



UNIVERSITY OF
LIVERPOOL

**Expression of FABP6 and FABP9 in prostate cancer
and their relationship to malignant progression**

THESIS SUBMITTED IN ACCORDANCE WITH THE REQUIREMENT OF
THE UNIVERSITY OF LIVERPOOL
FOR THE DEGREE OF DOCTOR IN PHILOSOPHY

By:

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DEDICATION

To the soul of my father

To my great mother

To my wife and kids

Last but not least, to my sisters and brothers

ABSTRACT

The increased expression of fatty acid binding protein 4 (FABP4) and 5 (FABP5) play a crucial role in promoting tumorigenicity and metastasis of prostate cancer. FABP4 was suggested to be a therapeutic target for metastasis of prostate cancer. FABP5 was also proven to be a prognostic marker and potential treatment target. In contrast to numerous studies conducted to investigate the crucial role of FABP4 and FABP5 in prostate cancer, investigations on the possible involvement of other FABPs in the malignant progression of prostate cancer are rare. To find out whether any other FABP family proteins can also be used as diagnostic or prognostic markers for prostate cancer as FABP5, we measured the mRNA levels of 10 FABPs in benign and malignant prostate cell lines. Results showed that *FABP4*, *FABP5*, *FABP6*, *FABP9* and *FABP12* exhibited clearly higher levels in all tested malignant cell lines compared to their levels in the benign PNT-2 cells. Although, FABP4 and FABP5 have been associated with human prostate cancer as revealed in studies of our group and other groups, the finding of potential roles of FABP6, FABP9 and FABP12 have not been addressed. FABP12 is the most recently discovered member and no antibody was available for this candidate, thus FABP12 will be studied in a separate project of the group. Thereafter we assessed the expression status of FABP6 and FABP9 in both human prostate cell lines and tissues at the protein level. FABP6 protein was overexpressed only in 1 of the 5 malignant cell lines and its immunohistological staining intensities were not significantly different between the benign and the malignant prostate tissues. In contrast, FABP9 protein was highly expressed in highly malignant cell lines PC-3 and PC3-M, but its level in the benign PNT-2 and other malignant cell lines was not detectable. When analysed in an

Abstract

archival set of human prostate tissues, immunohistological staining intensity for FABP9 was significantly higher in carcinomas than in benign cases and the increase in FABP9 was significantly correlated with reduced patient survival times. Moreover, the increased level of staining for FABP9 was significantly associated with the increased joint Gleason scores (GS) and androgen receptor index (AR). Suppression of FABP9 expression in highly malignant PC3-M cells inhibited their invasive potential, but did not affect their growth rate, anchorage-independent growth and migration rate. Our results suggest that FABP9 is a valuable prognostic marker to predict the outcomes of prostate cancer patients, perhaps by playing an important role in prostate cancer cell invasion.

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DECLARATION

No portion of the work referred to in the 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 May 2013 and December 2016. All the experiments presented in the result chapter were performed by me under the supervision of my supervisor, Professor Youqiang Ke and Dr Shiva S. Forootan, a senior research associate in Molecular Pathology department. The thesis was written by me with their guidance.

List of Publications

Majed Saad Al Fayi, Xiaojun Gou, Shiva S. Forootan, Waseem Al-Jameel, Zhengzheng Bao, Philip R. Rudland, Philip A. Cornford, Syed A. Hussain, Youqiang Ke. *The increased expression of fatty acid-binding protein 9 in prostate cancer and its prognostic significance*. Oncotarget, 2016. Published online on October 13, 2016. DOI:10.18632/oncotarget.12635.

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Table of Contents

ABSTRACT.....	3
ACKNOWLEDGMENTS.....	5
DECLARATION.....	6
LIST OF PUBLICATIONS.....	7
LIST OF CONTENTS.....	8
LIST OF FIGURES.....	16
LIST OF TABLES.....	19
ABBREVIATIONS.....	21
1. INTRODUCTION.....	25
1.1 CANCER INCIDENCE.....	25
1.2 EPIDEMIOLOGY OF PROSTATE CANCER	26
1.2.1 Incidence.....	26
1.2.2 Mortality	27
1.2.3 Survival.....	28
1.2.4 Factors affecting prostate cancer risk	29
1.3 THE PATHOLOGY OF PROSTATE CANCER	31
1.3.1 Prostate anatomy.....	31
1.3.2 Normal function of the prostate	32
1.3.3 Normal prostatic cells.....	33
1.3.4 Prostate cancer initiation.....	35
1.3.5 Prostate cancer cell lines.....	38
1.4 ANDROGENS AND ANDROGEN RECEPTORS IN PROSTATE CANCER	40
1.4.1 Androgens and androgen receptor in normal prostate	40

List of Contents

1.4.2	AR and prostate carcinogenesis	42
1.5	POTENTIAL PROGNOSTIC AND DIAGNOSTIC MARKERS FOR PROSTATE CANCER ...	43
1.5.1	Over-diagnosis and over-treatment of prostate cancer	43
1.5.2	Biomarkers in prostate cancer.....	45
1.6	FATTY ACIDS, FATTY ACID BINDING PROTEIN FAMILY AND PROSTATE CANCER ..	52
1.6.1	Fatty acids	52
1.6.2	Fatty acids and prostate cancer	53
1.6.3	Fatty acid binding protein family (FABPs)	53
1.6.4	General functions of FABPs.....	54
1.6.5	Fatty acid binding proteins (FABPs) affinity and structure.....	56
1.6.6	Fatty acid binding proteins in health and disease	60
1.6.7	Therapeutic target of FABPs.....	66
1.7	HYPOTHESIS	67
1.8	AIM AND SCOPE	67
2.	MATERIALS AND METHODS.....	70
2.1	CELL CULTURE	70
2.1.1	INITIATE CELL CULTURE FROM FROZEN STOCK	70
2.1.2	CELL LINES AND CULTURE CONDITION	70
2.1.3	TRANSFECTED CELLS CULTURE	71
2.1.4	CRYOPRESERVATION OF CELLS.....	71
2.2	EVALUATING MRNA TRANSCRIPTION IN CELL LINES USING QPCR..	72
2.2.1	RNA ISOLATION	72

List of Contents

2.2.2	CALCULATION OF DNA AND RNA CONCENTRATIONS	72
2.2.3	INTEGRITY AND QUALITY OF TOTAL RNAs	73
2.2.4	POLYMERASE CHAIN REACTION.....	74
2.2.4.1	PCR primer design	74
2.2.4.2	Blast	75
2.2.4.3	First strand cDNA synthesis	76
2.2.4.4	Relative real-time PCR	76
2.2.4.5	Relative quantitation analysis	77
2.3	EVALUATING PROTEIN EXPRESSION IN CELL LINES USING WESTERN BLOTTING.....	78
2.3.1	ISOLATION OF PROTEIN EXTRACT FROM CELL CULTURE.....	78
2.3.2	BRADFORD ASSAY	78
2.3.3	SODIUM DODECYL SULPHATE POLYACRYLAMIDE PROTEIN GEL ELECTROPHORESIS (SDS-PAGE)	79
2.3.4	TRANSFER PROTEINS FROM SDS GEL TO PVDF MEMBRANE	79
2.3.5	IMMUNODETECTION	80
2.4	EVALUATING PROTEIN EXPRESSION IN TISSUES USING IMMUNOHISTOCHEMISTRY.....	81
2.4.1	TISSUE SAMPLES AND PATIENT DATA.....	81
2.4.2	TISSUE SECTIONS	82
2.4.3	IMMUNOHISTOCHEMISTRY.....	82
2.4.4	CONTROLS FOR IMMUNOHISTOCHEMISTRY	84
2.4.5	SCORING IMMUNOREACTIVITY	84

List of Contents

2.5	MOLECULAR BIOLOGY	85
2.5.1	RNA INTERFERENCE:	85
2.5.2	DESIGNING siRNA SEQUENCES	85
2.5.3	TRANSIENT TRANSFECTION.....	86
2.5.4	DESIGNING SHORT HAIRPIN RNA (shRNA) SEQUENCES	87
2.5.5	MOLECULAR CLONING.....	89
2.5.5.1	Cloning silencing sequences into vector	89
2.5.5.2	Annealing sense and anti-sense oligos.....	90
2.5.5.3	Competent bacteria cells preparation	90
2.5.5.4	Double digestion of plasmid DNA.....	91
2.5.5.5	Ligation shRNA insert into psiRNA.....	92
2.5.5.6	Transformation	93
2.5.5.7	Transformation efficiency.....	94
2.5.5.8	Plasmid DNA isolation	94
2.5.6	STABLE TRANSFECTION.....	97
2.5.6.1	Ring cloning of transfected cells.....	97
2.6	IN-VITRO ASSAYS.....	99
2.6.1	MTT CELL PROLIFERATION ASSAY	99
2.6.1.1	Growth curve preparation	99
2.6.1.2	Cell number determination.....	100
2.6.2	INVASION ASSAY.....	100
2.6.3	SOFT AGAR ASSAY	102
2.6.4	WOUND HEALING ASSAY	103

List of Contents

2.7	STATISTICAL ANALYSIS.....	104
2.7.1	STATISTICAL PACKAGE FOR SOCIAL SCIENCES (SPSS).....	104
2.7.2	CHI-SQUARE (X ²)	104
2.7.3	SURVIVAL ANALYSIS	104
2.7.3.1	Kaplan-Meier curve	104
2.7.3.2	Log Rank test	105
2.7.3.3	Mann-Whitney U test.....	105
2.7.3.4	Student t-test	105
3.	RESULTS	107
3.1	MEASUREMENTS OF LEVELS OF FABP MRNAS IN PROSTATE CELL LINES	107
3.1.1	THE QUALITY AND INTEGRITY OF THE TOTAL RNAs	107
3.1.2	RELATIVE LEVELS OF MRNAS OF ALL FABPS IN PROSTATE CELLS	110
3.1.2.1	Expression Pattern of FABP1, FABP2, FABP3, FABP7 and FABP8	110
3.1.2.2	FABP4.....	110
3.1.2.3	FABP5.....	111
3.1.2.4	FABP6.....	111
3.1.2.5	FABP9.....	111
3.1.2.6	FABP12.....	112
3.2	EXPRESSION OF FABP6 AND FABP9 IN PROSTATE CELLS AT THE PROTEIN LEVEL	114
3.2.1	EXPRESSION OF FABP6 PROTEIN.....	114

List of Contents

3.2.2	EXPRESSION LEVEL OF FABP9 AT PROTEIN LEVEL	115
3.3	EXPRESSION OF FABP6 AND FABP9 IN PROSTATE TISSUES	118
3.3.1	EXPRESSION OF FABP6 IN PROSTATE TISSUES.....	119
3.3.2	EXPRESSION OF FABP9 IN PROSTATE TISSUES.....	119
3.4	CORRELATION OF FABP6 AND FABP9 EXPRESSION TO GLEASON SCORES.....	124
3.5	CORRELATIONS WITH PATIENT SURVIVAL	125
3.5.1	RELATIONSHIP BETWEEN FABP9 AND PATIENT SURVIVAL.....	125
3.5.2	RELATIONSHIP BETWEEN AR INDEX AND PATIENT SURVIVAL	126
3.5.3	RELATIONSHIP BETWEEN PSA AND PATIENT SURVIVAL	128
3.5.4	RELATIONSHIP BETWEEN GS AND PATIENT SURVIVAL.....	129
3.6	CORRELATION OF FABP9 EXPRESSION WITH LEVELS OF GS, AR INDEX AND PSA	130
3.6.1	CORRELATION OF FABP9 AND AR INDEX.....	130
3.6.2	CORRELATION OF FABP9 AND PSA LEVEL.....	131
3.7	SUPPRESSION OF FABP9 EXPRESSION BY SIRNA.....	132
3.7.1	SELECTION OF MOST EFFICIENT SIRNA FOR FABP9 SUPPRESSION	132
3.7.2	ESTABLISHMENT OF STABLY FABP9-SUPPRESSED PC3-M CELL LINES	134
3.7.3	STABLE TRANSFECTION	138
3.8	EFFECT OF FABP9-SUPPRESSION ON TUMOUR CELLS IN VITRO.....	141
3.8.1	EFFECT OF FABP9-SUPPRESSION ON CELLULAR PROLIFERATION	141

