Increased FABP12 expression in prostate cancer and its possible promoting role in tumorigenicity

THESIS SUBMITTED IN ACCORDANCE WITH THE REQUESTMENTS OF

THE UNIVERSITY OF LIVERPOOL

FOR THE DEGREE OF DOCTOR IN PHILOSOPHY

By:

Asmaa Imad AL-Bayati

March, 2021

Dedication

To the soul of my father, who lost his life due to cancer. I hope by my work I can contribute to beat this disease.

To my mother, who made my dreams come true.

Abstract

Prostate Cancer is the most common male cancer in the developed world. The growth and spread of prostate cancer cells are caused by the increased activity of cancer-promoting genes. Thus, identification of these genes is an essential step for discovering reliable therapeutic targets, an example of these genes is the *fatty acids binding proteins 5* (*FABP5*) gene. FABPs are a family of 12 cytosolic fatty acid-transporters. In contrast to the extensive research work in the tumorigenicity-promoting role of FABP5, the studies in other FABPs is relatively rare and the possible role of other FABPs was not fully understood. In this PhD project, I have identified another FABP family gene *FABP12*'s role in prostate cancer. Preliminary analysis showed that the level of FABP12 mRNA is increased by up to 105- times in prostate cancer cell lines compared with that in benign prostatic epithelial cells. It is increased by 3.6 - to 16.3- times at the protein levels. To assess systematically the functional role of FABP12 in malignant progression of the cancer cells and hence to validate it as a novel therapeutic target, I first immunohistochemically stained an archival set of prostate tissues and found that FABP12 is greatly increased in prostate cancer when compared with the benign tissues and the increased FABP12 is significantly associated with increased degree of malignancy. I also found that the increased FABP12 is significantly correlated with the reduced time of patient survival. When the effect of FABP12 on malignant progression of the cancer cells was fully investigated using gene-editing technique CRISPR/Cas9 to knockout *FABP12*, it was found that the FABP12-knockout cells exhibited a significant reduction in proliferation, invasion, migration, and anchorage-independent growth. These results suggested that FABP12 is novel promoting factor, a diagnostic and prognostic marker, and a possible therapeutic target for prostate cancer.

ACKNOWLEDGMENTS

First all thanks and praise are due to God

I would like to offer my sincerest thanks to my supervisor, Prof. Ke Youqiang. If it is not you, I would not have completed my PhD. Prof. Ke is not only a supervisor, but also like a second father. Being always there for me to encourage and to guide me in all aspects. Thank you, Prof. Ke, for believing in me and for taking me as a PhD student in your group.

I would like to thank my family, my dad, I wish you were alive to celebrate our lifelong dream, I am pretty sure you feel us. I only wish that you were still alive to see me when I earn my PhD title and graduate, you waited for this moment during your life. I know you would have been very proud of me.

My mum, who sacrificed everything for my sake, her support and prayers make this dream come true. Thank you, mom, for being the strength I always needed and the love I realize I always had. You are simply extraordinary. Thank you immensely, mom, from the bottom of my heart.

Special thanks to my lovely sister, who enlighten every day of my life, the person whom I always rely on throughout my academic journey and helped me a lot during my PhD.

I am indebted to many of my colleagues: Juliana Jamal and Liza Ishak. Also, I would like to thank my group who supported me: Dr. Jiacheng Zhang , Mr. Bandar Alenezi, Mr. Mohammed Alsubhi, Mr. Abdulghani Naeem, and Mr. Saud Abdulsamad. Finally, I'd like to express my appreciation to all staff members of University of Liverpool, for their help in my study.

DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

DECLARATION OF ORIGINALITY

This thesis is a product of my own work which has been carried out during my PhD study in the Department of Molecular and Clinical Cancer Medicine (Pathology), University of Liverpool, between March 2016 and September 2019. All experiments presented in the result chapter were performed by me under the supervision of my supervisor, Professor Youqiang Ke .

Publications

-Waseem Al-Jameel, Gang He, Xi Jin, Jiacheng Zhang, **Asmaa Al-Bayati,** Angela Platt-Higgins, Philip S Rudland, and Youqiang Ke. Inactivated Fatty Acid-Binding Protein 5 (FABP5) is a Suppressor for Prostate Cancer. Prime Archives in Genetics 2020 (e-Book) Chapter One, Page 1-34. https://videleaf.com/inactivated-fatty-acid-binding-protein-5-fabp5-is-a-suppressor-for-prostate-cancer/

- Waseem Al-Jameel, Xiaojun Gou, Xi Jin, Jiacheng Zhang, Qiang Wei, Jianzhong Ai, Hong Li, **Asmaa Al-Bayati,** Angela Platt-Higgins, Andrew Pettitt, Philip S. Rudland, and Youqiang Ke. Inactivated FABP5 suppresses malignant progression of prostate cancer cells by inhibiting the activation of nuclear fatty acid receptor PPARγ. Genes & Cancer 2019, **10:** 80-96. https://doi.org/10.18632/genesandcancer.192

- **Asmaa AL-Bayati,** MajedAL-Fayi, Waseem Al-Jameel, Jiacheng Zhang, Youqiang Ke. Increased FABP12 expression in prostate cancer and its possible promoting role in malignant progression. Eur. J Surg Oncol. 2017, **43:** 2208. https://doi.org/10.1016/j.ejso.2017.10.091.

- **Asmaa AL-Bayati**, Majed AL-Fayi, Waseem Al-Jameel, Zhang Jiacheng, Ke Youqiang. Increased FABP12 expression in prostate cancer and its possible promoting role in malignant progression. A Poster Presentation in the NCRI Conference. (5-8 Nov. 2017, Liverpool, UK).

Table of contents

List of abbreviations

Abbreviations	Full name
AR	Androgen receptor
AREs	Androgen response elements
APS	Ammonium persulphate
BPH	Benign prostatic hyperplasia
BRCA1/BRCA2	Breast cancer mutated gene
CZ	Central zone.
χ^2	Chi-square
CK	Cytokeratin
DHT	Dihydrotestosterone
DRE	Digital rectal examination
DMSO	Dimethyl sulphoxide
ERSPC	European Randomized study of Screening for Prostate Cancer
EDTA	Ethylene diamine tetra acetic acid
FABP	Fatty acid binding protein
GS	Gleason score
HCC	Hepatocellular carcinoma
hK3	Human kallikrein 3
IL-6	Interleukin-6
LH	Luteinizing hormone
LUTS	Lower urinary tract symptoms
PSA	Prostate-specific-antigen

PZ	Peripheral zone
PAP	Prostatic acid phosphatase
PIN	Prostatic Intraepithelial Neoplasia
PCA3	Prostate cancer antigen 3
PPAR	Peroxisome proliferator-activated receptor
PVDF	PolyVinylidene DiFluoride
SPSS	Statistical Package for Social Sciences
TURP	Transurethral resection of the prostate
TZ	Transition zone

CHAPTER ONE:

INTRODUCTION

1. INTRODUCTION

1.1 Epidemiology of prostate cancer

1.1.1 Incidence

Prostate cancer (PCa) is the 2nd most common malignancy internationally. On 2012, there were 1.1 million new cases diagnosed in the world (72). In the United Kingdom, PCa is considered the most common cancer in males. Statistical analysis on the period from 2014 to 2016 showed that there were approximately 47,700 new cases of PCa diagnosed per year. In 2016, PCa accounts for 26% of all new cancer cases in males in the UK. The number of cases had been kept increasing in the past 20 years. As shown in Figure 1, amongst the 20 most frequently occurred cancer types, prostate cancer ranked the second highest cancer type diagnosed each year (25).

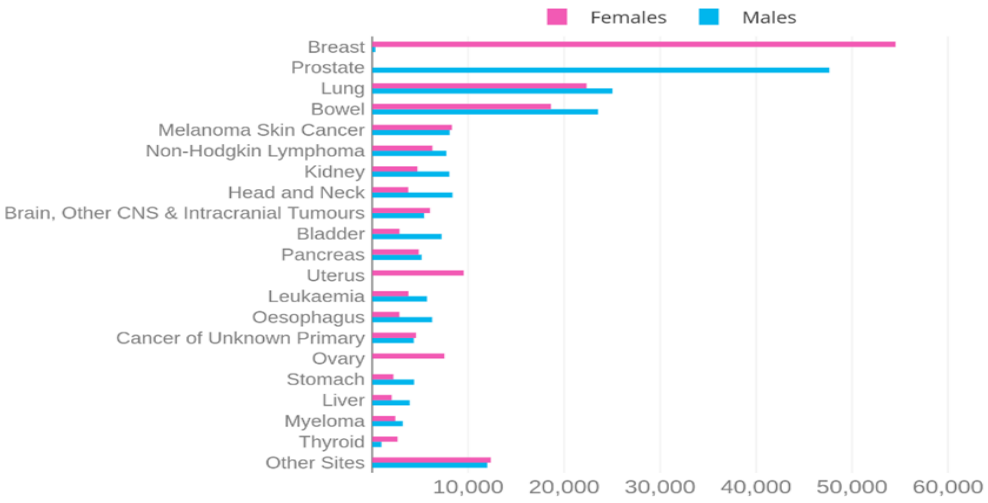


Figure 1: Incidence amongst male and female for the twenty most common cancers in the UK (25).

PCa was largely regarded as an old man disease, although the start age being diagnosed appeared to decrease in the recent years. An American study reported that likelihood to

develop PCa rose with aging. Thus, men aged 39 years had a 0.005% risk to develop PCa, whereas this percentage increased to 2.2% and 13.7% for age groups of 40 to 59 and 60 to 79, respectively (170).

As shown in Figure 2, the incidence of PCa occurred most frequently among men aged 75-79. It is documented in the past record that the incidence of PCa increases as the increasing age of the patients until 79 year old; then the incidence starts to decrease as the increasing ages of the patients until 90 (25).

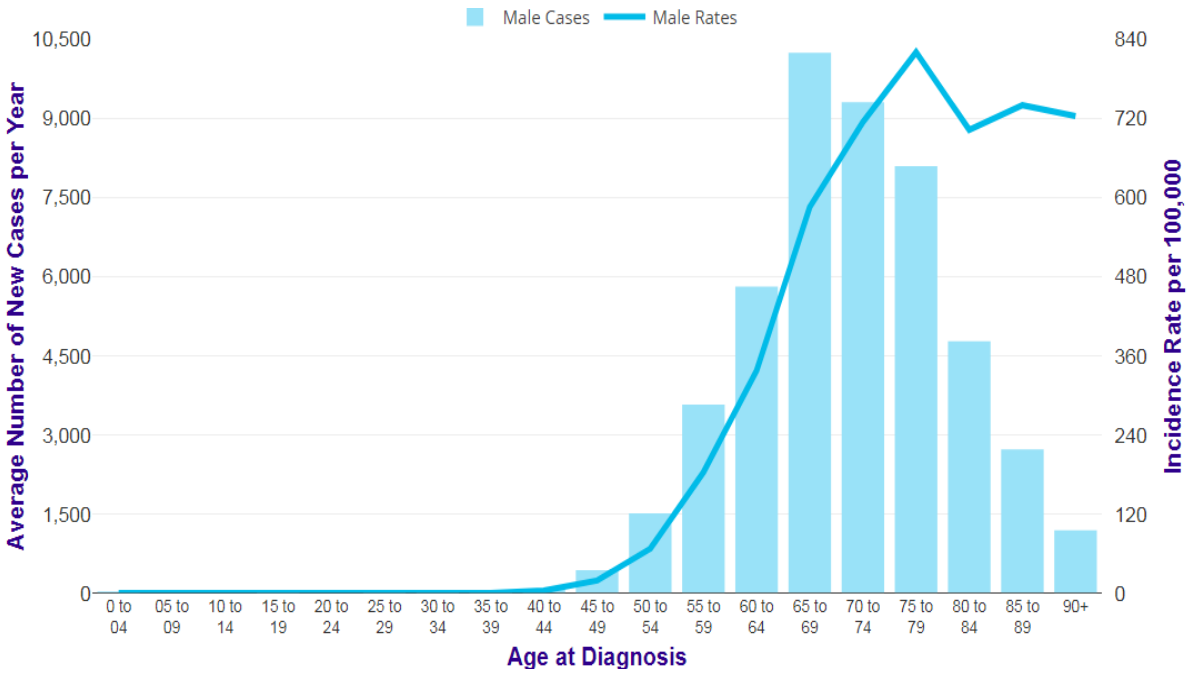


Figure 2: Age-based incidence of PCa in the UK during 2014-2016 (25). This graph illustrates new cases categorized by age groups.

Most PCa cases are diagnosed in the developed countries, such as those in North America and in West Europe. As shown in Figure 3, highest rate of PCa was found in North America, Oceana, West and North Of Europe (37).

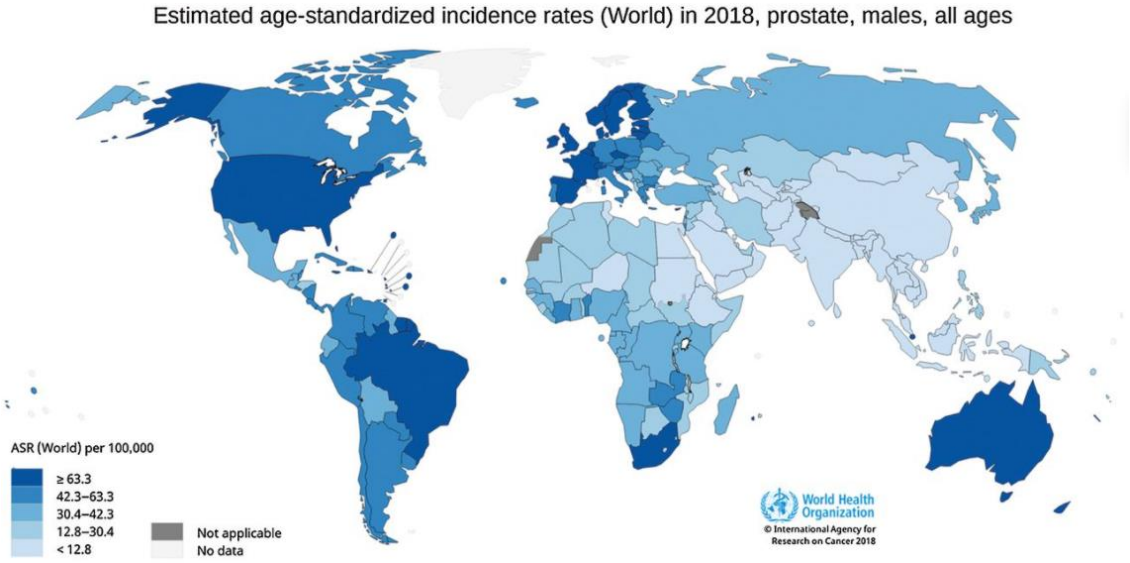


Figure 3: worldwide prostate cancer incidence in 2018 (72). ASR: Age-Standard Rate/100, 000.

1.1.2 Mortality

In UK men, PCa is the second most common cause of cancer related death. There are approximately 11,700 deaths yearly, corresponding to 32 deaths per day (25). There is a direct relationship between age and prostate cancer mortality, as reflected by the highest mortality in elderly as shown in figure 4.

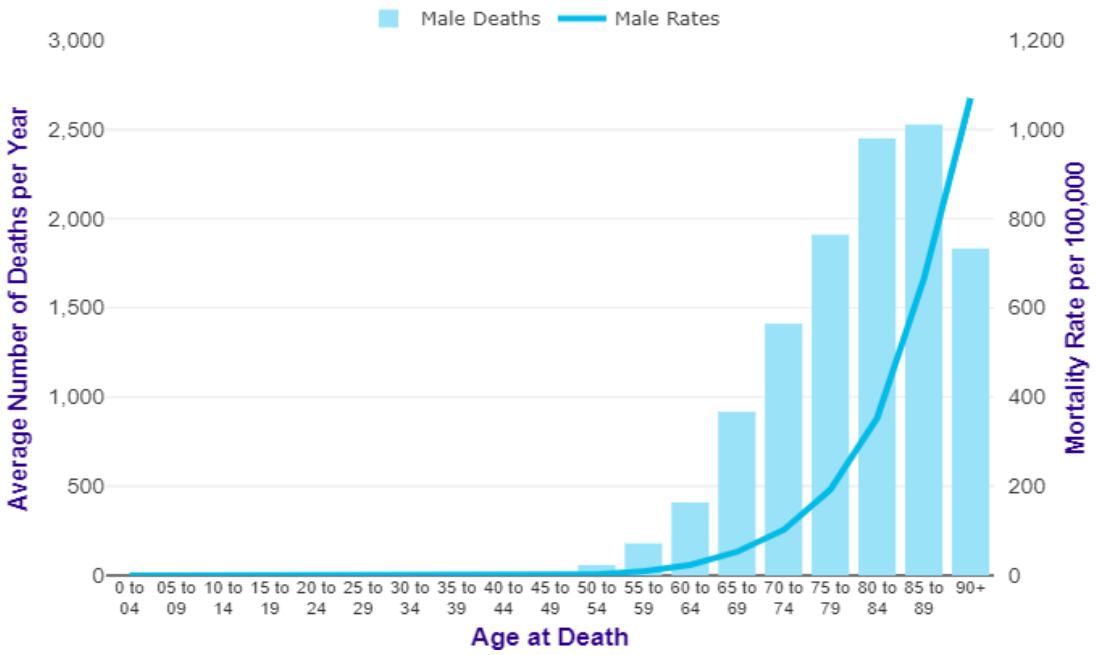


Figure 4: Worldwide PCa morality. Death is more common in poor countries like Africa, South America and the Caribbean Countries (37, 25).

1.1.3 Survival

During 2010, it was projected that ten-year survival for PCa is 84%. The ten-year survival time for this cancer had been increased since 1970, as illustrated in Figure 5. Over a 40 years period, from 1971 to 2010, there was a 59% increase in the 10-year survival rate. Nowadays in England, more than 8 in 10 men are expected to live after initial PCa diagnosis for at least ten years. This could be attributable to early diagnostic tests: prostate-specific antigen (PSA) and transurethral resection of prostate (TURP) (25, 97). When 20 most frequently occurred cancers in Britain are compared, the ten-year survival for PCa stands at the 3rd highest position (25).

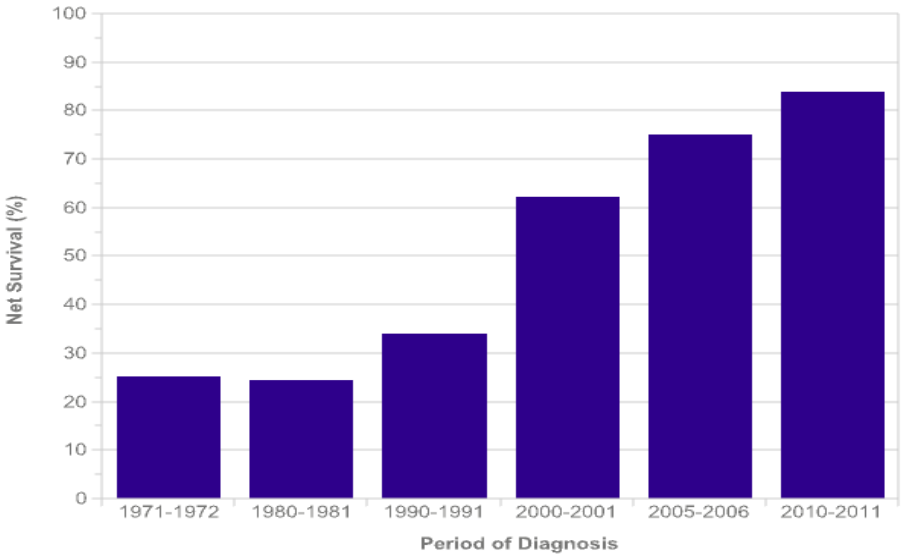


Figure 5. The ten-year survival of PCa (25). The bar charts highlight the ten-year survival in British PCa cases in those aged 15 to 99 years.

1.1.4 Risk factors

1.1.4.1 Age

Ageing was observed to be associated with PCa risk by many studies. It was reported that the group of 75 – 79-year-old men was at the highest risk to develop PCa (25). While in the younger group of less than 50 years of age, PCa affected just 1% of the population (233, 243). Carter et al. suggested that the likelihood to have malignant histological changes in prostate increased with increasing age and it is about 20% in the age group of 50-60, rising up to 50% in the age group of 70-80 years (77).

1.1.4.2 Ethnicity

The risk of PCa is different between different ethnic groups. The African American men have the highest PCa risk, followed by the Caucasian men. The Asian men have the lowest PCa risk. Regarding to the causes of different risks amongst the different ethnic groups, numerous epidemiological studies were performed. It is still undecided whether this is attributable to socioeconomic, genetics or environmental factors (131, 25). One of explanations for lower PCa incidence in Asian population than in American was dietary variations. It was speculated that intake of Isoflavonoids products such as soybean by Japanese men might have protected them from developing PCa (47).

Many studies speculated that African American men have higher rates of PCa prevalence and mortality compared to other ethnic groups. In these studies, researchers found the family history of PCa may play a role in higher prevalence rate among African American group. However, Makinen and associates suggested that family history of PCa is not significantly correlated with higher prevalence rate in African American men (134, 226). Another explanation for increased PCa in certain ethnic groups was genetic involvement. A genetic locus 8q24 was suggested to be a risk factor for PCa. Researchers found that this locus was correlated with an 8 % higher risk of PCa among the white men while in African men had a 16 % higher risk (202).

More extensive research is needed to understand the effect of ethnic origin in development of PCa.

1.1.4.3 Family history

Previous studies reported increased risk of PCa with those whose father, brother, or second-degree relatives have had the disease, with ratios of 2.4, 3.3 and 1.9 respectively (25). It was reported that men with positive family history of PCa, will develop the disease by 6-7 years earlier than those with negative family history of PCa. Causes behind PCa clustering in families are still under studies, however studies hypothesized that in addition to the possibility of gene involvement, there could be a likelihood that those family members all exposed to same environmental effects (47).

A study conducted by Smith et al. (1996) revealed that the presence of HPC1 (hereditary prostate cancer 1) locus (which lies on long arm of chromosome 1), in Swedish and American high risk families, in which they develop PCa at younger age, and about 5 or more members affected with PCa. However, a large meta-analysis involved 772 families with inherited PCa suggested that the association with HPC1 was merely 6% of families (171, 47).

1.1.4.4 Diet

Numerous studies have attempted to explain the role of fat intake in PCa. High fat food and consumption of red meat were linked to development of PCa. Researchers have presumed a correlation between PCa and animal fat intake. A previous study reported that men who consumed linoleic acid were at a 5-folded higher risk to develop PCa. An anticipated hypothesis for increased PCa with high fat intake was that fatty acids exert their effect on serum sex hormone level, which affects the PCa occurrence (96, 176).

Studies on Japanese immigrants to USA may provide a clue to the effect of diet and environmental factors, as it was thought that these men maintained low PCa risk, however, they shift to high risk group, and this risk was correlated with time they lived in a new country . A study on total fat intake and its risk in PCa between African Americans and Asian Americans revealed 15% higher incidence of PCa (46, 87, 195, 188, 227). Red meat may be correlated with PCa, although more studies needed.

Another pathway, through which fatty diet can affect PCa cancer development is through stimulation of insulin like growth factor-1 (IGF-1), which is secreted by liver and stimulated by high fat intake. IGF-1 can promote proliferation and suppress apoptosis, and this was proved by cohort studies in which men with higher IGF-1 concentration had a 4.3-fold higher risk to develop PCa (39, 47).

1.2 The pathology of prostate cancer

1.2.1 Prostate anatomy

The prostate is a fibro muscular male reproductive gland, which encircles the male urethra. It is surrounded by a thin sheath forming the true capsule. Exterior to this is a pseudo-capsule which originates from 3 coverings layers, these layers cover the gland from the frontal, behind and side aspects. Areas from above the gland run with the bladder neck as shown in figure 6. The prostate gland constitutes of ducts and a basement membrane and is contained by stroma; the ducts have a columnar and secretory cell lining. Prostate gland's main function is the provision of proteins and electrolytes to secrete prostatic fluid that aids in sperm transportation (110, 126).

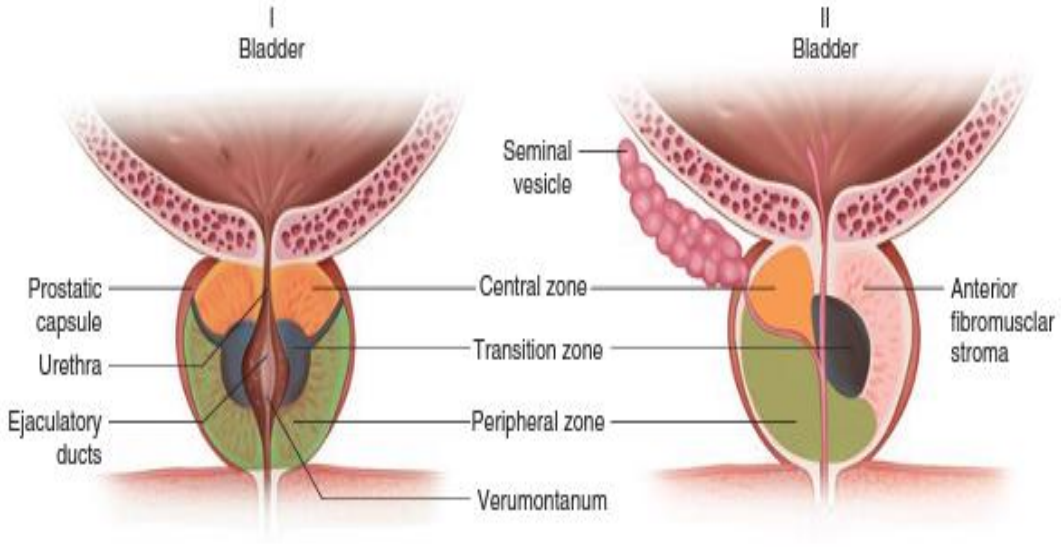


Figure 6: Prostate anatomy showing the three zones of the prostate and their relation with the bladder (110).

Zones of the prostate gland include: the transitional zone, the central zone, and the peripheral zone. The bulk of the prostate includes glandular tissue (70 %) and the remaining 30 % is fibromuscular stroma tissue. Ejaculatory ducts surround the central zone. Twenty-five percent of the central zone consists of glandular tissue. Prostatic adenocarcinoma is very rare to develop in this zone (only around 1-5%). In contrast, the peripheral zone has 70 % glandular tissue, its position is in the lateral and posterior of the prostate, most of prostate adenocarcinomas occur in this zone (70%). This is the area that can be recognized by digital rectal examination (DRE) test. The central zone of the prostate lies as a triangle which has its base in continuity with seminal vesicles and its tip is at verumontanum (148,144, 65, 110).

1.2.2 Epithelial cells of prostate

Three distinct cells entities were identified in the prostatic epithelium, each of which is characterized by its own shape, function and relation to cancer development. As represented in Figure 7, the main type is the secretory luminal cells, which secrete prostatic proteins and are androgen dependent. Therefore, the secretory luminal cells express androgen receptor (1).

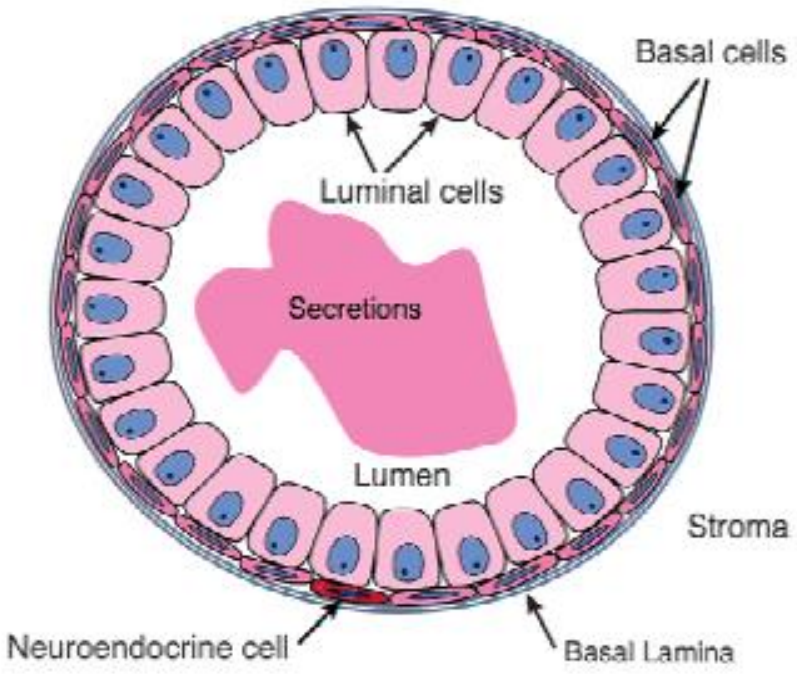


Figure 7: Cell types in a prostate duct (1).

The second type is the basal cells, which are positioned as a continuum sheet between luminal cells and the basement membrane. The type less frequently seen is the neuroendocrine cells. With their ambiguous embryology origin, neuroendocrine cells may assist in luminal cell growth through signaling. They are androgen-independent, often found discretely in basal layer, and express neuropeptides, such as serotonin. In spite of their scarcity number, whenever they aggregate, or acquire their features, neuroendocrine cells constitute a distinctive characteristic of aggressive form of PCa (1).

1.2.3 Prostate cancer

1.2.3.1 Benign prostatic hyperplasia

When prostatic epithelial cells that surround the urethra proliferate and overgrow, this eventually results in benign prostatic hyperplasia (BPH). BPH refers to a condition in which the prostate gland enlarges and both stromal and glandular structures grow in a non-malignant manner. Its incidence increases with age. The causes behind BPH are unknown; however, it was thought to result from hormonal or growth factor stimuli. Clinically, patients with BPH experience urination abnormalities such as high frequency, urgency, etc. BPH is usually diagnosed through DRE, or through PSA. BPH was found to start at the transitional zone, specifically from submucosal layer. Since this area surrounds the urethra, the compression will result in lower urinary tract symptoms (141, 57, 110).

1.2.3.2 Prostatic Intraepithelial Neoplasia

When epithelial cells proliferate non-invasively within the ducts, the condition is called Prostatic Intraepithelial Neoplasia (PIN), which is regarded as a cancer precursor (110). PIN is a pre-neoplastic state in which epithelium cells proliferate with significant cytoplasmic and architectural atypia, restricted to ducts and acini. PIN includes both low and high grades, the latter is prevalent in prostate cancer cases. Whenever it is diagnosed on biopsy, it elevates the risk of having PCa in next biopsies, while the former is not associated with elevated risk of PCa in later biopsy. Histological pattern of high-grade PIN can be subdivided into: tufting, micropapillary, cribriform and flat (1).

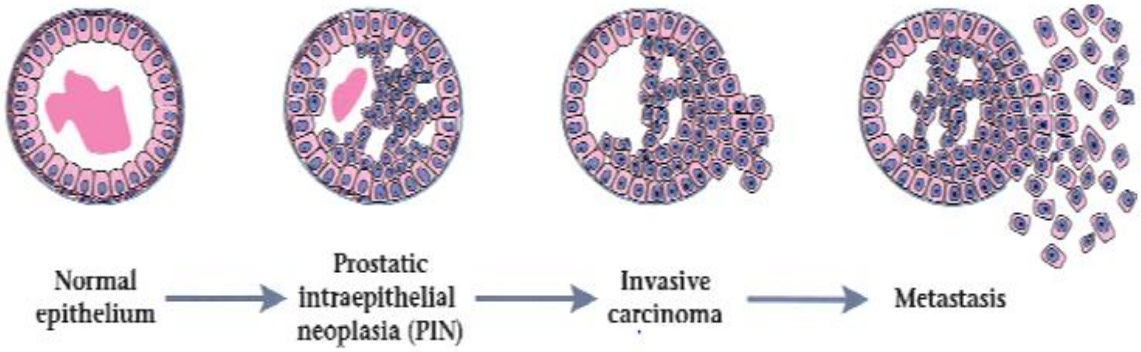


Figure 8: illustration of development of PIN and metastasis in prostate epithelium (1).

Studies have considered high-grade PIN as pre-malignant lesion for several reasons: being juxtapositionally encountered to lethal cancer in peripheral zone, these lesions antedate carcinomas by a decade of time. HGPIN bears an analogy to cancer in structural characteristics as it disrupts the basal layer. Conversely, there are 2 differentiating

features that assist to diagnose PIN represented by the inability of PIN to go through stroma as it has an integral membrane. Moreover, PIN does not affect PSA; hence it is identified solely in tissue samples (1).

1.2.3.3 Gleason score

For histopathological evaluation of prostate adenocarcinoma, the system suggested by Donald F. Gleason has been used widely in world for last 50 years. Donald F. Gleason developed this classification based on structural patterns and increased mitosis. Carcinomas are graded from 1 to 5; representing different degrees of malignancies of lesions. Thus, Gleason grade 1 represents the lowest malignant carcinoma cases, whereas Gleason grade 5 represents the highest malignant carcinoma cases (58), as shown in figure 9.

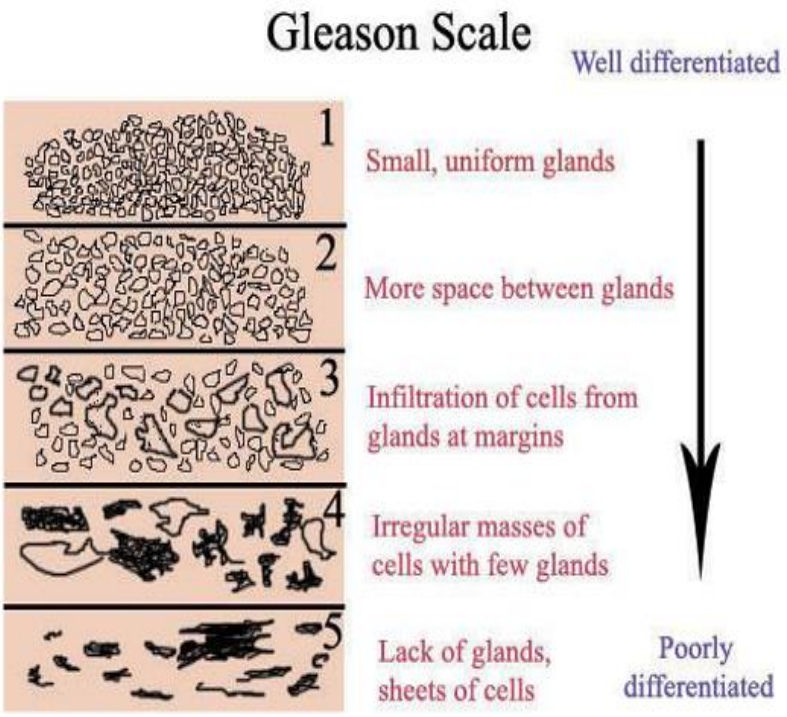


Figure 9: Gleason grading ranging from 1 as the lowest malignant to 5 as the highest malignant changes (98).

Considering the heterogeneity nature of PCa, multiple lesions were often found in the same case. Since Gleason grading system cannot completely reflect the heterogenic nature, Gleason scoring system was introduced. The combined Gleason scores of a prostate carcinoma are the Gleason grade of the main lesion plus the Gleason grade of the second largest lesion of the same case. Thus, the lowest combined Gleason scores are 2. Rising in combined scores indicates increase of malignancy of the disease and the highest combined Gleason scores, or score 10, represents the most malignant cases which usually

have a very poor prognosis (1). The most common lesion in a PCa case is called ‘primary lesion’ and the second most common lesion is called ‘secondary lesion’ .PCa is categorised or diagnosed according to the histological appearance of the cancer cells combined with the clinical behaviour (233).

1.2.4 Prostate cell lines

Six prostate epithelial cell lines were used in this study. They are the benign prostatic cell line PNT2, the weakly malignant cell line LNCaP, the moderately malignant cell line 22Rv1 and the highly malignant cell lines DU145, PC3 and PC3-M.

The benign cell line PNT2 was established from the normal prostatic tissue of a 33-year old man without the history of any prostatic diseases. In order to immortalize the cells, Simian virus 40 plasmid was transfected into the cells to generate a stable cell line. These cells preserve normal epithelial tissue features, indicated by expressing Cytokeratins 8, 18 and 19, which are members of the Keratin family (138,187, 194).

