is not the cause behind the formation of prostate neoplasia. The tumors needs association with other genetic aberration which includes increase of function mutation in MYC, EZH2 oncogenes, suppressed PTEN, NKX 3.1 and T P53 and AKT pathway which is involved in PC so in case

TABLE 4.5: Results, Column 1 Frequency, Column 2 Fusion Type, Column 3 RT-PCR, Column 4 5/6 Fold Ratio to 1/3 Exon.

SAMPLE ID	TMPRSS-ERG FUSION	TMPRSS-ERG TRANSCRIPT TYPE	TMPRSS-ERG RT-PCR	ERG EXON5/6: EXONS ½ RT-PCR
PT 001	NEGATIVE	-	-	
PT 002	POSITIVE	TYPE I	33.54	0.687770909
PT003	POSITIVE	TYPE IV	34.41	28.84
PT004	NEGATIVE	-	-	
PT005	POSITIVE	TYPE IV	35.05	1200.982988
PT006	NEGATIVE	-	-	
PT007	NEGATIVE	-	-	
PT008	POSITIVE	Type I	41.35	0.020474897
PT009	NEGATIVE	-	-	
CONTROL 1	NEGATIVE	-		
CONTROL 2	NEGATIVE	-		

for defining association between fusion and clinicopathological data these factors should be kept in mind. In this study we found TMPRSS-ERG fusion is most frequently in patient who had tumor stage 2 and 3 GS 6 or 8 and WHO either 3 or 4, which shows that patients who had fusion harbored more progressive PC. The overall PSA in the fusion positive patient was above 10ng/ ml. Results also reported by Rajput Airtel [134] in which he analyzed 196 PC cases of which 101 patient harbored fusion. Fusion was confirmed by RT PCR and association of sequencing among the patient fusion status and prostate stage was noteworthy. In this study statistical importance can be established between positive TMPRSS-ERG fusion patient and clinicopathological data as the PC had severe stages of the tumor and high PSA. The studies reported before also show similar association between the clinicopathological data and fusions. However, the study can be further strengthened by including other biomarkers as well and larger sample can be better correlation between the clinicopathology and fusion. Which can be designed in the future.

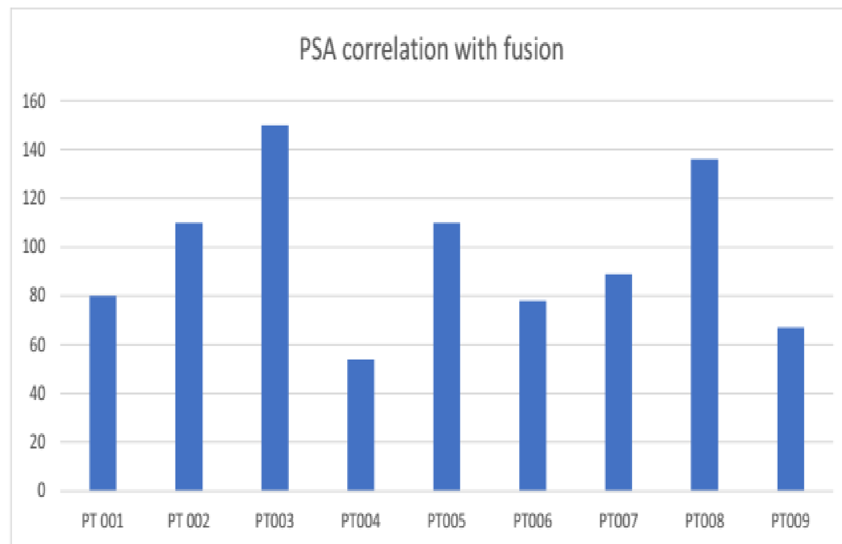

FIGURE 4.14: PSA and Fusion Correlation

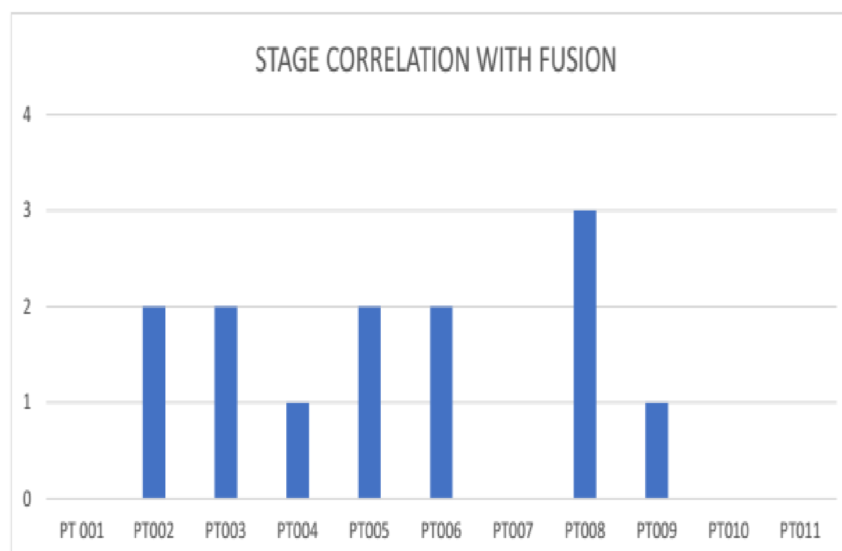

FIGURE 4.15: Stage and Fusion Correlation

Chapter 5

Conclusion

The studies confirm that the presence of TMPRSS -ERG fusion gene is a very common marker in prostate cancer. We were successful in finding TMPRSS fusion in Pakistani population. However, data is consistent with the idea that the isoforms of the fusion messenger RNA expressed and their expression level may affect disease progression. The TMPRSS is a trans membrane-bound same protease that is localized to prostate and is overexpressed in prostate epithelium. It was reported by Nelson that TMPRSS promoter region contains the androgen response element which shows androgen inducible expression of gene. In our study we found no evidence of TMPRSS-ERG Fusion in benign prostate tissue and that expression of specific messenger RNA is associated with aggressive disease. Our study indicate that prostate cancer tissue contains the miscellany of fusion transcript which are the key in prostate cancer disease aggressiveness. These fusions must be evaluated at transcription level to unfold heterogeneity of disease because prostate cancer is a heterogenous disease and there are no reliable marks for prostate cancer detection prostate specific antigen in Pakistan it is clear that a combination of marker will be very helpful in prostate Cancer diagnosis treatment.

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Appendices

CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY

GENETIC STUDY OF PROSTATE CANCER TISSUE IN PAKISTANI POPULATION

File ID: ______________ Date: ______________________

Sample ID: ______________ Hospital No. ________ Ward: ________ Bed No.____

Name: ______________________________ **Status:** *Normal* ☐ *Patient* ☐

Address: ____

Contact No.____

Personal Information:

Age: _______

Gender: _________

Ethnicity: _________

Height____________

Family History:

Diseased Parents (Father): ____(Mother)____

Diseased Children (Boys):_______(Girls):____

Diseased Siblings (Male):_______(Female):_______

Clinical and Laboratory Parameters

1. *PSA level:* ___________
2. *Stage (I to IV):*_________
3. *Chemotherapy:* ______________
4. *Androgen Inhibitors:* ______________

Refered by:

Name: _________________________

Contact: _______________________

Informed Consent:

I am donating my tissue for research purposes only and not for commercial use.

میں اپنا خون صرف تحقیقی مقاصد کے لیے عطیہ کر رہا / رہی ہوں۔

Signature:

Genetic Study of Prostrate Cancer Tissue in Pakistan Population