List of Contents

3.8.2	EFFECT OF FABP9 SUPPRESSION ON INVASIVENESS OF PROSTATE CANCER CELLS	144
3.8.3	EFFECT OF FABP9 SUPPRESSION ON ANCHORAGE-INDEPENDENT GROWTH OF PROSTATE CELLS	147
3.8.4	EFFECT OF FABP9 SUPPRESSION ON CELL MIGRATION	149
4	DISCUSSION	153
4.1	FABP6 AND FABP9 WERE DIFFERENTIALLY EXPRESSED AT MRNA LEVEL	154
4.2	FABP9 WAS OVEREXPRESSED IN PROSTATE CANCER CELLS AND TISSUES	156
4.3	POOR PATIENT SURVIVAL WAS CORRELATED WITH INCREASED EXPRESSION OF FABP9 AND GS	158
4.4	INCREASED EXPRESSION OF FABP9 WAS CORRELATED WITH GS AND AR	160
4.5	SUPPRESSION OF FABP9 REDUCED PROLIFERATION AND INVASIVENESS OF PROSTATE CANCER CELLS	162
5	CONCLUSION	163
6	FUTURE WORK	165
7	REFERENCES	168
8	APPENDIX A:	195
8.1	EQUIPMENT	195
8.2	REAGENTS SUPPLIER	200
8.2.1	Reagents for cell culture	200
8.2.2	Reagents for Western blot	201
8.2.3	Reagents for IHC	201

List of Contents

8.2.4	Reagents for RT-PCR	202
8.2.5	Reagents for general molecular biology	202
8.2.6	Reagents for cell proliferation assay.....	204
8.2.7	Reagents for cell invasion assay	204
8.2.8	Reagents for soft agar assay.....	204
8.3	BUFFERS.....	204
8.3.1	Cell Culture.....	204
8.3.2	Western Blot	205
8.3.3	Immunohistochemistry	207
8.3.4	Molecular Biology	208
9	APPENDIX B: PUBLICATIONS, AWARDS, CONFERENCES	212

LIST OF FIGURES

Chapter 1

FIGURE 1.1: AVERAGE NUMBER OF NEW CASES DIAGNOSED FOR ALL CANCERS IN THE UK IN 2013.	25
FIGURE 1.2: MALE CANCER INCIDENCE IN THE UK IN 2013.....	26
FIGURE 1.3: PROSTATE CANCER RELATIVE SURVIVAL RATES, ENGLAND AND WALES 1971-2011.	28
FIGURE 1.4: ANATOMY OF THE MALE REPRODUCTIVE AND URINARY SYSTEM.....	31
FIGURE 1.5: ILLUSTRATION OF PROSTATE GLAND ZONES; PZ = PERIPHERAL ZONE, TZ = TRANSITION ZONE, CZ = CENTRAL ZONE.....	32
FIGURE 1.6: THREE DIFFERENT CELL TYPES CAN BE DETECTED IN PROSTATE EPITHELIUM: LUMINAL CELLS, BASAL CELLS AND NEUROENDOCRINE (NE) CELLS	34
FIGURE 1.7: BENIGN PROSTATIC HYPERPLASIA (BPH).	35
FIGURE 1.8: GLEASON SCORE GRADING SYSTEM..	37
FIGURE 1.9: LIGAND-DEPENDENT ACTIVATION OF THE ANDROGEN RECEPTOR.	41
FIGURE 1.10: PUTATIVE FUNCTIONS OF FABPs IN THE CELL.	55
FIGURE 1.11: CRYSTAL STRUCTURE OF LIGAND-BOUND FABPs.	57
FIGURE 1.12: FINGERPRINT FOR FATTY ACID BINDING PROTEINS.....	58

Chapter 2

FIGURE 2. 1: psiRNA-h7SK-GFPZEO VECTOR MAP.....	89
FIGURE 2. 2: PRINCIPLE OF INVASION ASSAY PERFORMED IN CHAMBER WITH 8 μ M PORE SIZE WHICH COATED WITH MATRIGEL MATRIX.	102

Chapter 3

FIGURE 3. 1: THE QUALITY AND QUANTIFICATION OF TOTAL RNA WERE ASSESSED USING THE RNA 6000 NANO LABCHIP ON AGILENT 2100 BIOANALYZER.....	108
FIGURE 3. 2: THE QUALITY AND QUANTIFICATION OF TOTAL RNA WERE ASSESSED USING THE RNA 6000 NANO LABCHIP ON AGILENT 2100 BIOANALYZER.....	109
FIGURE 3. 3 : QUANTITATIVE PCR ANALYSIS OF RELATIVE LEVELS OF FABP MRNAs IN BENIGN AND MALIGNANT PROSTATE EPITHELIAL CELLS.....	113
FIGURE 3. 4: EXPRESSION OF FABP6 IN BENIGN AND MALIGNANT PROSTATE CELLS.	116
FIGURE 3. 5: EXPRESSION OF FABP9 IN BENIGN AND MALIGNANT PROSTATE CELLS.....	117
FIGURE 3. 6: SUMMARY OF PATIENT TISSUE NUMBERS USED IN THIS STUDY FOR BOTH FABP6 AND FABP9.....	118
FIGURE 3. 7: EXAMPLES OF IMMUNOHISTOCHEMICAL DETECTION OF FABP6 IN BENIGN AND MALIGNANT PROSTATE TISSUES.	120
FIGURE 3. 8: EXAMPLES OF IMMUNOHISTOLOGICAL DETECTION OF FABP9 IN BENIGN AND MALIGNANT PROSTATE TISSUES	122
FIGURE 3. 9: KAPLAN-MEIER SURVIVAL CURVES OF PATIENTS WITH PROSTATE CANCER....	126
FIGURE 3. 10: KAPLAN-MEIER SURVIVAL CURVES OF PATIENTS WITH PROSTATE CANCER	127
FIGURE 3. 11: KAPLAN-MEIER SURVIVAL CURVES OF PATIENTS WITH PROSTATE CANCER	128
FIGURE 3. 12: KAPLAN-MEIER SURVIVAL CURVES OF PATIENTS WITH PROSTATE CANCER	129
FIGURE 3. 14: BOX PLOT OF CORRELATION BETWEEN STAIN INTENSITY FOR FABP9 AND ANDROGEN RECEPTOR (AR) INDEX IN 3 GROUPS OF PATIENTS WITH PROSTATE CANCER: WEAK, MODERATE AND STRONG STAINING FOR FABP9.	130
FIGURE 3. 15: BOX PLOT OF CORRELATION BETWEEN STAIN INTENSITY FOR FABP9 AND PROSTATIC SPECIFIC ANTIGEN (PSA) LEVEL IN 3 GROUPS OF PATIENTS WITH PROSTATE CANCER: WEAK, MODERATE AND STRONG STAINING FOR FABP9.	131

List of Figures

FIGURE 3. 16: KNOCKDOWN OF FABP9 mRNA IN HIGHLY MALIGNANT PROSTATE CANCER CELLS. A), THE EFFECT OF MRNA	133
FIGURE 3. 17: DIGESTION OF psiRNA-h7SKGFPZEO PLASMID.	134
FIGURE 3. 18: CONFIRMATION OF THE CORRECT DNA INSERTION.	136
FIGURE 3. 19: KNOCKDOWN OF FABP9 mRNA IN HIGHLY MALIGNANT PROSTATE CANCER CELLS.....	139
FIGURE 3. 20: A) WESTERN BLOT ANALYSIS OF FABP5 IN TRANSFECTANTS EXPRESSING REDUCED LEVELS OF FABP9. B) THE RELATIVE LEVELS OF FABP5 IN PC3M-FABP9-siM AND PC3M-FABP9-siH WERE SIMILAR TO THAT IN THE PARENTAL PC3-M CELLS.	140
FIGURE 3. 21: STANDARD CURVES OF PARENTAL PC3-M AND DIFFERENT TRANSFECTANT CELL LINES.....	142
FIGURE 3. 22: THE IMPACT OF FABP9 SUPPRESSION ON THE PROLIFERATION RATE OF TRANSFECTANT CELLS.	143
FIGURE 3. 23: THE EFFECT OF FABP9 SUPPRESSION ON INVASIVENESS OF TRANSFECTANT CELLS.....	145
FIGURE 3. 24: THE IMPACT OF FABP9 SILENCING ON THE ANCHORAGE-INDEPENDENT GROWTH OF TRANFECTANT CELLS.....	148
FIGURE 3. 25: THE IMPACT OF FABP9 SILENCING ON MIGRATION RATE OF TRANFECTANT CELLS.....	151

Chapter 4

FIGURE 4. 1: PROJECT SUMMARY AND FINDINGS.	166
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LIST OF TABLES

Chapter 1

TABLE 1.1: PROSTATE CANCER MORTALITY RATE PER 100,000 POPULATION IN THE UK, 2014 (CRUK 2016B).....	27
TABLE 1.2: INCIDENCE AND MORTALITY RATE PER 100,000 BY RACE 2003–2007 (BRAWLEY 2012).....	30
TABLE 1.3: CHROMOSOMAL LOCALIZATION OF ALL HUMAN FABP GENES AND THEIR DISTRIBUTION.	59

Chapter 2

TABLE 2. 1: PCR FORWARD AND REVERSE PRIMER SEQUENCES FOR ALL <i>FABP</i> GENES. ...	75
TABLE 2. 2: REAL-TIME PCR PROGRAM FOR AMPLIFICATION OF SHORT TARGET DNAs ..	77
TABLE 2. 3: PRIMARY ANTIBODIES USED FOR IMMUNOHISTOCHEMISTRY STAINING.....	83
TABLE 2. 4: FABP9 TARGET AND siRNA SEQUENCES. HIGHLIGHTED REGIONS ARE TARGETED BY siRNAs AGAINST FABP9 GENE.	86
TABLE 2. 5: SEQUENCES OF shRNAs TARGETING FABP9 AND THE NEGATIVE CONTROL.	88
TABLE 2. 6: ANNEALING REACTION.	90
TABLE 2. 7: DOUBLE DIGESTION REACTION.	91
TABLE 2. 8: LIGATION REACTION.....	93

Chapter 3

TABLE 3. 1: IMMUNOHISTOLOGICAL STAINS OF TISSUES WITH ANTIBODY AGAINST FABP6.	121
TABLE 3. 2: EXPRESSION STATUS OF FABP9 IN BENIGN AND MALIGNANT PROSTATE TISSUES.	123
TABLE 3. 3: A PART OF PSI-RNA-h7SKGFPZEO PLASMID SEQUENCE MAP.....	137
TABLE 3. 4: CELL COUNTS OF PARENTAL AND TRANSFECTANT PC3-M CELLS AT THE 3 RD DAY OF PROLIFERATION ASSAY IN THREE SEPARATE EXPERIMENTS. USING STUDENT'S T-TEST, <i>P</i> VALUES WERE OBTAINED BY COMPARING DATA FROM TEST GROUPS TO PARENTAL GROUP.	142
TABLE 3. 5: NUMBER OF INVADDED CELLS (MEAN ± SD) PRODUCED IN THREE SEPARATE EXPERIMENTS. DIFFERENCES OF INVASIVENESS BETWEEN DIFFERENT GROUPS WERE ASSESSED BY STUDENT'S T-TEST. <i>P</i> VALUES WERE OBTAINED BY COMPARING DATA IN TEST GROUPS TO CONTROL GROUP.	146
TABLE 3. 6: COLONY COUNTS OF TRANSFECTED CELLS IN AT 4 TH WEEK OF TRIPPLICATE SOFT AGAR ASSAY. DIFFERENCES OF NUMBER OF COLONIES BETWEEN DIFFERENT GROUPS WERE ASSESSED BY STUDENT'S T-TEST. <i>P</i> VALUES WERE OBTAINED BY COMPARING SAMPLE GROUPS TO CONTROL GROUP DATA.	147
TABLE 3. 7: MIGRATION RATES OF TRANSFECTED CELLS AT 24 HOURS IN WOUND HEALING ASSAY.	149

List of abbreviations

Abbreviations	Full name
AR	Androgen receptor
ACTH	Adrenocorticotropic hormone
AREs	Androgen response elements
APS	Ammonium persulphate
BPH	Benign prostatic hyperplasia
BRCA1/BRCA2	Breast cancer mutated gene
CZ	Central zone.
Chr	Chromosome
cDNA	Complementary DNA
χ^2	Chi-square
CK	Cytokeratin
CRH	Corticotrophin releasing hormone
DHT	Dihydrotestosterone
DRE	Digital rectal examination
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulphoxide
ERSPC	European Randomized study of Screening for Prostate Cancer
EPCA2	Early prostate cancer antigen 2

List of Abbreviations

EPA	Eicosapentaenoic acid
EDTA	Ethylene diamine tetra acetic acid
FABP	Fatty acid binding protein
GS	Gleason score
GFR	Growth Factor Reduced
GnRH	Gonadotropin releasing hormone
HSCs	Hepatic stellate cells
HCC	Hepatocellular carcinoma
<i>hK3</i>	Human kallikrein 3
IL-6	Interleukin-6
LH	Luteinizing hormone
LUTS	Lower urinary tract symptoms
NMR	Nuclear magnetic resonance
NSCLC	Non-small cell lung cancer
OD	Optical density
PSA	Prostate-specific-antigen
PZ	Peripheral zone
PAP	Prostatic acid phosphatase
PIN	Prostatic Intraepithelial Neoplasia
PCA3	Prostate cancer antigen 3
PSCA	Prostate stem cell antigen

List of Abbreviations

PPAR	Peroxisome proliferator-activated receptor
PVDF	PolyVinylidene DiFluoride
RNAi	RNA interference
RIN	RNA integrity number
SV40	Simian virus 40 genome
shRNA	Short hairpin RNA
siRNA	Small or short interfering RNA
SPSS	Statistical Package for Social Sciences
TURP	Transurethral resection of the prostate
TZ	Transition zone
tPSA	Total PSA
TGF- β 1	Transforming growth factor- β 1

CHAPTER ONE:

Introduction

1. Introduction

1.1 Cancer incidence

Cancer is the most serious health threat to human life (McGuire 2016). In 2012, it was estimated that 14.1 million new cancer cases were diagnosed in the world: 7.4 million in males (53%) and 6.7 million in females (47%) (Torre et al. 2015). In 2013, there were about 350,000 new cancer cases were diagnosed in the UK, including 179,000 in males and 173,000 in females (CRUK 2013) (**Figure 1. 1**). Cancers of Lung, colon, breast and prostate account for 53% of all new diagnosed cases in the UK (CRUK 2013). About 50% of all cases diagnosed during the period of 2011 to 2013 in the UK were in people aged 70 and over. The cancer incidence is more common in black and white males than in Asian males, whereas more common in white females than in black and Asian females. Prostate cancer is the most common male cancer in the UK, accounting for 26% of all new cancer cases in males (De Angelis et al. 2014; Torre et al. 2015).