The weakly malignant cell line LNCaP was established from a metastasis in the left supraclavicular lymph node, which was originated from the primary PCa in a 50-year-old Caucasian male in 1980. The cells were initially removed from the metastasis via needle aspiration biopsy. This cell line expresses AR and PSA; hence, it is regarded as an androgen-responsive cell line (94). The androgen responsive feature of LNCaP cell line enabled these cells to be a model for androgen study; this can be accomplished when cells are grown in culture medium with fetal bovine serum that contains testosterone. On the other hand, It has been reported that in the androgen absent environment (for

example, when charcoal-striped fetal bovine serum, instead of serum albumin, was used), these cells will grow independently without androgen (95, 32, 248).

The moderately malignant PCa cell line 22Rv1 was established from a bone metastasis in 1999 through a xenograft collected from a patient with a bone cancer metastasized from the original prostate cancer. These cells expressed both PSA and AR (194, 235, 248).

The highly malignant PCa cell line DU145 was established in 1975 from a brain metastasis of a 69-year-old Caucasian male who had a widely spread PCa. DU145 cells do not express AR or PSA. When nude mice were injected subcutaneously with cells from this line, the resultant tumor preserved both genotypic and phenotypic features of prostate (115, 248, 196).

The highly malignant prostate cancer cell line PC-3 was established in 1979 from a 62-year-old Caucasian man, who had a rib metastasis originated from PCa .Similar to DU145, these cells do not produce AR or PSA. Moreover, some reports suggested that these cells had features of small cell carcinomas or neuroendocrine cancers (108, 198, 248, 176).

The highly malignant cell line PC3-M was established from the most malignant subpopulation of the PC3 cells. When PC-3 xenografts of athymic mice were generated by injection of PC-3 cells, part of the xenografts were removed and subjected to primary tissue culture. This process was performed repeatedly, and PC3-M cells were established from those mice xenografts, thus the PC3-M cell line is presumed to be more malignant than its parental cell line PC3 (147, 248).

1.3 Androgens and prostate cancer

Androgens and their receptor have fundamental roles in men's sexual characteristics (200). Natural androgens in the body are steroids: testosterone (T) and 5α-dihydrotestosterone (DHT). The former is generated mostly by testes and small amount by adrenal glands. The 5 α-reductase enzyme which is found in prostate, skin, and scalp, is responsible for converting testosterone into a potent androgen DHT. T and DHT will bind to androgen receptor (AR). *AR* gene belongs to the nuclear receptor family, which has eight exons, located at chromosome X (62).

In prostate, both normal and neoplastic cells depend on androgens for their growth. The ratio of the number of cells growth to the number of cells death is controlled by the interactions between androgens and AR (50). The subsequent growth in PCa is attributable to the increased cell proliferation proportion over the programed cell death proportion. Studies showed that AR is an important factor to promote prostate cancer and some studies hypothesized that the development of PCa was a result of the increasing level of AR, which can promote cell proliferation and invasiveness. It was reported that there were 1029 mutations occurred in *AR* gene and 159 of them were related with PCa (77, 200).

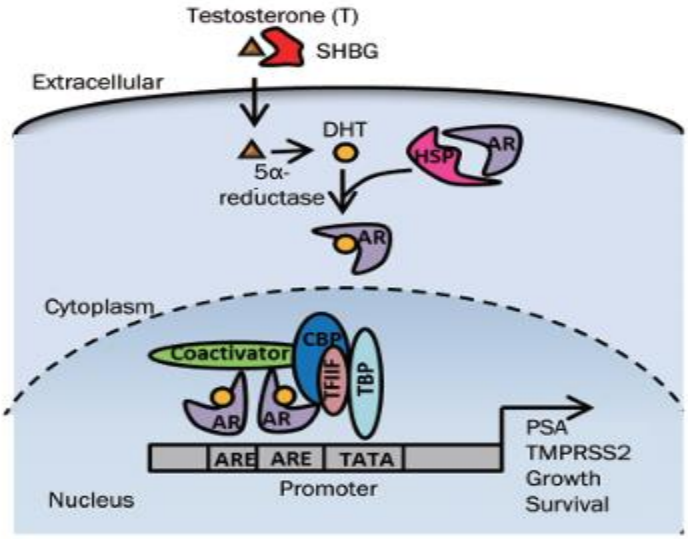


Figure 10: Androgens and androgen receptor in prostate cancer (200).

As shown in Figure 10, the activation of androgen receptor depends on ligand-based reaction. Once T is produced in testes, it is transported into the blood by a carrier protein called sex hormone binding globulin (SHBG). T is entered the cell by passive diffusion (163) .At the prostate, T is converted into DHT by 5-α-reductase intracellularly. DHT attaches to ligand-binding pocket of the AR and initiates the detachment of the heat-shock proteins (HSP) from AR. Then AR is transported to the nucleus, dimerized and attached to the androgen response element (ARE). ARE, located at promoter region of target genes, such as *PSA*, can initiate the recruitment of basal transcription machinery [such as TATA-box-binding protein (TBP) and transcription factor IIF (TFIIF)] with other coregulators such as members of the p160 family of coactivators, and cAMP-response

element-binding protein (CREB)-binding protein (CBP), to regulate the transcription process (200).

The growth and expansion of PCa depends on DHT stimulation at the early stage. Those PCa cells, whose growth is dependent on androgen supply, were defined as androgen dependent cells, which is different from another type of PCa cells that are defined as androgen sensitive cells. These cells do not depend on androgen for their growth, but they show some response to androgen stimulation. At the advanced stage, PCa cells grow independently, they are defined as androgen-independent cells. It was reported that the cells become independent of AR is due to changes in *AR*, either mutated, amplified, or totally lost (62, 247).

Androgen deprivation treatment started since 1940s, when Charles Huggins proved that PCa respond considerably to the changes caused by surgical testes removal. This work indicated that the growth and expansion of PCa depend on peripheral blood androgen supply. Following this discovery, androgen deprivation was introduced as standard therapy for PCa, either in the form of surgical castration or through medication. These modalities helped the prognostic and survival aspects of PCa patients (62).

Medication-based androgen blocking has been adopted in prostate cancer treatment and proved to suppress cancer growth. Nevertheless, within less than three years, patients will develop resistance which leads ultimately to castration-resistant prostate cancer (CRPC) which is considered as fatal and does not respond to conventional androgen deprivation treatment (200).

Several attempts have been made to understand the mechanism underlying castration resistance but are still futile, however, there is a consensus among scientists that various factors are involved, the most common of these are: AR mutation, increased AR sensitivity to agonists, independent ligand stimulation of AR, or other causes (23). It was reported that AR gene mutations may be attributable to amino acid substitutions in ligand binding domain region at AR gene, the mutant AR binds to other steroidal hormone, which activates AR transcription and prostate cancer proliferation (200).

1.4 PSA and prostate cancer

Prostatic specific antigen (PSA) is a serine protease encoded by one of the human Kallikrein family gene (*KLK3*), *KLK3* gene is located in chromosome 19 (176). PSA, secreted by prostate epithelial cells, has been employed broadly as an early marker to detect PCa. As a biomarker, PSA helped PCa diagnosis and hence reduced patient death. Until now, PSA is still a most commonly used biomarker world widely in detecting PCa. When compared to another PCa diagnostic test DRE, PSA test has more chance to detect an organ-confined disease (73, 34).

For any screening tests, sensitivity can be defined as the ability of the test to detect the true positive cases; while specificity is the ability of the test to detect true negative cases and hence to identify all people who do not have the disease (11). For PCa screening, PSA was introduced in previous years as a screening tool to detect PCa cases. Since that time, efforts were ongoing to identify a cutoff point, which is a PSA level that can distinguish cases that need further testing from those who don't need further assessment (16).

It was reported that PSA blood level of 4 ng/ml is associated with sensitivity of 20.5% and the specificity is 93.8%. However, a PSA screen program failed to find highly sensitive and specific PSA cutoff point (206). Thus, the exact cutoff point for PSA test is still a matter of argument.

It is still unclear whether using PSA screen to identify the PCa cases in an early stage in the last 20 years had resulted in reducing PCa mortality rate. A screening trial hypothesized that there was no beneficial result in terms of mortality when PSA is used for screening (10). Adding to this, another American study, which was conducted in 2012, suggested a limitation on the use of PSA test, excluding high-risk cases. This conclusion was based on the fact that adverse effects of PSA test that include infection, bleeding, sexual dysfunction and urinary problems. Furthermore, the test had only resulted in low reduction of mortality (151, 7).

Using PSA test to make diagnosis of PCa, there are several drawbacks. First of all, current assays are company-based, and each manufacturer has its own operation protocol in kit application. This makes it difficult to compare the effectiveness achieved by kits manufactured by different companies. Sometimes, test repetition is necessary when a laboratory fails to obtain reliable value due to absence of a standardised operation procedure. Moreover, blood PSA level may increase in conditions other than PCa, such as old age, infection, instruments application, and ongoing medications such as non-steroidal anti-inflammatory drugs. Apart from that, PSA can't recognize active or inert disease. In a clinical trial, it was observed that 15% of men with PSA level lower than 4ng/ml had high-grade disease (207, 73). It was suggested that the major drawbacks for PSA as a biomarker is its both limited specificity and over diagnosis (63).

1.4.1 PSA screening controversy

PSA based screening has been a controversial subject for years, as it leads to an elevation in diagnosed and treated cases, some of which may never manifested into clinically harmful disease (10, 182). It is still debatable whether PSA screening for men with risk of PCa had reduced mortality rate or not (73). Moreover, PSA has led to over diagnosis in a range of 1.7- 67 % and this wide range value is affected by study design and population characteristics (132, 7). In 1968, 10 screening criteria were published by Wilson and Jungner. These were set out to regulate any screening test as shown in box 1. Under these regulations, the screening test should identify serious medical issues; the disease should have diagnostic and therapeutic strategies, a comprehensive understanding of the medical condition, and assessment of cost-effectiveness of both diagnosis and treatment (53). For PCa screening, two points of Wilson and Jungner criteria are considered: availability of treatment (represented by radical prostatectomy) and establishing PSA to be used as a PCa biomarker. However, one important criterion remains unattainable, which is to assess if advantages of screening outweigh the harms (172, 100, 114).

Box 1. **Wilson and Jungner classic screening criteria**[1]

1. The condition sought should be an important health problem.
2. There should be an accepted treatment for patients with recognized disease.
3. Facilities for diagnosis and treatment should be available.
4. There should be a recognizable latent or early symptomatic stage.
5. There should be a suitable test or examination.
6. The test should be acceptable to the population.
7. The natural history of the condition, including development from latent to declared disease, should be adequately understood.
8. There should be an agreed policy on whom to treat as patients.
9. The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole.
10. Case-finding should be a continuing process and not a "once and for all" project.

Box 1. Wilson and Jungner classic screening criteria (9).

The test starting age, the age to terminate the test, and the frequency of the test, all these are issues of debate (34). Persistent increases in serum PSA levels in patients with previous negative biopsy remain a clinical dilemma. Approximately 75% of patients with PSA between 2 and 10 ng have a negative result of PCa in biopsy test. Nevertheless, a significant false-negative detection rate must be considered because sampling errors are frequent, involving 10%–20% of clinically significant PCa (28, 63).

1.5 Prostate cancer biomarkers

Biomarkers are identified as molecules that whenever measured or assessed, they will provide valuable disease data ahead of standard clinical examinations. They can be measured from different sources like tissue biopsy, blood samples, and urine (7). Some biomarkers studied and applied in PCa detection are described below.

1.5.1 4k score

Kallikreins are a family of 15 serine proteases, which involved in changing cell regulatory, elevating extracellular matrix modification, and leading to an increased invasiveness and angiogenesis (12).

This test is based on plasma assessment of 4 prostate derived kallikrein proteins, with some clinical features added to assess the risk of high-grade Gleason score at biopsy (7). This test has been established by The OPKO medical company through merging four kallikrein molecules (tPSA, fPSA, intact PSA, and kallikrein-related peptide 2 (hK2)). The result of the test is combined with clinical data such as age and previously negative specimen to decide the disease progression status (73). This test was widely used to identify those early-stage patients who had not shown any clinical symptoms yet. This test is aimed to identify those cases with risks to develop high-grade cancer. Since the biopsies can be confined to the high-risk cases only, the 4K score test may result in reduction of the number of biopsies (63). According to current guidelines, the 4K score is considered as diagnostic/prognostic test to detect aggressive prostate cancer risk (107).

The 4K score leads to an 8% decline in unnecessary biopsies for the first time, screened cases (7). According to a retrospective Swedish study, the 4K test was found to be superior to combined PSA and age parameters in predicting the accuracy in diagnosed PCa cases (73, 220). European retrospective studies reported that 4K score is accurate in predicting for aggressive cancer (29). Furthermore, this score is cost-effective as its application can decrease the biopsies by 48-56 % of the cases (222, 73).

According to NCCN guidelines, 4K score could be helpful in men with no previous biopsy or those after a negative biopsy. But they advised against its application as a first line screening. Moreover, FDA had not approved its application (73). This test is contraindicated in men within 96 hours after DRE, those who are taking 5α-reductase inhibitor, or those who received medical or surgical treatment for BPH, therefore, this constraint its feasibility to be applied in wide range of population (7).

1.5.2 PCA3 assay

This assay was developed by The Hologic Company as Progensa assay (73). It is based on measurement of both Prostate Cancer Gene 3 (PCA3) and PSA and calculation the ratio of PCA3 RNA to PSA RNA in male urine samples. In this assay, the PCA3 level is correlated with the degree of PCa malignancy (7).

This test was approved by FDA in 2012 for biopsy-negative patients (those PCa patients with a negative result in previous prostate biopsy). Since there was a strong disagreement on what is the exact PCA3 cut-off level and on whether it had true prognostic value, it had not been approved yet to be used as a screening test in the population with a raised PSA level, although it was agreed to be applied for men tested repeatedly with biopsy. PCA3 is more specific than PSA as it is not affected by prostate infection or inflammation (122, 7).

Comparing with other PCa diagnostic assays, PCA3 is preferable in predicting patient outcome. Sensitivity and specificity ranged between 53% -69% and between 71% -83%, respectively (7). Some studies reported an association of PCA3 score with the highly

malignant PCa, whereas some other studies (66, 92) suggested a lack of correlation between this score and disease severity. Moreover, whether PCA3 assay is correlated with the combined Gleason scores is still a matter of debate (63, 7).

1.5.3 TMPRSS2 and ERG.

The fusion of transmembrane protease, serine 2 (TMPRSS2) and erythroblast transformation-specifically related gene (ERG) resulted in gene derangement that was first reported in blood-originated tumours in 2005, and then reported in solid tumours, including PCa. The level of this fusion protein can be measured in men via collecting urine samples after a prostate massage (208, 63).

According to the genome atlas of PCa, the most frequent type of this derangement is E26 ETS derangement, which was reported in 58% of tumours (26, 63).It was suggested that *TMPRSS2:ERG* is specific to PCa and has predictive value of 94% (179). It was suggested from the European Randomized Study of Screening for PCa (ERSPC) that the combination of PCA3 and TMPRSS2: ERG, increased the predictive value of picking up high risk PCa cases (63). On the other hand, some other researchers had reported that this fusion gene was absent in the aggressive PCa disease (166, 73).

1.5.4 Michigan prostate score (MiPS)

This score was developed in 2013. It measures the levels of blood, urine PCA3, and urine TMPRSS2: ERG. This score was mainly designed for cases with a raised blood PSA level before a biopsy test, in addition to men who had previous negative biopsy test result (7).

It was reported by Salami et al. (178) that Michigan score of combining 3 diagnostic assays can raise distinctive ability for PCa diagnosis as compared to each test alone. Leyton et al. (122) reported reduction of unnecessary biopsies for men with PCa risk by 35% when MiPS score is evaluated ahead. However, a recent work by Gopalan et al. shows absence of significant correlation between TMPRSS2: ERG gene fusion and patient outcome. Moreover, the assay cut-off value is still debated (76, 7).

1.5.5 IL-6

IL-6 belongs to cytokines family; and is considered the powerful proinflammatory cytokine that has various functions. IL-6 mediates inflammatory response and affects cell signalling. It is involved in cell growth and distant metastasis. Reports showed that it is associated with cancer in lung, breast, head and neck, and interestingly, prostate (177, 184, 156, 15, 186). Numerous cells are involved in production of IL-6 including fibroblasts, keratinocytes, mast cells, macrophages, T and B cells. This cytokine is involved in monocyte differentiation, regulation of dendritic cell maturation (139, 218).

A raised IL-6 level was found in autopsies of PCa patients, in three PCa cell lines (PC3, DU145, and LNCaP) and in blood tests from PCa patients who were resistant to treatment. Although IL-6 was expressed in CRPC, its correlation with patient survival is still undefined (186, 73).

Nguyen and associates reported that high IL-6 level promoted prostate cancer cell growth and distant spread by affecting cellular proliferation, invasiveness, epithelial to mesenchymal transition (EMT). However, when anti-IL-6 antibody was introduced, it showed an opposite effect, as it reduced growth rate of PCa cell lines by inhibiting the effect of IL-6. It was reported that IL-6 induces PCa invasiveness through EMT. As EMT activates fibroblasts, these fibroblasts secrete matrix metalloproteinases (MMPs) under IL-6 stimulation, which ultimately leads to invasiveness in PCa cells (74, 156, 186).

IL-6 may be used in future to predict response of chemotherapy. Mahon et al, reported that IL-6 and other 6 cytokines levels were changed in PCa patients that were treated with docetaxel. After one cycle of treatment with docetaxel, those cases with an increased IL-6 expression level was associated with a worse prognosis (133, 73).

1.5.6 CTCs

Circulating tumour cells (CTCs) are those cancer cells that are capable of penetrating blood vessels and moving into the circulation. CTCs may initiate a metastatic spot. These cells have been detected in many carcinomas cases through blood tests. A ratio of normal blood cells to CTCs was presumed to be 10^6-10^9:1. Consequently, the detection of these cells requires a very sensitive laboratory method (240, 63). The

CellSearch® system is applied to enumerate these tumour cells through blood samples. The protocol depends on immunomagnetic enrichment of cells, labelling them through fluorescent dyes, then detection through fluorescence-based microscopy (148).

It was reported that the percentage of these cells rises in more advanced PCa disease. A study reported that these cells have heterogeneous characteristics. In addition to their low number (210, 63), it was suggested that this heterogeneity could be correlated with PCa treatment resistance, and therefore CTCs may be a factor to monitor treatment management in PCa (181).

One study reported CTCs association with overall survival in PCa patients, however, more clinical trials are needed to evaluate these cells before they can be an established biomarker. Since sensitive method is needed to detect CTCs, nowadays only CellSearch®, that is the FDA approved system to carry out this test (125, 63). This again takes its tolls on limitation of this test.

1.5.7 Immune checkpoints and regulators PD-1 and PD-L1

The programmed cell-death receptor 1 (PD-1) is a transmembrane glycoprotein of immunoglobulin family which has 288 amino acids. It is considered as an inhibitory receptor of immune system expressed in many immune cells, especially cytotoxic T-cells. PD-L1 is a PD-1 ligand, which is found in T cells, B cells and macrophages. When PD-L1 binds with PD-1 in normal tissues, this conjugate maintains immune system stability and control immune mechanism during an infection or inflammation (204, 52). It was

found that PD-L1 is expressed by many tumor cells such as breast carcinoma, lung carcinoma and melanoma (239).

Taube et al, reported that PD-1 and PD-L1 interaction in tumor microenvironment can inhibit cytotoxic T-cell, which may contribute to the immune escape for cancerous cells, in which the immune system will be incapable to detect and to eradicate these cells. However when PD-1 and PD-L1 interaction is blocked, tumor cells may be killed by cytotoxic T-cells. Thus, development of medications that prevent PD-1 and PD-L1 interaction may be a future hope for cancer treatment (204).

A breakthrough in immunology field was by James Allison and Tasuku Honjo who were awarded Nobel prize in 2018 for their efforts on immune checkpoints, they discovered a way to stimulate immune system to eradicate cancer, and this breakthrough led to introducing many immune checkpoint inhibitors which showed optimistic results on the treatment of Melanoma, kidney, and lung cancers (209, 61).

In PCa, the first immune check point inhibitor tried was Ipilimumab, which was assessed through two clinical trials in patients with CRPC. But the trials were unsuccessful in improving patient survival. Another PD-1 inhibitor Pembrolizumab was assessed in PCa cases through phase 2 trial and the trial reported that 4 out of 20 PCa patients had a significant PSA response (78). In 2018, the largest study was conducted to assess PD-1 expression status in 539 primary prostate carcinomas and 57 CRPC. It was reported that PD-1 expression was detected in 8% of primary PCa and 32% of CRPC (86, 61).

Another immune checkpoint member, B7-H3 (CD276), was found to have an unfavourable prognosis in PCa and it was linked to a more aggressive disease and a

worse outcome. Studies showed that B7-H3 was elevated when cancer was treated with hormonal therapy after prostatectomy (73).

Further study is required to fully understand the mechanisms of immune regulator actions and to improve the immunotherapy based on immune check point in PCa.

1.6 Fatty acid-binding proteins

The various actions of adipocytes, fatty acids and proteins in cancer progression have been revealed. Their roles range from being utilized as energy providers and cell signalling molecules to the regulations of cancer metabolisms, hence, to promote more aggressive cancer phenotypes by enabling the cancer cell relocation, infiltration, and self-renewal (55).

Fatty acid-binding proteins (FABPs) are proteins expressed in different configurations in numerous tissues. They aid in the transportation of lipids. In humans, FABPs are classified as part of the intracellular lipid binding family. This family is made up of 10 members: FABP1 to FABP9 and FABP12. FABP10 and FABP11 are not found in humans, but they are expressed in other species, such as zebrafish (Danio Rerio) and teleost fish (Solea senegalensis) (84, 90).

It has been previously thought that FABPs, like other lipid metabolism factors, are only involved in the transportation of lipids. However, their importance in cancer pathogenesis was recognized with the findings of the differential expression patterns of FABPs in cancer tumorigenesis and progression (84, 105).

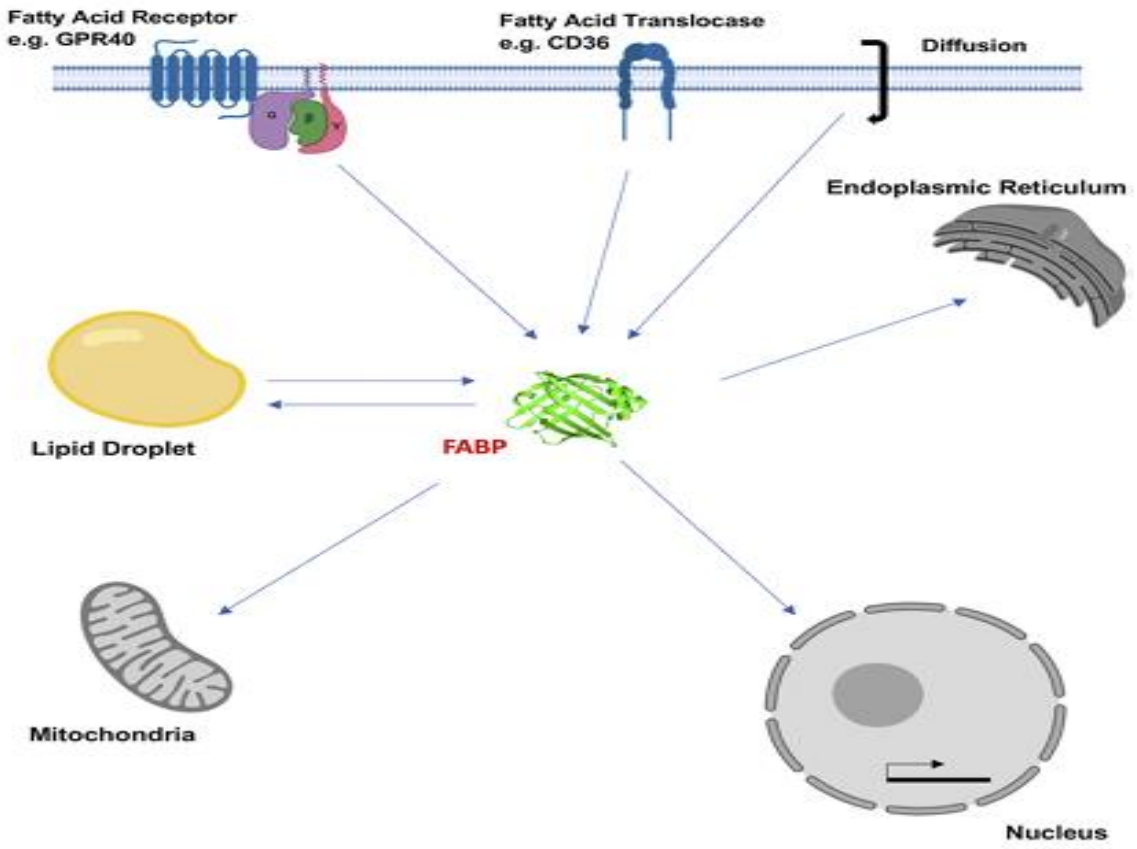


Figure 11: FABPs diverse functions (140). Transportation of fatty acids into cells is achieved through membrane diffusion and via help of receptors like G-protein. FABPs act as chaperone regulating all functions of cell lipid transportation and storage. Inside cells, FABPs send lipid to endoplasmic reticulum (ER) for signalling; to mitochondria for oxidative process, and to nucleus for transcription activity (140).

All members of FABPs can bind long chain fatty acids, however; they differ in their selectivity, affinity and binding mechanism (192). Sequences of human FABPs are shown in the figure 12.

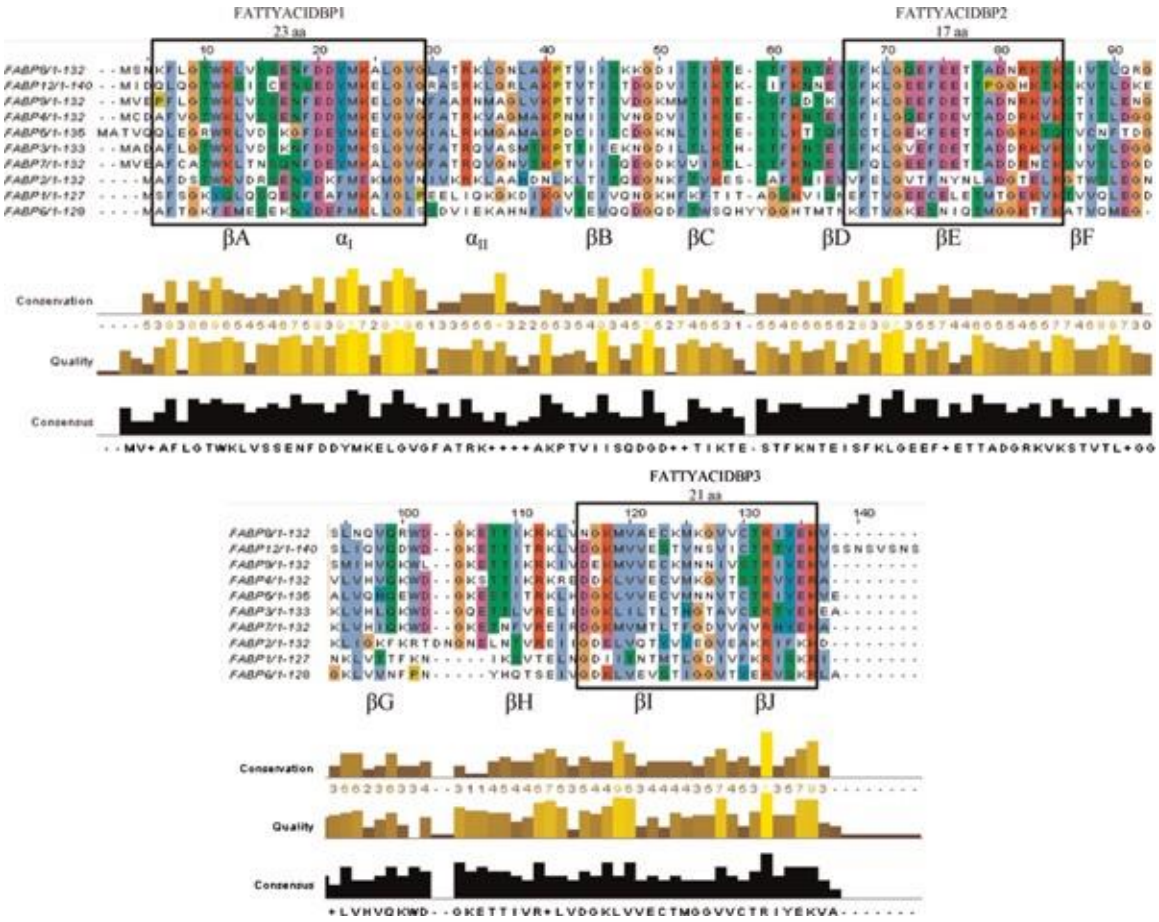


Figure 12: Amino acid sequences of human FABPs. The annotation was created by analysis of multiply aligned sequences (AMAS), data obtained from (NCBI) website (www.ncbi.nlm.nih.gov/) (192).

FABP1

Id	Chromosome	Strand	Exon Start	Exon End
2168	2	-	88122982	88123053

FABP2

Id	Chromosome	Strand	Exon Start	Exon End
2169	4	-	119317250	119319040

FABP3

Id	Chromosome	Strand	Exon Start	Exon End
2170	1	-	31360418	31360797

FABP4

Id	Chromosome	Strand	Exon Start	Exon End
2167	8	-	81478497	81478864

FABP5

Id	Chromosome	Strand	Exon Start	Exon End
2171	8	+	81280483	81280595

FABP6

Id	Chromosome	Strand	Exon Start	Exon End
2172	5	+	160187367	160187454

FABP7

Id	Chromosome	Strand	Exon Start	Exon End
2173	6	+	122779501	122779794

FABP8

Id	Chromosome	Strand	Exon Start	Exon End
5375	8	-	81440326	81443397

FABP9

Id	Chromosome	Strand	Exon Start	Exon End
646480	8	-	81458383	81458433

FABP12

Id	Chromosome	Strand	Exon Start	Exon End
646486	8	-	81524981	81525045

Figure 13 :Exon structure of FABPs (245).

FABP1	14,208 Da
FABP2	15,207 Da
FABP3	14,858 Da
FABP4	14,719 Da
FABP5	15,164 Da
FABP6	14,371 Da
FABP7	14,889 Da
FABP8	14,909 Da
FABP9	15,092 Da
FABP12	15,565 Da

Table 1 : Molecular weight of FABPs (244).

Chromosomal localization and tissue distribution of each FABP is summarised in table 2, and discussed in the following section.

Gene	Common name	Aliases for proteins	Previous symbols	Localisation	Chromosomal location	OMIM ID/ HGNC ID	Number of amino acids
FABP1	Liver FABP	L-FABP, hepatic FABP, Z protein, heme-binding protein		Liver, intestine, pancreas, kidney, lung, stomach	2p11	134650/ 3555	127
FABP2	Intestinal FABP	I-FABP, gut FABP (gFABP)		Intestine, liver	4q28-q31	134640/ 3556	132
FABP3	Heart FABP	H-FABP, O-FABP, mammary-derived growth inhibitor (MDGI)	*FABP11*	Cardiac and skeletal muscle, brain, kidney, lung, stomach, testis, adrenal gland, mammary gland, placenta, ovary, brown adipose tissue	1p33-p31	134650/ 3557	133
FABP4	Adipocyte FABP	A-FABP, aP2		Adipocytes, macrophages, dendritic cells, skeletal muscle fibres	8q21	600434/ 3559	132
FABP5	Epidermal FABP	E-FABP, keratinocyte-type FABP (KFABP), psoriasis-associated-FABP (PA-FABP)		Skin, tongue, adipocyte, macrophage, dendritic cells, mammary gland, brain, stomach, intestine, kidney, liver, lung, heart, skeletal muscle, testis, retina, lens, spleen, placenta	8q21.13	605168/ 3560	135
FABP6	Ileal FABP	Il-FABP, Ileal lipid-binding protein (ILLBP), intestinal bile acid-binding protein (I-BABP), gastrophin		Ileum, ovary, adrenal gland, stomach	5q23-q35	600422/ 3561	128
FABP7	Brain FABP	B-FABP, brain lipid-binding protein (BLBP), MRG		Brain, central nervous system (CNS), glial cell, retina, mammary gland	6q22-q23	602965/ 3562	132
FABP8	Myelin FABP	M-FABP, peripheral myelin protein 2 (PMP2)		Peripheral nervous system, Schwann cells	8q21.3-q22.1	170715/ 9117	132
FABP9	Testis FABP	T-FABP, testis lipid-binding protein (TLBP), PERF, PERF 15		Testis, salivary gland, mammary gland	8q21.13	--/3563	132
FABP12	---	---		Retinoblastoma cell,[a] retina (ganglion and inner nuclear layer cells),[b] testicular germ cells,[b] cerebral cortex,[b] kidney,[b] epididymis[b]	8q21.13	--/34524	140

Table 2: Human *FABP* genes nomenclature and localization as listed in Human Gene Nomenclature Committee (HGNC) and OMIM website (192).

1.6.1 FABP1

FABP1 is found mainly in the liver. It is also present in smaller amounts in intestines, kidneys and the stomach. Several ligands can bind to FABP1 to achieve its significant functions of cellular activities. Ligands include a variety of fatty acids and their metabolites to bilirubin and heme (217).

It is thus clear that the roles of FABPs are various and include the transportation of fatty acids to cells, participating in PPAR signal transduction, regulating enzyme activity, and controlling gene expression and cell growth. FABP1 has a proven relation to steatotic liver and non-alcoholic fatty liver disease. The down regulation of FABP1 activates the quiescent stellate cells in the liver leading to the secretion of collagen and proteins by the stellate cells and the subsequent hepatic fibrogenesis (41, 84).

1.6.2 FABP2

FABP2 is expressed exclusively in the small intestine, in which dietary lipids are absorbed. The part of the small intestine with the highest levels of FABP2 is the jejunum. Saturated and unsaturated fats are known to be used for the triglyceride synthesis. When excessive fatty acids accumulate, FABP2 controls fatty acid transferring in order to prevent the alteration of membrane characteristics by un-esterified fatty acids build up (84).

A number of FABP2 polymorphism studies have been carried out and revealed clues of the roles it has in the human intestine. A threonine substitution at amino acid 54 was identified and it resulted in disturbed lipid metabolism. Increased insulin resistance,

hypertriglyceridemia and increased accumulation of triglycerides were described in the threonine variant (84).

1.6.3 FABP3

FABP3 is expressed predominantly in the heart and muscle tissue, as well as the tissue of the lung, ovary, brain, placenta, mammary gland, and stomach (217). In order to provide enough energy to these tissues with high energy expenditures, FABP3 transfers fatty acids to mitochondria to produce energy. However, elevated levels of FABP3 may evoke cardiac dysfunction by reducing the calcium levels in the sarcoplasmic reticulum (123).

FABP3 accumulates in the brain tissue 10-fold more than the brain FABP (FABP7). This indicates its significant action of neurological performances. When compared to other FABPs of the brain, FABP3 is found in the later development stage of the brain. It acts in the production of neurites and the maturation of synapses. Lower levels of FABP3 may be associated with some neurological disorders such as Down syndrome and Alzheimer's disease, which are caused by deficiencies in signal transduction and alterations in membrane structure (42).

1.6.4 FABP4

FABP4 secretion leads to several physiological effects including greater glucose production in hepatic cells, augmented insulin secretion and reduced cardiomyocyte contraction (118, 27). It was reported that FABP4 has a paracrine/endocrine signalling

function; thus it acts on nearby or distant organs after it is released from adipose tissue to change metabolism and cell function (140).

Recent studies have revealed that FABP4 can participate in the elaboration of atherosclerosis in heart disease via inflammation and the accumulation of lipids in the macrophages or foam cells. A study on mice revealed that a more than 60% reduction in the blockage of coronary arteries is observed with the absence of both ApoE and FABP4 when compared to the absence of ApoE alone. This shows an important role of FABP4 in the development of atherosclerosis (135, 215).

1.6.5 FABP5

FABP5 has a wide range distribution in the body: epidermis, mammary gland, brain, liver, kidney, lung, adipocyte, macrophage, tongue, and testis (192, 84).

Like other FABP family members, it binds and traffics fatty acids, in addition to the keratinocyte differentiation. One study (234) suggests its association with obesity, abnormal lipid and insulin levels (192).