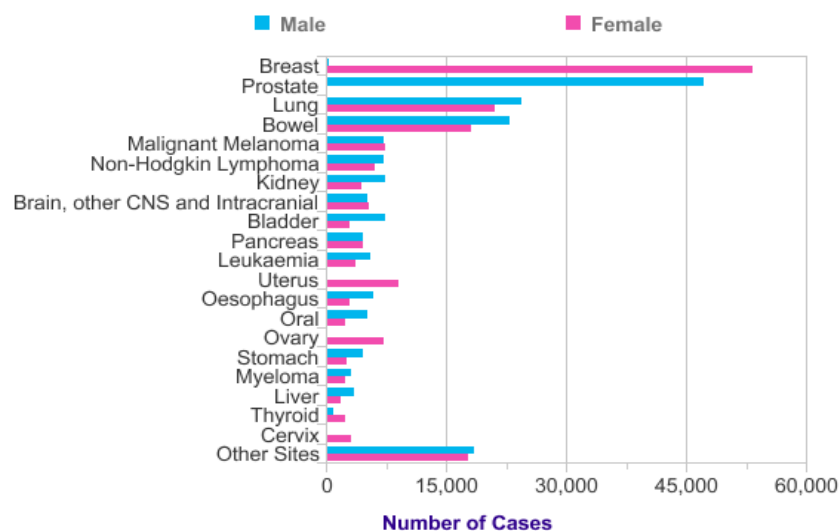


Figure 1.1 : Average number of new cases diagnosed for all cancers in the UK in 2013 (CRUK 2013).

1.2 Epidemiology of prostate cancer

1.2.1 Incidence

Prostate cancer is the second most common cancer and the sixth leading cause of cancer death in the developed countries (CRUK 2013). In 2013, there were around 1.4 million cases of prostate cancer diagnosed and 293,000 deaths worldwide (Global Burden of Disease Cancer 2015). Prostate cancer is the most common male cancer in the UK, accounting for 26% of all new cases of cancer in males (CRUK 2016b) and 13% of all new cases in the UK (Ferlay J 2013) (**Figure 1.2**). In 2013, there were 47,300 new prostate cancer cases diagnosed in the UK. The incidence of prostate cancer increased through the entire 1980s and peaked in 1991 in United states (Brawley 2012). Since 1992, the serum prostate-specific-antigen (PSA) screen was used on a wide population and this marker helped early diagnosis of prostate cancer and thus contributed to improvements in subsequent 5-year survival rates (Adamson et al. 2003; Barry 2001; Moore et al. 2009).

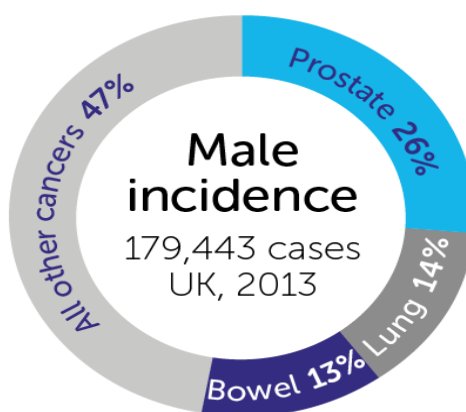


Figure 1. 2: Male cancer incidence in the UK in 2013 (CRUK 2013).

Introduction

1.2.2 Mortality

In 2014, the number of patients died from prostate cancer was recorded as 11,300 in the UK, accounting for 7% of all cancer deaths as shown in **Table 1.1**. Prostate cancer is the 4th most common cause of cancer death in the UK, accounting for 13% of all cancer deaths in males. Several separate studies have shown that mortality among patients with prostate cancer varies according to such factors as where they live (Papa et al. 2014; Yu et al. 2014), clinical management (Eggerer et al. 2011; Shikanov et al. 2012) and marital status (Abdollah et al. 2011; Tyson et al. 2013).

Table 1.1: Prostate cancer mortality rate per 100,000 population in the UK, 2014 (CRUK 2016b).

	England	Wales	Scotland	Northern Ireland	UK
Deaths	9,529	613	906	239	11,287
Crude Rate	35.6	40.3	34.9	26.5	35.5
Age-standardised (AS) Rate	48.2	50.2	46.7	42.4	48.1
AS Rate – 95% lower confidence limit (LCL)	47.2	46.2	43.7	37.0	47.2
AS Rate – 95% upper confidence limit (UCL)	49.1	54.2	49.8	47.8	49.0

Introduction

1.2.3 Survival

In England and Wales during 2010-2011, 94% of prostate cancer patients survived for 1-year, then the survival rate fell to 85% for 5-years. Early detection via tests such as transurethral resection of the prostate (TURP) and PSA played a crucial role in this improved survival. One-year survival for prostate cancer has increased from 66% during 1971-1972 to 94% during 2010-2011 in England and Wales (**Figure 1.3**) (CRUK 2016b). Five-year survival for patients aged 15-49 was 91%, and this was increased to 94% in patients aged 60-69-year-old; Five-year survival rate reached to its lowest point at 66% for patients aged 80-99 in England during 2009-2013. Moreover, stage of prostate cancer at diagnosis is strongly correlated with patient survival. For instance, 2012 data in England show that 85% of patients diagnosed at stage IV survived for at least one year, versus 100% of patients diagnosed at stage I (ONS 2016b).

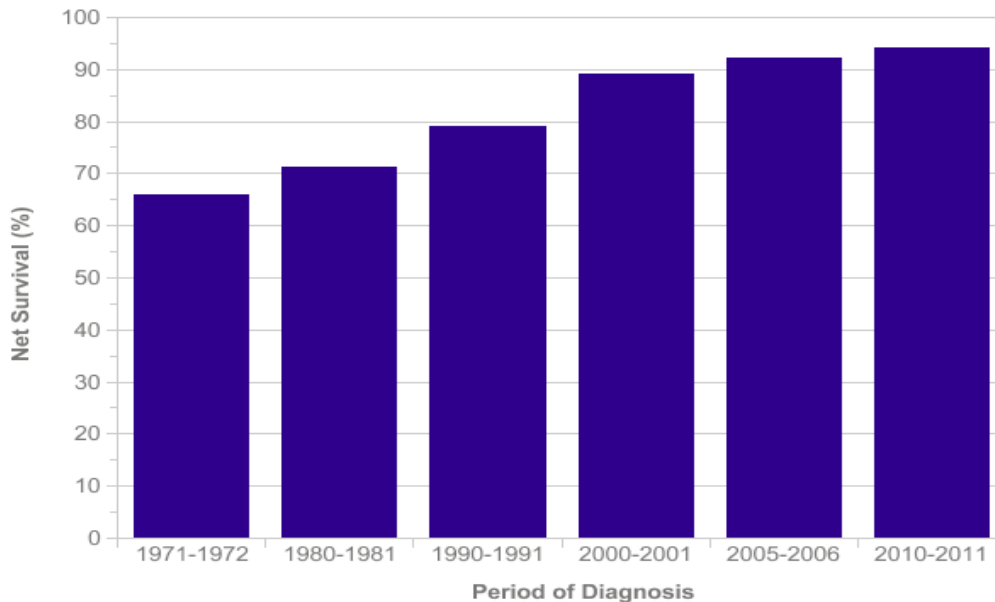


Figure 1. 3: Prostate cancer relative survival rates, England and Wales 1971-2011 (CRUK 2016b).

Introduction

1.2.4 Factors affecting prostate cancer risk

1.2.4.1 Age

In the UK, around 54% of prostate cancer cases diagnosed are in people aged 70 and over; and only 1% are diagnosed in men under 50s. Also in the US, about 65% of all prostate cancer cases are diagnosed in patients aged 65 and over. Therefore, the risk increases with the increased age. When the age is over 65, the chance of developing prostate cancer is more common than any other cancer type in men (ONS 2016a).

1.2.4.2 Family history

There is an increased risk of prostate cancer in men with a history of prostate cancer in their family (Bruner et al. 2003; Johns & Houlston 2003). The risk of developing prostate cancer is 2.1-2.4 times higher in men whose father has or had the disease. The risk increases to 2.9-3.3 times in men whose brother has or had the disease (ACS 2016). Around 5-10% of prostate cancer cases are correlated with family history and hereditary factors. Also, it has been reported that the risk increase in men whose mothers has or had breast cancer by 19-24% (Chen et al. 2008; Hemminki & Chen 2005).

1.2.4.3 Race

The incidence and mortality rate of prostate cancer have been reported amongst different races worldwide. On the basis of the latest England data, it is clear that black men have higher risk of developing prostate cancer than their white counterparts (CDC 2016; Jones & Chinegwundoh 2014). African American patients develop more aggressive disease and have the highest rate of 275.3 per 100,000 which is 60% higher than the whites with a rate of 172.9 per 100,000 (Kumar 2005). Asians have the lowest incidence rate of prostate cancer; however, in recent years, some Asian countries have reported a

Introduction

rapidly growing trend due to changes in lifestyle (Baade et al. 2013; Chen et al. 2014) (Table 1.2).

Table 1.2: Incidence and mortality rate per 100,000 by race 2003–2007 (Brawley 2012).

Race/ethnicity	Incidence	Mortality
All races	156.0	24.7
White	149.5	22.8
Black	233.8	54.2
Asian/Pacific Islander	88.3	10.6
American Indian/Alaskan Native	75.3	20.0
Hispanic	107.4	18.8

1.2.4.4 Other cancers

Previous studies have shown that urological carcinomas increase the risk of prostate cancer. For instance, renal (Liu, Hemminki & Sundquist 2011; Neuzillet, Lechevallier & Coulangue 2007) and bladder (Kellen et al. 2007; Lehnert et al. 2012) carcinomas have been shown to increase prostate cancer risk by 69% and 15%, respectively. Also, it has been reported that previous lung cancer and melanoma increased the risk of prostate cancer by 56% and 50%, respectively (Bradford et al. 2010; Hayat et al. 2007).

Introduction

1.3 The pathology of prostate cancer

1.3.1 Prostate anatomy

Prostate gland is a part of male's reproductive and urinary system. Prostate is an oval shaped gland located at the base (or neck) of the bladder, surrounding the urethra as shown in **Figure 1.4**. It is surrounded by a capsule of connective tissue called prostatic capsule. The prostate gland can be divided either by lobe or by zone. What is important clinically is the division of the prostate into 3 zones: the central zone surrounds the ejaculatory ducts and makes up to 25% of the total gland volume; the transitional zone surrounds the urethra accounting for 5% of the total prostate mass and is the primary site of benign prostatic hyperplasia (BPH), the peripheral zone is the body of the gland makes up 70% of prostate volume and is the most common location for adenocarcinomas (**Figure 1.5**) (Ashley Evan Ross 2016).