1.6.6 FABP6

FABP6 is also known by its alternate name: intestinal bile acid binding protein (I-FABP), because of its high affinity for bile acid (242). FABP6 is expressed mainly in the ileum, binding bile acid to give its major role as a surfactant to facilitate in lipid digestion, thus

controlling bile acid and lipid homeostasis. The lack of expression of FABP6 in male mice renders them more susceptible to fatty liver disease (2).

1.6.7 FABP7

FABP7 is expressed in glia cells of the nervous system (8), it was hypothesized that it contributes to central nervous system development by supplying fatty acids during cellular maturation (192). One study suggested FABP7 association with several psychiatric disorders, specifically, Down syndrome and schizophrenia (225). More research is needed to understand its role. These neurological diseases may develop as a result of fatty acids binding and energy supply.

1.6.8 FABP8

FABP8 is called myelin protein (M-FABP) due to its predominance in peripheral nervous system myelin (216). Despite decades of research, the role of M-FABP is unidentified; few studies had reported that it may be essential for myelin stabilizing and biogenesis (192, 241).

1.6.9 FABP9

It was proposed that FABP9 is one of major protein components of mammalian sperm (60); it was assumed that it attributes to sperm protection (113, 192), however more studies are needed to understand its role.

1.6.10 FABP12

It is the most recently discovered member of FABP family, little information is currently available, and it was detected in high level in human retinoblastoma cells (217).

1.7 Role of FABPs in cancer

1.7.1 FABP1 and cancer

FABP1 is expressed in Liver, intestine and Kidney as described in the previous section. Studies were performed to find out whether FABP1 was related to cancer in these organs. At least one study reported an association between FABP1 and colon cancer; significant downregulation of FABP1 found in circulating colon cancer cells (162). Loss of FABP1 was identified as major contributing factor to microsatellite unstable colorectal carcinomas (228, 84). These two studies seemed to suggest that FABP1 may be a tumour suppressor.

In contrast, another study on the role of FABP1 in hepatocellular carcinomas (HCC) suggested that FABP1 may have a tumour promoting role. FABP1 induced vascular endothelial growth factor (VEGF) expression through its interaction with the VEGF receptor, leading to new blood vessels formation (angiogenesis). This investigation showed that the role of FABP1 in enhancing migration properties of the cancer cells via the VEGFR2 pathway, indicating a promoting role of FABP1 in the metastasis of HCC

(117). FABP1 was found to be highly upregulated in the HCC cells. The increased level of FABP1 is correlated to lymph node metastasis and the stage of the malignant progression (103, 84).

1.7.2 FABP2 and cancer

Fatty acid binding protein 2, also known as intestinal fatty acid binding protein, is related to the formation of some diseases that may progress to the development of cancers. These diseases include diabetes, myocardial infarction, stroke, and gallbladder diseases (69).

Very few studies were carried out to detect the role of FABP2 in cancer progression. However, a study conducted in 2010 to investigate the association of FABP2 expression with dietary habits and lipid uptake in colorectal carcinoma. This study drew the attention to the negative correlation between FABP2 and fat uptake. Therefore, FABP2 is unlikely to be an accurate predictor of the risk of colorectal cancer (111).

1.7.3 FABP3 and cancer

The role of FABP3 in cancer is not yet fully understood, there are debates on whether it promotes or suppresses cancer. Previous studies found that FABP3 was overexpressed in 4 types of cancers: non-small cell lung carcinoma (203), gastric cancer (88), leiomyosarcoma (49) and melanoma (127). Conversely, FABP3 was found to facilitate the suppression of breast cancer (99, 155), lung adenocarcinoma (159), lymphomas (229), and embryonic cancers (203).

1.7.4 FABP4 and cancer

A number of studies had demonstrated FABP4 involvement in the aggressiveness of various cancers such as prostate cancer (211), breast cancer (82), cholangiocarcinoma (157), glioblastoma (35), and leukaemia (157).

Recent evidence suggests that FABP4 can be used as a novel molecular marker for the investigation, prediction, and the monitoring of bladder cancer during therapy as well as a potential novel therapeutic target (19).

The role of FABP4 in the epithelial-mesenchymal transition (EMT) of cancer cells was reported. FABP4 overexpression has been correlated to EMT transition in cholangiocarcinoma (157) and cervical cancer (104).

1.7.5 FABP5 and cancer

FABP5 or E-FABP is expressed in high level in cancer cells, contributing to the aggressive phenotypes of cancer, promoting proliferation, invasion, tumorigenicity and metastatic ability of the cancer cells. High level of FABP5 expression was also related to the resistance to therapy and poor prognosis in various cancers such as gastric cancer (88, 238), melanoma (120), cervical cancer (224), breast cancer (168), prostate cancer (112, 152, 6), cholangiocarcinoma (102), oral cancer (59) and HCC (112). Amongst all different cancer types, the role of FABP5 in promoting prostate cancer was studied most.

Numerous studies proved FABP5 promoted tumor invasiveness both in vitro and in vivo (140).

A cohort study reported expression of FABP5 in triple negative breast cancer, the high level was associated with aggressive disease and low survival. The authors hypothesized that FABP5 exerted its effects via altering extracellular matrix to allow the tumor cells invading nearby organs (168).

Our research group had been studying FABP5 since 2000. After identifying FABP5 as a differentially expressed gene between benign and the malignant cells, we compared and measured the relative levels of FABP5 between the benign and the malignant prostate and breast cancer cell lines. When comparing to benign counterparts, FABP5 expression levels were higher in breast and prostate cancers by 6.5- fold and 5-17-fold, respectively (150, 68).

Morgan *et al.* reported significantly high FABP5 levels in both PCa cells and prostate carcinoma tissues. It was found that the increased FABP5 expression was significantly associated with reduced patient survival time. When the level of FABP5 was suppressed via RNAi in PCa cells, their tumorigenicity and metastatic ability was greatly suppressed both *in vitro* and *in vivo* (150). During the extensive studies on the molecular mechanisms involved in the malignancy-promoting role of FABP5, a novel signal transduction pathway initiated by the stimulation of fatty acids transported by FABP5 was discovered. The detailed route for this signal transduction pathway is like following: the increased level of FABP5 transports a large amount of fatty acids into the cytoplasm, and most of the fatty acids were used as new sources of energy supply for the cells,

whereas some excessive amount of fatty acids were delivered to their nuclear receptor PPARγ. The activated PPARγ can trigger a series of molecular events or a chain of molecular reactions, including up-regulating some cancer promoting genes, such as VEGF; and down-regulating possible tumor-suppressor genes, and hence to facilitate the malignant progression of the cancer cells (150).

1.7.6 FABP6 and cancer

Several attempts have been made to examine FABP6's role in colorectal cancer. Since it is expressed widely in ileum, one study identified that FABP6 was highly expressed in colorectal carcinomas comparing to benign tissues. However, tissues from metastatic sites had low FABP6 levels (84). Thus, more studies are required to determine the exact role of FABP6 in colorectal cancer and other cancer cells.

1.7.7 FABP7 and cancer

FABP7 plays an important role in Notch1 signalling pathway. It has also been widely examined in breast cancer, in which a FABP7-positive cohort was associated with the triple negative breast cancer group. This correlated with poor survival outcome, high tumour grade and increased proliferation (230).

In 2008, Slipicevic et al. published a paper on melanoma in which they described high expression of FABP7 in both primary and metastatic tissues that was correlated to

increased tumour size and a decreased relapse-free survival period (191, 84). However, the true role of FABP7 is not yet clear.

1.7.8 FABP8 and cancer

Until recently, there has been no reliable evidence of FABP8's involvement in any types of carcinomas; more work is necessary to decide the role of FABP8 in cancer cells.

1.7.9 FABP9 and cancer

FABP9 has been studied by our group in 2016. It was reported that FABP9 is overexpressed in both PCa cells and tissues. This expression was correlated with increased malignancy and poor survival time. When FABP9 knockdown cells were compared to parental PCa lines, the suppression of FABP9 expression produced significant inhibition on cell proliferation, it did not seem to have significantly affected the invasion and the anchorage-independent growth which is an indication of tumorigenicity (5). More investigation is needed to decide whether FABP9 has a possible role in cancer.

Questions and Hypothesis

In this study, I hypothesize that FABP12 may be an important diagnostic and prognostic factor for PCa patients, and the increased FABP12 may play an important role in promoting malignant progression. In this work, I plan to investigate the expression status and its prognostic significance of FABP12 in PCa cells and tissues; to study the functional role of FABP12 in malignant progression of the cancer cells and to validate whether the increased level of FABP12 is a novel therapeutic target and a diagnostic (or prognostic) marker. I intend to achieve the proposed goal through experimental work to address the following questions:

- Does FABP12 overexpressed in prostate cancer cell lines and tissues?
- Does FABP12 expression in prostate carcinomas correlate with patient Gleason score?
- Does FABP12 expression in prostate carcinomas correlate with AR index and PSA levels?
- Is the increased FABP12 expression in prostate carcinomas correlated with patient survival time?
- Does FABP12 expression affect the malignant progression of the PCa cells?

Malignant progression can be identified as a change in cellular behavior from benign to malignant, thus, the cells will acquire uncontrolled growth features, in addition to invasion ability (236). Malignant progression can occur in cultured cell as in the previous study on human prostate epithelial (HPE) cells. In that study malignant transformation of

HPV-18 immortalized HPE was achieved after multiple exposures to a chemical carcinogen (166).

Aims

- To examine FABP12 expression status in benign and malignant prostate cancer cell lines by Western blot analysis to assess whether FABP12 is overexpressed in cancer cells and whether the increased level of expression is associated with the increased malignancies of the cancer cells.

- To examine FABP12 expression status in an archive set of benign and malignant prostate tissues by immunohistochemical staining to assess whether FABP12 is overexpressed in malignant prostate tissues and whether this increase is correlated with some common diagnostic markers.

- To functionally characterize the role of FABP12 by knockout this gene in a highly malignant prostate cell model to assess whether FABP12 knockout can affect the malignant characteristics of the cells, so as to determine the role that FABP12 play in malignant progression.

CHAPTER TWO

METHODOLOGY

2. MATERIALS AND METHODS

2.1 Cells and Culture

In this study, a set of six benign and malignant prostate epithelial cell lines was used. PNT2 is a benign prostate epithelial cells line . LNCaP is a weakly malignant PCa cell line; 22Rv1 is a moderate malignant PCa cell line; While DU145, PC3 and PC3M are highly malignant PCa cell lines (196, 205, 214). The cell culture work performed in a tissue culture hood (LabGard, USA) following university safety guidelines. All cells were cultured in RPMI 1640 medium supplied with 10% (vol/vol) foetal calf serum, penicillin (100 U/ml) (Bioser, East Sussex, UK), streptomycin (100ug/ml) and L glutamine (20mM) (Invitrogen). LNCaP cells were supplied with sodium pyruvate (100ug/ml) (Sigma, Grillingham, UK) in addition to the above supplements . Cells were grown and maintained as monolayer cultures and were kept in a humidifier incubator (Borolabs, UK) at 37ºC with 5% CO_2.

2.1.1 Cell thawing

Cell vials in different racks stored in a tank of liquid nitrogen were taken out of the tank and placed in a 37 ºC water bath to thaw slowly. Then cells were diluted with 20ml of fresh medium to remove the effect of DMSO, followed by centrifugation for 3 minutes at 91×g. Supernatant was discarded, and cell pellet was resuspended in fresh medium. Cells were calculated by haemocytometer and cultured in a 25-cm^2 flask. Cells were monitored and medium was changed every 3 days to replace supplements.

2.1.2 Cell Subculture

Cells in the medium were poured into a flask and monitored daily under the microscope. When the cells grew to about 70-80% confluent, they were sub-cultured in tissue culture hood under aseptic conditions. The medium was aspirated from the flask, cells were washed with phosphate buffer saline (PBS), treated with trypsin in versine (2.5%: 97.5%, v/v) for 3 minutes inside the incubator. Thereafter, cells were checked under microscope to ensure they were rounded up and detached. To deactivate trypsin action, double volume of fresh medium that contained 10% FBS was added, then centrifuged for 3 minutes at 91×g. The pellet was resuspended with RPMI medium and split to 3 new flasks.

2.1.3 Cells cryopreservation

The storage of cells was done by placing cells in cold temperature. The growing and dividing cells were prepared for freezing storage by discarding medium, washing with PBS, trypsinzing and resuspension with fresh medium. Then cells were transferred to Universal tube, and counted by haemocytometer, centrifuged for 3 minutes at 91 ×g before the pellet was resuspended in freezing medium that was prepared by adding DMSO to RPMI at a ratio of 7.5 %. Special type of vials was used to prevent the explosion when exposed to liquid nitrogen. One ml of cells was added into each cryovials, a special cool box (Mr. Frosty™) was used to keep the cells at -80 for one night and then eventually moving the cryovials to liquid nitrogen.

2.2 Polymerase chain reaction

This part of work was done by our group as described in (5). The first step of experiment was to isolate RNA from each PCa cell lines by using RNAeasy Mini Kit (Qiagen, USA). Cell pellet was prepared as described in 2.1. Then cells were lysed and centrifuged at 1008 xg. The mixture was added to RNAeasy mini column attached to collection tube, to be centrifuged at 8000 xg for 15 seconds.

This was followed by 2 steps of washing with buffer. Then RNA was eluted through centrifuging at 1008 xg for 1 minute.

NanoDrop ND-1000 spectrophotometer (Labtech, UK) was used to calculate RNA yield and purity.

The integrity of total RNAs was assessed by Agilent 2100 bioanalyzer. The work was started by decontamination of one of electrode while filling the electrode with RNase-free water, and placing it in the Agilent 2100 bioanalyzer. Agilent RNA 6000 Nano gel matrix was placed in spin filter and centrifuged for 10 minutes at 1792 xg. The gel was then aliquoted and stored. RNA 6000 Nano dye was added to filtered gel and vortexed. RNA Nano chip was placed on the chip priming station, and gel-dye mix was added into the chip station. RNA Nano marker was added into the ladder for each sample. Then, the chip was placed in the bioanalyzer. The minimum RIN accepted in this experiment was 9.0.

The primers used for FABP12 are shown in table 3.

gene	Forward	Tm	reverse	Tm
FABP12	GGAAGGAAATAGCAACAGTGG	61.29	CTACACGCTCACCATATAAGC	60.66

Table 3: forward and reverse primers for FABP12 used in PCR experiment.

These primers were designed by using the website (perlprimer.sourceforge.net/) While those for β-actin were purchased from Life Technologies Ltd (Paisly, UK). The table shows characteristics that applied when designing the primers: melting temperature between 57ºC and 63ºC, the G+C content between 30-80%, length between 18-22 bp, less than 2ºC difference in melting temperature (Tm) between the forward and reverse primers.

First strand cDNA was synthesized using total RNA from 6 PCa cell lines to be used as a template for qPCR. Total RNA was mixed with Oligo (dT) primer, dNTP mixture, and nuclease-free water. The mixture was incubated for 5 minutes at 65 ºC, then chilled on ice for 1 minute, followed by mixing with DTT, first strand buffer, SuperScript reverse transcriptase and incubating at 50 ºC for 1 hour. Then incubating at 70 ºC for 15 minutes to deactivate the reaction.

A mixture was prepared for real-time PCR containing: SYBR Green qPCR mix, forward primer, reverse primer, cDNA and nuclease-free water. The mixture was centrifuged and placed in thermocycler (Peltier thermal cycler PTC-200, GMI, USA). Then, the relative differences of FABP12 mRNAs between benign and malignant cells were calculated. Relative fold differences of all samples were calculated according to ΔCt = Ct (gene of interest) – Ct (housekeeping gene), housekeeping gene was β-actin. While ΔΔCt =ΔCt of sample -ΔCt of the calibrator sample (5).

2.3 Detection of protein expression in cell lines by Western blot

2.3.1 Collection of cell pellets

The pellets of the cultured cells were collected when cells were 80% confluent or above. The old medium was discarded, and cells were washed with PBS, trypsinized in the way as described in cell subculture, and centrifuged. The pellet collected was lysed with a mixture of the CelLytic reagent plus protease inhibitor in a universal tube. The tube was then placed on a roller mixture shaker to shake for 15 minutes, followed by centrifuging at 11,200 × g for 10 minutes, and the supernatant was collected for Western blot analysis.

2.3.2 Bradford assay

Bradford assay was used for measurement of protein in each sample loaded for Western blot analysis. When protein binds to Coomassie Blue G-250 dye, it can change dye's color from brown to blue. The color correlated with protein concentration and can be detected by measuring absorbance with a spectrometer (Biotech, UK). Quantification of protein concentration can be made through comparing protein absorbance with a standard curve of serial dilutions of a common protein called bovine serum albumin (BSA) (91).

Bradford dye was filtered through a Whatman paper, 1 ml was added to each tube of serial dilutions and the protein tubes. Followed by transferring the serial dilutions and cell proteins in triplicate into microplate reader to measure the absorbance at 595 nm by spectrometer. A standard linear curve of protein concentration vs corresponding absorbance values was plotted and used to define unknown sample concentrations.

2.3.3 Western blot

The total proteins obtained from each source were separated by electrophoresis with a Bio-Rad Mini-Protean gel system (Bio-Rad, UK). All buffer reagents required were prepared ahead according to recipes as shown in the appendix. A, protein concentration from each sample was calculated based on Bradford assay results and an equal amount of total protein in each sample was loaded into a well of the gel for PAGE. Sample buffer (Laemmli buffer) was prepared in an Eppendorf tube. β-mercaptoethanol was added to each protein sample to stop the formation of the disulfide bonds and hence to prevent the protein aggregation. Then proteins dissolved in buffer at the reduced condition were

denatured by heating on a hot plate for 10 minutes at 95 °C. Then the mixture was placed on ice immediately for 3 minutes. The gel was positioned in a clamping frame and ensured that it is locked well. Running buffer was added to the tank. Molecular weight marker was loaded as a reference, and cell protein (20 µg/20 µl) was loaded into each separate wells. The electrophoresis was conducted for 1 hour with 100 volt.

To transfer the separated proteins in the polyacrylamide gel onto a PolyVinylidene DiFluoride (PVDF) membrane for further immune-detection, the protein gel was placed on the membrane to form a sandwich and wrapped with a cassette. The cassette was prepared by sponges, filter papers, gel, and then membrane and last is filter paper and sponge as shown in figure 14. Bubbles were removed by a roller to prevent displacement of gel. Cold ice was used to prevent overheating and gel break. PVDF membrane (Immobilon-P, Millipore, and USA) was activated by incubation in methanol for 2 minutes. Then rinsed in cold transfer buffer before assembled it into the cassette with Whatman filter papers and sponges, the transfer is done at $4^{o}C$ in a transfer buffer prepared in advance (appendix. A). Electroblotting was conducted for 1 hour at 100v, with ice pack changed after a half hour. One hour later, both gel and membrane were disassembled and washed in distilled water.

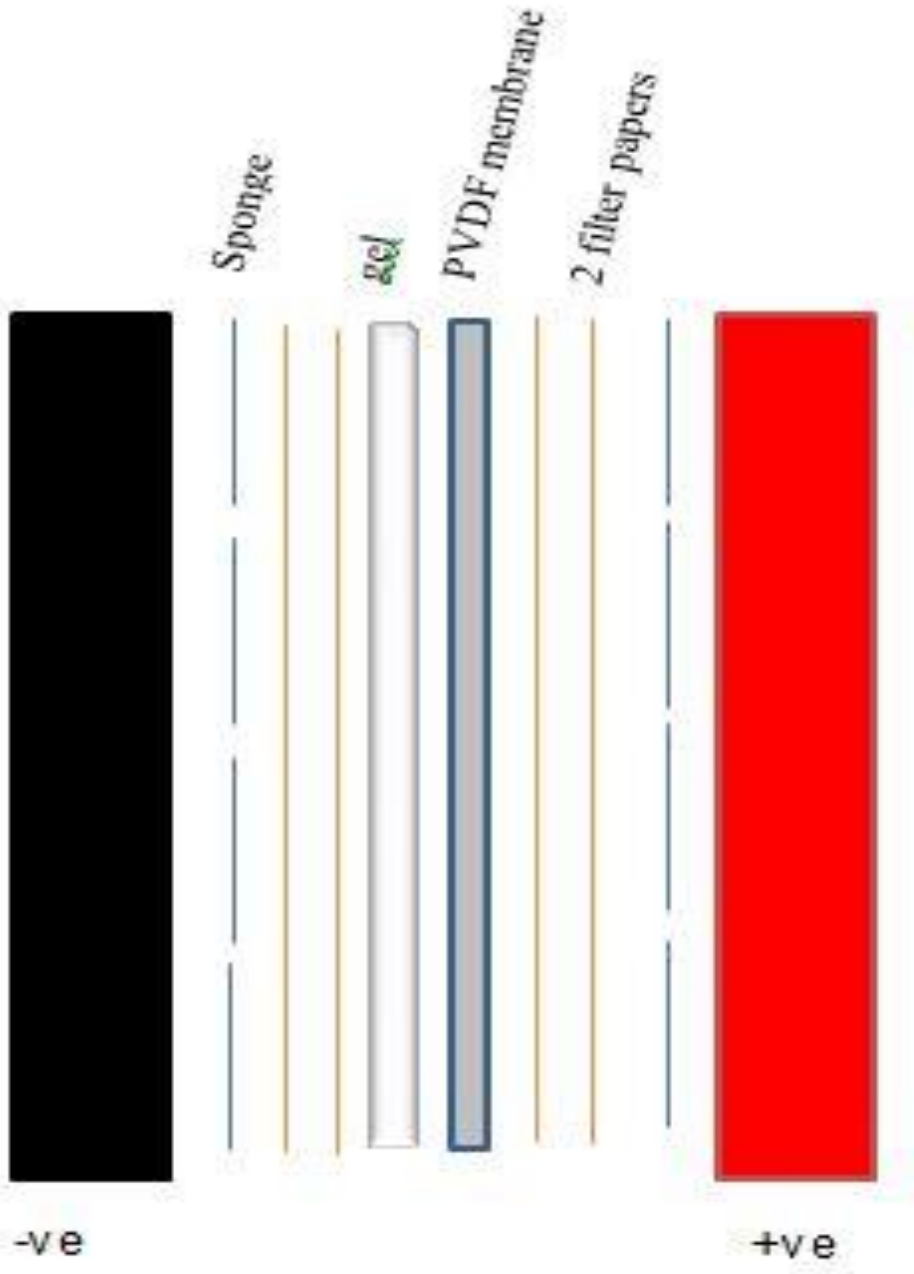


Figure 14. Diagrammatic illustration of Western blot transfer cassette. Arranged from black to red: sponge, 2 filter papers, gel, PVDF membrane, 2 filter papers and sponge. The current migrates from the negative black electrode to the positive red electrode.

An additional step was taken to check the efficiency of the protein transfer. The gel was stained with Coomassie blue, followed by washing with distilled water overnight and checked to ensure that the transfer was completed. The membrane was stained with Ponceau S red for 3-5 minutes to ensure transfer was successful by the presence of protein bands. Then the membrane was washed with TBS buffer for few times on a shaker plate to remove the stain. The membrane was stored at $4^{o}C$ for antibody hybridization analysis later.

2.3.4 Immunodetection

The membrane was first washed with TBS-T buffer to remove any possible gel residuals and then incubated with the primary anti-FABP12 antibody (Abcam, ab155089) for one hour on a shaker, followed by 4 washes (each 5 minutes) with 1× TBS-T buffer to remove the unbound antibody. The membrane was then incubated with the secondary antibody conjugated with horseradish peroxidase for one hour on a shaking plate, followed by other 4 washes (5 minutes each) to remove any unbound secondary antibody residuals. Afterwards, the membrane was first incubated in Chemiluminescence ECL in dark condition. The protein bands were visualized by the ChemiDoc imaging system, optimizing exposure and timing to get the optimal images.

The membrane was hybridized with an anti-β-actin to correct any possible loading discrepancies. Firstly, the membrane was blocked in TBS-T milk for 30 minutes to prevent nonspecific binding. Secondly the membrane was incubated with primary anti-β-actin mouse antibody (1:50000) in 20 ml milk for 30 minutes, followed by 4 washes (5

minutes each) with TBS-T buffer. The membrane was then incubated with a secondary rabbit anti-mouse antibody (1:10000) for 30 minutes followed by washing and the actin band was visualized using ECL system as described above.

2.4 Evaluation of FABP12 expression in prostate tissues

2.4.1 Sample collection

All human prostate tissues used in this work were obtained from an archival set of prostate cancer cases from Royal Liverpool Broadgreen University hospital. The cases were selected from diagnosed PCa patients in the period 1995-2001. These patients were aged between 67 and 73 years and all had transurethral resection of prostate (TURP). The work was conducted under the Medical Research Council guidelines and the project was approved by the National Science Ethics Committee (Project reference number: Ke; 02/019). The formalin-fixed paraffin-embedded (FFPE) samples were processed in our Molecular Pathology Laboratory with the standard FFPE tissue processing procedures described previously (150). Cases were re-examined by two qualified pathologists and categorized according to their combined Gleason score into weakly, moderate and highly malignant carcinomas. Levels of PSA were classified into two groups of low (<10 ng/ml) and high (≥ 10 ng/ml). All patient data were obtained from the hospital patient records in an anonymous manner. AR index (a parameter used to measure the intensity of AR staining (5, 165) was categorized into low AR level (AR index below 3), moderate AR level (AR index 4-6), and high AR level (AR index above 6).

2.4.2 Tissue sections

Tissue blocks from BPH and carcinoma cases were cooled on microtome cold plate. The tissue sections from each block were cut at 4 µm thickness with a microtome. The tissue slice was flattened by placing it in a water bath before it was mounted on an adhesive glass slide (Leica, UK). The slides were labeled carefully and placed into a 37 °C oven in an upright position in a slide rack to dry off any water over night.

2.4.3 Immunohistochemistry

The immunohistochemical staining technique was used to detect protein expression status in tissues and to detect their subcellular localizations. This is achieved through binding a specific antibody to the tissue antigen via initiation of antigen-antibody reaction and then, the slides were processed through different steps according to protocols to detect FABP12 expression in PCa tissue. Since FABP12 is a newly discovered molecule, the protocol was optimized in laboratory by multiple tests to get the highest binding affinity of antibody. The slides were first deparaffinized in 2 racks of xylene for 5 minutes respectively. Then rehydrated in 2 changes of 100% ethanol (5 minutes each) sequentially. The slides were passed to the fifth rack which was 3% hydrogen peroxide mixed with methanol for 12 minutes to block the endogenous peroxidase. The slides were then washed in tap water.

To expose the target protein (antigen) sites, both enzymatic and heat-induced antigen retrieval were evaluated to assess the most effective retrieval methods for the specific antigen. In this work, the slides were immersed in a sodium citrate buffer. To prevent water evaporation and tissue drying out, the slides were placed in a plastic slide rack, sealed partially, and heated in a microwave for 15 minutes with a close monitoring. Then, they were left to cool down for 10 minutes and washed with tap water.

2.4.4 Antibody incubation

The hybridization of the antibody with the antigen was performed in a special incubation chamber that was filled with water to make humidified conditions to prevent drying out. The slides were washed in TBS-T 3 times, then anti-FABP12 antibody (Abcam, rabbit polyclonal FABP12 antibody) diluted in TBS was added to slides in humidified chamber which was then incubated in fridge overnight. Then the slides were washed 3 times in TBS-T. For the hybridization with the secondary antibody, Dako HRP system plus the linker was added, incubated for one hour, and washed with TBS-T three times. Then the secondary antibody diluted in TBS-T was added and incubated for 1 hour at RT, washed 3 times in TBS-T, and then incubated with Envision™ FLEX DAB^{+} chromogen mixed with Envision™ FLEX substrate (Dako) for 15min followed by rinsing with tap water.

The slides were counterstained in hematoxylin, washed with tap water, then rinsed with acid alcohol and blue Scott water, followed by washing in water, immersed 3 times in xylene and mounted on glass slides with cover slip using DPX mountant.

2.4.5 Evaluating immunoreactivity

The selection of controls was based on the recommendation of the stain kit manufacturer (Abcam, UK). For positive control, normal human testis tissue was used. For the negative control, the sample was stained with the same procedures except the step of primary antibody which was substituted with PBS. Since the applied antibodies were all polyclonal, the isotope control could not be applied.

For slides examination, light microscope was used, for image acquisition, Nikon dual headed microscope was used.

FABP12 immunoreactivity was scored by two independent observers and the staining intensity was evaluated through 40 × magnification and using scores of 10 fields. The staining intensity classification is shown as that in the table below:

Unstained	-ve or 0
Weak staining	+ or 1
Moderate staining	++ or 2
Strong staining	+++ or 3

Table 4: Classification system to evaluate staining intensities of PCa tissues.

2.5 Gene editing

To study the possible functional role of FABP12 in promoting malignant progression of the PCa cells, gene editing technique CRISPR Cas9 was used to knockout the *FABP12* gene from prostate cancer cells to assess the effect of the diminished expression of this gene in tumorigenicity of the cancer cells. Since CRISPR Cas9 technique is a new state-of-the-art method, the original protocol had to undergo several optimizations. The plasmid was purchased from GeneArt® CRISPR Nuclease (Invitrogen, U.K). It incorporates both a guided RNA and Cas9 nuclease to cleave the specifically targeted gene and to allow the development of clones of PCa cells with the absence of the target gene from both chromosomes. The techniques can be divided into two main processes: the knockout of the target gene and the establishment of cell lines originated from separated single colony with diminished expression of the target gene. The gene knockout was achieved by following steps:

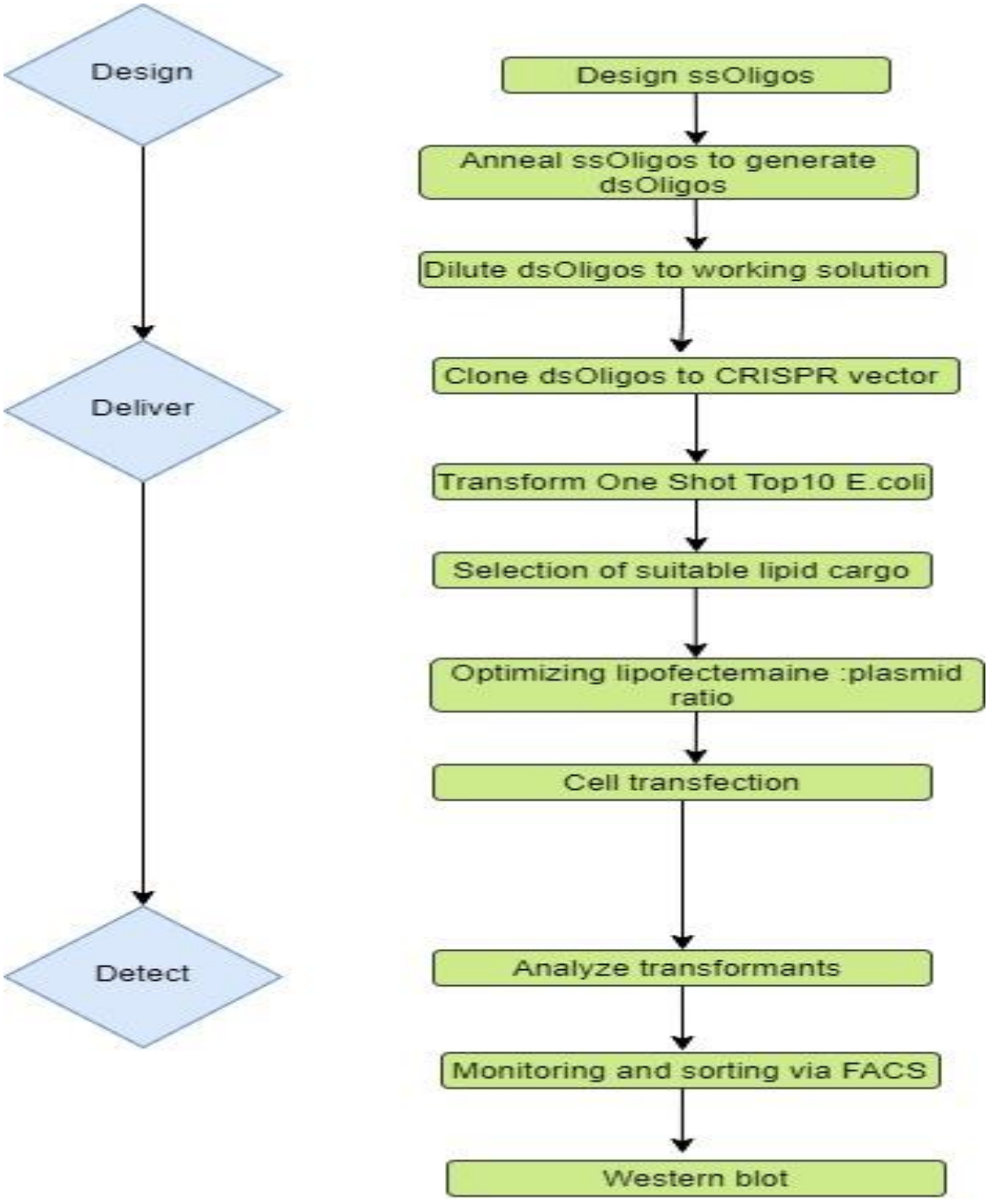


Figure 15: scheme represents knock out steps starting g from designing single stranded oligonucleotides. Three main steps in FABP12 KO include: Design, deliver and detect.

2.5.1 Guided RNA sequence design

The sequence designed inserted into the plasmid and used as guiding sequence which guided Cas9 to the crucial location of the target gene to initiate the knockout effect. Once the guiding oligonucleotide is designed, a complementary strand was made to form a double stranded DNA segment which will be inserted into the cloning site of the plasmid. The short DNA molecule is 20 base pair long. Additional over-hanging nucleotides, which were complementary to the linearized plasmid over-hangs, were flanked to both ends so that it can be ligated with the vector as shown in figures 16, 17. The choice of target sequence was based on the following guidelines:

1. The target sequence is 20 nucleotides in length that is adjacent to NGG proto-spacer adjacent motif (PAM) sequence, which is essential for cleavage.

2. No significant homology with other genes (assessed on online sequence designing tool).

3. The target sequence is designed in right orientation so that it meets PAM requirements as shown in figures 17.

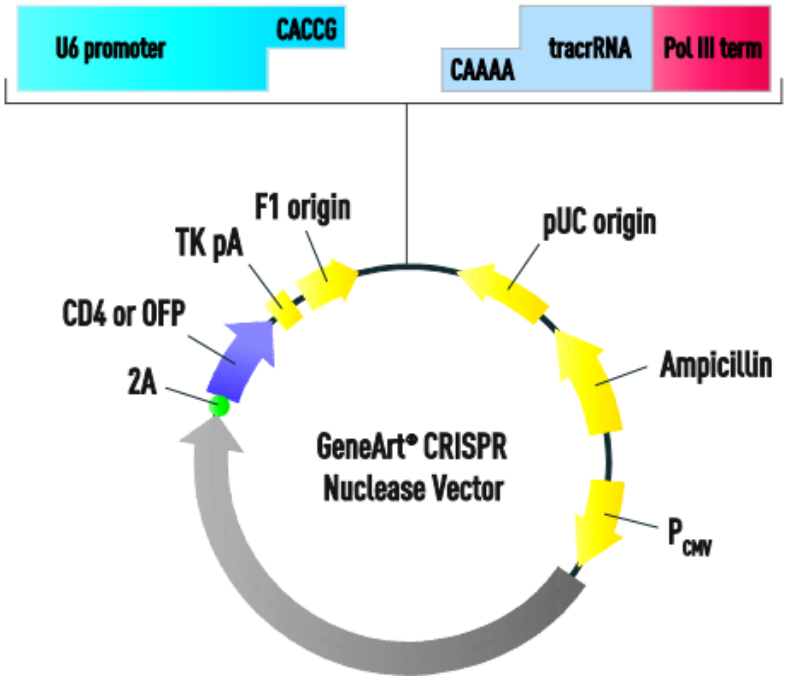
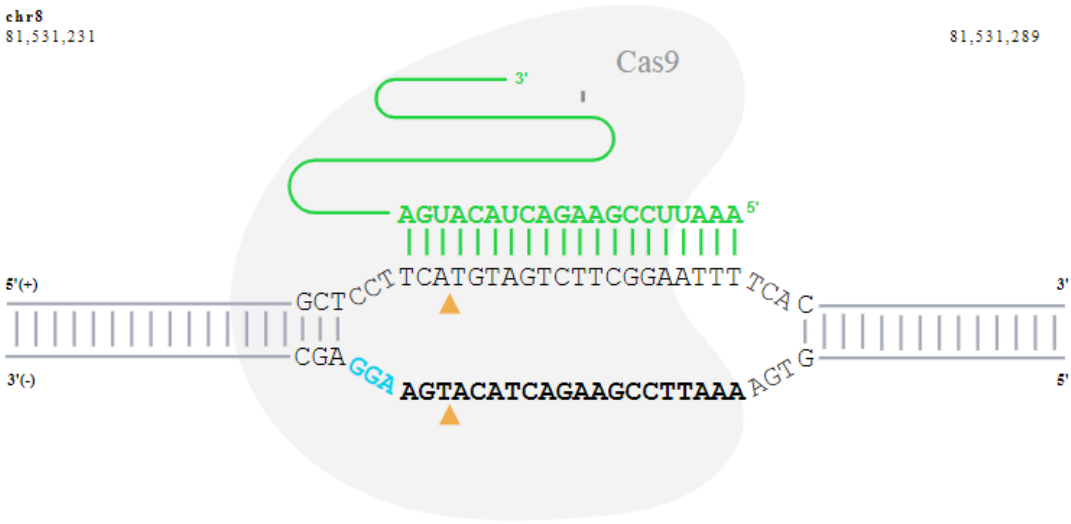


Figure 16 : map of Geneart CRISPR nuclease vector (Invitrogen, U.K).

The crRNA and tracrRNA of the GeneArt® CRISPR Nuclease Vector are expressed together as a guide RNA that mimics the natural crRNA-tracrRNA hybrid in bacterial systems. The guide RNA expression is driven by a U6 polIII type promoter. The vector is supplied with (OFP) to monitor transfection efficiency with 5 base pair 3′ overhangs on each strand as indicated. The ds oligos inserted as explained in 2.5.1, Additional over-hanging nucleotides, which were complementary to the linearized plasmid over-hangs, were flanked to both ends so that it can be ligated with the vector.

A



B

Figure 17. A. A scheme represents localization of guide sequence. Multiple guides were screened to identify the one that is efficiently active**. B.** Location of gRNA in FABP12 genome. This guide will target a sequence in the gene FABP12 in the Homo sapiens genome. The Cas9 RNP will bind to the sense strand (+) of the gene. It will create a double-stranded break at location 81,531,255.

(Scheme was generated using Synthego CRISPR design tool https://www.synthego.com/).

2.5.2 Annealing

Once both oligonucleotides sequences were designed, the products were purchased from the bio-synthetic company (Thermofisher, UK). The dried oligonucleotides products were suspended in T.E buffer separately. To anneal the two oligonucleotides to form a double-stranded DNA, A mixture was prepared of equal volume of the forward- and reverse- strand oligos with 10× annealing buffer with DNase/RNase-free water. The mixture was first heated on a heating block for 4 minutes at 95 °C and then cooled to 25°C gradually and stay at 25°C for 10 minutes. The double stranded oligos were then diluted in DNase/RNase-free water to make a 500 nM stock solution that was aliquoted and stored at -20°C. A working solution, the 500 nM oligos were diluted into 5nM ds oligos and the freshly prepared 5nM solution was used for experimental work.

2.5.3 Ligation reaction and transformation

To insert the ds oligonucleotides into the linearized plasmid (Invitrogen, UK) by DNA ligase: The ds oligos ligated to the plasmid in a reaction mixture of oligos, linearized plasmid, ligation buffer (final concentration 1×) and T4 DNA ligase. The reaction mixture was incubated at 25°C for 10 minutes to complete the ligation reaction. The ligation mixture was stored at 4 ^{o}C , ready for transformation to the One Shot® TOP10 competent E. coli cells supplied with the plasmid kit (Thermofisher, UK).

At least one day before the transformation experiment, agar plates were prepared for bacterial growth. The heat melted LB agar was cooled down and ampicillin (100 µg/mL) was added and mixed. The mixture was poured into Petri dishes and stored in 4 °C fridge. The LB plates were pre-warmed for 30 minutes in a 37 °C incubator before used for transformation.

A vial of One Shot® TOP10 competent E. coli was thawed on ice, the ligation reaction mixture (5µl) was added into that vial, the tube tapped gently, placed on ice for 30 minutes, the step of heat shock was performed by placing the tube for 30 seconds at 42 °C and immediately placing it on ice. S.O.C medium was added to the tube before it was taken to a shaking incubator and incubated for 60 minutes at 37°C with a shaking speed of 200 rpm. The E.coli cells in the S.O.C medium was then pipetted and spread with a spreader onto a pre-warmed LB agar plate and incubated in a 37°C oven overnight. The transformation efficiency was calculated by the number of colonies produced per µg of DNA.

2.5.4 Mini-preparation of DNA and sequencing

To confirm the guiding RNA sequence was successfully inserted into the plasmid in a correct orientation, DNA was extracted from each of the individual E. coli transfectant colonies with a mini-prep procedure. For each mini-DNA preparation, several colonies grown up from the ampicillin resistant LB agar plate were picked by a flame-sterilized loop, each was separately cultured in a flask containing LB medium with 100ug/ml ampicillin and incubated overnight in a shaking oven at 37 °C. The plasmid DNA was

isolated from the bacterial pellet using a DNA Mini-Prep kit (PureLink® HQ Mini Plasmid Purification Kit, Invitrogen, UK), following the manufacturer's instruction. The plasmid DNA was sent to Source BioScience (U.K) for nucleotide sequence analysis (the full sequence is presented in results, figure 3.14) to confirm the guiding sequence is inserted to the plasmid and the inserted fragment was in a correct orientation.

2.5.5 Delivery of transfection component

Once the successful cloning and the correct insertion orientation of the guiding sequence were confirmed, sufficient quantity of plasmid DNA was produced and purified from the E.coli cells and transfected into the highly malignant PCa cell line PC3M to yield the genetic deletion of the *FABP12* gene. Before the transfection, the purity of plasmid DNA was checked by the NanoDrop™ One spectrophotometer (Thermo Scientific, UK) to ensure that it was free from contamination. we used the cationic lipid based Lipofectamine® 2000 Reagent (Cat. no. 11668-027, Invitrogen, UK) as a delivery cargo. The cells were grown in 6 wells plate and when they became about 70 % confluent; the mixture of plasmid and Lipofectamine was added to cell lines and monitored.

2.5.6 Identifying of cells harboring plasmid

Transfection efficiency for PC3M cell lines was monitored by fluorescence microscope (Zeiss Axio Observer Z.1. Zeiss, Germany). The cells harboring the plasmid were sorted

by fluorescence activated cell sorting (FACS) machine (BD FACSAria™ III, BDbiosciences , USA). Since the plasmid used in this work contains an *orange fluorescent protein (OFP)* gene, any cells that had successfully taken up the plasmid should express OFP and were picked up by FACS. Thus, using the FACS machine, –ve OFP cells were discarded, +ve OFP cells were collected in FACS buffer, suspended in culture medium with 100µg/ml ampicillin (Ampicillin is a broad antibiotics against bacterial growth thus preventing potential bacterial contamination in cell culture), and incubated in incubator at 37^{o}C.

2.5.7 Generation of single cell colonies

To generate a knockout colony originated from a single transfectant cell, two methods were used: machine based FACS sorting and limited cell dilution. A summary of the procedures was illustrated in following flowchart:

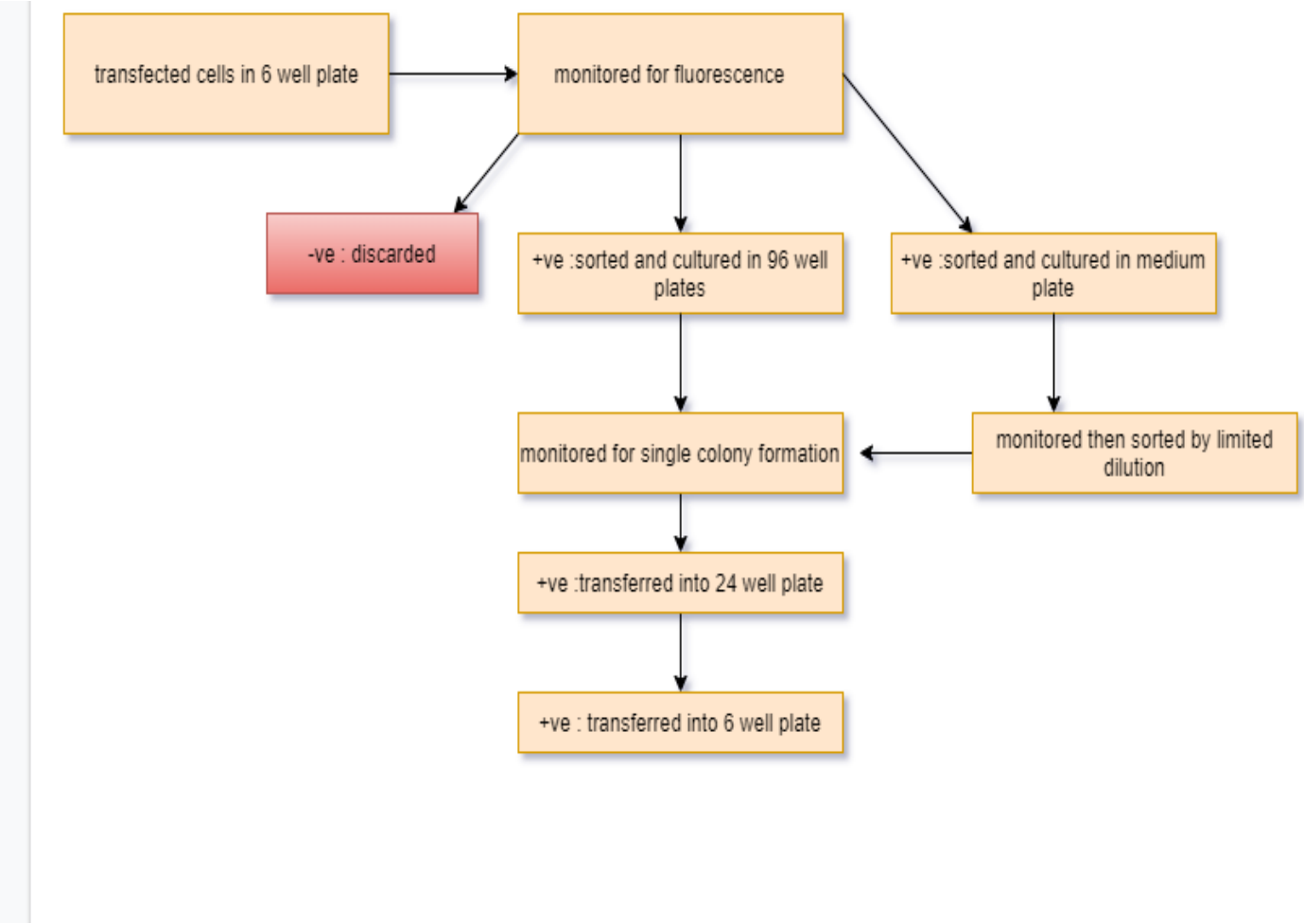


Figure 18: flowchart represents different steps applied in cell transfection.

2.6 In vitro tests for malignant characteristics

2.6.1 Proliferation assay

A proliferation assay was used to test the effect of gene knockout on the growth of the PCa cells. The numbers of cells at different experimental time points were calculated through evaluating cell viability which was based on the measurement of cell metabolic activity with a Resazurin assay kit (Thermofisher, UK) which was designed specifically for detections of cell viability and cell death. Once this compound enters live cells, the cytosol of live cells will chemically reduce resazurin (a blue, non-fluorescent compound) to red resorufin. Live cells continue the chemical reducing reaction. This reducing activity is proportional to metabolically active cells. Which will result in colour change that can be detected either by absorbance or fluorescence plate readers, to measure cell viability quantitatively. Since the dead cells cannot reduce the resazurin, the colour change can be used as an indicator of live cells only. The PrestoBlue® Cell Viability Reagent is time-saving viability reagent, as it provides robust data in only 10 minutes following reagent addition.

The assay was performed in a 96-well plate, in which cells with 100% gene KO, cells with 50% gene KO (only one allele was knocked out), and control cells were growing in different wells for 6 days. On each day, the PrestoBlue reagent was added to wells and incubated in cell culture incubator for two hours, then the absorbance at 570 nm was read by a spectrophotometer (Labtech, UK), the absorbance at 600 nm was read and used as a baseline wavelength.

The assay was run in triplicate, and two controls were used: the parental cells growing in the wells; and those wells which only culture medium, but no cells were added.

2.6.2 Motility assay

This assay is used to assess the effect of the gene knockout on cell migration ability. In this experiment, Ibidi culture-Insert (Ibidi TM, Germany) was used to evaluate cell migration behaviour. These insertions consist of two cell culture reservoirs, with a wall in between, whenever cell suspension added to reservoirs, cell growth will be followed. Then the insert will be removed after cell attached, this will generate a gap of 500 µm. The cell migration ability is measured by monitoring the speed of the wound-healing.

Cell suspension was prepared as described in cell culture section, 70 µl of suspension was added carefully to each well of cell cultures, followed by incubation in cell culture incubator in humidified condition at 37 °C and 5% CO2 for at least 24 hours. Cells were allowed to grow to fully confluent and a complete monolayer is formed, sterile tweezers was then used to remove the insert gently without disturbing cell layer. The dish was observed under microscope and pictures acquisition done at certain time points to assess cell migration by the sizes of the narrowing gaps. The images were obtained by microscope and quantitatively analysed by Image J software.

2.6.3 Invasion assay

The invasion assay was used to measure the ability of cells to invade extracellular matrix. This was achieved through a Boyden chamber system. It came in the shape of chambers with plate and inserts, the latter has 8-micron pore size membrane with the Matrigel basement membrane matrix. Cells were placed in upper chamber, and their migratory capability into lower chamber was assessed. Cells with invasive properties were able to detach and invade through Matrigel pores; however, non-invading cells were blocked by pores.

On day of the assay, chambers were pre-warmed by adding warm medium and placing in a cell culture incubator at 37^{o}C for two hours. Then medium was aspirated carefully. Medium with FBS was added to lower chamber, the FBS in the medium works as a chemoattractant. Cell suspension, prepared as $1.25x10^{5}$ cells /ml, was added with serum free medium into upper chamber, and incubated for 22 hours in a cell culture incubator. On the following day, cells that did not invade through the membrane were removed from upper chamber by scrubbing gently using cotton swab; the invaded cells on the opposite side of the membrane were stained using crystal violet. The invaded cells were counted under a microscope (EVOS, Thermo Scientific, UK). Nine random fields were counted for cell quantification analysis.

2.6.4 Soft agar assay

To test the anchorage-independent growth of the cells, the soft agar assay was used to assess effect of the gene knock out on the colony formation ability (as an indication of tumorigenicity) of the cells in soft agar.

The experimental procedures included preparation of two agarose layers, bottom, and top layers; the former is prepared by dissolving agarose powder in distal water and mixed it with FBS and 10× DMEM solution. The bottom layer mixture was added to each well of 96 well plates before it was transferred to 4°C fridge for 15 minutes to solidify the agarose. The second layer was prepared by mixing agarose in dH2O, FBS, 10× DMEM and the cells in 1X DMEM/10% FBS. The second layer mixture was added onto the solidified bottom layer, the plate was kept in a fridge until the second layer solidifies. The test was run in triplicates with control included. The plate was eventually incubated in cell incubator for 10 days, and colonies were viewed and counted under the microscope.

2.7 Data analysis

In this work, data analysis was carried out using the following software: Microsoft Excel, ImageJ, and Statistical Package for Social Sciences (SPSS). In all statistical tests: the significance was determined by p value < 0.05. The following tests were used accordingly:

A. Chi-square ($\chi 2$)

This test is used measure the association between two categorical variables (212). It was used in this study to assess if the difference is significant between the expected frequencies and the observed frequencies. Correlation between benign and malignant PCa tissues staining was assessed using $\chi 2$.

B. Kaplan-Meier curve

This test is used to assess the probability of surviving in a given period of time (75). In this work, Kaplan-Meier survival curve was used to analyze correlation between survival and expression of FABP12, GS, AR level and PSA level.

C. Log Rank test

This test is used to assess the null hypothesis that there is no difference between the populations in the probability of survival (17). In this study, log rank test was applied to assess the significance association between survival time and FABP12 expression.

D. Student's t-test

This test is one of the most frequently used statistical tool to analyse data. It compares the means between two groups when the variables follow normal distribution (149). In this work, Student's t-test was used to compare the difference between experimental and control groups in western blot, proliferation assay, invasion assay, soft agar and motility assays.

CHAPTER THREE:

RESULTS

3.1: Assessment of FABP12 expression at the mRNA level

The previous work by our group measured FABP family RNA levels in benign and malignant prostate cell lines; it was found that *FABP12* was one of the RNAs that is highly expressed in PCa cells.

The result confirmed that the differences in levels of the mRNAs for *FABP 1,3,7, and 8* between benign and malignant cell lines were not detectable (Fig 3.1.D). For *FABP4* mRNA, its level was higher in two high malignant cells (PC-3 and PC3-M) but was not different between the benign and other malignant cell lines. *FABP5* mRNA level was significantly increased in all 5 prostate cancer cell lines compared with that detected in the benign PNT-2 cells. FABP6 showed similarities to FABP4 in their mRNA levels, thus, their mRNA levels were highly expressed in malignant cells, while no expression in benign cell line was detected. This led to conclusion that FABP6 is predominantly expressed in prostate cancer cells, as that showed in previously (5). FABP9 mRNA level showed an elevation of 5 to 47-fold in cancer cell lines when compared to that in the benign cell line PNT-2.

As shown in Figure 3.1, the total RNA was in high quality, the quality and integrity of mRNA were assessed using Agilent 2100 Bio-analyzer (A), the RNA integrity numbers (RIN) obtained from different cell lines were either equal or above 9 (B). The levels of *FABP12* mRNA detected in different cell lines were shown in (C). When the level of *FABP12* mRNA in the benign PNT-2 cells was set at 1, the levels of the transcripts in the weakly malignant cell line LNCaP, moderately malignant cell line 22Rv1 and the highly

malignant cell lines DU145, PC3 and PC3-M were remarkably increased by 45 to 108 fold.

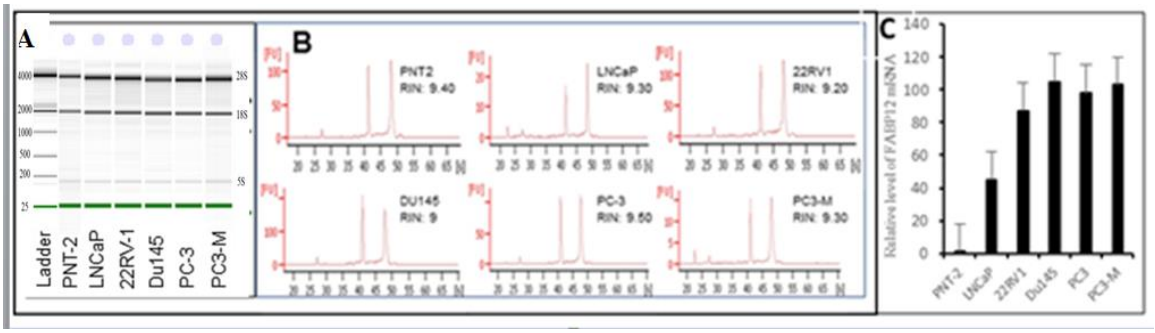


Figure 3.1: *FABP12* mRNA level is increased in all prostate cancer cell lines measured.

Quantitative PCR analysis of *FABP12* mRNA levels in the benign cell line PNT-2, weakly malignant cell line LNCaP, moderately malignant and androgen-responsive cell line 22Rv-1 and highly malignant androgen-independent cell lines DU145, PC-3 and PC3-M. **A)** The electropherograms of mRNAs plot from 6 cell lines and from the RNA ladder markers (green band). **B)** The fluorescence plots with double peaks representing ribosomal RNA 18S and 28S sub-units and the RNA integrity numbers (RIN) of the samples from different cell lines. Total RNA quantification was assessed using Agilent 2100 bioanalyzer. **C)** Relative levels of *FABP12* mRNA detected in benign and malignant prostate cell lines (5).

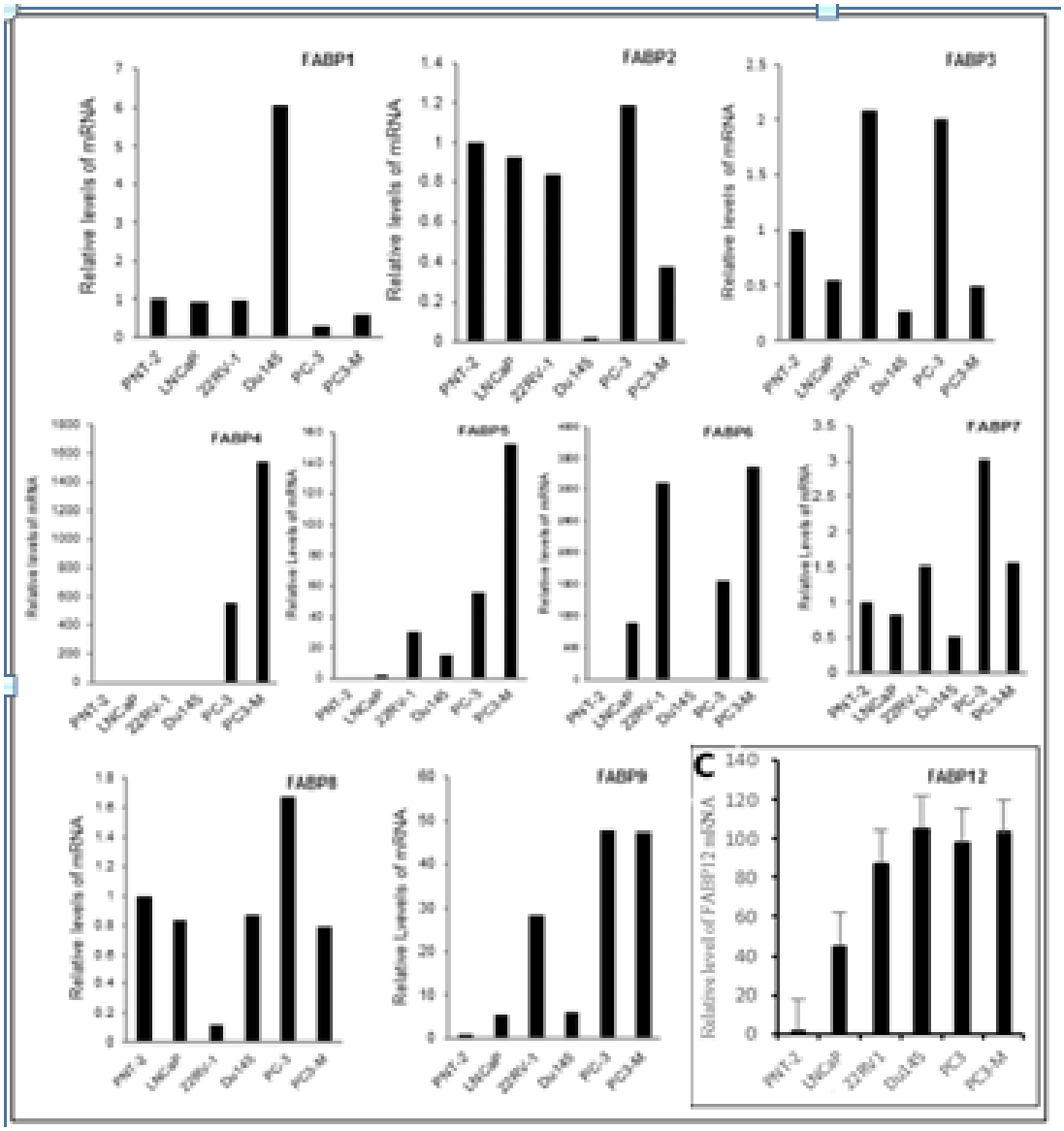


Figure 3.1. D. Quantitative PCR analysis of relative levels FABPs mRNAs in both benign and malignant PCa. cell lines (5).

The x axis shows the prostate cell lines PNT-2, LNCaP, 22Rv-1 , DU145, PC-3 and PC3-M. While the Y axis shows the relative level of mRNA of FABPs family.

3.2: Expression of FABP12 in prostate cells at protein level

Western blot was performed to analyse the level of FABP12 expressed in the benign PNT-2 cells, and the malignant PCa cell line LNCaP, the moderately malignant cell line 22Rv1 and the highly malignant cell lines DU145, PC3, PC3-M. As shown in Figure 3.2, the FABP12 protein band at 15 kDa was detected in all six cell lines analysed. The intensities of the bands seem to be different amongst the different cell lines.

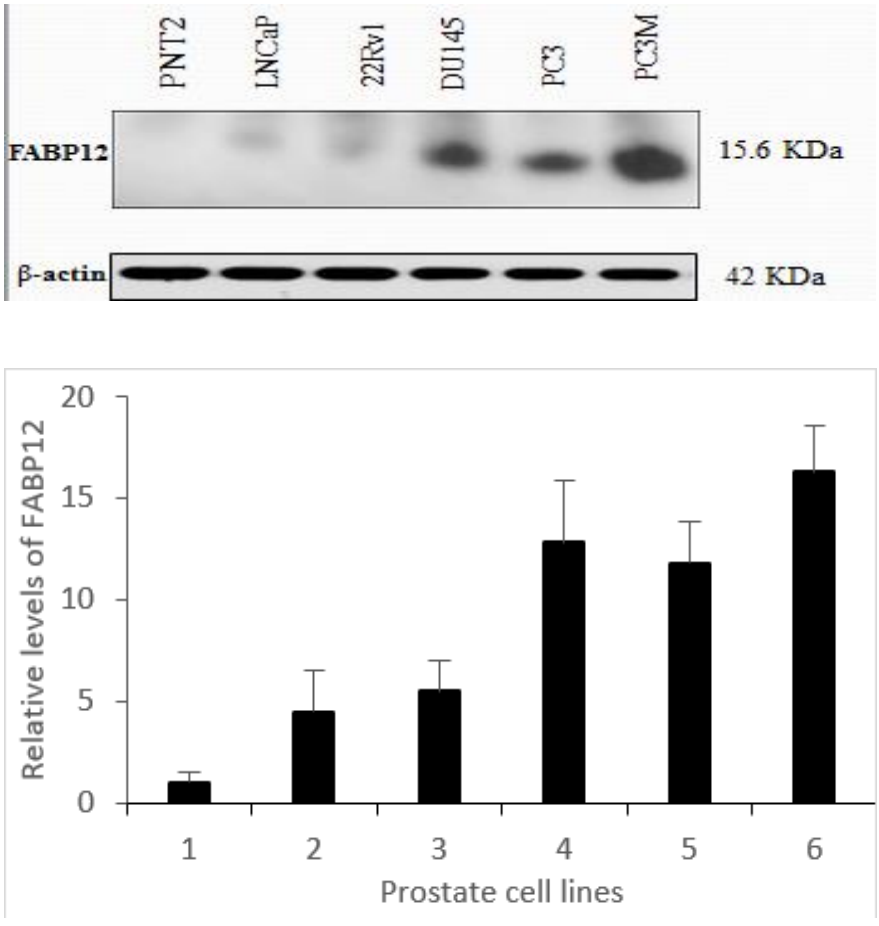


Figure 3.2: Expression of FABP12 in prostate cancer cell lines. A) Western blot analysis of FABP12 in benign and malignant prostate cancer cell lines. **B)** Quantitative assessment of the intensities of the peak area of bands on the blot by densitometry

scanning. The results (Mean ± SD) were obtained from 3 separate measurements. Relative levels of FABP12 were analysed by measuring the band intensities with the ImageJ.The specificity of antibody was evaluated by different methods by manufacturer.

The levels of FABP12 in malignant cells are higher than that in the benign PNT-2 cells, and this level was gradually increased as the increasing malignancies of the PCa cell lines. When the level of FABP12 protein in the benign PNT-2 cells was set at 1, its relative levels in the weakly (LNCaP), moderately (22Rv1), and highly (DU145, PC3, PC3-M) malignant prostate cancer cells were increased to 4.5 ± 2, 3.6 ± 1.5, 12.8 ± 3.1, 10.8 ± 2.1, 16.3 ± 2.3, respectively. Thus, at the protein level, the expression of FAB12 in PCa cells was increased by 4.5 to10.8 times.

3.2.1 Blocking the anti-FABP12 antibody by recombinant human FABP12

FABP12 is a newly discovered protein and the antibody is a new product. To exclude the possibility that a non-specific band has been detected, three separate Western blot analyses were performed on cell extracts from PC3-M cells which expressed the highest level of FABP12. One analysis was performed as normal. In the other two Western analyses, recombinant human (rh) FABP12 (Abcam, UK) was used to hybridize the anti-FABP12 for overnight at 4^{o}C at a concentration of 0.5μM. Both rhFABP12 (product No: ab126660) and anti-FABP12 (Product No: ab155089) were purchased from Abcam (UK). As shown in Figure 3.3, pre-hybridization with rhFABP12 for two hours blocked the most anti-FABP12 (Figure 3.3b), and pre-hybridization with RhFABP12 for overnight

completely blocked the anti-FABP12 (Figure 3.3c). When rhFABP12 was hybridized with the anti-FABP12 antibody for 2 hours before Western blot, most of the antibody was blocked. Whereas overnight pre-hybridization with rhFABP12 almost blocked all antiFABP12 antibody. This result indicated that the band detected in our preliminary study is indeed FABP12, not a non-specific molecule.

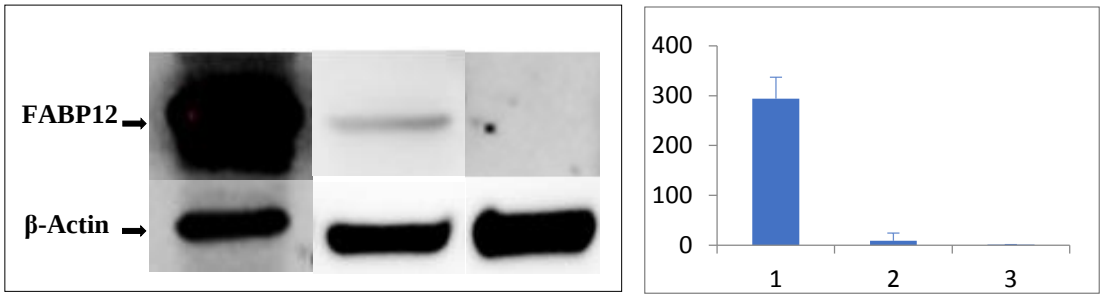


Figure 3.3: Blocking of anti-FABP12 antibody by rhFABP12. Western blot analysis of anti-FABP12 antibody specificity and the quantitative analysis of the blocking effect of the rhFABP12. The FABP12 level in 3 was set at 1. **1)** No rhFABP12 is added to hybridize the first antibody anti-FABP12. **2)** RhFABP12 is added to hybridize the anti-FABP12 for 2 hours before Western blot analysis. **3)** RhFABP12 is added to hybridize the anti-FABP12 for over/night before Western blot analysis.

3.3: FABP12 expression at tissue level

Formalin fixed paraffin embedded (FFPE) tissues that were obtained from an archival set of prostate cancer as described in methodology, the cases were categorized according to their Gleason scores as shown in Table 3.

Anti-FABP12 antibody immunohistochemical staining was conducted to examine the expression status of FABP12 in different categories of prostate tissues. Slides were examined under microscope by two independent examiners. The results showed that FABP12 is expressed mostly in cytoplasm. No nuclear staining was found except that one case (GS 6) exhibited a weakly staining. A representative slide from each category of the stained tissues was displayed in Figure 3.5 and appendix C. As showed in the figure, FABP12 cytoplasmic staining was observed in all cases and the staining intensity of each case was recorded and shown in Table 5.

Tissue	No. of cases	Cytoplasmic staining intensities				Nuclear stain intensities			
		-ve	+	++	+++	-ve	+	++	+++
BPH	27	25	2	_	_	27	_	_	_
Carcinoma (total)	88	19	23	28	18				
GS 6 and below	37	14	10	7	6		1	_	_
GS 7	18	4	3	8	3		_	_	_
GS 8-10	33	1	10	13	9		_	_	_

Table 5: Immunohistochemical staining of human prostate tissues with an antibody against FABP12. The tissues are classified as benign and malignant. Malignant tissues or carcinomas are further classified as weakly, moderately, and highly malignant carcinomas according to their combined Gleason scores (GS).

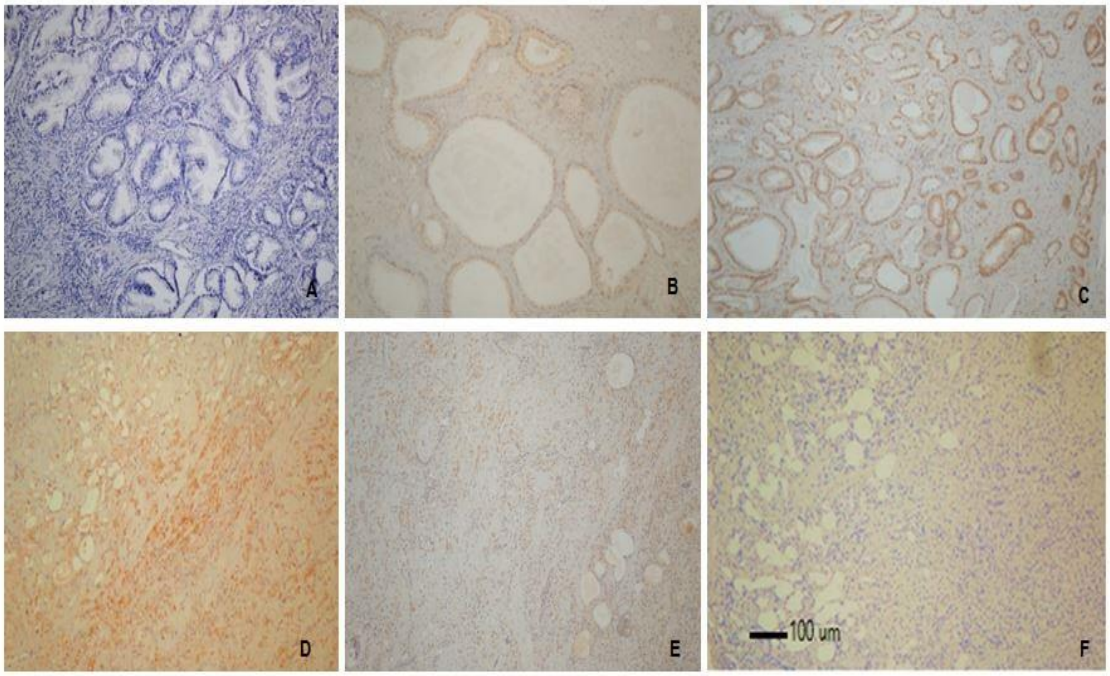


Figure 3.5. Representative anti-FABP12-stained slides from different categories of the prostate tissues. A) BPH tissue stained negatively in both cytoplasm and the nucleus. **B)** A weakly malignant carcinoma tissue (GS6) was stained weakly positive. C) a moderately malignant carcinoma (GS7) was stained moderately. D) A highly malignant carcinoma (GS10) was strongly stained. E) A highly malignant carcinoma was stained strongly in cytoplasm but stained negatively in nucleus. F) Negative control.

3.4: Association of FABP12 expression to combined Gleason scores

When the relationship between the FABP12 staining intensity and the combined GS was assessed, it was noticed that the staining intensity was the highest in the highly malignant carcinoma cases, as shown in figure 3.6. For the highly malignant cases, 27% were stained strongly positive (+++), 39% were moderately positive (++), and 30% were weakly positive (+). There was a small percentage of highly malignant carcinomas did not express FABP12 and thus, stained negatively.