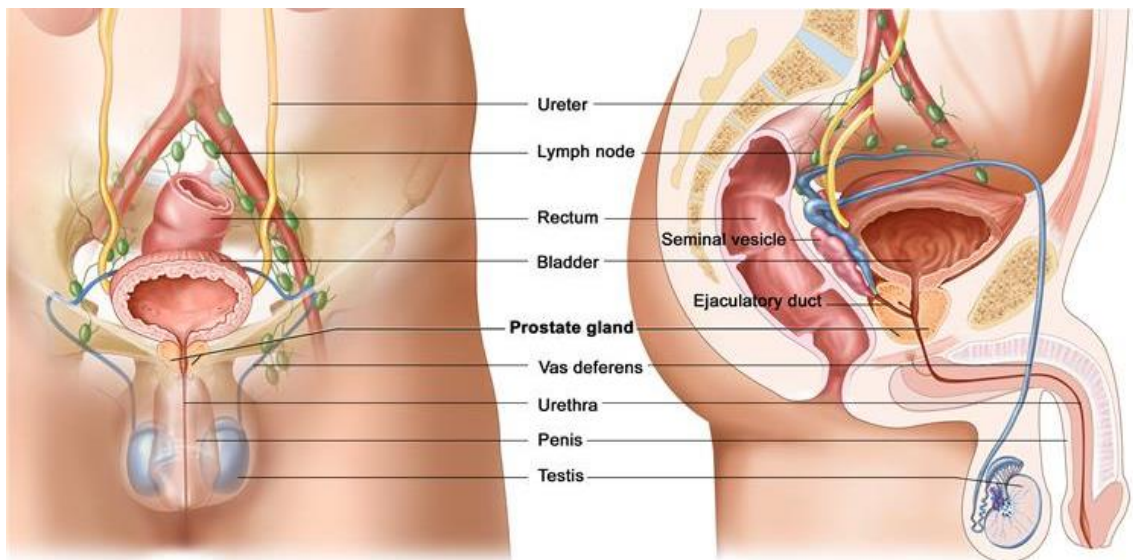


Figure 1.4: Anatomy of the male reproductive and urinary system (Bethesda 2016).

Introduction

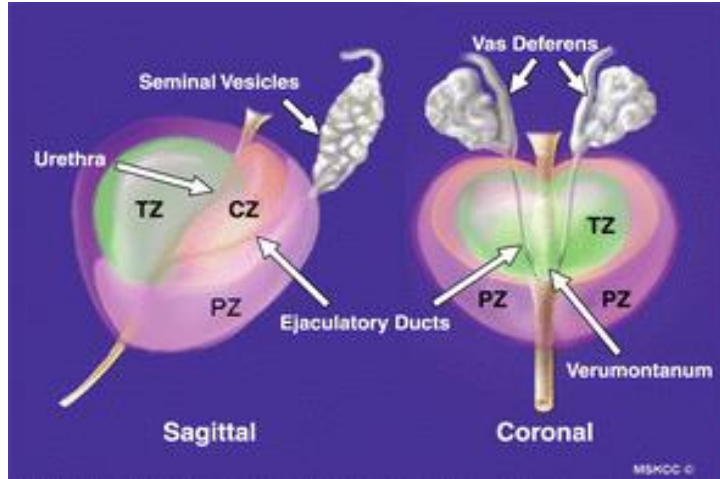


Figure 1. 5: Illustration of Prostate gland zones; PZ = peripheral zone, TZ = transition zone, CZ = central zone. (Curran et al. 2007)

1.3.2 Normal function of the prostate

Sex accessory tissues including prostate, seminal vesicles, bulbourethral glands and ampulla glands are believed to play a major role in reproductive processes. The main important function of prostate gland is the production of a fluid that, together with sperm cells testicles, fluid from the seminal vesicle and fluids from other glands such as bulbourethral gland makes up the semen. Then, prostate muscles press the semen into the urethra for ejaculation (Plan & Michael 2015). One third of the total volume of semen contains several enzymes (such as PSA), citric acid and zinc. The fluid produced by prostate gland is acidic; but another component of semen from seminal vesicles makes semen alkaline to protect sperms and prolong their life time. Prostatic secretion is also important for proper functioning of fertility in men (Alan et al. 2015; Sadava & Freeman 2008).

Introduction

1.3.3 Normal prostatic cells

There are three main prostatic epithelium cell types: luminal cell, basal cell and neuroendocrine cells. Basal cell population acts as a stem cell reservoir to generate these three types of other cells (**Figure 1.6**) (Humphrey 2012).

Luminal cells are the most predominant cells and constitute the exocrine compartment of prostate, secreting prostatic proteins such as PSA and prostatic acid phosphatase (PAP). These cells are androgen dependent cells and require androgen for their survival and viability, expressing high levels of androgen receptor (AR) (Foster et al. 2000). They also express cytokeratin (CK) 8 and CK18. As 95% of prostate adenocarcinomas were luminal phenotype, the increase expression of specific markers including PSA, PAP, CK8 and CK18 is expected in the majority of cancer cells (Denmeade, Lin & Isaacs 1996).

Basal cells are the second large population epithelial cell type in prostate located between basement membrane and luminal cells. Unlike luminal cells, these cells lack secretory activity and are unable to express AR and do not undergo apoptosis in response to androgen ablation (Bonkhoff & Remberger 1993; Okada et al. 1992). However, basal cells are able to either express high molecular weight protein markers such as CK14, CK5 and CD44 or factors that can protect against DNA damage such as free-radical scavenger GST- π (Harper et al. 1998).

Neuroendocrine cells are the third cell type in prostate and located in all the epithelium of acini and ducts of prostate gland. These cells are androgen independent and can be distinguished by producing thyroid stimulating hormone, serotonin and chromogranin A (Abate-Shen & Shen 2000). Also, neuroendocrine cells express neuroendocrine factors

Introduction

and neuropeptides that support proliferation of luminal cells (Harper et al. 1998). Several studies have suggested a positive correlation between the increase level of neuroendocrine factors and the advanced stage of prostate adenocarcinomas (Ruscica et al. 2007; Sagnak et al. 2011).

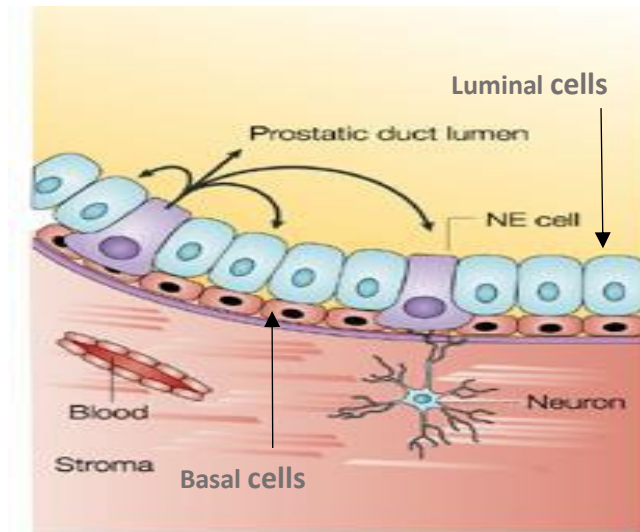


Figure 1. 6: Three different cell types can be detected in prostate epithelium: Luminal cells, basal cells and neuroendocrine (NE) cells (Bok & Small 2002).

Introduction

1.3.4 Prostate cancer initiation

1.3.4.1 Benign prostatic hyperplasia (BPH)

Benign prostatic hyperplasia (BPH) is also known as benign prostate enlargement which is a non-malignant overgrowth of prostate cells and histologically diagnosed by proliferation of the cellular elements of the prostate (**Figure 1.7**). BPH is not a precursor for prostate cancer and is not caused by cancer (Bok & Small 2002). However, both BPH and prostate cancer have some similarities such as both require androgen stimulation for their growth and both are age related diseases (Sausville & Naslund 2010). BPH occurs in transition zone, a ring of tissue around the urethra and its growth is inward toward the prostate's core, constantly tightening around the urethra and associated with lower urinary tract symptoms (LUTS). BPH is hormonally dependent on dihydrotestosterone (DHT) and testosterone production occurring in aged men (Wise & Md 2001) (50% of men diagnosed with BPH are by age 60 years and it increases to 90% by age 85 years (Deters 2016).

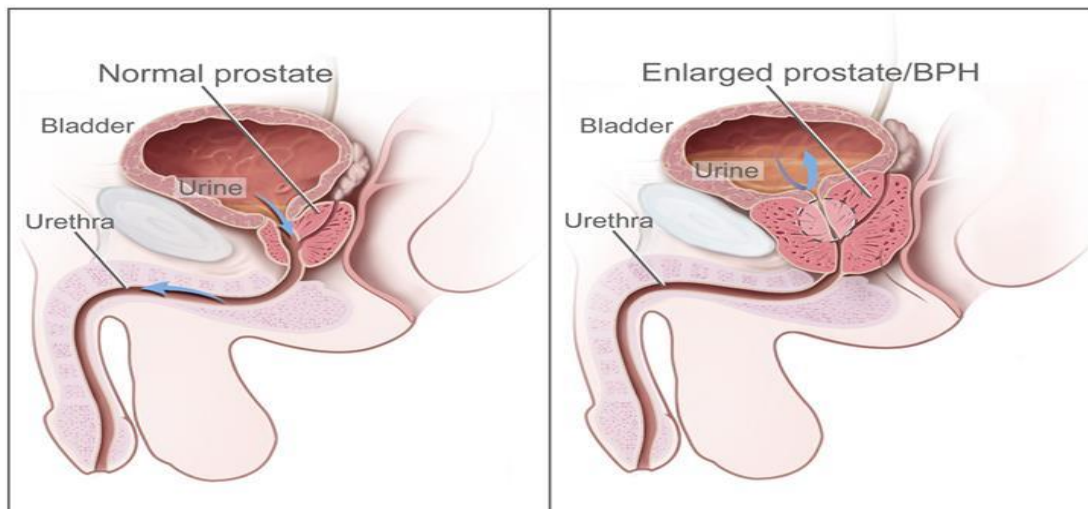


Figure 1. 7: Benign prostatic hyperplasia (BPH) (Bethesda 2016).

Introduction

1.3.4.2 Prostatic Intraepithelial Neoplasia (PIN)

Prostatic Intraepithelial Neoplasia (PIN) is a precancerous condition and sub stage of cellular transformation from a normal condition to malignant prostatic epithelium. PIN is identified by abnormal proliferation of cancer cells without invasion to surrounding stroma resulting in minimal changes that makes a variety of architectural and cytological aspect undistinguishable from carcinoma (School 2011).

According to their cellular features and nuclear enlargement, PIN can be divided into two grades: low grade PIN and high grade PIN. Low grade PIN lesion is a well differentiated early invasive tumour with an intact or rare interrupted basal layer (Roehrborn 2008). High grade PIN lesion is poorly differentiated with secretory luminal cell population and frequent interruption of basal layer (Bostwick 1989). This lesion was divided into four different groups: Tufting which is the most common type, Micropapillary, Cribriform and flat (Foster & Ke 1997). Several studies have shown correlation between PIN and prostate cancer and suggested that high grade PIN is an important marker for detection of prostate cancer (Ayala & Ro 2007).

1.3.4.3 Gleason grading system of prostate cancer

Gleason score (GS) is a histological grading system used to describe the morphological changes in prostate cancer. GS proposed by Dr. David Gleason in 1960s and become the most common grading system to evaluate the prognosis of prostate cancer (Gleason & Mellinger 1974).

In the Gleason grading, prostate cancer was classified according to their morphological appearance ranging from least aggressive (grade1) to the most aggressive cancer (grade 5), representing the degree of loss of normal glandular tissue as shown in **Figure 1.8**.

Introduction

Combined Gleason scores are a sum of primary score that represents the majority of tumour (>50%) and secondary score which represents the minority of tumour (<50%). Thus, the combined GS is a number ranging from 2-10, the highest GS is 10 and the lowest is 2. According to the differentiation, Gleason grading system has classified the scores into three groups: well differentiated (Gleason scores <6), moderately differentiated (Gleason scores 6-7) and poorly differentiated (Gleason scores 8-10). Combined GS appears to be the most commonly prognostic indicator in prostate carcinomas (Roehrborn 2008).

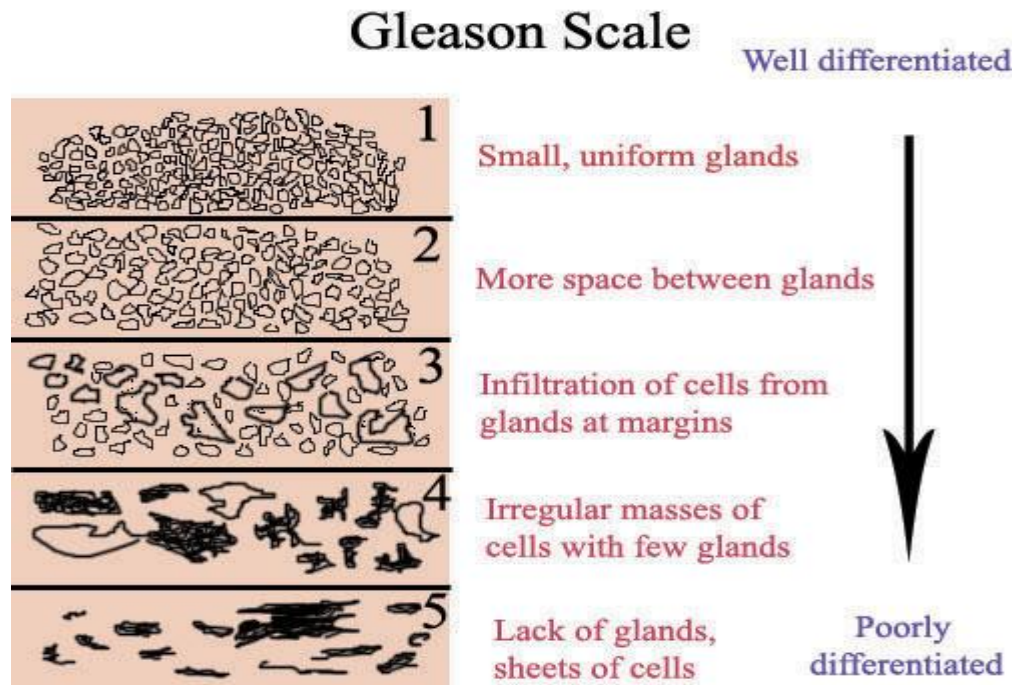


Figure 1. 8: Gleason score grading system. Microscopic determination of the loss of normal prostate glandular, ranging from least aggressive (grade 1) to the most aggressive cancer (grade 5), representing the degree of loss of normal glandular tissue (Gleason & Mellinger 1974).

Introduction

1.3.5 Prostate cancer cell lines

There are six different prostate cell lines widely used in laboratory studies including the work described in this study. These cell lines, which represent different spectrums of prostatic conditions, varied from benign PNT2, through low malignant LNCaP, moderately malignant 22RV1, to highly malignant cell lines Du145, PC-3 and PC3-M.

1.3.5.1 PNT-2

PNT-2 cell line is a normal human prostate cell line derived from prostate epithelial cells of a 33-year-old man and established by immortalization through transfection with plasmid containing simian virus 40 genome (SV40) (Cussenot et al. 1991; Sobel & Sadar 2005). Successful immortalisation was achieved only with SV40 expressing both large T and small t oncogenes, while attempts to immortalise with a vector expressing SV40 large T alone have given a few strains showing no extended lifespan and no cells which overcame the crisis (Cussenot et al. 1991). This cell line has a well differentiated morphology, expressing PSA, PAP, CK8, 18 and 19. PNT-2 has no tumorigenic effect when inoculated in nude mice (Berthon et al. 1995).

1.3.5.2 LNCaP

LNCaP cell line is derived from a lymph node metastasis of prostate cancer from a 50-year-old man in 1977 (Sobel & Sadar 2005). This cell line is androgen sensitive expressing high level of AR (in order increase growth rate), PSA and PAP. Previous studies in mice showed that LNCaP did form tumours and no metastatic spread was observed in any of the mice given injections (Tuxhorn et al. 2002; Zheng et al. 2010).

Introduction

1.3.5.3 22RV-1

22RV-1 is a moderately malignant, androgen responsive prostate epithelial cell line derived from a human prostatic carcinoma xenograft. These cells are being proliferated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft. This cell line express high level of PSA. The cell population doubling time is approximately 49-56 hours (Sramkoski et al. 1999).

1.3.5.4 Du145

Du145 is derived from moderately-differentiated prostate cancer that metastasized to central nervous system of a 69-year-old man (Sobel & Sadar 2005). This cell line express filament protein, PAP, CK5, 8 and 18; however, PSA, AR and glandular kallikrein are not present (Carpten et al. 2002; Nabha et al. 2005). Several animal studies have shown that tumours were formed when Du145 cells were injected subcutaneously in nude mice. The cell population doubling time is approximately 33 hours (Van Bokhoven et al. 2003).

1.3.5.5 PC-3 and PC3-M

PC-3 cells are poorly-differentiated bone marrow metastasis of prostate cancer (Kaighn et al. 1979; Sobel & Sadar 2005). PC3-M cell line was isolated from the most aggressive featured sub-population from liver metastasis produced in nude mice when injected with parental cell line PC-3 (Shevrin, Gorny & Kukreja 1989). Therefore, there are some similarities between PC-3 and PC3-M, but PC3-M is more aggressive than other prostate malignant cell lines. Animal studies show that tumour growth in female nude mice due to the possibility that PC-3 and PC3-M are androgen-independent. Evidence showed that

Introduction

these cell lines can exhibit high rate of tumorigenicity and metastasis in nude mice (Kozlowski et al. 1984).

1.4 Androgens and androgen receptors in prostate cancer

1.4.1 Androgens and androgen receptor in normal prostate

Androgen receptor (AR) is a 110 kDa ligand-activated nuclear transcription factor that belongs to a large family of so-called nuclear receptor proteins and structurally similar to the estrogen receptor (Shah et al. 2004; Stanbrough et al. 2001). The current model of action of the androgens and the AR is demonstrated in **Figure 1.9** (Tan et al. 2015). AR is expressed by secretory cells in prostate and its function relies on the action of androgens. The hypothalamus releases gonadotropin releasing hormone (GnRH) leading to secretion of luteinizing hormone (LH) from pituitary gland. After that, LH stimulates Leydig cells in testes to secrete testosterone which is the main androgen. Also, the release of corticotrophin releasing hormone (CRH) from the hypothalamus stimulates the pituitary gland to produce adrenocorticotrophic hormone (ACTH) which can secrete and stimulate the production of other androgens. Dihydrotestosterone (DHT) is a mediator of prostate growth. DHT is synthesized by the enzyme 5α -reductase from testosterone in the prostate, testes, adrenal gland and hair follicles. DHT has a stronger binding affinity for the AR than testosterone. Then, the complex translocate to the nucleus and regulate the expression of target genes through binding to androgen response elements (AREs) (Heinlein & Chang 2002, 2004; Roy et al. 1999).

Introduction

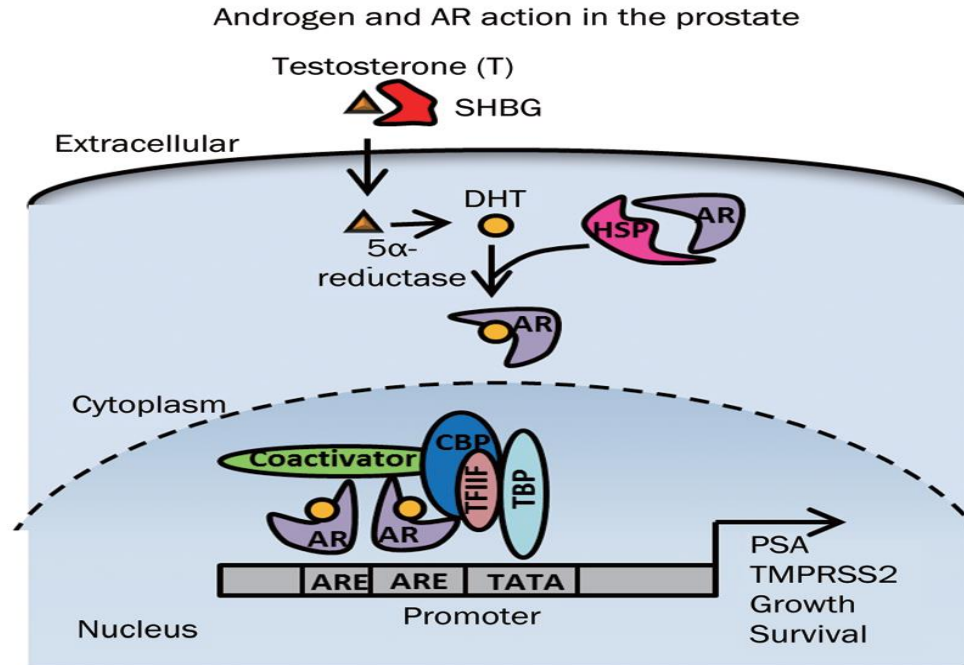


Figure 1. 9: Ligand-dependent activation of the androgen receptor. Testosterone is transported to target tissues such as the prostate and becomes converted to dihydrotestosterone (DHT) by 5- α -reductase. DHT binds to the ligand-binding pocket and promotes the dissociation of heat-shock proteins (HSPs) from the AR. The AR then translocates into the nucleus, dimerizes and binds to the androgen response element (ARE) in the promoter region of target genes such as prostate-specific antigen (PSA) and TMPRSS2. At the promoter, the AR is able to recruit members of the basal transcription machinery [such as TATA-box-binding protein (TBP) and transcription factor IIF (TFIIF)] in addition to other coregulators such as members of the p160 family of coactivators and cAMP-response element-binding protein (CREB)-binding protein (CBP). SHBG: serum sex hormonebinding globulin (Tan et al. 2015).

Introduction

1.4.2 AR and prostate carcinogenesis

Growth of prostate cancer cells requires androgen, similar to normal prostate cells. Androgens and AR are main mediators for the ratio of cell proliferation to cell death (Denmeade, Lin & Isaacs 1996; Feldman & Feldman 2001). It has been shown that androgen depletion had led to regression of prostate cancer (Feldman & Feldman 2001). The increased expression of AR is associated with diagnosed, but untreated prostate cancer. Similarly, reduced patient survival has been found to correlate with overexpression of AR (Ruizeveld de Winter et al. 1994; Schatzl et al. 2002). However, Lee and Chang reported that higher AR was correlated with low GS. The expression of ARs throughout the progression of prostate cancer remain controversial which might be because of variable responses to endocrine therapy (Karantanos, Corn & Thompson 2013; Lee & Chang 2003; Marques et al. 2010). Generally, AR expression may not correlate with prostate cancer development.

Nearly 50 years ago, Charles Huggins found that castration induced the regression of prostate cancer (Huggins 1967). Until now androgen depletion has been used as therapeutic intervention to treat prostate cancer either by surgical removal of the testes or by interfering with the release of the pituitary LH that stimulate the secretion of androgens in Leydig cells. This therapy is very effective in androgen-dependent cancer, but some cells proliferate and grow relatively or completely independent of androgens and go on to metastasize and eventually become androgen-independent cancer (Debes & Tindall 2004). This might be because of incomplete blockage of AR, AR mutations, AR amplifications or AR splice variant expression (Scher & Sawyers 2005). AR deprivation might be useful for some patients, but has not been yet shown to prolong survival (Fang,

Introduction

Merrick & Wallner 2010; Mayor 2015). Recently, a study showed the association between androgen ablation in the treatment of prostate cancer and the increased risk of Alzheimer's disease in a general population cohort (Nead et al. 2016).

1.5 Potential prognostic and diagnostic markers for prostate cancer

Most prostate cancer diagnosis and treatment decisions are first based on screening with serum PSA level or digital rectal examination (DRE) and subsequent biopsies for histopathological grading (Bretton 1994; NHS 2015b). However, each procedure has its limitations leading to an over-biopsy for diagnosis, an over-treatment of low-risk patients and non-essential surgery such as radical prostatectomies (Caram, Skolarus & Cooney 2016; Moyer 2012; Schroder 2011; Schroder et al. 2009). Available biomarkers can be used for making speedy and correct treatment strategy for individual patients to detect advance disease at an earlier stage and to predict the outcomes of patients. Blood, urine, semen and tissues can be used to detect biomarkers for prostate cancer. Therefore, identification of novel biomarkers should be used to predict the severity of malignancy and its progression, to distinguish between the benign and the malignant nature of each case, to monitor response to treatment and to predict the probability of recurrence. It should be non-invasive, consistent, economical and easily accessible (Velonas et al. 2013).

1.5.1 Over-diagnosis and over-treatment of prostate cancer

Although the widespread screening and diagnostic practices did lead to a degree of decline in mortality rates in some countries, many prostate cancer cases were not picked up in other countries and it has now become the most common male cancer in the developed world (Carter et al. 2013). In oncology, over-diagnosis is a widespread

Introduction

problem in prostate (Klotz 2013), lung (Esserman et al. 2014) and breast cancer (Welch & Black 2010). Over-diagnosis can lead to over-treatment and cause significant anxiety and unnecessary decrements in patient quality of life, particular with regarding to sexual dysfunction and urinary incontinence (Capitanio et al. 2008; Draisma et al. 2009). In the European Randomized study of Screening for Prostate Cancer (ERSPC), it was revealed that 20% mortality was reduced among the 240,000 men in the European screening program. However, for one life saved, 48 men were over treated (Schröder et al. 2009). To address this problem, there are 4 strategies to support decision-making about screening and treatment; to identify the highest risk patient, to minimize treatment for minimal-risk patients, to targeting preventive interventions and development and to validate new biomarkers that can help to identify progression of cancer and distinguish between benign and malignant lesion to minimal-risk cancers (Klotz 2013). Also, patient education is considered as one of the main critical solutions for this problem.