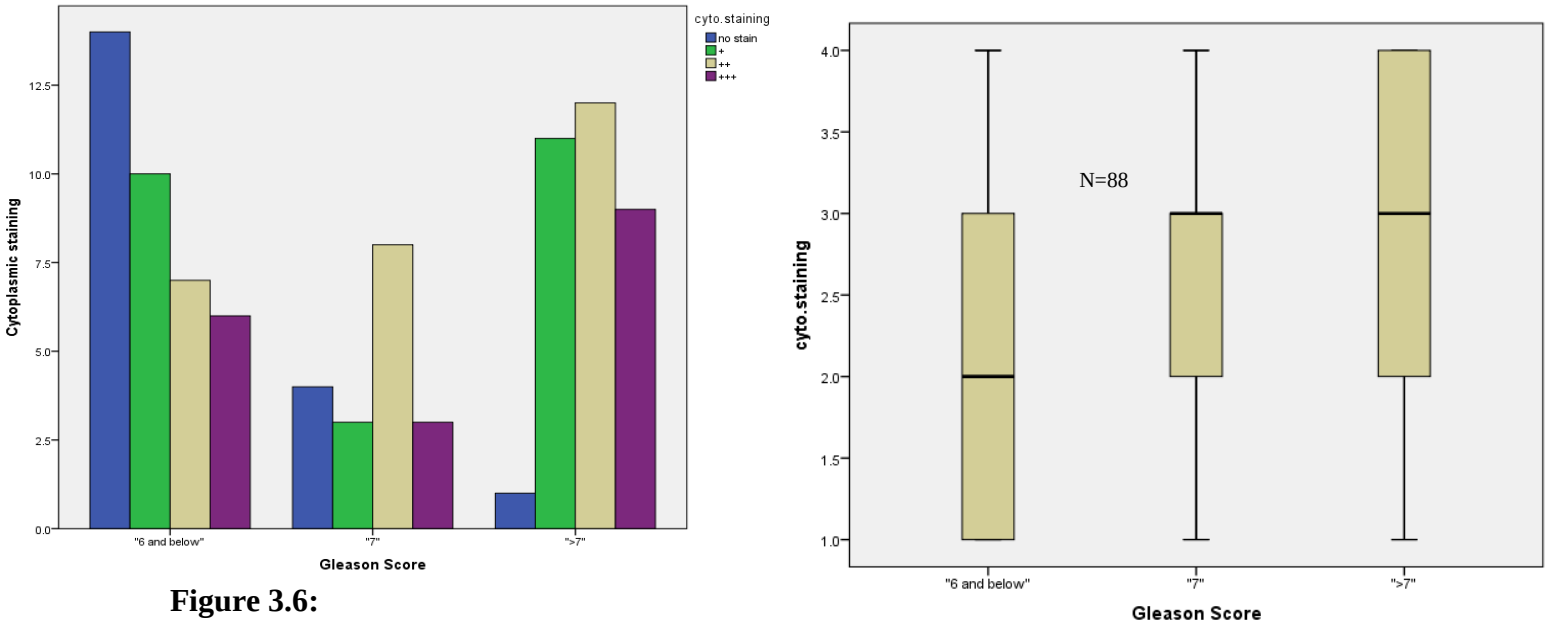


Figure 3.6:

A. FABP12 staining intensities in prostate carcinomas with different categories of the combined GS.

B. Box plot of FABP12 cytoplasmic staining intensity of the carcinoma cases of low (≥6), moderate (7), and high GS (8-10). X axis represents Gleason score while Y axis represents cytoplasmic staining.

For those moderately malignant carcinomas with a combine GS 7, 16.6% were stained strongly positive, 44.4% were moderately positive, and 16% were weak positive. There were 23% of the moderately malignant prostate carcinomas did not express FABP12 and thus, stained negatively. A decreased FABP12 cytoplasmic staining was observed in weakly malignant carcinomas with a combined GS $\leq$ 6. Only 6 % of the weakly malignant carcinomas were stained strongly positive. Nineteen and 27% of the weakly malignant carcinomas stained moderately and weakly positive, respectively. Fifty two percent of the weakly malignant carcinomas did not express FABP12 and thus, stained negatively. Amongst the 27 prostate BPH tissue samples, only 2 (7.4%) stained weakly positive, 25 (92.6%) samples were unstained.

Comparing FABP12 expression in BPH to that in prostate carcinomas with χ^2 test (χ^2 = 44.0931), the FABP12 staining intensities of the carcinomas was significantly higher than that in BPH tissues (χ^2 test, $p<0.00001$). Comparing the staining intensities amongst carcinoma tissues, a significantly higher staining intensity was observed in high malignant carcinomas than that observed in weakly malignant carcinomas ($\chi 2$ test, $\chi 2$ = 12.55, $p<0.0005$). The staining intensity of the highly malignant carcinomas was significantly higher than that observed in the moderately malignant carcinomas (χ^2 test, $\chi^2=4.8514$, $p<0.05$). Although the staining intensity observed in the moderately malignant carcinomas was higher than that observed in the weakly malignant carcinomas, this difference was not significant (χ^2 test, $\chi^2=1.34$, $p>0.1$).

3.5: Association of FABP12 expression with patient survival

As showed in figure 3.7, Kaplan-Meier surviving curve was used to assess the relationship between the level of FABP12 expression and the patient survival time. Those patients with a high FABP12 expression had a median survial time of 25 months, whereas those cases with a low FABP12 expression had a median survival of 60 months. Thus, the increased FABP12 expression in prostate carcinoma has siginificantly (log rank test, $p<0.05$) reduced the patient survival time by an average of 35 months.

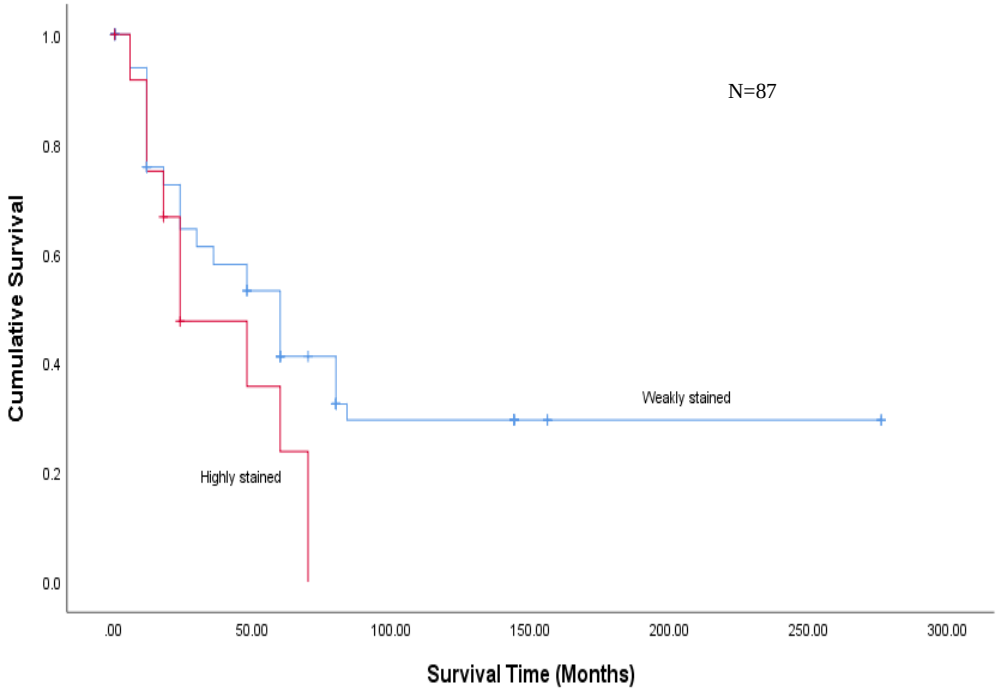


Figure 3.7 : Kaplan-Meier survival curve of stained cases. X axis and Y axis represent the cumulative survival time in months against FABP12 expression levels, for two groups, highly and moderately stained groups. Log-rank test, *P* value < 0.05.

3.6: Association of the combined GS with patient survival

Kaplan-Meier survival curve of prostate cancer patients was plotted according to their Gleason score and the result was shown in Figure 3.8. The low malignancy cases with GS ≤6 had survived more than 80 months. While those cases with moderate GS 7 had survived for 80 months. The high malignant cases with GS ≥ 8 had survived for 18 months only. The combined GS was significantly correlated with reduced patients' survival time (log-rank test, $p < 0.0001$).

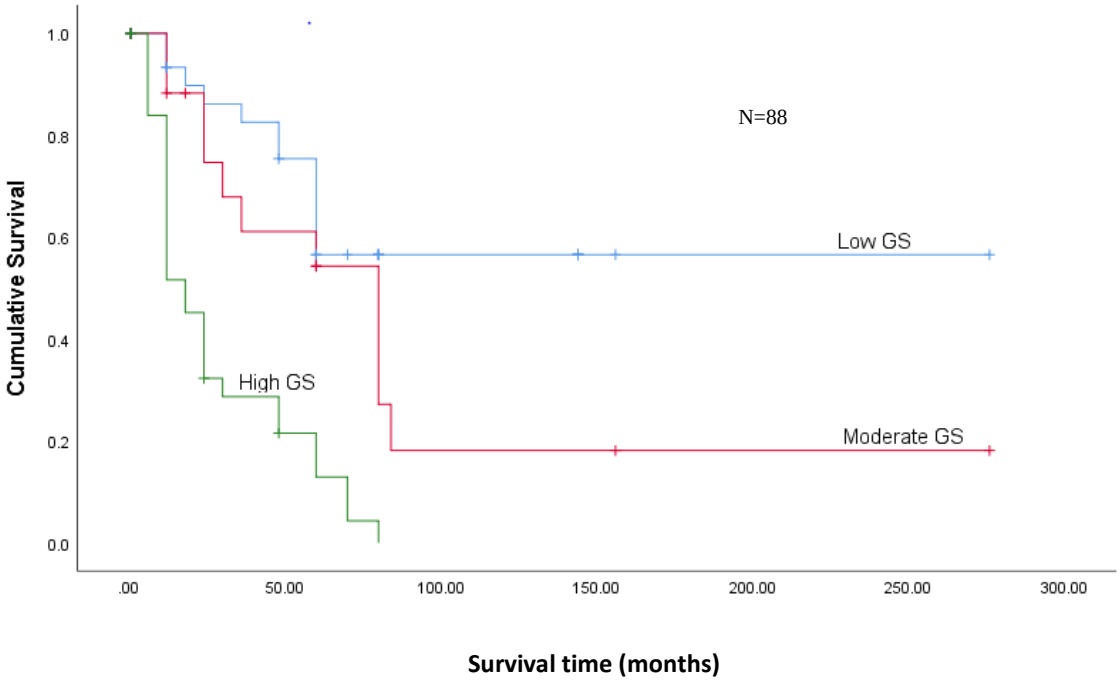


Figure 3.8: Kaplan-Meier surviving curve of patients with prostate cancer. This curve shows the patients cumulative survival in months for different combined GS, 3 groups are presented, GS<6, GS 7, and GS ≥ 8 (Log-rank test, *P* <0.0001). X axis represents survival (in months) while Y axis represents cumulative survival.

3.7: Association of AR index with patient survival

The association of AR index with PCa patient survival was assessed and the result is shown in Figure 3.9. Cases were categorized into three groups according to AR index: low AR level (AR index below 3), moderate AR level (AR index 4-6), and high AR level (AR index above 6). Patients with a low AR level had a median survival of 60 months. Patients with high AR levels had median survival of 24 Months. Although AR level had borderline correlation with patient survival, the differences in survival time are not statistically significant (Log-rank (Mantel Cox) test, p=0.06).

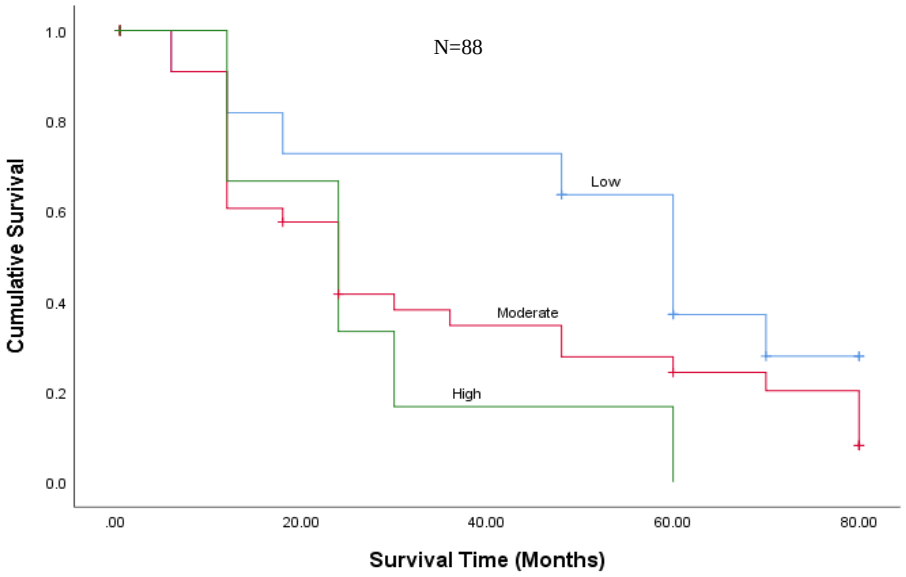


Figure 3.9: Kaplan-Meier survival curve for prostate cancer cases. The association between the AR level and the patient survival. X axis represents survival (in months) while Y axis represents cumulative survival.

3.8: Association of PSA with patient survival

PCa patients' survival association to PSA was assessed through Kaplan-Meier survival curve. The cases were categorized according to PSA levels, into low PSA group (<10 ng) and high PSA group (>10 ng). In the low PSA group, the average patient survival time is 60 months. Whereas in the high PSA group, the average patient survival time was reduced to 48 months. Although the average survival was different between the two group with low and high levels of PSA respectively, this difference was not statistically significant (Log-rank test, p=0.4). Thus the patient PSA level is not significantly correlated with the length of the patient survival time.

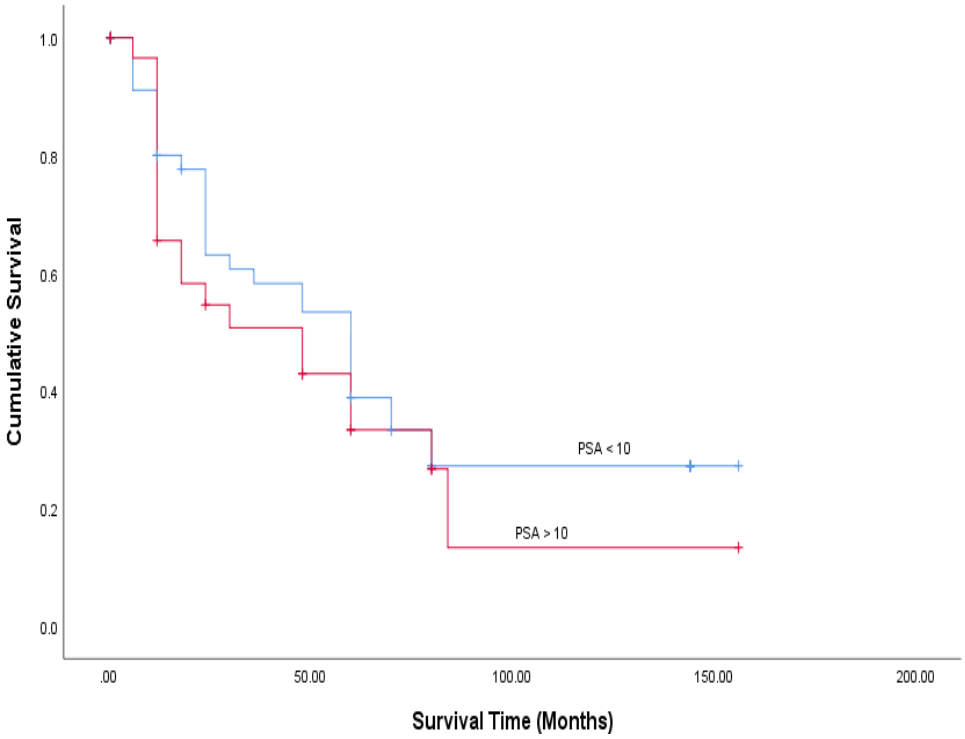


Figure 3.10: Kaplan-Meier survival curve of PCa patients with low and high levels of PSA. Patients' cumulative survival was plotted against time in months for PSA. Two groups are presented: group 1, PSA level < 10 ng/ml, and group 2, PSA level >10 ng/ml.

3.9: Correlation of FABP12 staining intensity to AR

Box plot was used to analyze the correlation between FABP12 staining intensity and AR index levels. The FABP12 staining intensities were categorized into weak-, moderate- and high- staining groups as shown in Figure 3.11. Mann Whitney U test showed that AR levels were not correlated with the FABP12 staining intensities in all three different group. The differences in AR indices either between low and moderate FABP12 stains (p=0.4), between moderate and high stains (p=0.6), or between low and high stains (p=0.1) are not significantly different. Thus, the FABP12 staining intensities of the carcinomas were not significantly correlated to their AR indices.

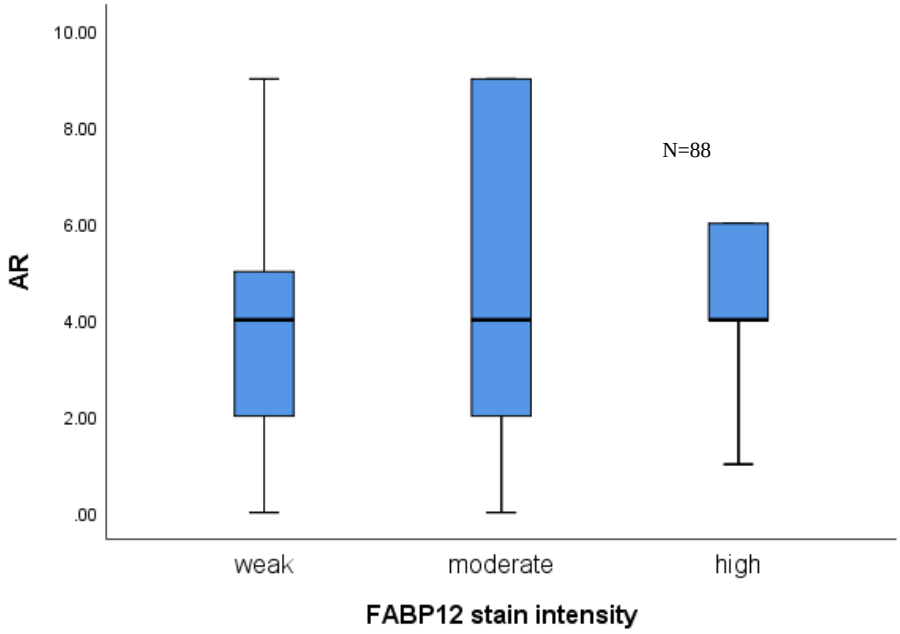


Figure 3.11: Box plot of FABP12 staining intensities against their AR indices. X axis represents 3 categories of FABP12 staining (weak, moderate and high), while Y axis represents AR index.

3.10: Correlation of FABP12 staining to PSA

PSA levels of the carcinoma cases against the FABP12 staining intensities are shown by box plot in Figure 3.12. The correlation of FABP12 staining intensities with PSA levels was assessed by Mann-Whitney U test. The differences in the PSA levels between the low and moderate FABP12 stains (p=0.05), between moderate and high stains p=0.06, or between the low and high stains (p=0.08) were not significant. Thus, the FABP12 staining intensities were not significantly correlated to the PSA levels of these tested carcinoma cases.

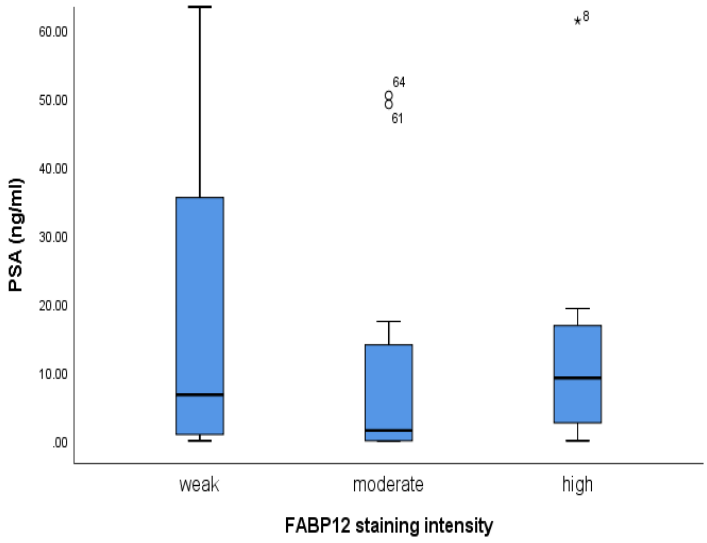
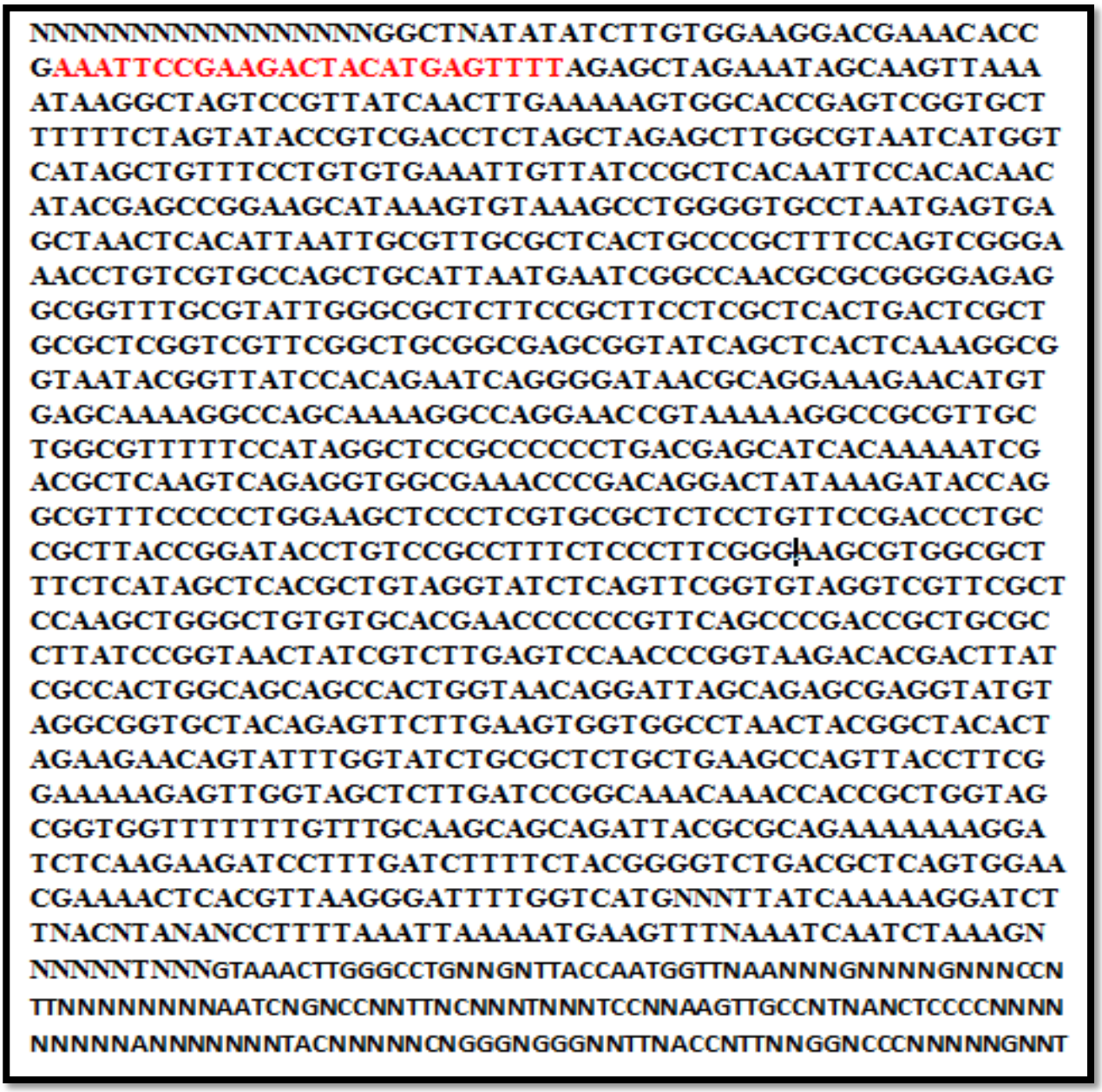


Figure 3.12: PSA levels of the prostate carcinomas are not significantly correlated with FABP12 staining intensities. X axis represents FABP12 staining intensity while Y axis represents PSA in (ng/ml).

3.11 Gene knockout

FABP12 gene knockout (KO) in PC3-M cells was achieved with CRISPR-Cas9 method , which is a state-of-the-art technique in gene editing. The gRNA was purchased from (Life Technologies, UK) and KO procedures were performed following the instructions of the manufacturer in the company protocol as described in methodology.

Sequences were analysed to select the one which had the edited genomic material. The selected sequence that is shown in figure 3.13 was used for following experiments. It was used to transfect high malignant prostate cancer cell line PC3M to knockout *FABP12* gene.



.

Figure 3.13: Nucleotide sequence of the CRISPR nuclease plasmid. The red letters are the guide sequence. This sequence was obtained from the plasmid DNA of a positive transformant colony and this sequencing analysis result confirmed that the guide sequence is successfully inserted into the plasmid in a correct orientation.

The transfection process was monitored through a fluorescence microscope (Zeiss Axio Observer Z.1. Zeiss, Germany). As shown in Figure 3.14, cells harbouring the plasmid exhibited an orange fluorescence due to the expressed orange fluorescent protein (OFP).

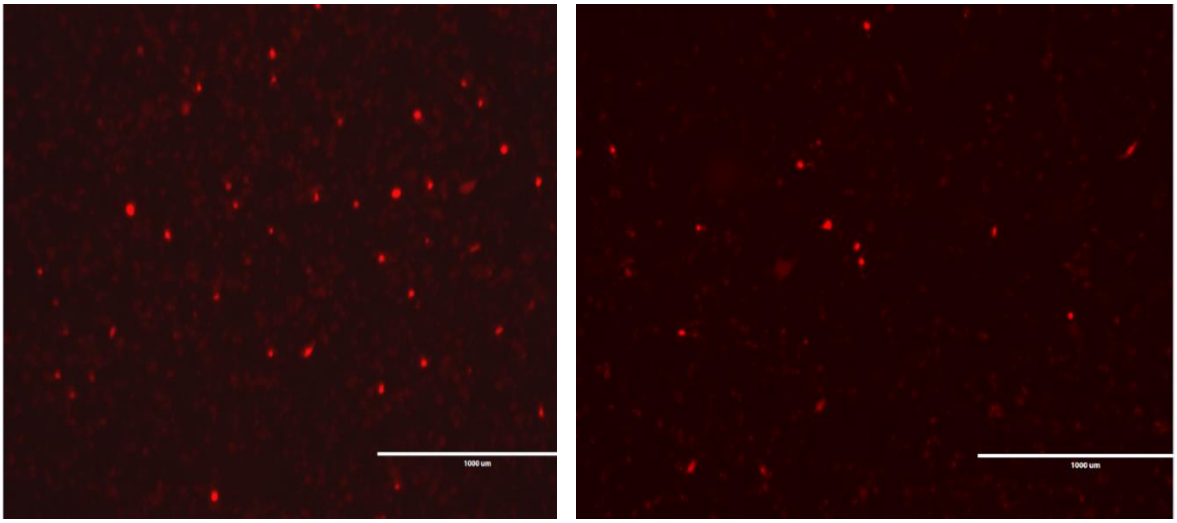
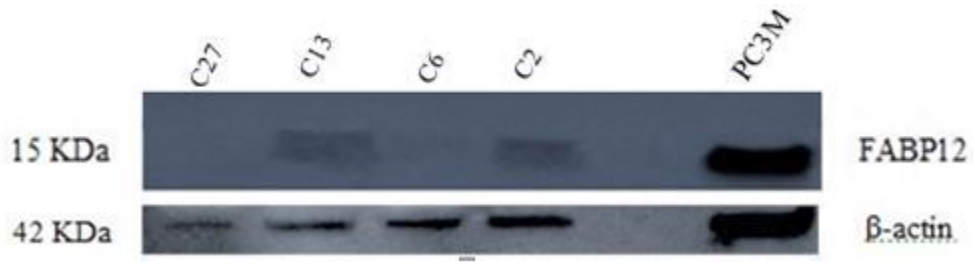


Figure 3.14: Transfection of the PC3-M cells with plasmid containing the *OFP* gene. The appearance of the positively transfected cells expressing OFP in an orange colour. These images were obtained with a fluorescence microscope (Zeiss Axio Observer Z.1. Zeiss, Germany).

Western blot was performed to assess FABP12 expression in parental PC3M cells and in the 4 sub-lines, named C2, C6, C13 and C27, respectively. As shown in Figure 3.15.A, parental PC3-M cells expressed a very high level of FABP12. For the sub-lines, C6 and C27 did not express FABP12, but both C2 and C13 expressed FABP12, although the

levels were much lower than those seen in the parental control cells. The quantitative analysis of the intensities of the peak areas of the bands on the blot was shown in Figure 3.16.B. When the level of FABP12 expression in PC3-M cells was set at 1, the relative FABP12 levels of C2 and C13 were 0.49±0.18 and 0.48±0.17, respectively. Thus, sub-lines C2 and C13 expressed about 50% of the level of FABP12 detected in the parental PC3-M cells. For C6, the expression of FABP12 was completely abrogated.



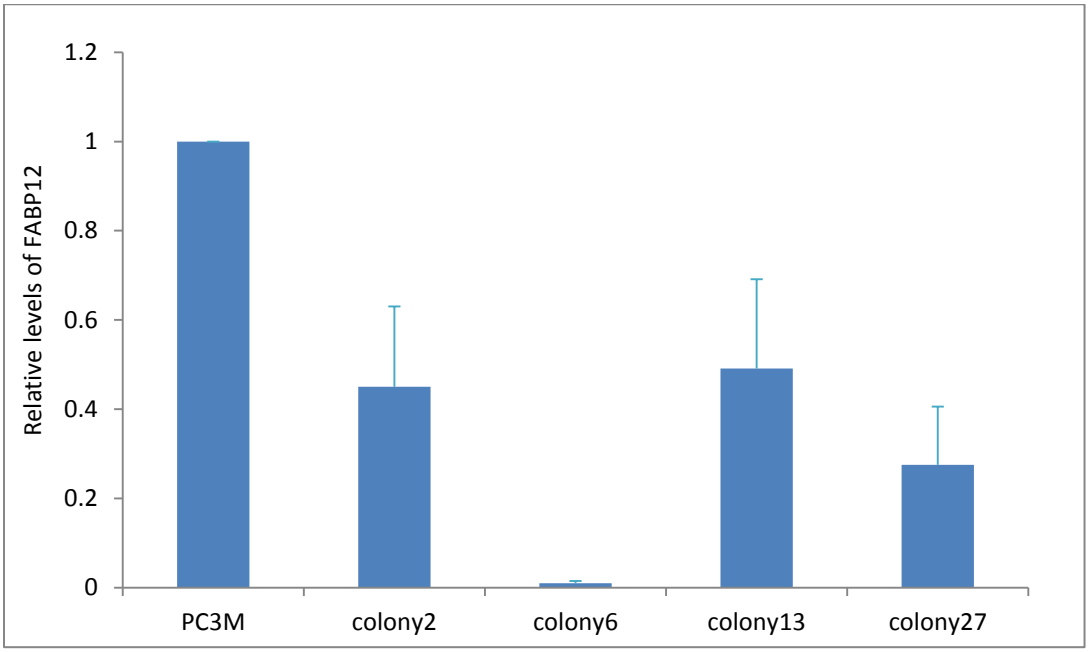


Figure 3.15: Detection of the FABP12 expression in the parental PC3-M cells and in different sub-lines.

A. Western bot analysis of the FABP12 expression in different cell lines
B. Quantitative analysis of the relative levels of FABP12 in the control and in different sub-lines by densitometry scanning of the peak areas of the bands on the blot. Results were obtained from three different measurements (mean ± SD).

3.12 In Vitro assays

3.12.1 Proliferation assay

Presto blue proliferation assay was performed to study the effect of *FABP12* gene KO on cell proliferation. Each of two sub-lines C6 and C13, and the parental control cell line PC3-M were assessed. The results were shown in Figure 3.16. When the number of cells (5000 cells) at the start of the assay was set at 1 for each cell line, the relative cell number reached the maximum at day 4. Whereas the maximum relative number of PC3-M cells at day 4 was 26000 ± 5468, the relative cell numbers of complete KO cell line C6 and the 50% KO line C13 were 18827 ± 8350, 20980 ± 3882, respectively. Comparing with the cell number of PC3-M, the numbers of C6 and C13 cells were reduced by 1.38 and 1.24 fold, respectively. It was significant for C6 (Student t-test, p=0.013, p=0.18). Comparing mean cell numbers of PC3M throughout the 6 days, the reductions of growth rates of both C6 and C13 were significant for C6 (P value =0.020, p =0.32), respectively. Moreover; the difference between C6 and C13 were also significant (Student t-test, p=0.030).

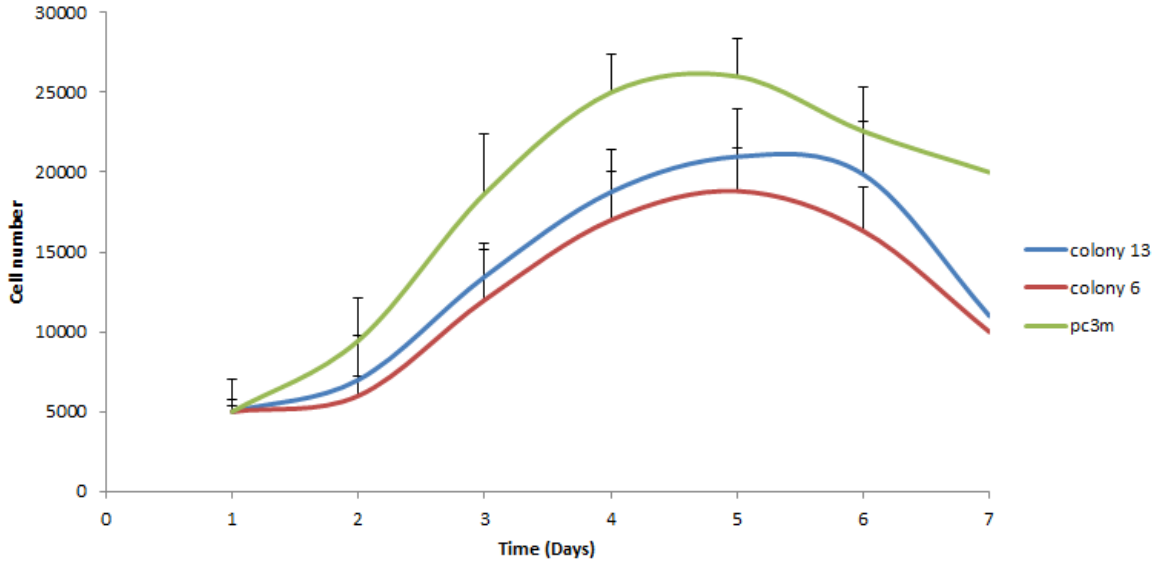


Figure 3.16. Cell proliferation assay: 5000 from each cell line was cultured in triplicate in six 96 well plates and in each day one plate were removed to make cell number count. Assay was carried in triplicates for 6 days and the 3 tested cell lines were the parental PC3-M, C6 and C13, respectively. The difference was assessed by Student t-test. X axis shows the time (in days), while Y axis shows cell number.

3.12.2 Soft agar assay

The assay to test cell anchorage independently growth in soft agar as an indication of tumorigenicity was performed using sensitive kit (Abcam, U.K) with the control PC3-M cells , C6 and C13 cells. At the end of the test, colonies formed by different cells in soft agar were scanned with a colony counter (Oxford Optronix, UK). As shown in Figure 3.17, the number of colonies produced by the parental control cells PC3M was strikingly higher than those produced by the *FABP12* KO cell lines C6 and C13. PC3M cell lines produced 80±20 colonies, whereas C6 and C13 produced only 7±3 and 15 ± 2 colonies, respectively (Figure 3.20). When assessed with Student t-test, the differences between the colony number produced by PC3M and those produced by C13 and C6 are highly significant (p=0.009, *p*=0.01). However, when the colony numbers produced by (C6) and (C13) cells were tested, the difference is not statistically significant (Student t-test, p= 1).

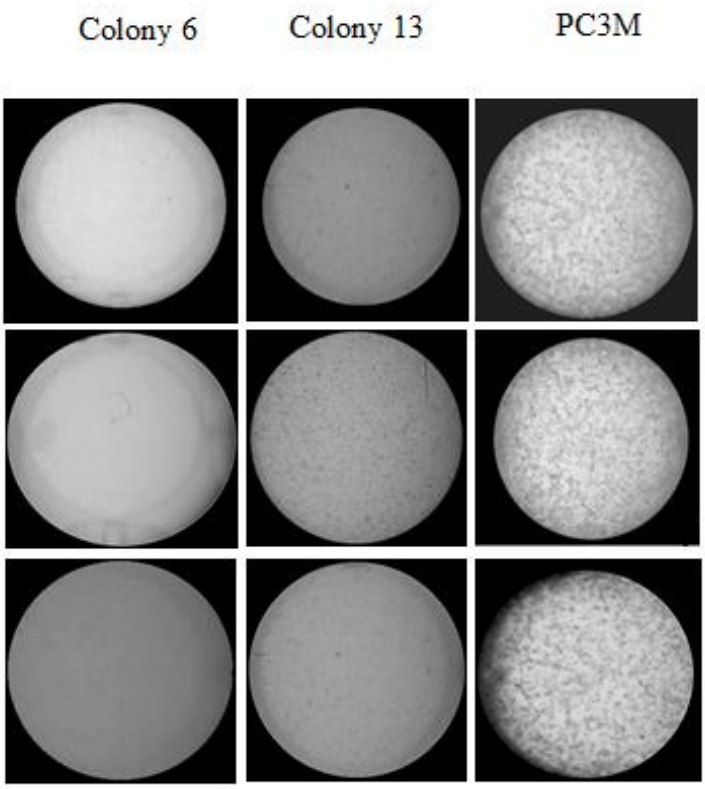
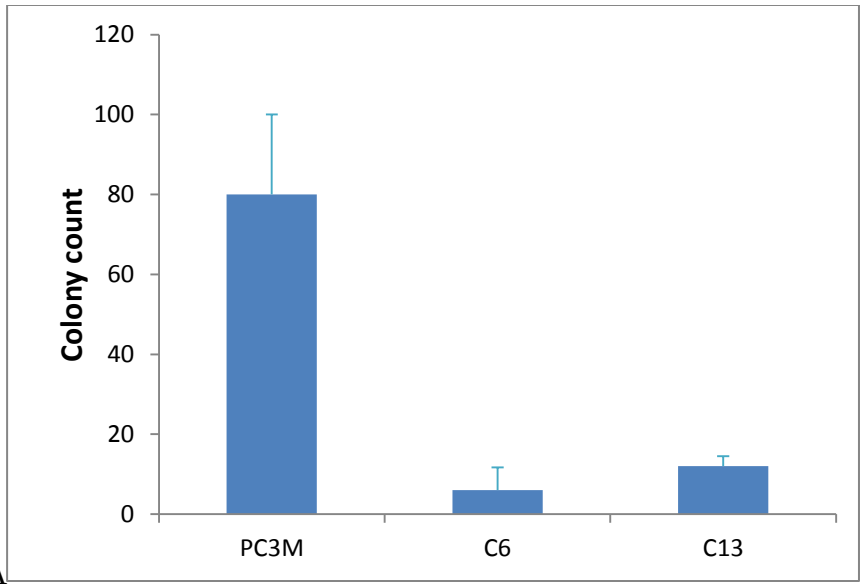


Figure 3.17. Plates of soft agar colony formation assay. Colonies formed by 3 different cell lines: PC3M cells, C13 cells and C6 cells.



A

Cell line	No of colonies + SD	*P* value
Control PC3M	80 ± 20	
C6	7 ± 3	0.01
C13	15 ± 2	0.009

B

Figure 3.18: Soft agar assay data analysis

A. Quantification of soft agar data.

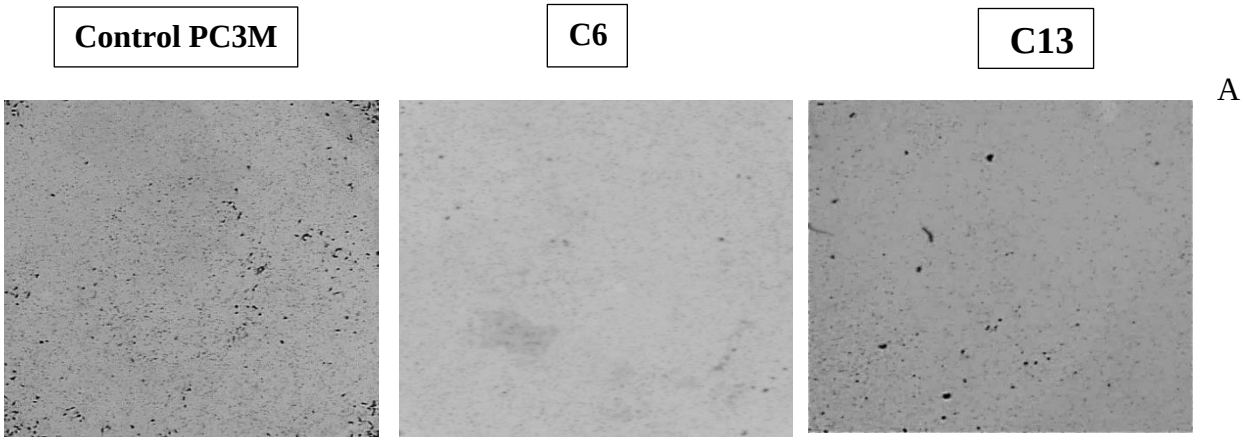
B. Number of colonies by three different cell lines.

3.12.3 Invasion assay

The invasiveness of the 3 cell lines was tested with the cell invasion assay and the number of the invaded cells from each of the 3 cell lines was shown in Figure 3.19. As shown in the table, the number of the invaded cells from the parental PC3-M line was 31 ±9. The numbers of invaded cells from C6 and C13 were 14 ±7 and 10 ±6, respectively. Comparing with the number of the invaded cells from PC3-M, the invaded cells from both C6 and C13 were significantly reduced (student t-test, p=0.04 and p=0.02). But the difference between the number of the invaded cells produced by C6 and that produced by C13 is not statistically significant (student t-test, p=0.47).

Cell line	**No. of invaded cells**	***P* value**
Control PC3M	31	-----
Colony 6	14	*P* value equals 0.04
Colony 13	10	*P* value equals 0.02 when comparing to PC3M

Table 6. Number of invaded cells from three different cell lines. The results are from three separate tests (Mean ±SD).



A

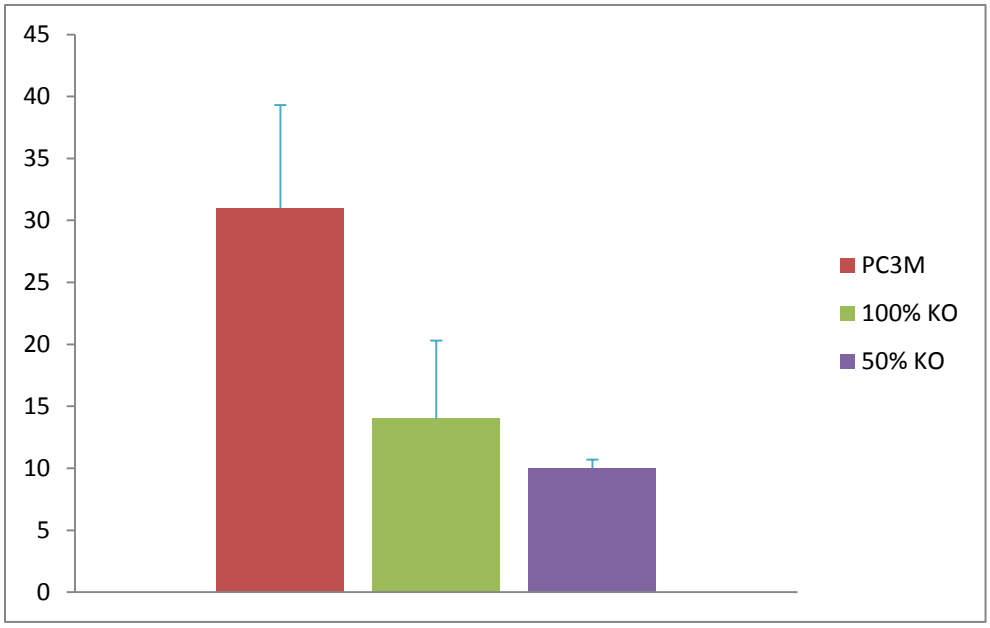


Figure 3.19: Invasion assay

A. Invaded cells under microscope, representing three groups of cell lines: PC3M, C6 and C13 cells.

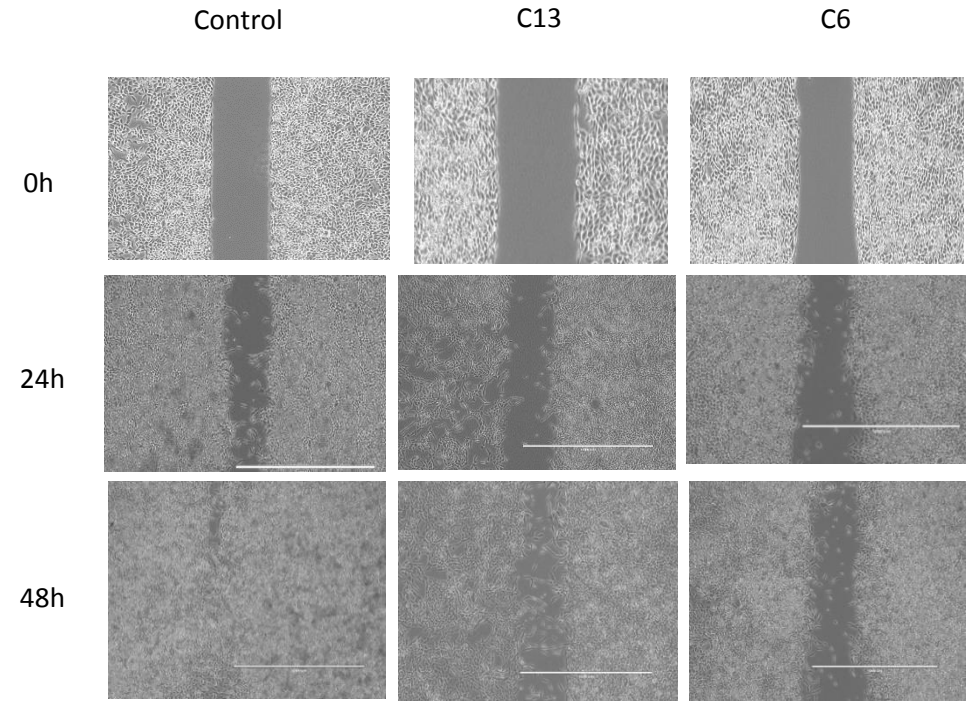
B. Numbers of the invaded cells from 3 cell lines.

3.12.4 Motility assay

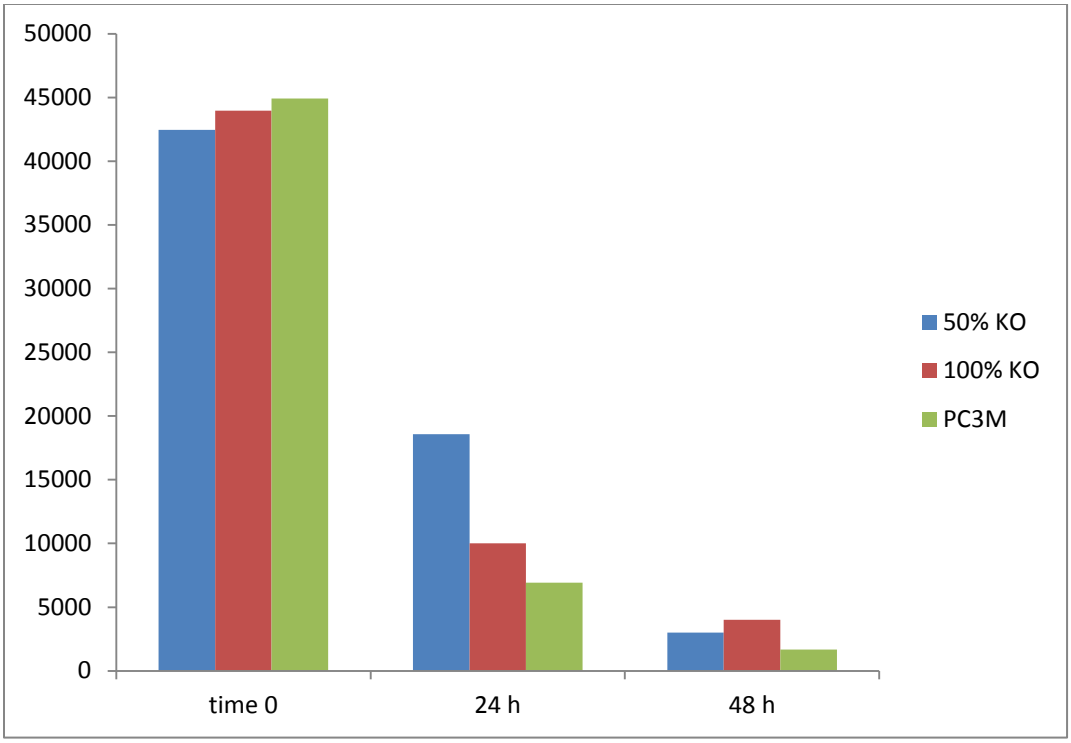
Migration is a fundamental characteristic for live cells. High migration ability is an indication of the metastatic potential of the cancer cells (106). In this work, Ibidi inserts were used to mimic the natural wound healing process. Then wound area was assessed in three different time points and quantified to evaluate cell migration ability of PC3M, C13 and C6 cells. As shown in Figure 3.20 A, the wound healing speeds of the 3 cell lines were different.

Data analysis at 24 hours showed significantly higher migration rate for PC3M compared to C6 cells (Student t-test, p=0.0014). Similarly, Migration rate for PC3M was significantly higher than C13 cell (Student t-test, p=0.0052).

After 48 hours, the gap in PC3M cells was almost completely closed, whereas in C13 and C6 cells, the gaps were only partially closed. The migration rate of the PC3-M cells is significantly higher that of the C6 Cells (Student t-test, p=0.02), its migration rate is not significantly higher than that of the C13 cells (Student t-test, $p = 0.08$).



A



B.

Figure 3.20: Motility assay

A. Speed of wound healing in three cell lines (PC3-M, C6 and C13), measured in three time points: 0, 24 and 48 hours.

B. Quantitative assessment of the wound healing data with ImageJ software. X axis shows time (in hours), while Y axis shows wound area.

Discussion

The growth and spread of prostate cancer (the most common male cancer) is caused by the increased activity of cancer-promoting genes. Thus, identification of these genes is an essential step for discovering reliable treatment targets. FABPs are a family of cytoplasmic proteins that act as lipid chaperones. They facilitate fatty acids transport; regulate cellular signaling and transcriptional activity (48). Over the past decade, involvements of FABPs in cancer pathogenesis have been investigated by different groups of scientists. It was proved that FABP1 played a role in hepatocellular carcinomas (41). FABP3 was suggested to play a role in non-small cell lung carcinomas (203), gastric cancer (88), leiomyosarcoma (49) and melanoma (127). FABP5 was proved to act as a PCa promoter and metastasis inducer (84). And it was detected in urine samples in cases with bladder cancer (48).

Extensive studies are ongoing to target metabolic pathways that were disrupted in PCa. It was reported that PCa is associated with increased expression of the enzyme fatty acid synthase (FASN). This enzyme regulates palmitate synthesis and had a role in energy balance. It was detected in all types of PCa (6, 64), suggesting possible role of fatty acids in PCa.

PCa may evolve to incurable CRPC. Previous research provided evidence on the association between these severe forms of PCa with disturbed fatty acids metabolism. It was found that tissues from CRPC patients showed high expression of FASN. One of characteristic features of cancer cells is their ability to produce endogenous fatty acids and cholesterol. These fatty acids will then be used by cancerous cells to promote growth,

and to resist treatment. Therefore, targeting fatty acids pathway could be a promising way for treatment of PCa (64, 246).

Previous work of our group found that *FABP4, FABP5, FABP6, FABP9* and *FABP12* exhibited clearly higher levels in part or all tested malignant cell lines compared to the level in the benign PNT-2 cells. Amongst these differentially expressed *FABPs*, FABP4 was previously shown to be involved in promoting tumorigenicity of prostate cancer cell line DU145 (211). Further investigation in human tissues showed levels of *FABP4*, *FABP6* were not significantly different between BPH and prostate carcinomas. The study also showed that the increased FABP9 is significantly associated with the increasing malignancy of the carcinomas, and its increased expression is significantly correlated to the reduced patient survival. However, *FABP9* knockdown by siRNA failed to show a significant suppression on tumorigenicity and metastasis (5). Despite the previous work on FABPs involvement in carcinogenesis, no studies were conducted to investigate the possible role of FABP12 in prostate cancer. This may be partially because *FABP12* gene is the most recently discovered member of the FABP family, and its functional role has not yet been explored. The current study conducted a systematic assessment of the expression status of FABP12 in benign and malignant prostate tissues and proved FABP12 overexpression in PCa cells and tissues. Moreover, this study demonstrated the possible functional role of FABP12 in PCa pathogenesis.

4.1 Technical considerations

This part will discuss the technical consideration in relation to gene editing technique and motility experiment.

A. CRISPR/Cas9

RNA Interference (RNAi) has been used for years to transiently repress gene expression (213). The discovery of CRISPR/Cas9 has revolutionized the biomedical research for being robust, efficient and precise gene editing tool (18). Both techniques can be useful depending on the experimental design and question to be addressed.

The aim of the current work was to assess the malignant role of FABP12 in PCa. According to (Figure 3.1.D), qPCR analysis shows relative level of FABP12 mRNA is higher than any other FABPs expression in PCa cell lines, if we chose to knockdown FABP12, it will be very difficult to reduce its expression or may leave residual expression, thus; the choice of CRISPR-Cas9 technique was to achieve knockout of the gene effect and study its functional role in PCa. Previous studies suggested that loss-of-function phenotype may not be achieved through decreased protein levels, while knockout technique will lead to fully depleted functional protein (18).

CRISPR technique was required to functionally characterize the role of *FABP12* by knockout this gene in a highly malignant prostate cells model to assess whether *FABP12* knockout can affect the malignant characteristics of the cells, so as to determine the role that *FABP12* plays in malignant progression.

Moreover, CRIPSR is widely used technique in research, particularly cancer research. There is a need to establish it in our lab and assess its functionality in PCa to pave the

way for more advanced gene manipulations research in PCa. After this work on FABP12, future work will explore the knockout of more than one gene of FABPs family and assess their correlation with PCa.

B. Motility assay

In this work, motility assay was applied to assess *FABP12* KO effect on cell migration in PCa. After the inserts were removed, cells were monitored for wound closure. In order to ensure cell migration is due to cell migration not proliferation, cells were serum starved before the start of assay, to inhibit cells' ability to proliferate. Protocol was optimized to choose the most suitable duration, and serum free medium was used to rule out proliferative effects.

In addition to the 48 h analysis, images were analyzed at 24 h to ensure the proliferation rate at lowest level. 24 h data analysis gives significant result as shown in section 3.12.4.

4.2 FABP12 was overexpressed at mRNA and protein levels

The preliminary study using q-PCR showed that its mRNA level is 3.6- to 105- fold higher in malignant cell lines than that in the benign PNT-2 cells. Western blot analysis showed that FABP12 is expressed in all cell lines detected. Although at the protein level, the increase of FABP12 in cancer cells are not as dramatic as that observed at the mRNA level, the results showed a strikingly high expression of FABP12 in three malignant cell lines (DU145, PC3 and PC3M) when compared to benign PNT2 cells. With values of 12.8 ± 3.1, 10.8 ± 2.1, 16.3 ± 2.3; respectively. Both the weakly and the moderately malignant LNCaP and 22Rv1 cells had relatively lower FABP12 increases of 4.5 ± 2 and 3.6 ± 1.5, respectively (figure 3.2). These results did not only confirm that FABP12 level was greatly increased in all PCa cell lines as compared to the benign PNT-2 cells, but also suggested that the level of FABP12 expression was correlatively increased as the increasing malignant malignancies of the cancer cells. This result showed that in cell lines, the difference in FABP12 levels between benign and malignant phenotypes is as great as that observed in FABP5, whereas the general expression of FABP12 is more abundant than FABP5, since FABP5 is hardly detectable in PNT2 and LNCaP cells at the protein level (67).

4.3 FABP12 was overexpressed in PCa tissues

The immunohistochemical staining of PCa cases showed that FABP12 is highly expressed in prostate carcinomas tissues. Compared to the BPH cases, FABP12 expression was significantly higher in prostate carcinoma tissues (χ^2 -test, $p< 0.00001$). The increased expression of FABP12 was correlated with the increasing GS, moreover; highly malignant and low malignant cases showed striking correlation significance ($\chi^2 = 12.55$, $P=0.000396$) (Figure 3.6). Thus, this may reflect the feasibility to use FABP12 alone or with GS as an indicator of high malignant cases.

The association of the increased FABP12 expression with the high level of malignancy indicated that the elevated FABP12 may play a promoting role in malignant progression of the prostate carcinoma cells.

The immunohistochemical staining pattern of FABP12 in prostate carcinoma tissue was almost all cytoplasmic staining. FABPs are lipid transporters and most of them are in cytoplasm. The example for this is FABP9 reported recently, which is also located in cytoplasm and which was proved to be associated with the prostate carcinoma tissues (5). However, in most studies, one member of the FABP family: FABP5 was stained in both cytoplasm and nucleus (67, 150), indicating that FABP5 may play a more complicated role in cancer cells than other members of the FABP family.

4.4 Poor patient survival was correlated with FABP12 overexpression and GS

The results in this work showed that the average survival time of the low FABP12 staining (most are weakly malignant carcinomas) was 60 months; whereas that of the carcinoma cases with relatively high FABP12 staining (most are highly malignant carcinomas) was 25 months (Figure 3.7). Thus, the patients with a low FABP12 expression level survived longer than those with a high FABP12 level by an average of 35 months. The increased FABP12 expression was significantly associated with poor patient survival (log rank test, P=0.05). These results suggested that FABP12 is a prognostic factor which can be used as a biomarker to predict the patient survival. Although previous work found a few members of FABP family were highly expressed in prostate cancer tissues, only FABP5 and FABP9 were significantly correlated with patient survival (67, 5). In this study, we found FABP12; the most recently discovered member of the FABP family, FABP12, is also a prognostic marker for prostate cancer.

The roles of AR as both diagnostic and prognostic markers for PCa have been in dispute for a long time. In the current study, our results in this specific archival set of tissue samples showed that the AR index was increased in prostate carcinomas (figure 3.12). Although the increased AR index had a borderline correlation with patient survival, this correlation was not statistically significant (Log-rank test, p=0.06).

A previous study suggested that for PCa patients undergoing androgen deprivation therapy, PSA level, may be an indication of better survival (126). However, some other studies suggested that PSA can predict survival only when combined to other factors forming a nomogram, in which several factors were collected to predict patient survival.

PSA was not considered as an ideal marker. Since PSA can be produced and secreted by both benign and malignant prostate cells, high serum PSA level can only reflect the enlargement of prostate size; it cannot discriminate benign from malignant disease (73). Thus, previous work showed that as a prognosis marker, PSA needs to be combined with other factors. In this current study, we found that the average survival time for low PSA (<10 ng) was 60 months (Figure 3.10). Whereas the high PSA (>10 ng) cases was 48 months. Although it seemed to have a 12 months difference, statistical assessment showed that the increased PSA level was not significantly correlated with the patient survival (Log-rank test, P =0.4).

Apart from the problem of lacking biomarker for prostate cancer diagnosis and prognosis, over biopsies is a problem in initial diagnosis of PCa. GS remains one of the major valuable factors for PCa prognosis (63). More accurate biomarkers which may be used to predict the malignant tendency of the cases are urgently required to replace GS and hence to avoid this invasive biopsy procedure. To study further the prognostic value of FABP12, we assessed the possible correlation between the increased FABP12 expression and the GS. When FABP12 staining intensity was analyzed among low and high GS cases of prostate carcinomas (Figure 3.6/B), a significant correlation was found between GS and FABP12 staining intensity (Mann Whitney P≤0.003). This result suggested that FABP12 may have a potential to be used as an independent biomarker for diagnosis and prognosis of PCa cases. It may provide a foundation for development of a new diagnosis or prognosis method, independent of the biopsies and GS diagnostic procedures.

Our analysis of correlation of staining intensities between AR and FABP12 or between PSA and FABP12 in PCa cases yielded non-significant correlations (P=0.8) and

(*P*=0.08). These results, in combination with the assessment that the expression of FABP12 was significantly correlated with the increasing GS, suggested that FABP12 is closer to GS as a biomarker, which is far better in predicting the patient outcome than either AR index or PSA level.

4.5 Role of FABP12 in PCa metastasis

The development of cancer metastasis is a multi-step process in which different cellular characteristics are modified. Moreover, cells will acquire new features. Examples of these features of the cancer cells are the increases in cellular proliferation, migration, invasion, and colony formation (249). To assess the possible promoting role of FABP12 in PCa carcinogenesis, we have systematically assessed the effect of *FABP12* KO on these malignant characteristics of the highly malignant PCa cells. Since each gene has two alleles, we hypothesized that our KO work has produced a colony (C6) with both alleles being knocked out and produced approximately 100% FABP12 suppression. Another colony (C13) exhibited about 50% reduction in FABP12 level, it was hypothesized that only one allele of the *FABP12* gene was knocked out. Proliferation assay results showed that the cell numbers from C6 and C13 at day 6 of the assay were reduced by 1.38 and 1.24 fold (p=0.02, p=0.32), respectively, compared with the control PC3-M cells. These results indicated that even a single allele KO or partially suppressed FABP12 expression had inhibited the cell proliferation. This proved that FABP12 was promoting growth in highly malignant PCa cells and targeting FABP12 by either a complete KO or a single allele KO will result in reduced cancer cell proliferation. It was proved previously that

cancer cells can synthesize endogenous fatty acids to promote cancer cell growth via glycolysis and other pathways (250). This previous work combined with our results in this study suggested that FABP12 plays a promoting role to cancer cells and it promotes the cancer cells growth by transporting fatty acids into cancer cells.

Comparison of the cell migration showed that cells migrated through wound gap were reduced significantly in C6 compared to PC3M (p=0.02). Analysis of invasion assay showed that cellular invasion was highly affected by FABP12 KO. C6 cells produced 54.8 % decrease in invaded cells comparing to PC3M and C13 cells produced 67% decrease in invaded cells as compared to PC3M cells. When tested in soft agar for anchorage independent growth, both C6 and C13 cells produced significant reductions in colony formation as compared to parental PC3M (p=0.01, p=0.009). Like that from proliferation assay, all these results obtained from invasion assay, migration assay and the soft agar assay confirmed a significant suppressive effect by *FABP12* KO in PCa cells. Since the suppression on colony formation in soft agar is so great, the colonies produced by C6 and C13 cells is only 7 and 15 respectively, it is unlikely that these cells are able to produce tumors in nude mouse. These results firmly established a promoting role of FABP12 in PCa cells. Although further investigation is needed on the exact molecular mechanisms of how FABP12 promoted tumorigenicity, it is reasonable to hypothesize that the greatly increased FABP12 expression may transport large amount of intra- and extracellular fatty acids to cytoplasm of the cancer cells to enhance the fatty acid-signal transduction and eventually lead to the promotion of the malignant progression of the cancer cells. Thus, FABP12 is a novel therapeutic target for treatment intervention.

4.6 Mechanistic model of FABP12 involvement in PCa

Previous studies on other FABPs family members identified an association of peroxisome proliferator-activated receptors (PPARs) in PCa pathogenesis. PPARs are fatty acids receptors of three isotypes (PPARα, PPARβ/δ and PPARγ). Their main function is to control lipid metabolism (7, 119, 136). Their role in carcinogenesis was previously explored, particularly PPARγ. Increased expression of PPARγ was correlated with bladder and prostate cancers (137, 190).

It was speculated that highly expressed FABP5 interacts with increased PPARγ in a coordinated manner to induce progression of PCa (67). Numerous studies showed the positive association between PCa and PPARγ overexpression. Moreover, elevated level of PPARγ drives PCa metastasis (4, 129).

The correlation between the newly discovered FABPs member FABP12 and PPARγ was assessed in a recent study, which hypothesized that FABP12 promotes PCa through PPARγ pathway. Additionally, FABP12 induces EMT which increases cellular motility and migration (129).

Liu et al. suggested the role of FABP12 in PCa metastasis as the following: FABP12 overexpression $\longrightarrow$activated PPARγ $\longrightarrow$ disrupted lipid metabolism $\longrightarrow$ EMT, increased migration and invasiveness (129).

Both PPARγ and AR are nuclear receptor that involve in normal prostate development and PCa, both influence each other within prostate cells. Previous study suggested that

PPARγ activation is induced by suppressing AR signaling in PCa (60,160, 161). It was hypothesized that CRPC development in late stage PCa. may be attributable to FABP12 expression and PPARγ pathway activation (129). However, further studies are required to fully understand the correlation of FABP12 and PPARγ in PCa.

Conclusion

In this study, I proved that FABP12 expression level is significantly increased in PCa cell lines and its level of increase is associated with the increasing malignancies of the cells. The results of this work also showed that the level of FABP12 in prostate carcinomas is significantly increased when compared with that in the BPH tissues. While the level of FABP12 in moderately malignant prostate carcinomas (GS 7) is significantly higher than that expressed in weakly malignant carcinomas cases (GS 2-6), its level in moderately malignant carcinoma is significantly lower than that expressed in the highly malignant carcinoma cases (GS 8-10).

When it was correlated with other currently used diagnostic biomarkers, the increased FABP12 in prostate carcinomas is significantly associated with the increased combined GS, but not with the increased levels of PSA and AR index. Similar to the increased level of combined GS, the increased FABP12 is significantly associated with a shorter patient survival time. However, the increased PSA or AR index are not significantly associated with the patient survival time. Thus, like the combined GS, FABP12 may be used as a new marker for PCa diagnosis and a novel prognostic marker to predict the PCa patient outcomes.

In this work, I have characterized the FABP12's functional role in PCa cells and found that the increased FABP12 promoted the malignant progression of the cancer cells and the malignant progression of prostate cancer can be suppressed by either completely knocking out the *FABP12* gene or partially suppressing its level of expression. Thus FABP12 is a novel target for PCa therapeutic intervention.

Future Work

-To investigate the molecular mechanisms on how FABP12 promoted the malignant progression of the cancer cells by studying its possible association with PPAR-γ or other metabolic pathways.

-To measure the levels of FABP12 in blood and urine samples of healthy men and PCa patients to assess whether serum or urine FABP12 level can be used as a novel diagnostic or prognostic biomarker.

-To investigate the possible relationship between FABP5 and FABP12 and to assess whether these two FABPs promote tumorigenicity of PCa cells in a coordinated manner.

-To identify or synthesize possible FABP12 inhibitors and to study the possibility of using these inhibitors as therapeutic agents to suppress the malignant progression of PCa cells.

References

1. Abate-Shen C, Shen MM. Molecular genetics of prostate cancer. Genes & Development. 2000;14(19):2410-34.
2. Agellon LB, Drozdowski L, Li L, Iordache C, Luong L, Clandinin MT, et al. Loss of intestinal fatty acid binding protein increases the susceptibility of male mice to high fat diet-induced fatty liver. Biochim Biophys Acta. 2007 Oct;1771(10):1283-8.
3. Agnihotri S, Mittal RD, Kapoor R, Mandhani A. Raising cut-off value of prostate specific antigen (PSA) for biopsy in symptomatic men in India to reduce unnecessary biopsy. The Indian journal of medical research. 2014;139(6):851-56.
4. Ahmad I, Mui E, Galbraith L, Patel R, Tan EH, Salji M, et al. Sleeping Beauty screen reveals Pparg activation in metastatic prostate cancer. Proc Natl Acad Sci U S A. 2016 07;113(29):8290-5.
5. Al Fayi MS, Gou X, Forootan SS, Al-Jameel W, Bao Z, Rudland PR, et al. The increased expression of fatty acid-binding protein 9 in prostate cancer and its prognostic significance. Oncotarget. 2016 Dec;7(50):82783-97.
6. Al-Jameel W, Gou X, Jin X, Zhang J, Wei Q, Ai J, et al. Inactivated FABP5 suppresses malignant progression of prostate cancer cells by inhibiting the activation of nuclear fatty acid receptor PPARgamma. (1947-6019 (Print)).
7. Alford AV, Brito JM, Yadav KK, Yadav SS, Tewari AK, Renzulli J. The Use of Biomarkers in Prostate Cancer Screening and Treatment. Rev Urol. 2017;19(4):221-34.
8. Amiri M, Yousefnia S, Seyed Forootan F, Peymani M, Ghaedi K, Nasr Esfahani MH. Diverse roles of fatty acid binding proteins (FABPs) in development and pathogenesis of cancers. (1879-0038 (Electronic)).
9. Andermann A, Blancquaert I, Beauchamp S, Déry V. Revisiting Wilson and Jungner in the genomic age: a review of screening criteria

over the past 40 years. Bull World Health Organ. 2008 Apr;86(4):317-9.
10. Andriole GL, Crawford ED, Grubb RL, Buys SS, Chia D, Church TR, et al. Mortality results from a randomized prostate-cancer screening trial. N Engl J Med. 2009 Mar;360(13):1310-9.
11. Antenor JA, Han M, Roehl KA, Nadler RB, Catalona WJ. Relationship between initial prostate specific antigen level and subsequent prostate cancer detection in a longitudinal screening study. J Urol. 2004 Jul;172(1):90-3.
12. Avgeris M, Mavridis K, Scorilas A. Kallikrein-related peptidases in prostate, breast, and ovarian cancers: from pathobiology to clinical relevance. Biol Chem. 2012 Apr;393(5):301-17.
13. Baier LJ, Sacchettini JC, Knowler WC, Eads J, Paolisso G, Tataranni PA, et al. An amino acid substitution in the human intestinal fatty acid binding protein is associated with increased fatty acid binding, increased fat oxidation, and insulin resistance. J Clin Invest. 1995 Mar;95(3):1281-7.
14. Barry MJ. Clinical practice. Prostate-specific-antigen testing for early diagnosis of prostate cancer. N Engl J Med. 2001 May 3;344(18):1373-7.
15. Bharti R, Dey G, Ojha PK, Rajput S, Jaganathan SK, Sen R, et al. Diacerein-mediated inhibition of IL-6/IL-6R signaling induces apoptotic effects on breast cancer. Oncogene. 2016 07;35(30):3965-75.
16. Bianco FJ, Wood DP, Grignon DJ, Sakr WA, Pontes JE, Powell IJ. Prostate cancer stage shift has eliminated the gap in disease-free survival in black and white American men after radical prostatectomy. J Urol. 2002 Aug;168(2):479-82.
17. Bland JM, Altman DG. The logrank test. BMJ. 2004;328(7447):1073.
18. Boettcher M, McManus MT. Choosing the Right Tool for the Job: RNAi, TALEN, or CRISPR. Mol Cell. 2015 May 21;58(4):575-85.
19. Boiteux G, Lascombe I, Roche E, Plissonnier ML, Clairotte A, Bittard H, et al. A-FABP, a candidate progression marker of human transitional cell carcinoma of the bladder, is differentially regulated

by PPAR in urothelial cancer cells. Int J Cancer. 2009 Apr;124(8):1820-8.
20. Bostwick DG. Gleason grading of prostatic needle biopsies. Correlation with grade in 316 matched prostatectomies. Am J Surg Pathol. 1994 Aug;18(8):796-803.
21. Bratt O. Hereditary prostate cancer: clinical aspects. J Urol. 2002 Sep;168(3):906-13.
22. Brawer MK, Peehl DM, Stamey TA, Bostwick DG. Keratin immunoreactivity in the benign and neoplastic human prostate. Cancer Res. 1985 Aug;45(8):3663-7.
23. Buchanan G, Greenberg NM, Scher HI, Harris JM, Marshall VR, Tilley WD. Collocation of Androgen Receptor Gene Mutations in Prostate Cancer. Clinical Cancer Research. 2001;7(5):1273.
24. Network CRUaNCI. Cancer Incidence and Survival in Major Ethnic Group, England 2002-2006. 2009.
25. CRUK (2016) [online]Available from : https://www.cancerresearchuk.org/
26. Cancer Genome Atlas Research Network. The Molecular Taxonomy of Primary Prostate Cancer. *Cell*. 2015;163(4):1011-1025. doi:10.1016/j.cell.2015.10.025
27. Cao H, Sekiya M, Ertunc ME, Burak MF, Mayers JR, White A, et al. Adipocyte lipid chaperone AP2 is a secreted adipokine regulating hepatic glucose production. Cell metabolism. 2013;17(5):768-78.
28. Capitanio U, Pfister D, Emberton M. Repeat Prostate Biopsy: Rationale, Indications, and Strategies. Eur Urol Focus. 2015 Sep;1(2):127-36.
29. Carlsson S, Maschino A, Schroder F, Bangma C, Steyerberg EW, van der Kwast T, et al. Predictive value of four kallikrein markers for pathologically insignificant compared with aggressive prostate cancer in radical prostatectomy specimens: results from the European Randomized Study of Screening for Prostate Cancer section Rotterdam. Eur Urol. 2013 Nov;64(5):693-9.
30. Carpten J, Nupponen N, Isaacs S, Sood R, Robbins C, Xu J, et al. Germline mutations in the ribonuclease L gene in families showing linkage with HPC1. Nat Genet. 2002 Feb;30(2):181-4.