Recently, active surveillance has been used which is to manage patients with \leq GS6 or \leq GS7, serial PSA (<10 ng/ml) and periodic biopsy (Soloway et al. 2010). Although active surveillance become widely used (Bethesda 2011), this approach still depends on PSA assessment and re-biopsies. This strategy may lead to unnecessary biopsies (a very invasive procedure) that carry other side risks such as decrement in quality of life and subsequent infection (Nam et al. ; Nam et al. 2010). Therefore, the ideal biomarkers hold the promise to provide a new way of disease stratification for prostate cancer and hence the biopsy procedure and nonessential treatments can be avoided.

Introduction

1.5.2 Biomarkers in prostate cancer

1.5.2.1 Prostatic acid phosphatase (PAP)

Prostatic acid phosphatase (PAP) is a 100 kDa glycoprotein found in large amount in seminal fluid. It had been suggested that PAP was significantly higher in adult prostate cancer patients. In 1941, the correlation between prostate cancer and increased level of PAP was documented (Huggins & Hodges 1941; Huggins & Hodges 2002). Prior to the introduction of PSA, PAP was used as a biomarker to diagnose prostate cancer patients in the 1940s and 1950s (Heller 1987). Since PAP levels elevated in non-prostatic disease or benign conditions of prostate, PAP as prognostic marker was overlooked (Taira et al. 2007). Therefore, PAP was replaced by PSA due to its poor sensitivity for screening and diagnosing prostate cancer (Kontturi 1991).

1.5.2.2 Prostatic specific antigen (PSA)

PSA or human kallikrein 3 (*hK3*) is a 33 kDa serine protease of the tissue kallikrein family produced by the epithelial cells of prostate gland into the secretory duct to contribute to liquefy seminal fluid and increase the sperm mobility. In prostate cancer, the basal cell layer disrupted allowing PSA to leak into blood leading to elevated levels of PSA. The incidence and mortality rate of prostate cancer increased in the USA throughout the entire 1980s and peaked in 1991 (Brawley 2012). In 1980s, Roswell Park Memorial Institute showed that elevated levels of PSA were correlated with BPH and prostate cancer (Papsidero et al. 1980). After that, PAP was dropped as a screening tool. Since 1992, the serum PSA screen was used on a wide population. This marker helped in early diagnosis of prostate cancer and thus contributed to improvements in subsequent 5-year survival rates (Adamson et al. 2003; Barry 2001; Moore et al. 2009). Currently PSA

Introduction

is the most common marker used for both diagnosis and treatment management (Sharifi, Gulley & Dahut 2005). Although the widespread screening for PSA did lead to a degree of decline in mortality rates in some countries by at least 21% (Schröder et al. 2014), many prostate cancer cases were not picked up by PSA screening (Ilic et al. 2013; PHE 2016a). Using a PSA cutoff level of 4 ng/ml as an indicator for prostate biopsy, the specificity and sensitivity of PSA were estimated about 60-70% and 40-50%, respectively. However, several separate studies even suggested that PSA screening had only very limited or even no survival benefits, when a PSA cutoff level of 4 ng/ml was used to recommend a prostate biopsy (or not) (Andriole et al. 2009; Kim & Andriole 2015; Moore et al. 2009). Some other research studies show that using a cut-off point ≥ 10 ng/ml increases its specificity and sensitivity to 95% and 72%, respectively. The results highlight that there is no distinct PSA cut-off to distinguish between the presence and absence of prostate cancer, but the best results were obtained and used for PSA cut off of 10 ng/ml (Kim & Andriole 2015; Vukotic et al. 2005). PSA levels may also be increased in some benign prostatic diseases, such as prostatitis and BPH or some medications and environmental factors. Since PSA is produced and secreted by both benign and malignant prostate cells, the serum level of PSA can only reflect the size of the prostate gland, it cannot be used to distinguish the benign or malignant nature of the cells or to reflect different stage of prostate cancer. To increase the accuracy of using PSA as a screening tool, different molecular forms of PSA have been measured such as total PSA (tPSA) which is a sum of bound and unbound PSA. Unfortunately, it still produces false positives or negatives results and failed to meet the appropriate biomarker for prostate cancer. Thus the real benefit of the widespread use of PSA screening is still

Introduction

debatable and the reliability of PSA as a prostate cancer marker is in serious question to be used as foundation for making accurate diagnosis and therapeutic decisions. Therefore, a reliable and accurate diagnostic or prognostic marker (Adamson et al. 2003; Forootan et al. 2007; Jing et al. 2001) that can be used to distinguish between benign and malignant prostatic disease is urgently needed for making speedy and correct treatment decisions (Bhavsar, McCue & Birbe 2013; Moore et al. 2009; Sardana & Diamandis 2012). Apart from PSA, there are many different biomarkers have been reported and their prognostic significance was studied such as human kallikrein 2 (*hK2*), transforming growth factor- β 1 (TGF- β 1), Interleukin-6 (IL-6), prostate cancer antigen 3 (PCA3), early prostate cancer antigen 2 (EPCA2), TMPRSS2-ERG fusion gene, S100 family protein, prostate stem cell antigen (PSCA) and fatty acid binding protein 5 (FABP5).

1.5.2.3 Human Kallikrein 2 (*hK2*)

hK2 is a serum protease with trypsin-like specificity that belongs to the same family as PSA. *hK2* and PSA share approximately 80% amino acids in the sequence and both are regulated by a similar mechanism (Yousef & Diamandis 2001). *hK2* is expressed in a high level and is restricted to prostate. *hK2* was used as a biomarker for prostate cancer since the expression of *hK2* is higher in malignant cases when compared with benign tissues, however it is not superior to PSA (Catalona et al. 1995). Previous study has shown that *hK2* mRNA was upregulated in highly malignant cells, while the PSA mRNA was down-regulated (Lintula et al. 2005). In 1999, Partin et al. examined the correlation between *hK2* levels and prostate cancer using *hK2*/free PSA ratio and found that it could be detected in 40% of prostate cancer patients with PSA < 4 ng/ml. It has been suggested to be a promising biomarker for predicting the outcomes of prostate

Introduction

cancer patients (Partin et al. 1999). However, several studies showed that *hK2* cannot discriminate pathological stage or unable to prove additional value over existing variables (Stephan et al. 2006; Vaisanen et al. 2006). Therefore, further investigation is needed to confirm its ability to be used as prognostic marker (Hong 2014).

1.5.2.4 Transforming growth factor- β 1 (TGF- β 1)

TGF- β 1 is involved in the regulation of immune response, cellular proliferation, chemotaxis and angiogenesis. Elevated levels of TGF- β 1 have been found in various cancers and the cytokines in the TGF- β 1 family implicated in progression of several cancer types (dos Reis et al. 2011; Zarzynska 2014). Increased expression of TGF- β 1 in prostate cancer was associated with highly malignant cases, metastasis and invasion. No correlation was observed between plasma levels of TGF- β 1 and stage of prostate cancer (Qin et al. 2013; Shariat et al. 2001). More investigations are needed on the potential value of TGF- β 1 as a prognostic marker (Y 2010).

1.5.2.5 Interleukin-6 (IL-6)

IL-6 is involved in the regulation of immune defence mechanism and bone turnover (Paul 1991). Numerous studies showed that the increased expression of IL-6 is correlated with the progression of prostate cancer through inhibition of cancer cell apoptosis and drug resistance (Zhang & Adachi 1999). Elevated levels of IL-6 have been found in advanced prostate cancer as well as other different cancer types such as breast cancer (Zhang & Adachi 1999), colorectal cancer (Waldner, Foersch & Neurath 2012) and ovarian cancer (Isobe et al. 2015). Treatment response to chemotherapy was better in patients with low IL-6 than those with high levels of IL-6 (Giri, Ozen & Ittmann 2001). Therefore, IL-6 can be used as a potential therapeutic target by blocking IL-6

Introduction

signalling. Further studies are needed to assess the therapeutic efficacy of anti-IL-6 therapy.

1.5.2.6 Prostate cancer antigen 3 (PCA3)

PCA3 has been proven to be a useful biomarker that can be used alongside with PSA for more accurate diagnosis (Van Gils et al. 2007). This non-coding RNA is restricted to the prostate and can be detected in prostatic fluids. Some studies showed that increased expression of PCA3 in 90% of biopsies from prostate cancer patients compared to those in benign cases (Salagierski & Schalken 2012). Furthermore, using PCA3 urine tests increased specificity and sensitivity for the diagnosis of prostate cancer (Day et al. 2011; Luo et al. 2014). However, since the effectiveness of this test depends on a full massage of prostate gland before the urine sample is taken, its test results may be varied amongst different examiners (CRUK 2016a). Although, PCA3 is commercially available as a diagnostic marker, it does not appear to have any prognostic value.

1.5.2.7 Early prostate cancer antigen (EPCA)

EPCA is a nuclear structural protein expressed in prostate adenocarcinomas. Elevated levels of EPCA in blood has been correlated with tumour progression of prostate cancer (Zhao, Zeng & Zhong 2010). Immunohistochemistry study suggested that EPCA has the potential to predict prostate cancer 5 years earlier than current protocols. However, some other studies showed that tissue EPCA2 staining was not correlated with tumour stage and Gleason scores in cases with prostate cancer (Dhir et al. 2004; Zhao et al. 2012).

1.5.2.8 TMPRSS2-ERG gene fusion

TMPRSS2-ERG is a transmembrane serine protease which is the first prostate gene fusion and the most frequent gene fusion in prostate cancer (Hessels et al. 2007).

Introduction

biostatistical method called cancer outlier profile analysis (COPA) showed a strong outlier profiles for v-ets erythroblastosis virus E26 oncogene (ERG) in 57% of prostate cancer cases. This was in concordance with the results of a study where prostate cancer-associated ERG overexpression was found in 72% of prostate cancer cases. In >90% of the cases that overexpression of ERG, a fusion of the 5'-untranslated region of the prostate-specific and androgen-regulated transmembrane-serine protease gene (TMPRSS2) with this fusion was found (Petrovics et al. 2005). One study found that a strong correlation between TMPRSS2-ERG gene fusion and high Gleason scores (Rajput et al. 2007). However, another study showed that no association with Gleason score (Mosquera et al. 2007). Controversial results have been reported in many research studies for correlations between TMPRSS2-ERG and prognosis (De Muga et al. 2012). Therefore, this test is not available for clinical diagnosis and some other evidences did not support the prognostic significance of TMPRSS2-ERG.

1.5.2.9 S100 protein family

S100 protein family is also known as calcium binding protein family which is 10,000 kDa protein in 100% saturated ammonium sulphate. They involve in many biological activities such as inflammation, differentiation, invasion or migration (Donato 2003). Dysregulation of several S100 family members has been recognized in most of cancer such as S100P (Mousses et al. 2002). In prostate cancer, multiple S100 family member's overexpression was detected including S100A2, S100A4, S100A8, S100A9 and S100A11 (Ke et al. 1997; Rehman et al. 2005). Although increased expression of these S100 family members was detected in prostate cancer, no significant association with poor patient survival was observed.

Introduction

1.5.2.10 Prostate stem cell antigen (PSCA)

PSCA is a member of a family so-called Thy-lyLy-6 of glycosyl-phosphatidyl-inositol. It has been shown that PSCA is predominantly prostate specific and its upregulation in prostate cancer xenografts (Ramirez, Nelson & Evans 2008; Reiter et al. 1998). Another study shows that PSCA overexpression is associated with high Gleason scores and metastatic progression, but the cause for increased expression of PSCA is still unknown (Han et al. 2004). Since, PSCA is normally expressed in the basal cells of normal prostate, the expression of PSCA in prostate cancer can be an indication that cancer arise from transformation of basal cells (Bonkhoff & Remberger 1996).

1.5.2.11 Fatty acid binding protein 5 (FABP5)

FABP5 is a 14 kDa cytosolic protein which is a member of fatty acid binding protein family with high affinity to bind long chain fatty acids and other lipids. Previous studies showed that some FABPs were implicated in progression of several cancer types and could be used as tumour biomarkers (Boiteux et al. 2009; Hammamieh et al. 2005; Ohmachi et al. 2006). FABP5 is expressed most abundantly in the skin. However, its expression has been identified in a wide range of tissues including brain, liver, kidney, adipocyte, lung, testis, spleen, skeletal muscle and prostate. Our previous studies have showed that FABP5 is a cancer promoting gene (Bao et al. 2013; Kawaguchi et al. 2016a). Jing *et.al* found that FABP5 was overexpressed in malignant prostate epithelial cell lines and able to develop metastasis in vivo when transfected into non-metastatic rat cells. Furthermore, FABP5 played a crucial promoting role in tumorigenicity and metastasis of prostate cancer by facilitating angiogenesis through up-regulating *VEGF* gene (Jing et al. 2001; Jing et al. 2000). We also showed that the suppression of VEGF

Introduction

expression has been detected in these transfected cell lines. Further investigation with staining of FABP5 showed a positive association between the increase expression of FABP5 and the increase degree of malignancies. FABP5 was also involved in malignant progression of pancreatic cancer (Sinha et al. 1999).