31. Carter HB, Piantadosi S, Isaacs JT. Clinical evidence for and implications of the multistep development of prostate cancer. J Urol. 1990 Apr;143(4):742-6.
32. Castanares MA, Copeland BT, Chowdhury WH, Liu MM, Rodriguez R, Pomper MG, et al. Characterization of a novel metastatic prostate cancer cell line of LNCaP origin. Prostate. 2016 Feb;76(2):215-25.
33. Catalona William J, Antenor Jo Ann V, Roehl Kimberly A. Screening for Prostate Cancer in High Risk Populations. Journal of Urology. 2002 2002/11/01;168(5):1980-84.
34. Catalona WJ, Smith DS, Ratliff TL, Basler JW. Detection of organ-confined prostate cancer is increased through prostate-specific antigen-based screening. JAMA. 1993 Aug;270(8):948-54.
35. Cataltepe O, Arikan Mc Fau - Ghelfi E, Ghelfi E Fau - Karaaslan C, Karaaslan C Fau - Ozsurekci Y, Ozsurekci Y Fau - Dresser K, Dresser K Fau - Li Y, et al. Fatty acid binding protein 4 is expressed in distinct endothelial and non-endothelial cell populations in glioblastoma. (1365-2990 (Electronic)).
36. Celis JE, Ostergaard M, Basse B, Celis A, Lauridsen JB, Ratz GP, et al. Loss of adipocyte-type fatty acid binding protein and other protein biomarkers is associated with progression of human bladder transitional cell carcinomas. Cancer Res. 1996 Oct;56(20):4782-90.
37. Center MM, Jemal A, Lortet-Tieulent J, Ward E, Ferlay J, Brawley O, et al. International variation in prostate cancer incidence and mortality rates. Eur Urol. 2012 Jun;61(6):1079-92.
38. Chan DW, Bruzek DJ, Oesterling JE, Rock RC, Walsh PC. Prostate-specific antigen as a marker for prostatic cancer: a monoclonal and a polyclonal immunoassay compared. Clin Chem. 1987 Oct;33(10):1916-20.
39. Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, et al. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. Science. 1998 Jan;279(5350):563-6.
40. Chavin G, Sheinin Y, Crispen PL, Boorjian SA, Roth TJ, Rangel L, et al. Expression of immunosuppresive B7-H3 ligand by hormone-treated prostate cancer tumors and metastases. Clin Cancer Res. 2009 Mar;15(6):2174-80.

41. Chen A, Tang Y, Davis V, Hsu FF, Kennedy SM, Song H, et al. Liver fatty acid binding protein (L-Fabp) modulates murine stellate cell activation and diet-induced nonalcoholic fatty liver disease. Hepatology. 2013 Jun;57(6):2202-12.
42. Cheon MS, Kim SH, Fountoulakis M, Lubec G. Heart type fatty acid binding protein (H-FABP) is decreased in brains of patients with Down syndrome and Alzheimer's disease. J Neural Transm Suppl. 2003 (67):225-34.
43. Chevli KK, Duff M, Walter P, Yu C, Capuder B, Elshafei A, et al. Urinary PCA3 as a predictor of prostate cancer in a cohort of 3,073 men undergoing initial prostate biopsy. J Urol. 2014 Jun;191(6):1743-8.
44. Ching KZ, Ramsey E, Pettigrew N, D'Cunha R, Jason M, Dodd JG. Expression of mRNA for epidermal growth factor, transforming growth factor-alpha and their receptor in human prostate tissue and cell lines. Mol Cell Biochem. 1993 Sep;126(2):151-8.
45. Chmurzyńska A. The multigene family of fatty acid-binding proteins (FABPs): function, structure and polymorphism. J Appl Genet. 2006;47(1):39-48.
46. Cook LS, Goldoft M, Schwartz SM, Weiss NS. Incidence of adenocarcinoma of the prostate in asian immigrants to the united states and their descendants. The Journal of Urology. 1999 1999/01/01/;161(1):152-55.
47. Crawford ED. Epidemiology of prostate cancer. Urology. 2003 Dec 22;62(6 Suppl 1):3-12.
48. Das R, Hammamieh R, Neill R, Melhem M, Jett M. Expression pattern of fatty acid-binding proteins in human normal and cancer prostate cells and tissues. Clin Cancer Res. 2001 Jun;7(6):1706-15.
49. Davidson B, Abeler Vm Fau - Hellesylt E, Hellesylt E Fau - Holth A, Holth A Fau - Shih I-M, Shih IeM Fau - Skeie-Jensen T, Skeie-Jensen T Fau - Chen L, et al. Gene expression signatures differentiate uterine endometrial stromal sarcoma from leiomyosarcoma. (1095-6859 (Electronic)).

50. Denmeade SR, Lin XS, Isaacs JT. Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. Prostate. 1996 Apr;28(4):251-65.
51. Deras IL, Aubin SM, Blase A, Day JR, Koo S, Partin AW, et al. PCA3: a molecular urine assay for predicting prostate biopsy outcome. J Urol. 2008 Apr;179(4):1587-92.
52. Dermani FK, Samadi P, Rahmani G, Kohlan AK, Najafi R. PD-1/PD-L1 immune checkpoint: Potential target for cancer therapy. J Cell Physiol. 2019 Feb;234(2):1313-25.
53. Dobrow MJ, Hagens V, Chafe R, Sullivan T, Rabeneck L. Consolidated principles for screening based on a systematic review and consensus process. CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne. 2018;190(14):E422-E29.
54. Draisma G, Etzioni R, Tsodikov A, Mariotto A, Wever E, Gulati R, et al. Lead time and overdiagnosis in prostate-specific antigen screening: importance of methods and context. J Natl Cancer Inst. 2009 Mar;101(6):374-83.
55. Duong MN, Geneste A, Fallone F, Li X, Dumontet C, Muller C. The fat and the bad: Mature adipocytes, key actors in tumor progression and resistance. Oncotarget. 2017 Aug;8(34):57622-41.
56. Elix C, Pal SK, Jones JO. The role of peroxisome proliferator-activated receptor gamma in prostate cancer. Asian J Androl. 2018 May-Jun;20(3):238-43.
57. Emberton M, Fitzpatrick JM, Rees J. Risk stratification for benign prostatic hyperplasia (BPH) treatment. BJU Int. 2011 Mar;107(6):876-80.
58. Epstein JI, Allsbrook WC, Amin MB, Egevad LL, Committee IG. The 2005 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma. Am J Surg Pathol. 2005 Sep;29(9):1228-42.
59. Fang LY, Wong Ty Fau - Chiang W-F, Chiang Wf Fau - Chen Y-L, Chen YL. Fatty-acid-binding protein 5 promotes cell proliferation and invasion in oral squamous cell carcinoma. (1600-0714 (Electronic)).

60. Farkhondeh P, Tahereh K, Mahmood JT, Mojgan B, Jamileh G, Fatemeh SN. A Novel Human Lipid Binding Protein Coding Gene: PERF15, Sequence and Cloning. J Reprod Infertil. 2009 Oct;10(3):199-205.
61. Fay AP, Antonarakis ES. Blocking the PD-1/PD-L1 axis in advanced prostate cancer: are we moving in the right direction? Ann Transl Med. 2019 Mar;7(Suppl 1):S7.
62. Feng Q, He B. Androgen Receptor Signaling in the Development of Castration-Resistant Prostate Cancer. Front Oncol. 2019;9:858.
63. Filella X, Fernández-Galan E, Fernández Bonifacio R, Foj L. Emerging biomarkers in the diagnosis of prostate cancer. Pharmgenomics Pers Med. 2018;11:83-94.
64. Flavin, R., Zadra, G., & Loda, M. Metabolic alterations and targeted therapies in prostate cancer. The Journal of pathology. (2011); 223(2), 283–294.
65. Fine SW, Reuter VE. Anatomy of the prostate revisited: implications for prostate biopsy and zonal origins of prostate cancer. Histopathology. 2012 Jan;60(1):142-52.
66. Foj L, Milà M, Mengual L, Luque P, Alcaraz A, Jiménez W, et al. Real-time PCR PCA3 assay is a useful test measured in urine to improve prostate cancer detection. Clin Chim Acta. 2014 Aug;435:53-8.
67. Forootan FS, Forootan SS, Malki MI, Chen D, Li G, Lin K, et al. The expression of C-FABP and PPARγ and their prognostic significance in prostate cancer. Int J Oncol. 2014 Jan;44(1):265-75.
68. Forootan FS, Forootan SS, Gou X, Yang J, Liu B, Chen D, et al. Fatty acid activated PPARγ promotes tumorigenicity of prostate cancer cells by up regulating VEGF via PPAR responsive elements of the promoter. Oncotarget. 2016;7(8):9322-39.
69. Furuhashi M, Hotamisligil GS. Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. Nat Rev Drug Discov. 2008 Jun;7(6):489-503.
70. Furuhashi M, Saitoh S, Shimamoto K, Miura T. Fatty Acid-Binding Protein 4 (FABP4): Pathophysiological Insights and Potent Clinical

Biomarker of Metabolic and Cardiovascular Diseases. (1179-5468 (Print)).
71. Furuhashi M, Tuncman G, Görgün CZ, Makowski L, Atsumi G, Vaillancourt E, et al. Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein aP2. Nature. 2007 Jun 21;447(7147):959-65.
72. Globocan (2018): Globocan, Global cancer observatory [online] Available from: http://gco.iarc.fr.
73. Gaudreau PO, Stagg J, Soulières D, Saad F. The Present and Future of Biomarkers in Prostate Cancer: Proteomics, Genomics, and Immunology Advancements. Biomark Cancer. 2016;8(Suppl 2):15-33.
74. Giannoni E, Bianchini F, Masieri L, Serni S, Torre E, Calorini L, et al. Reciprocal activation of prostate cancer cells and cancer-associated fibroblasts stimulates epithelial-mesenchymal transition and cancer stemness. Cancer Res. 2010 Sep 1;70(17):6945-56.
75. Goel MK, Khanna P, Kishore J. Understanding survival analysis: Kaplan-Meier estimate. International journal of Ayurveda research. 2010;1(4):274-78.
76. Gopalan A, Leversha MA, Satagopan JM, Zhou Q, Al-Ahmadie HA, Fine SW, et al. TMPRSS2-ERG gene fusion is not associated with outcome in patients treated by prostatectomy. Cancer Res. 2009 Feb 15;69(4):1400-6.
77. Gottlieb B, Beitel LK, Nadarajah A, Paliouras M, Trifiro M. The androgen receptor gene mutations database: 2012 update. Hum Mutat. 2012 May;33(5):887-94.
78. Graff JN, Alumkal JJ, Drake CG, Thomas GV, Redmond WL, Farhad M, et al. Early evidence of anti-PD-1 activity in enzalutamide-resistant prostate cancer. Oncotarget. 2016;7(33):52810-17.
79. Green MR, Sambrook J. Polymerase Chain Reaction. Cold Spring Harb Protoc. 2019 06;2019(6).
80. Gronberg H. Prostate cancer epidemiology. Lancet. 2003 Mar 8;361(9360):859-64.

81. Gronberg H, Xu J, Smith JR, Carpten JD, Isaacs SD, Freije D, et al. Early age at diagnosis in families providing evidence of linkage to the hereditary prostate cancer locus (HPC1) on chromosome 1. Cancer Res. 1997 Nov 1;57(21):4707-9.
82. Guaita-Esteruelas S, Guma J, Masana L, Borras J. The peritumoural adipose tissue microenvironment and cancer. The roles of fatty acid binding protein 4 and fatty acid binding protein 5. (1872-8057 (Electronic)).
83. Gui-Zhong L, Libo M, Guanglin H, Jianwei W. The correlation of extent and grade of inflammation with serum PSA levels in patients with IV prostatitis. Int Urol Nephrol. 2011 Jun;43(2):295-301.
84. Gurung S, Chung KPS, Lee TK. Emerging role of fatty acid binding proteins in cancer pathogenesis. Histol Histopathol. 2019 Jan;34(1):1-12.
85. Haese A, de la Taille A, van Poppel H, Marberger M, Stenzl A, Mulders PF, et al. Clinical utility of the PCA3 urine assay in European men scheduled for repeat biopsy. Eur Urol. 2008 Nov;54(5):1081-8.
86. Haffner MC, Guner G, Taheri D, Netto GJ, Palsgrove DN, Zheng Q, et al. Comprehensive Evaluation of Programmed Death-Ligand 1 Expression in Primary and Metastatic Prostate Cancer. Am J Pathol. 2018 Jun;188(6):1478-85.
87. Harman SM, Metter EJ, Blackman MR, Landis PK, Carter HB, Aging BLSo. Serum levels of insulin-like growth factor I (IGF-I), IGF-II, IGF-binding protein-3, and prostate-specific antigen as predictors of clinical prostate cancer. J Clin Endocrinol Metab. 2000 Nov;85(11):4258-65.
88. Hashimoto T, Kusakabe T Fau - Sugino T, Sugino T Fau - Fukuda T, Fukuda T Fau - Watanabe K, Watanabe K Fau - Sato Y, Sato Y Fau - Nashimoto A, et al. Expression of heart-type fatty acid-binding protein in human gastric carcinoma and its association with tumor aggressiveness, metastasis and poor prognosis. (1015-2008 (Print)).
89. Haunerland NH, Spener F. Fatty acid-binding proteins--insights from genetic manipulations. Prog Lipid Res. 2004 Jul;43(4):328-49.

90. Haunerland NH, Spener F. Properties and physiological significance of fatty acid binding proteins. Advances in Molecular and Cell Biology. Elsevier; 2003. p. 99-122.
91. He F. Bradford Protein Assay. Bio-protocol. 2011 2011/03/20;1(6):e45.
92. Hessels D, van Gils MP, van Hooij O, Jannink SA, Witjes JA, Verhaegh GW, et al. Predictive value of PCA3 in urinary sediments in determining clinico-pathological characteristics of prostate cancer. Prostate. 2010 Jan;70(1):10-6.
93. HI S. Prostate Cancer. In: JT I, editor. 2nd ed. New York: Armitage JO; 2000.
94. Horoszewicz JS, Leong SS, Chu TM, Wajsman ZL, Friedman M, Papsidero L, et al. The LNCaP cell line--a new model for studies on human prostatic carcinoma. Prog Clin Biol Res. 1980;37:115-32.
95. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, et al. LNCaP model of human prostatic carcinoma. Cancer Res. 1983 Apr;43(4):1809-18.
96. Rose, D. P. Effects of dietary fatty acids on breast and prostate cancers: evidence from in vitro experiments and animal studies. Am J Clin Nutr 1997;66 (6 Suppl), 1513S-1522S.
97. Hsing AW, Chokkalingam AP. Prostate cancer epidemiology. Front Biosci. 2006 May 1;11:1388-413.
98. Humphrey PA. Gleason grading and prognostic factors in carcinoma of the prostate. Mod Pathol. 2004 Mar;17(3):292-306.
99. Huynh H, Alpert L Fau - Pollak M, Pollak M. Silencing of the mammary-derived growth inhibitor (MDGI) gene in breast neoplasms is associated with epigenetic changes. (0008-5472 (Print)).
100. Ilic D, Djulbegovic M, Jung JH, Hwang EC, Zhou Q, Cleves A, et al. Prostate cancer screening with prostate-specific antigen (PSA) test: a systematic review and meta-analysis. BMJ. 2018;362:k3519.
101. Isaacs JT, Coffey DS. Etiology and disease process of benign prostatic hyperplasia. Prostate Suppl. 1989;2:33-50.

102. Jeong CY, Hah YS, Cho BI, Lee SM, Joo YT, Jung EJ, et al. Fatty acid-binding protein 5 promotes cell proliferation and invasion in human intrahepatic cholangiocarcinoma. Oncol Rep. 2012 Oct;28(4):1283-92.
103. Jiang Z, Shen H, Tang B, Yu Q, Ji X, Wang L. Quantitative proteomic analysis reveals that proteins required for fatty acid metabolism may serve as diagnostic markers for gastric cancer. (1873-3492 (Electronic)).
104. Jin J, Zhang Z, Zhang S, Chen X, Chen Z, Hu P, et al. Fatty acid binding protein 4 promotes epithelial-mesenchymal transition in cervical squamous cell carcinoma through AKT/GSK3beta/Snail signaling pathway. (1872-8057 (Electronic)).
105. Jing C, Beesley C, Foster CS, Rudland PS, Fujii H, Ono T, et al. Identification of the messenger RNA for human cutaneous fatty acid-binding protein as a metastasis inducer. Cancer Res. 2000 May;60(9):2390-8.
106. Justus CR, Leffler N, Ruiz-Echevarria M, Yang LV. In vitro cell migration and invasion assays. Journal of visualized experiments : JoVE. 2014 (88):51046.
107. 4Kscore. 2021. [online] Available from: https://www.4kscore.com/
108. Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest Urol. 1979 Jul;17(1):16-23.
109. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med. 2010 Jul;363(5):411-22.
110. Kasivisvanathan Ve, Challacombe Be. The big prostate.
111. Kato I, Land S Fau - Majumdar AP, Majumdar Ap Fau - Barnholtz-Sloan J, Barnholtz-Sloan J Fau - Severson RK, Severson RK. Functional polymorphisms to modulate luminal lipid exposure and risk of colorectal cancer. (1877-783X (Electronic)).
112. Kawaguchi K, Kinameri A, Suzuki S, Senga S, Ke Y, Fujii H. The cancer-promoting gene fatty acid-binding protein 5 (FABP5) is

epigenetically regulated during human prostate carcinogenesis. Biochem J. 2016 Feb;473(4):449-61.
113. Kido T, Arata S, Suzuki R, Hosono T, Nakanishi Y, Miyazaki J, et al. The testicular fatty acid binding protein PERF15 regulates the fate of germ cells in PERF15 transgenic mice. Dev Growth Differ. 2005 Jan;47(1):15-24.
114. Kirollos M. Response to :Prostate cancer screening with prostate-specific antigen (PSA) test : a systematic review and meta-analysis. U.K2018.
115. Kozlowski JM, Fidler IJ, Campbell D, Xu ZL, Kaighn ME, Hart IR. Metastatic behavior of human tumor cell lines grown in the nude mouse. Cancer Res. 1984 Aug;44(8):3522-9.
116. Kruger NJ. The Bradford Method for Protein Quantitation. In: Walker JM, editor. Basic Protein and Peptide Protocols. Totowa, NJ: Humana Press; 1994. p. 9-15.
117. Ku CY, Liu YH, Lin HY, Lu SC, Lin JY. Liver fatty acid-binding protein (L-FABP) promotes cellular angiogenesis and migration in hepatocellular carcinoma. (1949-2553 (Electronic)).
118. Lamounier-Zepter V, Look C Fau - Alvarez J, Alvarez J Fau - Christ T, Christ T Fau - Ravens U, Ravens U Fau - Schunck W-H, Schunck Wh Fau - Ehrhart-Bornstein M, et al. Adipocyte fatty acid-binding protein suppresses cardiomyocyte contraction: a new link between obesity and heart disease. (1524-4571 (Electronic)).
119. Lemberger T, Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: a nuclear receptor signaling pathway in lipid physiology. Annu Rev Cell Dev Biol. 1996;12:335-63.
120. Levi L, Lobo G Fau - Doud MK, Doud Mk Fau - von Lintig J, von Lintig J Fau - Seachrist D, Seachrist D Fau - Tochtrop GP, Tochtrop Gp Fau - Noy N, et al. Genetic ablation of the fatty acid-binding protein FABP5 suppresses HER2-induced mammary tumorigenesis. (1538-7445 (Electronic)).
121. Levy E, Ménard D, Delvin E, Stan S, Mitchell G, Lambert M, et al. The polymorphism at codon 54 of the FABP2 gene increases fat absorption in human intestinal explants. J Biol Chem. 2001 Oct;276(43):39679-84.

122. Leyten GH, Hessels D, Jannink SA, Smit FP, de Jong H, Cornel EB, et al. Prospective multicentre evaluation of PCA3 and TMPRSS2-ERG gene fusions as diagnostic and prognostic urinary biomarkers for prostate cancer. Eur Urol. 2014 Mar;65(3):534-42.
123. Li W, Zhang S, Zhou S, Jiang L, Wang W. Cardiac Fatty Acid Binding Protein (FABP3) Depletes SR Calcium Load in Ventricular Myocytes. Biophysical Journal. 2017;112(3):424a.
124. Li Y, Alsagabi M, Fan D, Bova GS, Tewfik AH, Dehm SM. Intragenic rearrangement and altered RNA splicing of the androgen receptor in a cell-based model of prostate cancer progression. Cancer Res. 2011 Mar;71(6):2108-17.
125. Liberko M, Kolostova K, Bobek V. Essentials of circulating tumor cells for clinical research and practice. Crit Rev Oncol Hematol. 2013 Nov;88(2):338-56.
126. Lilja H, Ulmert D, Vickers AJ. Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. Nat Rev Cancer. 2008 Apr;8(4):268-78.
127. Linge A, Kennedy S Fau - O'Flynn D, O'Flynn D Fau - Beatty S, Beatty S Fau - Moriarty P, Moriarty P Fau - Henry M, Henry M Fau - Clynes M, et al. Differential expression of fourteen proteins between uveal melanoma from patients who subsequently developed distant metastases versus those who did Not. (1552-5783 (Electronic)).
128. Liu AY, True LD, LaTray L, Nelson PS, Ellis WJ, Vessella RL, et al. Cell-cell interaction in prostate gene regulation and cytodifferentiation. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(20):10705-10.
129. Liu RZ, Choi WS, Jain S, Dinakaran D, Xu X, Han WH, et al. The FABP12/PPARγ pathway promotes metastatic transformation by inducing epithelial-to-mesenchymal transition and lipid-derived energy production in prostate cancer cells. Mol Oncol. 2020 Dec;14(12):3100-20.
130. Liu RZ, Graham K Fau - Glubrecht DD, Glubrecht Dd Fau - Lai R, Lai R Fau - Mackey JR, Mackey Jr Fau - Godbout R, Godbout R. A fatty acid-binding protein 7/RXRbeta pathway enhances

survival and proliferation in triple-negative breast cancer. (1096-9896 (Electronic)).
131. Lloyd T, Hounsome L, Mehay A, Mee S, Verne J, Cooper A. Lifetime risk of being diagnosed with, or dying from, prostate cancer by major ethnic group in England 2008–2010. BMC Medicine. 2015 2015/07/30;13(1):171.
132. Loeb S, Bjurlin MA, Nicholson J, Tammela TL, Penson DF, Carter HB, et al. Overdiagnosis and overtreatment of prostate cancer. Eur Urol. 2014 Jun;65(6):1046-55.
133. Mahon KL, Lin HM, Castillo L, Lee BY, Lee-Ng M, Chatfield MD, et al. Cytokine profiling of docetaxel-resistant castration-resistant prostate cancer. Br J Cancer. 2015 Apr;112(8):1340-8.
134. Makinen T, Tammela TL, Stenman UH, Maattanen L, Rannikko S, Aro J, et al. Family history and prostate cancer screening with prostate-specific antigen. J Clin Oncol. 2002 Jun 1;20(11):2658-63.
135. Makowski L, Boord JB, Maeda K, Babaev VR, Uysal KT, Morgan MA, et al. Lack of macrophage fatty-acid-binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis. Nat Med. 2001 Jun;7(6):699-705.
136. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. Cell. 1995 Dec;83(6):835-9.
137. Mansure JJ, Nassim R, Chevalier S, Szymanski K, Rocha J, Aldousari S, et al. A novel mechanism of PPAR gamma induction via EGFR signalling constitutes rational for combination therapy in bladder cancer. PLoS One. 2013;8(2):e55997.
138. Marchiani S, Tamburrino L, Nesi G, Paglierani M, Gelmini S, Orlando C, et al. Androgen-responsive and -unresponsive prostate cancer cell lines respond differently to stimuli inducing neuroendocrine differentiation. Int J Androl. 2010 Dec;33(6):784-93.
139. Mauer J, Denson JL, Brüning JC. Versatile functions for IL-6 in metabolism and cancer. Trends Immunol. 2015 Feb;36(2):92-101.

140. McKillop IH, Girardi CA, Thompson KJ. Role of fatty acid binding proteins (FABPs) in cancer development and progression. Cellular Signalling. 2019 2019/10/01/;62:109336.
141. McLatchie GReoc, Borley NReoc, Chikwe Jeoc. Oxford handbook of clinical surgery. edited by Greg McLatchie, Neil Borley, Joanna Chikwe. Fourth edition / ed. 2013.
142. McNeal JE. Origin and development of carcinoma in the prostate. Cancer. 1969 Jan;23(1):24-34.
143. McNeal JE. Origin and evolution of benign prostatic enlargement. Invest Urol. 1978 Jan;15(4):340-5.
144. McNeal JE, Bostwick DG, Kindrachuk RA, Redwine EA, Freiha FS, Stamey TA. Patterns of progression in prostate cancer. Lancet. 1986 Jan 11;1(8472):60-3.
145. Merola R, Tomao L, Antenucci A, Sperduti I, Sentinelli S, Masi S, et al. PCA3 in prostate cancer and tumor aggressiveness detection on 407 high-risk patients: a National Cancer Institute experience. J Exp Clin Cancer Res. 2015 Feb;34:15.
146. Metcalfe C, Evans S, Ibrahim F, Patel B, Anson K, Chinegwundoh F, et al. Pathways to diagnosis for Black men and White men found to have prostate cancer: the PROCESS cohort study. British journal of cancer. 2008;99(7):1040-45.
147. Mickey DD, Stone KR, Wunderli H, Mickey GH, Vollmer RT, Paulson DF. Heterotransplantation of a human prostatic adenocarcinoma cell line in nude mice. Cancer Res. 1977 Nov;37(11):4049-58.
148. Miller MC, Doyle GV, Terstappen LW. Significance of Circulating Tumor Cells Detected by the CellSearch System in Patients with Metastatic Breast Colorectal and Prostate Cancer. J Oncol. 2010;2010:617421.
149. Mishra P, Singh U, Pandey CM, Mishra P, Pandey G. Application of student's t-test, analysis of variance, and covariance. Annals of cardiac anaesthesia. 2019 Oct-Dec;22(4):407-11.
150. Morgan EA, Forootan SS, Adamson J, Foster CS, Fujii H, Igarashi M, et al. Expression of cutaneous fatty acid-binding protein

(C-FABP) in prostate cancer: potential prognostic marker and target for tumourigenicity-suppression. Int J Oncol. 2008 Apr;32(4):767-75.
151. Moyer VA. Screening for prostate cancer: U.S. Preventive Services Task Force recommendation statement. Ann Intern Med. 2012 Jul 17;157(2):120-34.
152. Myers JS, von Lersner AK, Sang QX. Proteomic Upregulation of Fatty Acid Synthase and Fatty Acid Binding Protein 5 and Identification of Cancer- and Race-Specific Pathway Associations in Human Prostate Cancer Tissues. J Cancer. 2016;7(11):1452-64.
153. Nagle RB, Ahmann FR, McDaniel KM, Paquin ML, Clark VA, Celniker A. Cytokeratin characterization of human prostatic carcinoma and its derived cell lines. Cancer Res. 1987 Jan;47(1):281-6.
154. Institute NC. 2015. [online] Available from : https://seer.cancer.gov/
155. Nevo J, Mattila E, Pellinen T, Yamamoto DL, Sara H, Iljin K, et al. Mammary-derived growth inhibitor alters traffic of EGFR and induces a novel form of cetuximab resistance. Clin Cancer Res. 2009 Nov;15(21):6570-81.
156. Nguyen DP, Li J, Tewari AK. Inflammation and prostate cancer: the role of interleukin 6 (IL-6). BJU Int. 2014 Jun;113(6):986-92.
157. Nie J, Zhang J, Wang L, Lu L, Yuan Q, An F, et al. Adipocytes promote cholangiocarcinoma metastasis through fatty acid binding protein 4. J Exp Clin Cancer Res. 2017 Dec;36(1):183.
158. Ohata TA-Ohoo, Yokoo H, Kamiyama T, Fukai M, Aiyama T, Hatanaka Y, et al. Fatty acid-binding protein 5 function in hepatocellular carcinoma through induction of epithelial-mesenchymal transition. (2045-7634 (Electronic)).
159. Okano T, Kondo T, Fujii K, Nishimura T, Takano T, Ohe Y, et al. Proteomic signature corresponding to the response to gefitinib (Iressa, ZD1839), an epidermal growth factor receptor tyrosine kinase inhibitor in lung adenocarcinoma. Clin Cancer Res. 2007 Feb;13(3):799-805.

160. Olokpa E, Bolden A, Stewart LV. The Androgen Receptor Regulates PPARγ Expression and Activity in Human Prostate Cancer Cells. J Cell Physiol. 2016 Dec;231(12):2664-72.
161. Olokpa E, Moss PE, Stewart LV. Crosstalk between the Androgen Receptor and PPAR Gamma Signaling Pathways in the Prostate. PPAR Res. 2017;2017:9456020.
162. Onstenk W, Sieuwerts AM, Mostert B, Lalmahomed Z, Bolt-de Vries JB, van Galen A, et al. Molecular characteristics of circulating tumor cells resemble the liver metastasis more closely than the primary tumor in metastatic colorectal cancer. (1949-2553 (Electronic)).
163. Oren I, Fleishman SJ, Kessel A, Ben-Tal N. Free diffusion of steroid hormones across biomembranes: a simplex search with implicit solvent model calculations. Biophys J. 2004 Aug;87(2):768-79.
164. Parikh R, Mathai A, Parikh S, Chandra Sekhar G, Thomas R. Understanding and using sensitivity, specificity and predictive values. Indian J Ophthalmol. 2008 2008 Jan-Feb;56(1):45-50.
165. Pertschuk LP, Schaeffer H, Feldman JG, Macchia RJ, Kim YD, Eisenberg K, et al. Immunostaining for prostate cancer androgen receptor in paraffin identifies a subset of men with a poor prognosis. Lab Invest. 1995 Aug;73(2):302-5.
166. Pettersson A, Graff RE, Bauer SR, Pitt MJ, Lis RT, Stack EC, et al. The TMPRSS2:ERG rearrangement, ERG expression, and prostate cancer outcomes: a cohort study and meta-analysis. Cancer Epidemiol Biomarkers Prev. 2012 Sep;21(9):1497-509.
167. Pierorazio PM, Walsh PC, Partin AW, Epstein JI. Prognostic Gleason grade grouping: data based on the modified Gleason scoring system. BJU Int. 2013 May;111(5):753-60.
168. Powell CA, Nasser MW, Zhao H, Wochna JC, Zhang X, Shapiro C, et al. Fatty acid binding protein 5 promotes metastatic potential of triple negative breast cancer cells through enhancing epidermal growth factor receptor stability. Oncotarget. 2015;6(8):6373-85.

169. Technology pbCS. Phosphosite.org.2021. [online] Available from : https://www.phosphosite.org/
170. Rawla P. Epidemiology of Prostate Cancer. World journal of oncology. 2019;10(2):63-89.
171. Rebbeck TR. Prostate Cancer Genetics: Variation by Race, Ethnicity, and Geography. Semin Radiat Oncol. 2017 Jan;27(1):3-10.
172. Reiner WG, Walsh PC. An anatomical approach to the surgical management of the dorsal vein and Santorini's plexus during radical retropubic surgery. J Urol. 1979 Feb;121(2):198-200.
173. Rhim JS. In vitro human cell culture models for the study of prostate cancer. Prostate Cancer Prostatic Dis. 2000 Dec;3(4):229-35.
174. Roddam AW, Duffy MJ, Hamdy FC, Ward AM, Patnick J, Price CP, et al. Use of prostate-specific antigen (PSA) isoforms for the detection of prostate cancer in men with a PSA level of 2-10 ng/ml: systematic review and meta-analysis. Eur Urol. 2005 Sep;48(3):386-99; discussion 98-9.
175. Roehrborn CG, Boyle P, Gould AL, Waldstreicher J. Serum prostate-specific antigen as a predictor of prostate volume in men with benign prostatic hyperplasia. Urology. 1999 Mar;53(3):581-9.
176. Russel PJ, Jackson P, Kingsley EA. Prostate Cancer Methods and Protocols. 2003.
177. Sahin E, Baycu C, Koparal AT, Burukoglu Donmez D, Bektur E. Resveratrol reduces IL-6 and VEGF secretion from co-cultured A549 lung cancer cells and adipose-derived mesenchymal stem cells. Tumour Biol. 2016 Jun;37(6):7573-82.
178. Salami SS, Schmidt F, Laxman B, Regan MM, Rickman DS, Scherr D, et al. Combining urinary detection of TMPRSS2:ERG and PCA3 with serum PSA to predict diagnosis of prostate cancer. Urol Oncol. 2013 Jul;31(5):566-71.
179. Sartori DA, Chan DW. Biomarkers in prostate cancer: what's new? Curr Opin Oncol. 2014 May;26(3):259-64.
180. Schachtrup C, Emmler T, Bleck B, Sandqvist A, Spener F. Functional analysis of peroxisome-proliferator-responsive element motifs in genes of fatty acid-binding proteins. Biochem J. 2004 Aug;382(Pt 1):239-45.

181. Scher HI, Graf RP, Schreiber NA, McLaughlin B, Jendrisak A, Wang Y, et al. Phenotypic Heterogeneity of Circulating Tumor Cells Informs Clinical Decisions between AR Signaling Inhibitors and Taxanes in Metastatic Prostate Cancer. Cancer Res. 2017 10;77(20):5687-98.
182. Schröder FH, Hugosson J, Roobol MJ, Tammela TL, Ciatto S, Nelen V, et al. Screening and prostate-cancer mortality in a randomized European study. N Engl J Med. 2009 Mar;360(13):1320-8.
183. M. S. Encyclopedia of cancer [Internet]. 2011 Berlin, Heidelberg: Springer
184. Sen M, Johnston PA, Pollock NI, DeGrave K, Joyce SC, Freilino ML, et al. Mechanism of action of selective inhibitors of IL-6 induced STAT3 pathway in head and neck cancer cell lines. J Chem Biol. 2017 Jul;10(3):129-41.
185. Sfanos KS, Bruno TC, Meeker AK, De Marzo AM, Isaacs WB, Drake CG. Human prostate-infiltrating CD8+ T lymphocytes are oligoclonal and PD-1+. Prostate. 2009 Nov;69(15):1694-703.
186. Shao G, Liu Y, Ma T, Zhang L, Yuan M, Zhao S. GCN5 inhibition prevents IL-6-induced prostate cancer metastases through PI3K/PTEN/Akt signaling by inactivating Egr-1. Biosci Rep. 2018 12;38(6).
187. Sherwood ER, Berg LA, Mitchell NJ, McNeal JE, Kozlowski JM, Lee C. Differential cytokeratin expression in normal, hyperplastic and malignant epithelial cells from human prostate. J Urol. 1990 Jan;143(1):167-71.
188. Shimizu H, Ross RK, Bernstein L, Yatani R, Henderson BE, Mack TM. Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. Br J Cancer. 1991 Jun;63(6):963-6.
189. Shirai T, Asamoto M, Takahashi S, Imaida K. Diet and prostate cancer. Toxicology. 2002 Dec 27;181-182:89-94.
190. Sikka S, Chen L, Sethi G, Kumar AP. Targeting PPARγ Signaling Cascade for the Prevention and Treatment of Prostate Cancer. PPAR Res. 2012;2012:968040.