1.6 Fatty acids, fatty acid binding protein family and prostate cancer

1.6.1 Fatty acids

Fatty acids play a key role in metabolic regulation and are essential components for all membranes (Berg, Tymoczko & Stryer 2002; Rustan & Drevon 2001). In addition, fatty acids are important for mechanical protection, electrical and thermal insulation (Drevon 2005). Fatty acids can act through enzymatic and transcriptional network to control gene expression (Jump 2004; Pegorier, Le May & Girard 2004). The most common major fatty acids in plant and animal are linoleic acids and arachidonic acids. Also, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are considered to be major fatty acids in fish oil (Berg, Tymoczko & Stryer 2002). Free fatty acids are released during digestion and absorbed in the small intestine then re-esterified to triacylglycerols (Rustan & Drevon 2001). Free fatty acids are taken up into cells by protein carrier and transported intracellularly via fatty acid binding proteins (Rustan & Drevon 2001).

Introduction

1.6.2 Fatty acids and prostate cancer

Prostate cancer has become the most common male cancer in Western developed nations. Fat consumption is highest in men in Europe and North America showing around 40% of total energy intake is obtained from their diet (Micha et al. 2014; NHS 2015a). Several studies showed the correlation between dietary fat intake and prostate cancer (Crowe et al. 2008; Di Sebastiano & Mourtzakis 2014). The same was true when Western diet was introduced to Japanese diet, where their traditional diet is low in fat. The incidence of prostate cancer was significantly increased and the high levels of fat were consumed the more likely to develop aggressive prostate cancer (Marks et al. 2004). Most recent survey of 1,854 results on patients diagnosed with prostate cancer between 2004 and 2009 in North Carolina showed that the increased saturated fat intake was associated with aggressive prostate cancer (UoNCHCS 19 April 2016). Conversely, a study found that there is no correlation between prostate cancer risk and dietary fat intake (Crowe et al. 2008).

1.6.3 Fatty acid binding protein family (FABPs)

FABPs are intracellular lipids chaperones that control lipid responses in cells and linked to metabolic and inflammatory pathways. FABPs belong to a large family so-called iLPB which is divided into 4 subfamilies according to the type of ligands to which they bind. Since the first discovery of FABPs in 1972 (Ockner et al. 1972), 12 members of the FABP family have been identified so far and 2 of them are restricted to fish (Smathers & Petersen 2011). The nomenclature of each member was after the tissue in which they were first isolated or prominently expressed. However, FABPs can express in different human organs either singly or co-expressed with 1 or more other family

Introduction

members (Chmurzynska 2006; Furuhashi & Hotamisligil 2008; Smathers & Petersen 2011). FABPs serve to bind a variety of ligands such as saturated, unsaturated long chain fatty acids, retinoid and other lipids with high affinity. FABPs are abundantly expressed 14-15 kDa proteins in tissue that is highly active in fatty acid uptake. But for other tissues that are less active in fatty acid metabolism, FABPs are not highly expressed. FABPs expression is usually regulated at transcriptional level in a given cell type reflecting the capacity of their lipid-metabolising in adipocytes, hepatocytes and cardiomyocytes. It has been suggested that FABPs make up between 1% and 5% of all soluble cytosolic proteins (Chmurzynska 2006; Furuhashi & Hotamisligil 2008; Smathers & Petersen 2011).

1.6.4 General functions of FABPs

Several studies suggested that the presence of FABPs inside the cell is essential for fatty acids storage and import; making the fatty acids more soluble and preventing the detergent-like properties of high concentrations of fatty acid. Recently, numerous studies showed that FABPs are central regulators of lipid metabolism, immune response and inflammation. FABPs have been suggested to be involved in secretion, uptake and intracellular transport of lipids to subcellular-organelles, for example to the endoplasmic reticulum for signalling, to lipid droplet for storage or to the mitochondria for oxidation (**Figure 1.10**) (Furuhashi & Hotamisligil 2008; Smathers & Petersen 2011). In addition, they are involved in conversion of fatty acids to eicosanoid intermediates (Zimmer et al. 2004). FABPs have the ability to access to the nucleus targeting fatty acids for transcriptional regulation such as the peroxisome proliferator-activated receptor (PPAR) family PPAR- α , PPAR- δ and PPAR- γ in the nuclear lumen (Forootan et al. 2016;

Introduction

Hughes et al. 2015; Tan et al. 2002). Some FABP family members themselves are controlled by PPAR family which is liganded by fatty acids. FABPs also have been involved in a wide range of associated diseases such as atherosclerosis, obesity, diabetes and cancer (Thumser, Moore & Plant 2014). FABPs have also been suggested to control cancer growth through co-ordination with other fatty acid transporters and carcinogens (Schachtrup et al. 2004), steroids (Gao et al. 2010), hormones (Pinthus et al. 2007) and their derivatives (Chmurzynska 2006).

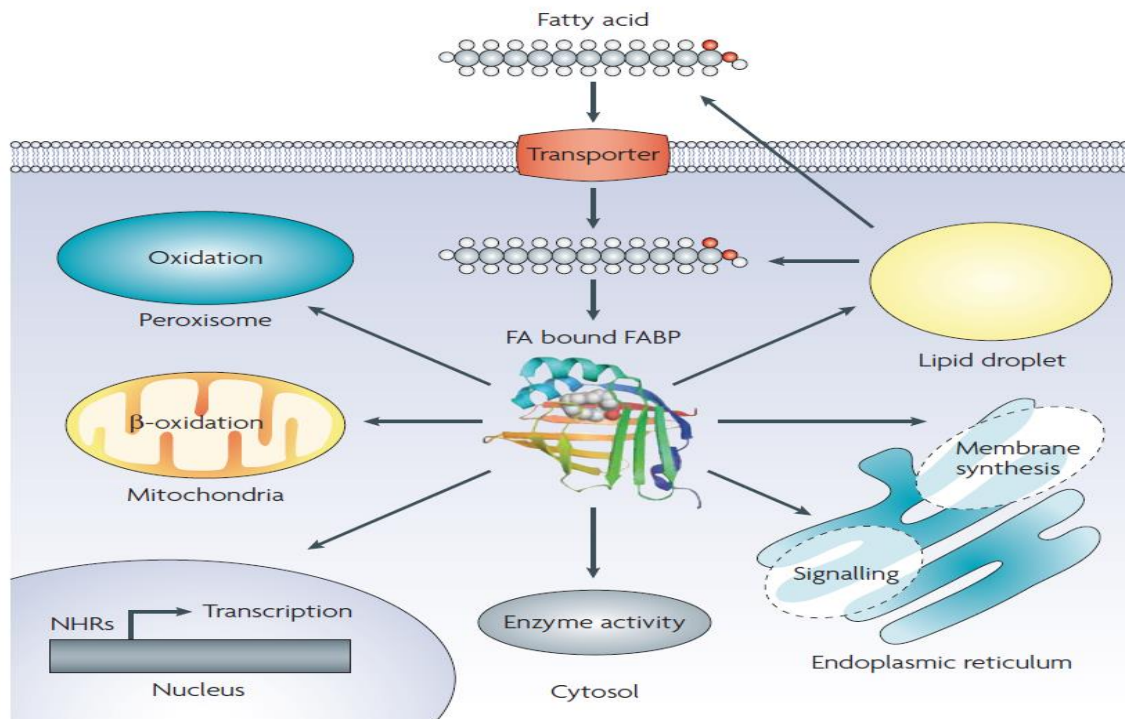


Figure 1. 10: Putative functions of FABPs in the cell. FABPs have been proposed to play a role in the transport of lipids to specific compartments in the cell: to lipid droplets for storage; to the endoplasmic reticulum for signalling, trafficking and membrane synthesis; to the mitochondria or peroxisome for oxidation; to cytosolic or other enzymes to regulate their activity; to the nucleus for the control of lipid-mediated transcriptional programs via nuclear hormone receptors (NHRs) (Furuhashi & Hotamisligil 2008).

Introduction

1.6.5 Fatty acid binding proteins (FABPs) affinity and structure

The structure of several FABPs was revealed based on X-ray crystallography, nuclear magnetic resonance (NMR) and other biochemical techniques. FABP family has 15% to 70% sequences identity between different members. Despite the differences in their protein sequences, all FABPs share identical three dimensional structures. Common to all FABPs is a 10-stranded antiparallel β -barrel structure, organized into two orthogonal five-stranded β -sheets. The binding pocket is located inside the β -barrel, the opening of which is capped on one side by the N-terminal helix loop helix motif, and fatty acids are bound to the interior cavity as shown in **Figure 1.11** (Chmurzynska 2006). The overall structure of *FABP* gene is highly conserved, consisting of 4 exons separated by 3 introns. For all FABPs, the position of exons and introns are the same. All *FABPs* share a conserved three-element fingerprint (**Figure 1.12**) (Smathers & Petersen 2011).

All *FABPs* bind a variety of ligands such as saturated, unsaturated long chain fatty acids, retinoid and other lipids with high affinity (Storch & Thumser 2010). Binding mechanism and binding affinity are based on the small structural differences between isoforms. Generally, the binding affinity increased with more hydrophobic ligands. Additionally, FABPs bind only one ligand per molecule of protein except *FABP1* which can bind two ligands simultaneously. *FABP7* is highly selective for long chain fatty acids, whereas *FABP1* has a binding capacity for different ligands from lysophospholipids to haem (Smathers & Petersen 2011). Chromosomal mapping of all *FABPs* show that *FABP1*, *FABP2*, *FABP3*, *FABP6* and *FABP7* exist on separate chromosome in the human, whereas *FABP4*, *FABP5*, *FABP8*, *FABP9* and *FABP12* are localized at chromosome (Chr) 8q21 as shown in **Table 1.3** (Chmurzynska 2006).

Introduction

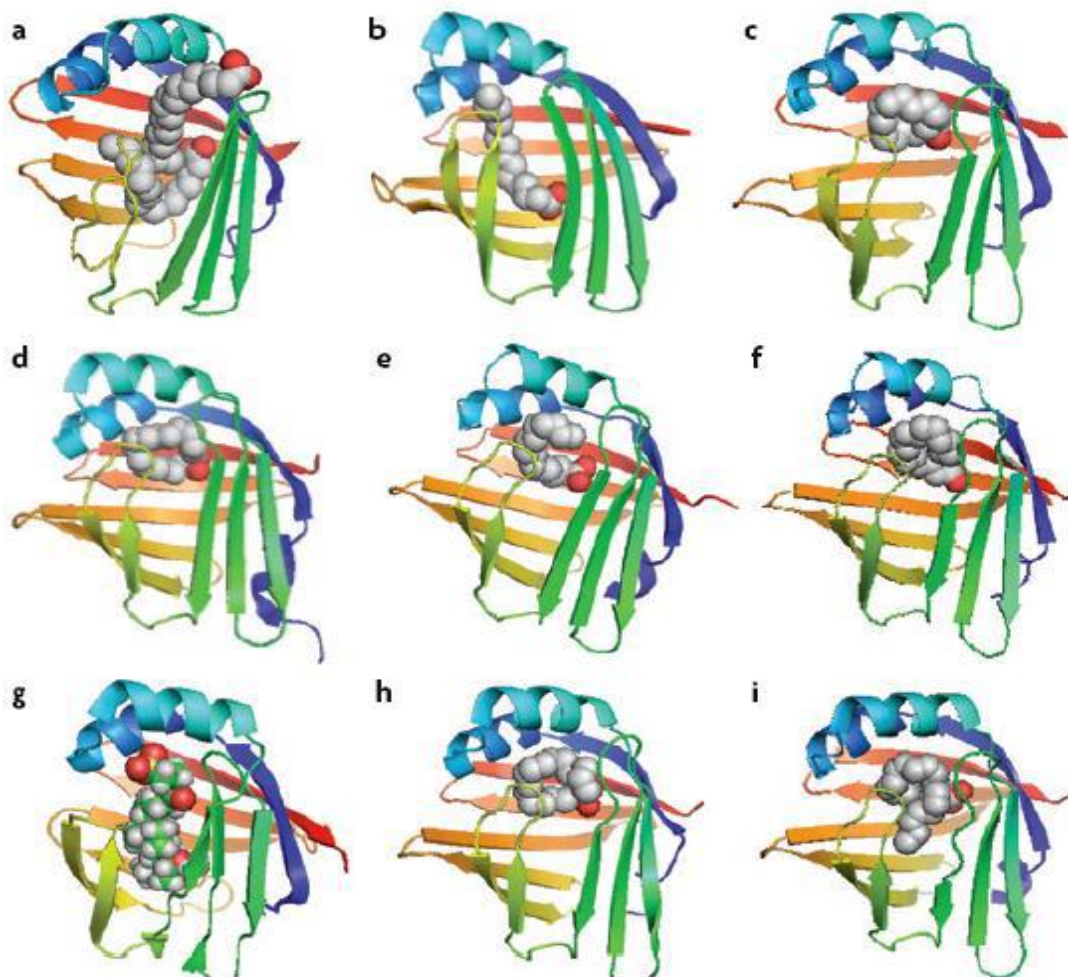


Figure 1. 11: Crystal structure of ligand-bound FABPs. Ligand-binding FABPs are shown above (The figures were created using PyMOL). (a) FABP1; (b) FABP2; (c) FABP3; (d) FABP4; (e) FABP5; (f) FABP8; (g) FABP6; (h& i) FABP7 (Furuhashi & Hotamisligil 2008).