191. Slipicevic A, Jørgensen K, Skrede M, Rosnes AK, Trøen G, Davidson B, et al. The fatty acid binding protein 7 (FABP7) is involved in proliferation and invasion of melanoma cells. BMC Cancer. 2008 Sep;8:276.
192. Smathers RL, Petersen DR. The human fatty acid-binding protein family: evolutionary divergences and functions. Hum Genomics. 2011 Mar;5(3):170-91.
193. Smith JR, Freije D, Carpten JD, Grönberg H, Xu J, Isaacs SD, et al. Major susceptibility locus for prostate cancer on chromosome 1 suggested by a genome-wide search. Science. 1996 Nov;274(5291):1371-4.
194. Sramkoski RM, Pretlow TG, Giaconia JM, Pretlow TP, Schwartz S, Sy MS, et al. A new human prostate carcinoma cell line, 22Rv1. In Vitro Cell Dev Biol Anim. 1999 1999 Jul-Aug;35(7):403-9.
195. Stattin P, Bylund A, Rinaldi S, Biessy C, Déchaud H, Stenman UH, et al. Plasma insulin-like growth factor-I, insulin-like growth factor-binding proteins, and prostate cancer risk: a prospective study. J Natl Cancer Inst. 2000 Dec;92(23):1910-7.
196. Stone KR, Mickey DD, Wunderli H, Mickey GH, Paulson DF. Isolation of a human prostate carcinoma cell line (DU 145). Int J Cancer. 1978 Mar;21(3):274-81.
197. Storch J, Corsico B. The emerging functions and mechanisms of mammalian fatty acid-binding proteins. Annu Rev Nutr. 2008;28:73-95.
198. Tai S, Sun Y, Squires JM, Zhang H, Oh WK, Liang CZ, et al. PC3 is a cell line characteristic of prostatic small cell carcinoma. Prostate. 2011 Nov;71(15):1668-79.
199. Taitt HE. Global Trends and Prostate Cancer: A Review of Incidence, Detection, and Mortality as Influenced by Race, Ethnicity, and Geographic Location. American journal of men's health. 2018;12(6):1807-23.
200. Tan MH, Li J, Xu HE, Melcher K, Yong EL. Androgen receptor: structure, role in prostate cancer and drug discovery. Acta Pharmacol Sin. 2015 Jan;36(1):3-23.

201. Tan NS, Shaw NS, Vinckenbosch N, Liu P, Yasmin R, Desvergne B, et al. Selective cooperation between fatty acid binding proteins and peroxisome proliferator-activated receptors in regulating transcription. Mol Cell Biol. 2002 Jul;22(14):5114-27.
202. Tan SH, Petrovics G, Srivastava S. Prostate Cancer Genomics: Recent Advances and the Prevailing Underrepresentation from Racial and Ethnic Minorities. Int J Mol Sci. 2018 Apr 22;19(4).
203. Tang Z, Shen Q, Xie H, Zhou X, Li J, Feng J, et al. Elevated expression of FABP3 and FABP4 cooperatively correlates with poor prognosis in non-small cell lung cancer (NSCLC). (1949-2553 (Electronic)).
204. Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, et al. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med. 2012 Mar 28;4(127):127ra37.
205. Tepper CG, Boucher DL, Ryan PE, Ma AH, Xia L, Lee LF, et al. Characterization of a novel androgen receptor mutation in a relapsed CWR22 prostate cancer xenograft and cell line. Cancer Res. 2002 Nov;62(22):6606-14.
206. Thompson IM, Ankerst DP, Chi C, Lucia MS, Goodman PJ, Crowley JJ, et al. Operating characteristics of prostate-specific antigen in men with an initial PSA level of 3.0 ng/ml or lower. Jama. 2005 Jul 6;294(1):66-70.
207. Thompson IM, Pauler DK, Goodman PJ, Tangen CM, Lucia MS, Parnes HL, et al. Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4.0 ng per milliliter. N Engl J Med. 2004 May 27;350(22):2239-46.
208. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science. 2005 Oct;310(5748):644-8.
209. Topalian SL. Targeting Immune Checkpoints in Cancer Therapy. Jama. 2017 Nov 7;318(17):1647-48.

210. Turajlic S, Swanton C. Metastasis as an evolutionary process. Science. 2016 Apr;352(6282):169-75.
211. Uehara H, Takahashi T, Oha M, Ogawa H, Izumi K. Exogenous fatty acid binding protein 4 promotes human prostate cancer cell progression. Int J Cancer. 2014 Dec;135(11):2558-68.
212. Ugoni A, Walker BF. The Chi square test: an introduction. COMSIG review. 1995;4(3):61-64.
213. Unniyampurath U, Pilankatta R, Krishnan MN. RNA Interference in the Age of CRISPR: Will CRISPR Interfere with RNAi? Int J Mol Sci. 2016 Feb;17(3):291.
214. van Bokhoven A, Varella-Garcia M, Korch C, Johannes WU, Smith EE, Miller HL, et al. Molecular characterization of human prostate carcinoma cell lines. Prostate. 2003 Nov;57(3):205-25.
215. Vasseur-Cognet M, Lane MD. Trans-acting factors involved in adipogenic differentiation. Curr Opin Genet Dev. 1993 Apr;3(2):238-45.
216. Veerkamp JH, Maatman RG. Cytoplasmic fatty acid-binding proteins: their structure and genes. (0163-7827 (Print)).
217. Veerkamp JH, Paulussen RJA, Peeters RA, Maatman RGHJ, van Moerkerk HTB, van Kuppevelt THMSM. Detection, tissue distribution and (sub)cellular localization of fatty acid-binding protein types. Molecular and Cellular Biochemistry. 1990 1990/10/01;98(1):11-18.
218. Velazquez-Salinas L, Verdugo-Rodriguez A, Rodriguez LL, Borca MV. The Role of Interleukin 6 During Viral Infections. Front Microbiol. 2019;10:1057.
219. Verhagen AP, Aalders TW, Ramaekers FC, Debruyne FM, Schalken JA. Differential expression of keratins in the basal and luminal compartments of rat prostatic epithelium during degeneration and regeneration. Prostate. 1988;13(1):25-38.
220. Vickers AJ, Gupta A, Savage CJ, Pettersson K, Dahlin A, Bjartell A, et al. A panel of kallikrein marker predicts prostate cancer in a large, population-based cohort followed for 15 years without screening. Cancer Epidemiol Biomarkers Prev. 2011 Feb;20(2):255-61.

221. Vlaeminck-Guillem V, Ruffion A, André J, Devonec M, Paparel P. Urinary prostate cancer 3 test: toward the age of reason? Urology. 2010 Feb;75(2):447-53.
222. Voigt JD, Zappala SM, Vaughan ED, Wein AJ. The Kallikrein Panel for prostate cancer screening: its economic impact. Prostate. 2014 Feb;74(3):250-9.
223. Wang G, Bonkovsky HL, de Lemos A, Burczynski FJ. Recent insights into the biological functions of liver fatty acid binding protein 1. J Lipid Res. 2015 Dec;56(12):2238-47.
224. Wang W, Chu HJ, Liang YC, Huang JM, Shang CL, Tan H, et al. FABP5 correlates with poor prognosis and promotes tumor cell growth and metastasis in cervical cancer. Tumour Biol. 2016 Nov;37(11):14873-83.
225. Watanabe A, Toyota T, Owada Y, Hayashi T, Iwayama Y, Matsumata M, et al. Fabp7 maps to a quantitative trait locus for a schizophrenia endophenotype. PLoS Biol. 2007 Nov;5(11):e297.
226. Weinrich SP, African American Hereditary Prostate Cancer Study N. Prostate cancer screening in high-risk men. Cancer.2006/02/15;106(4):796-803.
227. Whittemore AS, Kolonel LN, Wu AH, John EM, Gallagher RP, Howe GR, et al. Prostate cancer in relation to diet, physical activity, and body size in blacks, whites, and Asians in the United States and Canada. J Natl Cancer Inst. 1995 May 3;87(9):652-61.
228. Wood SM, Gill AJ, Brodsky AS, Lu S, Friedman K, Karashchuk G, et al. Fatty acid-binding protein 1 is preferentially lost in microsatellite instable colorectal carcinomas and is immune modulated via the interferon gamma pathway. (1530-0285 (Electronic)).
229. Wu C-H, Sahoo D, Arvanitis C, Bradon N, Dill DL, Felsher DW. Combined analysis of murine and human microarrays and ChIP analysis reveals genes associated with the ability of MYC to maintain tumorigenesis. PLoS genetics. 2008;4(6):e1000090-e90.
230. Xie M, Wu X, Zhang J, He C, Wei S, Huang J, et al. The Prognostic Significance of Notch1 and Fatty Acid Binding Protein 7 (FABP7) Expression in Resected Tracheobronchial Adenoid Cystic

Carcinoma: A Multicenter Retrospective Study. Cancer Res Treat. 2018 Oct;50(4):1064-73.
231. Xu J. Combined analysis of hereditary prostate cancer linkage to 1q24-25: results from 772 hereditary prostate cancer families from the International Consortium for Prostate Cancer Genetics. Am J Hum Genet. 2000 Mar;66(3):945-57.
232. Yan F, Shen N, Pang JX, Zhang YW, Rao EY, Bode AM, et al. Fatty acid-binding protein FABP4 mechanistically links obesity with aggressive AML by enhancing aberrant DNA methylation in AML cells. Leukemia. 2017 06;31(6):1434-42.
233. Yeldir N, Yildiz E, Dundar G. Gleason Score Correlation Between Prostate Needle Biopsy and Radical Prostatectomy Materials. Turk Patoloji Derg. 2019;35(3):185-92.
234. Yeung DC, Wang Y, Xu A, Cheung SC, Wat NM, Fong DY, et al. Epidermal fatty-acid-binding protein: a new circulating biomarker associated with cardio-metabolic risk factors and carotid atherosclerosis. Eur Heart J. 2008 Sep;29(17):2156-63.
235. Yorukoglu K. Current developments in uropathology. 1 ed: Turkiye Klinikleri J Med Pathol-Special Topics; 2016. p. 35-40.
236. Yuan H, Wei X, Zhang G, Li C, Zhang X, Hou J. B7-H3 over expression in prostate cancer promotes tumor cell progression. J Urol. 2011 Sep;186(3):1093-9.
237. Zang X, Thompson RH, Al-Ahmadie HA, Serio AM, Reuter VE, Eastham JA, et al. B7-H3 and B7x are highly expressed in human prostate cancer and associated with disease spread and poor outcome. Proc Natl Acad Sci U S A. 2007 Dec;104(49):19458-63.
238. Zhao G, Wu M, Wang X, Du Z, Zhang G. Effect of FABP5 gene silencing on the proliferation, apoptosis and invasion of human gastric SGC-7901 cancer cells. Oncol Lett. 2017 Oct;14(4):4772-78.
239. Zheng Y, Fang YC, Li J. PD-L1 expression levels on tumor cells affect their immunosuppressive activity. Oncol Lett. 2019 Nov;18(5):5399-407.
240. Zieglschmid V, Hollmann C, Böcher O. Detection of disseminated tumor cells in peripheral blood. Crit Rev Clin Lab Sci. 2005;42(2):155-96.

241. Zimmerman AW, van Moerkerk HT, Veerkamp JH. Ligand specificity and conformational stability of human fatty acid-binding proteins. Int J Biochem Cell Biol. 2001 Sep;33(9):865-76.
242. Zimmerman AW, Veerkamp JH. New insights into the structure and function of fatty acid-binding proteins. Cell Mol Life Sci. 2002 Jul;59(7):1096-116.
243. Zynger D, Parwani A, Suster S. Prostate Pathology. New York, UNITED STATES: Demos Medical Publishing; 2014.
244. Molecular weight of FABPs (2021)[online] powered by cell signaling technology Available from :https://www.phosphosite.org/ (Accessed 05/2021)
245. Genecard (2021) Exon structure of FABPs [online] Available from : https://www.genecards.org/
246. Wen S, Niu Y, Lee SO, et al. Targeting fatty acid synthase with ASC-J9 suppresses proliferation and invasion of prostate cancer cells. Mol Carcinog. 2016;55(12):2278-2290.
247. Scher, H. I., Buchanan, G., Gerald, W., Butler, L. M., & Tilley, W. D. Targeting the androgen receptor: improving outcomes for castration-resistant prostate cancer. Endocrine-related cancer 2004; 11(3), 459–476.
248. Namekawa T, Ikeda K, Horie-Inoue K, Inoue S. Application of Prostate Cancer Models for Preclinical Study: Advantages and Limitations of Cell Lines, Patient-Derived Xenografts, and Three-Dimensional Culture of Patient-Derived Cells. Cells. 2019; 20;8(1):74.
249. Van Zijl F, Krupitza G, Mikulits W. Initial steps of metastasis: cell invasion and endothelial transmigration. Mutat Res. 2011;728(1-2):23-34.
250. Zhu J, Thompson CB. Metabolic regulation of cell growth and proliferation. Nat Rev Mol Cell Biol. 2019;20(7):436-450.

Appendix. A

1. Equipment

BD BioCoatTM Growth Factor Reduced (GFR) Matrigel TM Invasion Chamber Carbon Steel

BD Biosciences, USA

……

CO2 incubator Model TC2323

Borolabs, Basingstoke, UK

..……

Cell culture filter cap flasks

Cell culture plates

Cryogenic vial

Nunc, Denmark

……

Coverslip 20×40mm

Shandon, UK

……

Carbon steel surgical blades

Swann-Morton, Sheffield, UK

…….

Falcon 2059 tube

Becton Dikinson, USA

……

Gel electrophoresis

Bio-Rad, UK

……

GelCount

OXFORD OPTRONIX, Oxford, UK

……

Haemocytometer slide

Weber scientific International, NJ, USA

……

Hot plate (Ori-Block 08-3)

Techne, England, UK

……

Haemocytometer

SLS Ltd., Nottingham, UK

……

Immobilon, Transfer membrane

Millipore, UK

……

Microtome HM355

Microm, Oxford, UK

……

Microtubes

Starlab, Milton Keynes, UK

……

Microslide

Surgipath, UK

……

Multiskan MS plate reader

Labsystem, Finland

……

Microcentrifuge

Beckman coulter, UK

……

Needle

BD Microlance, Ireland

……

NanoDrop spectrophotometer

Labtech International, Ringmer, UK

……

Pipette tips

QIAGEN, Crawley, UK

……

Syringes

BD Microlance, Ireland

……

Spectrophotometer

BioTec, Brigend, UK

……

Superior Adhesive slide

Leica, Germany

……

Cell culture pipettes 5-50 ml

Greiner Bio-One bio-one, UK

..……

Universal tube

Greiner Bio-One, UK

..……

Water bath

Grant Instruments, UK

..……

Whatman filter paper

Whatman, England, UK

2. Reagents

Reagents for cell culture

DMSO	Sigma-Aldrich, Germany
Foetal calf serum	Gibco, Invitrogen, Paisley, UK
L-Glutamine	Lonza, Belgium
Opti-MEM I medium	Gibco, Invitrogen, Paisley, UK
Penicillin/ Streptomycin	Lonza, Belgium
Phosphate buffered saline (tablet)	Gibco, Invitrogen, Paisley, UK

RPMI 1640	Gibco, Invitrogen, Paisley, UK
Sodium pyruvate	Sigma, USA
Trypsin	Gibco, Invitrogen, Paisley, UK
Versene	Gibco, Invitrogen, Paisley, UK
Zeocin	Invitrogen, CA, USA

Reagents for Western blot

β-mercaptoethanol	Sigma, USA
Ammonium persulfate (APS)	Sigma, USA
Bradford reagent	Sigma, USA
Bromophenol blue	Sigma, USA
CelLytic-M	Sigma, USA
Coomassie blue	Bio-Rad GmbH,UK
ECL detection kit	GE Healthcare, Buckinghamshire, UK
Glycine	Melford, UK
Methanol	Fisher scientific, Loughborough, UK

Reagents for IHC

EnVisionTM FLEX/DAB Chromogen	Agilent Technologies, UK
EnVisionTM FLEX/HRP	Agilent Technologies, UK
Ethanol	Sigma, UK
Haematoxylin	Sigma, UK
Hydrogen peroxide 30% (w/w)	Sigma, UK

Sodium citrate	Sigma, UK
Sodium chloride	Melford, UK
Tris base ultrapure	Melford, UK
Tween-20	Sigma-Aldrich, Germany
Xylene	GENTA, Tockwith, UK

Reagents for general molecular biology

Ampicillin	Sigma, USA
Agarose	Genflow, Fradley, UK
Glucose	Sigma, USA
Glycerol	Sigma, USA
Isopropanol BDH, England, UK	
LB agar	Sigma, USA
LB broth	Sigma, USA
Magnesium chloride	Sigma, USA
Magnesium sulphate	Sigma, USA
Zeocin	Invitrogen, CA, USA

Reagents for cell invasion assay

Crystal violet	Sigma, USA

Reagents for soft agar assay

Cell Transformation Assay Kit	Abcam, UK

3.BUFFERS

Cell Culture

Routine cell culture medium

RPMI medium 1640…….500ml

Foetal calf serum……. 10% (v/v)

Pen-Strep (5000 U/ml) ….. 5ml

L-Glutamine (20mM) …… 5ml

Sodium pyruvate (100mM) ... 5ml

Selective medium

Routine medium with ZeocinTM… (100µg/ml)

Trypsin/EDTA solution (T/E) (2.5%)

Versene……. 100ml ×1

Trypsin……2.5ml

MTT solution (5mg/ml)

MTT…….50mg

PBS……...10ml

PBS

PBS……1 tablet

dH2O…… 500ml

Autoclaved

Western Blot

M Tris pH 6.8

Tris base……...12.1gr

dH2O……. 100ml

pH adjusted with HCl

10% (w/v) SDS solution

Sodium Dodecyl Sulfate…10gr

dH2O……100ml

%10 (w/v) APS solution

Ammonium persulfate...100mg

dH2O……...1ml

SDS-PAGE sample loading buffer (SLB ×2)

M Tris-HCl (pH 6.8) …2.5ml

Glycerol 40% (v/v) ….. 4ml

Bromophenol blue 0.5% (w/v) …...0.8ml

SDS 10%.....2ml

β-mercaptoethanol…….0.5ml

dH2O……. 4.7ml

SDS-PAGE sample loading buffer (SLB ×5)

M Tris-HCl (pH 6.8) …1.25ml

Glycerol 40% (v/v) …….15ml

Bromophenol blue 0.5% (w/v) …2.5ml

SDS 10% …….5ml

β-mercaptoethanol …….1.25ml

Transfer buffer (pH 8.3)

Glycine ……14.4g (192mM)

Methanol …… 20% (v/v)

Tris base ……. 3.03g (25mM)

dH2O ……. up to 1Lit

pH adjusted with HCl

TBS buffer (pH 7.6 ×10)

Sodium chloride ……... 87.66gr (1500mM)

Tris base ……... 60.58gr (500mM)

dH2O …… up to 1 Lit

pH adjusted with HCl

Autoclaved

TBS-Tween 1% x1

TBS buffer …… 100ml x10

Tween 20 ……. 1ml

dH2O …….…. up to 1 Lit

TBS-T-milk 5%

Dried milk ……. 5gr

TBS-T ……... 100ml x1

<u>Immunohistochemistry</u>

Hydrogen peroxide-Methanol solution

Hydrogen peroxide 30% (w/w) ….12ml

Methanol ……... 400ml

Sodium citrate buffer (10mM)

Tris sodium citrate……. 29.41gr

dH2O ……. up to 10 Lit

pH 6; adjusted with HCl

EDTA buffer (pH 7)

EDTA ……37.2gr

Sodium hydroxide …… 3.2gr

dH2O ……. up to 10 Lit

TBS-Tween 5%

Sodium chloride ……... 87.66gr (1500mM)

Tris base ……... 60.58gr (500mM)

Tween 20 ……. 5ml

dH2O ……. up to 10 Lit

pH adjusted with HCl

Acid/alcohol 1%

HCl …… 20ml

IMS ……... 1400ml

dH2O ……. 60ml

Scott's tap water

MgSO4…….…. 20gr

NaHCO3……... 3.5gr

dH2O ……... up to 1 Lit

<u>Molecular Biology</u>

LB medium

LB broth ……20gr

dH2O …… 1 Lit

Autoclaved

LB agar

LB agar …….35gr

dH2O …… 1 Lit

Autoclaved

Glycerol …… 5ml

LB medium ……. 4ml

Bacteria culture ……... 3ml

TBE stock solution ×10

Tris base ……. 108gr (890mM)

Boric acid ……... 55gr (890mM)

EDTA 0.5M, pH 8 ……. 40ml (20mM)

dH2O ……. up to 1 Lit

Adjust the pH and sterilized by autoclave

50x TBE stock solution

Tris base …… 242gr

Glacial Acetic Acid …... 57.1gr

EDTA 0.5M, pH 8 …… 100ml

dH2O ……. up to 1 Lit

Adjust the pH and sterilized by autoclave

TE buffer (pH 7.6)

Tris-HCl …… 1.21gr (10mM

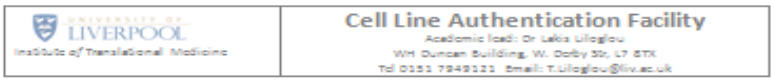
EDTA …… 0.3722gr (1mM

dH2O ……. up to 1 Lit

Adjust the pH and sterilized by autoclave

Appendix .B

STR profile for cell lines used in this project



Certificate of cell line authenticity

Customer	Asmaa Al-Bayati (Prof Ke)
Organisation	UoL ITM DMCCM
Tested Cell line	22RV1
Authentication method	GenePrint® 10, Promega Corporation
Database(s) used for comparison	DSMZ, ATCC
Authentication undertaken by	Pat Gerard/Andy Birss
Date	08/05/18

	Alleles		Match
TH01	6	9.3	√
D21S11	29	30	X
D5S818	11	13	√
D13S317	9	12	√
D7S820	9	10 11	√
D16S539	11	12	X
CSF1PO	10	11 12	√
AMEL	X	Y	√
vWA	15	21	√
TPOX	8	8	√

Comments (optional):
DSMZ 90% match
ATCC 94% match

Operator

Pat Gerard/Andy Birss
Research Technician

Academic Lead

Dr Lakis Liloglou
Senior Lecturer

UNIVERSITY OF LIVERPOOL
Institute of Translational Medicine

Cell Line Authentication Facility
Academic lead: Dr Lakis Liloglou
WH Duncan Building, W. Derby St, L7 8TX
Tel 0151 7949121 Email: T.Liloglou@liv.ac.uk

Certificate of cell line authenticity

Customer	Asmaa Al-Bayati (Prof Ke)
Organisation	UoL ITM DMCCM
Tested Cell line	PC3
Authentication method	GenePrint® 10, Promega Corporation
Database(s) used for comparison	DSMZ, ATCC
Authentication undertaken by	Pat Gerard/Andy Birss
Date	05/05/18

	Alleles		Match
TH01	6	7	√
D21S11	29	31.2	√
D5S818	13	13	√
D13S317	11	11	√
D7S820	8	11	√
D16S539	11	11	√
CSF1PO	11	11	√
AMEL	X		√
vWA	17	17	√
TPOX	8	9	√

Comments (optional):
DSMZ 100% match
ATCC 100& match

Operator

Pat Gerard/Andy Birss
Research Technician

Academic Lead

Dr Lakis Liloglou
Senior Lecturer

UNIVERSITY OF LIVERPOOL
Institute of Translational Medicine

Cell Line Authentication Facility
Academic lead: Dr Lakis Liloglou
WH Duncan Building, W. Derby St, L7 8TX
Tel 0151 7949111 Email: T.Liloglou@liv.ac.uk

Certificate of cell line authenticity

Customer	Asmaa
Organisation	UoL ITM DMCCM
Tested Cell line	PC3M
Authentication method	GenePrint® 10, Promega Corporation
Database(s) used for comparison	DSMZ, ATCC
Authentication undertaken by	Pat Gerard/Andy Birss
Date	08/05/18

	Alleles		Match
TH01	6	7	√
D21S11	29	31.2	√
D5S818	13	13	√
D13S317	11	11	√
D7S820	8	11	√
D16S539	11	11	√
CSF1PO	11	11	√
AMEL	X		√
vWA	17	17	√
TPOX	8	9	√

Comments (optional):
DSMZ 100% match
ATCC 100% match

Operator

Pat Gerard/Andy Birss
Research Technician

Academic Lead

Dr Lakis Liloglou
Senior Lecturer

Appendix C. Supplementary materials Western blot

Expression of FABP12 in PCa cells

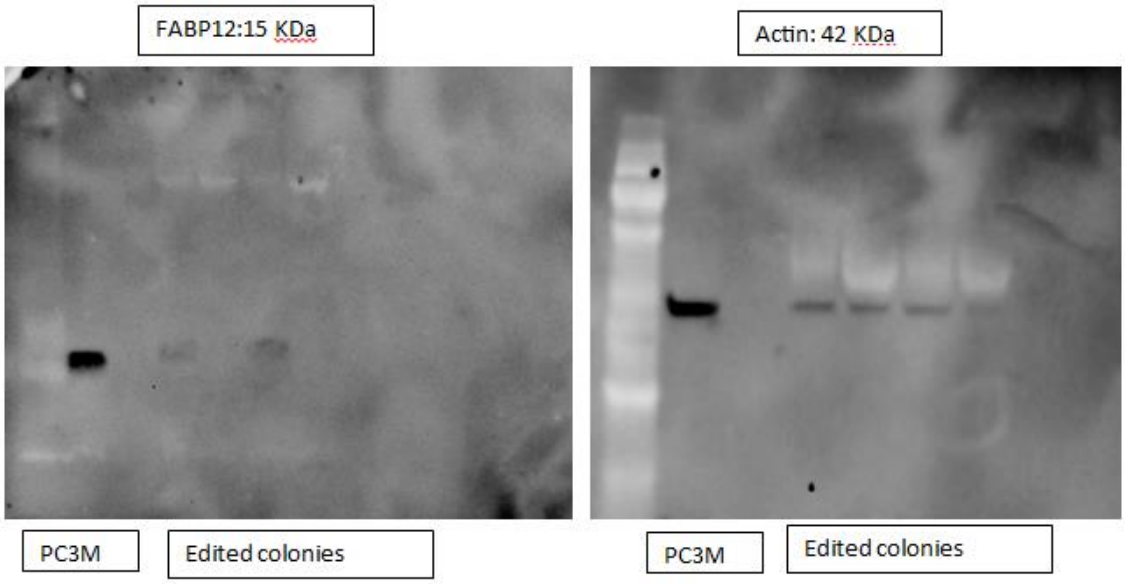


Figure C.1 This figure represents FABP12 expression in PCa cells. Anti-FABP12 antibody (Abcam, ab155089) was applied and PC3M highly malignant cell line was used as a control. This blot shows how antibody detects FABP12 (15 KDa) in PC3M cells, as compared to reduced exression in edited colonies . The image on left represents anti B-actin that was used as a loading reference as described in methods.

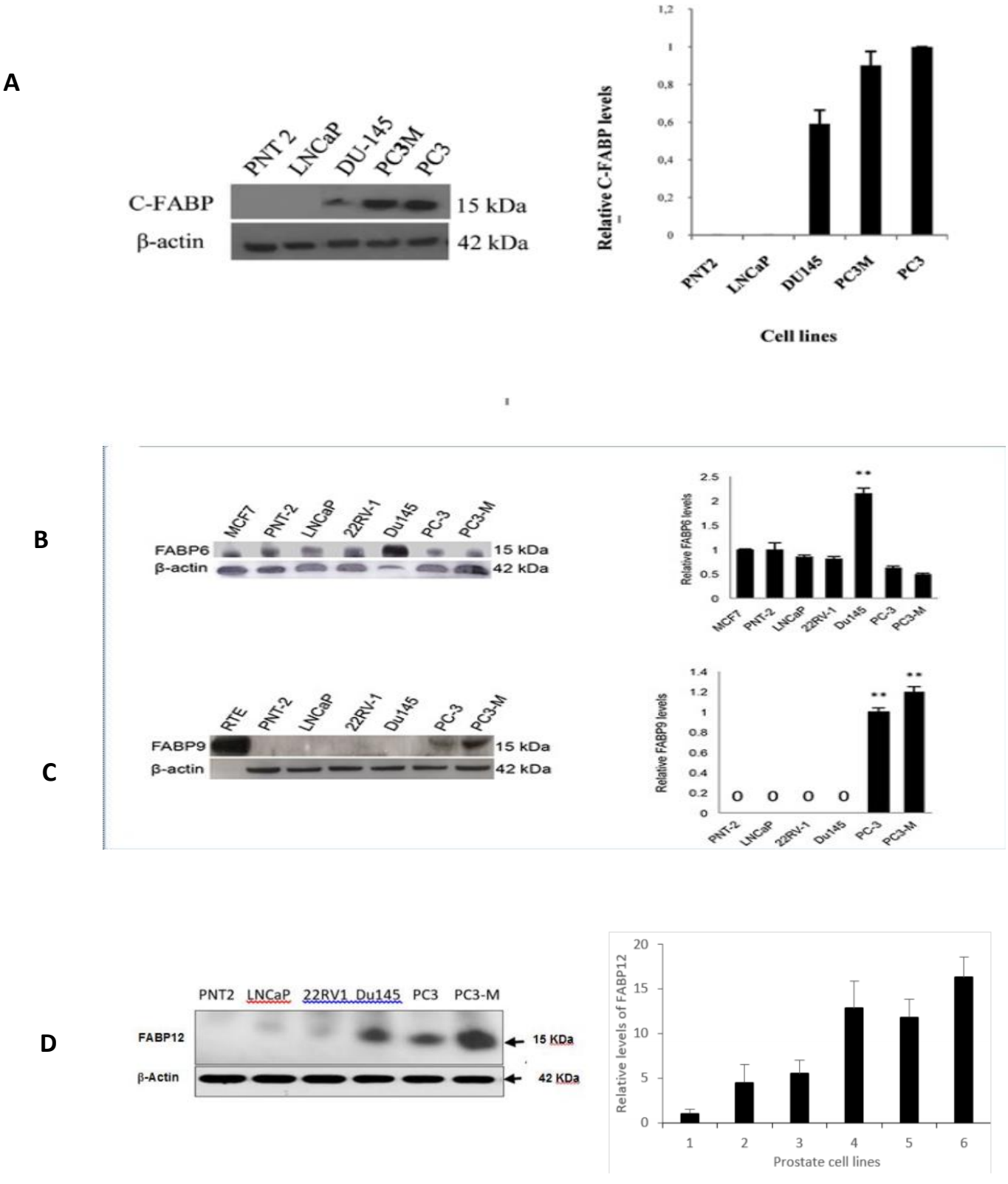


Figure C2 . Expression of different FABPs in PCa cell lines (5, 67).

This figure compares expression of different FABPs in prostate cell lines A.FABP5, B.FABP6, C. FABP9 , D. FABP12

The pattern of expression was different between the members of FABPs family; FABP5 expression in PNT2 and LNCaP cells was not detectable. While in FABP12, there was clear expression in these two cell lines.

FABP9 was expressed only in high malignant PCa cell lines PC3, PC3M. For FABP6, and although it was expressed in low and moderate cell lines, it was slightly expressed in high malignant PCa cells: PC3 and PC3M; while FABP12 showed high expression in high malignant cell lines.

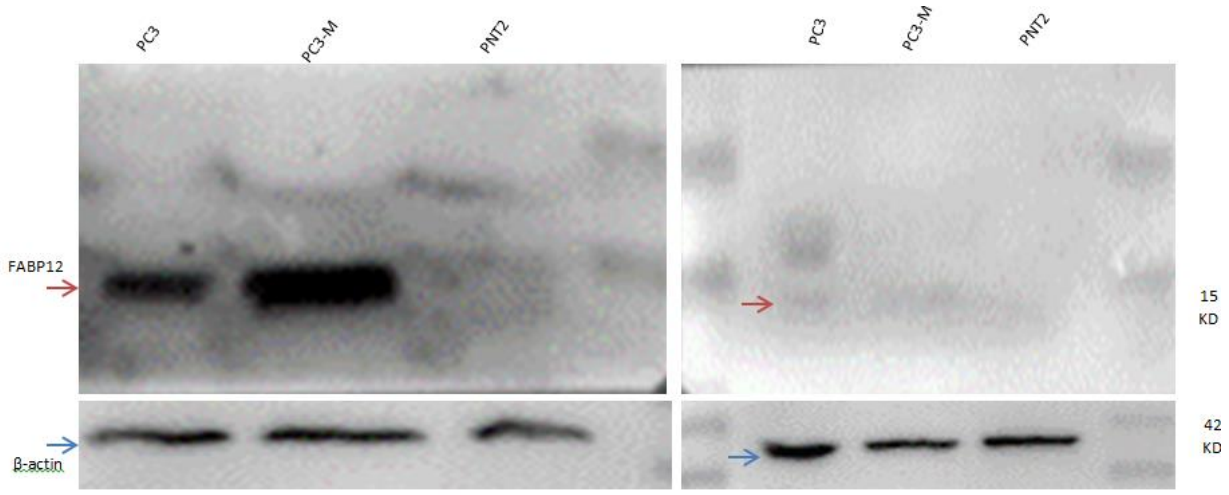


Figure C.3 Blocking of anti-FABP12 antibody by rhFABP12.

A supplementary image to figure 3.3 showed detection of FABP12 in control group (left image), in which no rhFABP12 is added to hybridize the first antibody anti-FABP12. Three cell lines were used PNT2, PC3, and PC3-M. FABP12 represented as single band (red arrow), β-actin band (blue arrow).

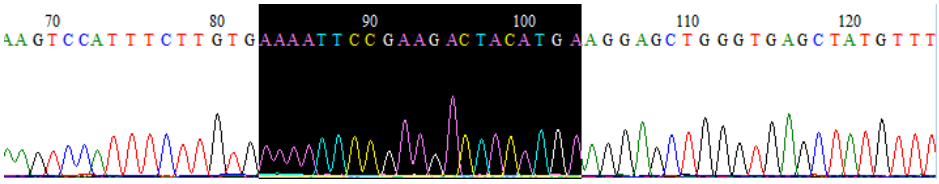
While the right image showed the suppression of FABP12 with rhFABP12 for 48 h. in 3 cell lines PNT2, PC3 and PC3-M.

Appendix D. Supplementary analysis for CRISPR/Cas9 gene KO

In this part, figures from genomic analysis of PCa KO clone are shown.

Sample from colony 6 (which is PC3M KO clone) was sent to GENEWIZ company to run PCR and sanger sequence, results from genomic analysis are presented below. The PCR done on a lysate of the prostate cell line colony 6, which showed the highest reduction in FABP12 expression as indicated in figure 3.15. Primers were supplied by company (not shared with consumer for commercial reasons). Then, amplicons sent for Sanger Sequencing using the same primers that have been used to generate the amplicons. The data were then analyzed as below :

A



B

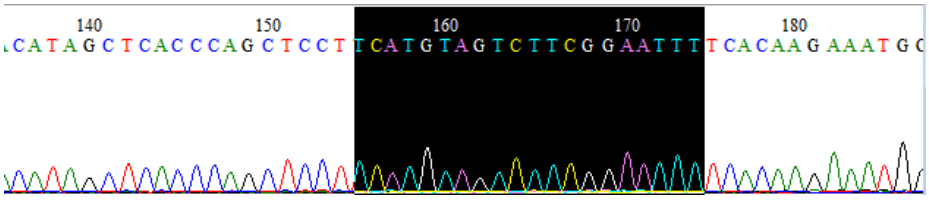


Figure D1: Genomic analysis of PC3M KO clone 6. (A more detailed schematic figure is presented Figure 17.A in methodology). A represents reverse strand while B. shows the forward strand, each consists of 20 nucleotides. The sequence inserted is clearly shown. The inserted sequence is attaching to PAM sequence (AGG) that will guide Cas9 to initiate the cut.