Introduction

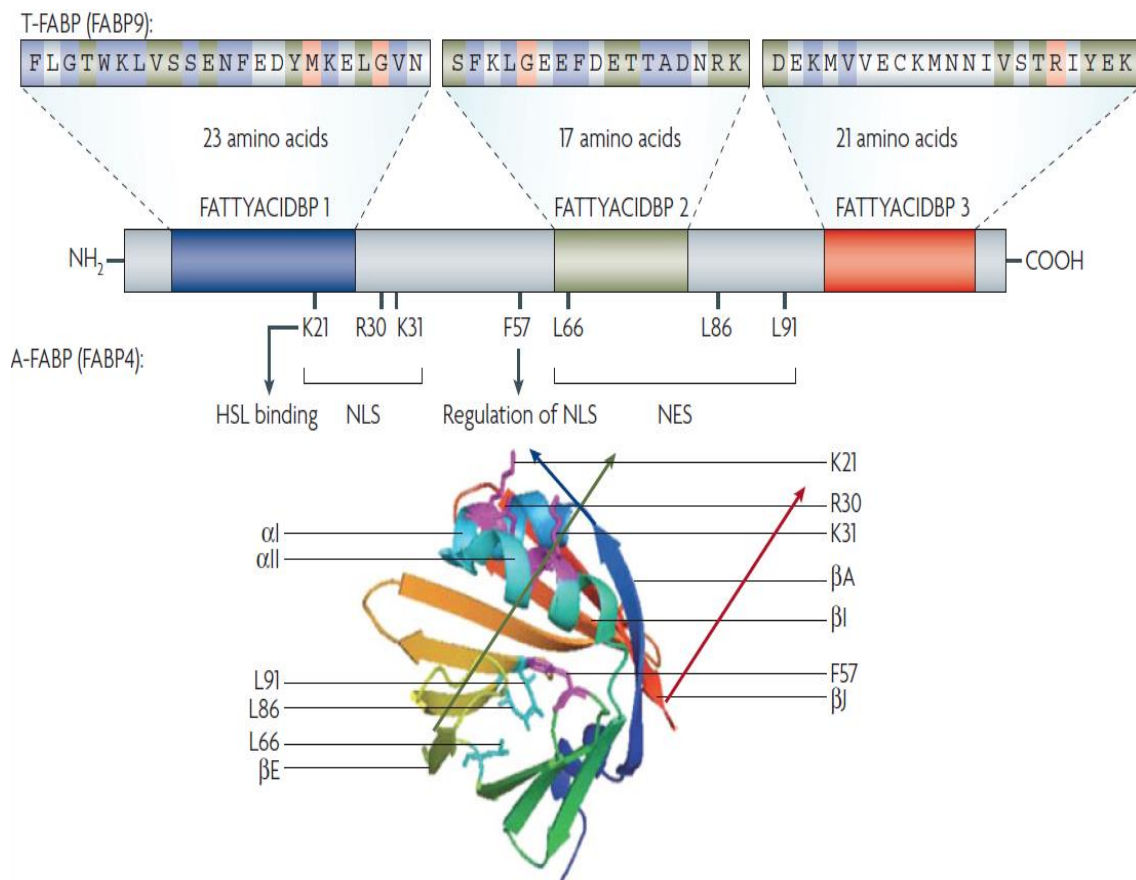


Figure 1. 12: Fingerprint for fatty acid binding proteins. potential functional domains include a nuclear localization signal (NLS) and its regulation site, nuclear export signal (NES) and a hormone-sensitive lipase (HSL) binding site 17,26,100. The primary sequence of A-FABP does not show a readily identifiable NLS. However, the signal could be found in the 3D structure of the protein and was mapped to three basic residues (K21, R30 and K31) located in the helix–loop–helix region, whose side chains shift their orientation upon ligand binding to form a functional NLS¹⁷. The NES is also not apparent in the primary sequence, but assembles in the tertiary structure from three nonadjacent leucine residues (L66, L86 and L91) to form a motif reminiscent to that of established NES (Furuhashi & Hotamisligil 2008).

Introduction

Table 1.3: chromosomal localization of all human FABP genes and their distribution.

Gene	Chromosomal location	No. of amino acids	Protein localization
<i>FABP1</i>	2p11	127	Liver, intestine, pancreas, kidney, lung and stomach.
<i>FABP2</i>	4q28–q31	132	Intestine and liver.
<i>FABP3</i>	1p33–p31	133	Cardiac and skeletal muscle, brain, kidney, lung, stomach, testis, adrenal gland, mammary gland, placenta, ovary, brown adipose tissue.
<i>FABP4</i>	8q21	132	Adipocytes, macrophages, dendritic cells, skeletal muscle fibres and prostate.
<i>FABP5</i>	8q21.13	135	Skin, tongue, adipocyte, dendritic cells, mammary gland, stomach, intestine, kidney, lung, heart, skeletal muscle, testis, retina, lens, spleen, placenta and prostate.
<i>FABP6</i>	5q23–q35	128	Ileum, ovary, adrenal gland, stomach
<i>FABP7</i>	6q22–q23	132	Brain, central nervous system (CNS), glial cell, retina, mammary gland
<i>FABP8</i>	8q21.3–q22.1	132	Peripheral nervous system, Schwann cells
<i>FABP9</i>	8q21.13	132	Testis, salivary gland, mammary gland.
<i>FABP12</i>	8q21.13	140	Retinoblastoma cell, a retina (ganglion and inner nuclear layer cells), testicular germ cells cerebral cortex.

1.6.6 Human fatty acid binding proteins in health and disease

1.6.6.1 FABP1

FABP1 is abundantly expressed in liver tissue representing 5% of all cytosolic protein in hepatocytes, but is also expressed in different tissues including those of intestine, kidney, pancreas and lung organs (Chmurzynska 2006). FABP1 plays a key role in the hepatic β -oxidation of unesterified fatty acids via the regulation of expression or fatty acid trafficking (Atshaves et al. 2010). It has been shown that FABP1 is required to maintain lipid droplet in hepatic stellate cells (HSCs). FABP1 downregulation results in HSC secretion of collagen and proliferation, activation and loss of lipid droplets leading to hepatic fibrogenesis (Chen et al. 2013). Unlike the other members, *FABP1* has a binding capacity for a wide range of ligands, uniquely binds to two ligands or various hydrophobic molecules such bile acids and cholesterol (Storch 1993).

A study addressed the positive correlation between serum FABP1 levels and obesity and insulin resistance in Chinese young adults (Shi et al. 2012). Increased expression of FABP1 has been recognised in several cancer types, including liver, colorectal, lung and gastric cancers (Ku et al. 2016; Lawrie et al. 2004; Wang et al. 2015). Furthermore, FABP1 play a key role in metastasis and invasiveness of breast cancer and serves as diagnostic biomarker for breast cancer. Recent study has shown a positive correlation between the expression of FABP1 and VEGF expression in hepatocellular carcinoma (HCC), suggesting that FABP1 could serve as a potential therapeutic target for HCC therapy (Dong et al. 2007; Ku et al. 2016).

Introduction

1.6.6.2 FABP2

In addition to FABP1, FABP2 is also mostly expressed in intestinal enterocytes, representing 1-2% of all cytosolic proteins. FABP2 is highly expressed in jejunum which play a key role in lipid adsorption (Agellon, Toth & Thomson 2002). Generally, FABP2 is involved in the binding and transport of hydrophobic fatty acids from cell membrane to endoplasmic reticulum. Also, FABP2 is guiding fatty acids to the mitochondria for energy sources in the enterocytes. However, some other reports recognised a lower fatty acids uptake by enterocytes characterized by a high level of FABP2 (Gajda & Storch 2015). Increased levels of FABP2 has been linked to increasing degrees of villous atrophy in coeliac disease (Adriaanse et al. 2013; Uhde et al. 2016). The overexpression of FABP2 affects the intracellular processes needed for adequate intestinal fat adsorption and transport. Most recent studies have linked FABP2 with atherosclerosis in type-2 diabetes mellitus patients and pneumatosis intestinalis disease (Khattab et al. 2016; Matsumoto et al. 2016).

1.6.6.3 FABP3

The main cells expressing FABP3 are myocytes, although it also expressed in a wide range of tissues including skeletal muscle, brain, testes and lung (Storch & Thumser 2010). FABP3 plays several key roles in fatty acids transport, cell signalling, cell growth and gene expression (Besnard et al. 2002). It has been suggested that FABP3 is a myokine that can contribute to regulating energy supply to heart (Binas et al. 1999), endocrine paracrine signalling (Thumser, Moore & Plant 2014). The main function for FABP3 is to traffic fatty acids towards the mitochondria (Smathers & Petersen 2011).

Introduction

FABP3 has been proven to be a reliable prognostic marker for the early diagnosis of myocardial infarction/acute coronary syndrome (McMahon et al. 2012). Recent studies have shown that FABP3 involve in progression of several cancer types. In embryonic cancer cells, the elevated expression of FABP3 inhibits cell growth leading to apoptosis (Song et al. 2012). FABP3 acts as a tumour suppressor for breast cancer, showing the complete absence of FABP3 in breast cancer samples (Dean & Rhodes 2014). In contrast, increased expression of FABP3 is correlated with the development and aggressiveness of gastric cancer. Most recent study showed that overexpression of FABP3 is associated with poor prognosis in non-small cell lung cancer (NSCLC) (Tang et al. 2016).

1.6.6.4 FABP4

FABP4, is highly expressed in adipocytes and macrophages, recognized as an adipokine in addition to its traditional role in guiding fatty acids within cells. A study supported the role FABP4 as an adipokine has shown that interaction between adipocytes and macrophages increases secretion of FABP4 into the culture medium (Falcao-Pires et al. 2012; Kralisch & Fasshauer 2013).

It has been suggested that there is a positive correlation between FABP4 and lipid metabolism or inflammation impacting on various diseases such as atherosclerosis, Non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome (Furuhashi et al. 2014). Numerous recent studies suggested that FABP4 has the potential to serve as a biomarker for acute myocardial infarction, as the presence of FABP4 in blood indicate that it is released from myocardium after cells are damaged (Pyati et al. 2016). FABP4 has the potential to serve as a prognostic marker for invasive bladder cancer (Ohlsson et al.

Introduction

2005), and as a therapeutic target for metastasis of prostate cancer (Uehara et al. 2014). Also, increased expression of FABP4 has been linked to the poor prognosis in non-small cell lung cancer (NSCLC) and it was also evidenced FABP4 as prognostic predictor for NSCLC (Tang et al. 2016).

1.6.6.5 FABP5

FABP5 was identified in endothelial cells of different tissues including skin, heart, liver, placenta, skeletal muscle and renal medulla (Makowski & Hotamisligil 2005; Masouye, Saurat & Siegenthaler 1996). The absence of FABP5 in the liver during perinatal development was compensated by increasing levels of FABP4 (Owada et al. 2002). FABP5 plays a crucial role in progression of breast cancer (Powell et al. 2015). Knockdown of FABP5 expression in highly malignant prostate cell line significantly inhibited tumorigenicity and; the details of the role of FABP5 in prostate cancer has been discussed in section 1.5.2.11 (Adamson et al. 2003; Forootan et al. 2010; Morgan et al. 2008). Increased expression of FABP5 was also recognized in other cancer types such as colorectal cancer, oral squamous carcinoma and pancreatic cancer (Fang et al. 2010; Kawaguchi et al. 2016b; Sinha et al. 1999).

1.6.6.6 FABP6

FABP6 is abundantly expressed in the ileum, but it is also expressed in placenta, ovaries and adrenal gland. FABP6 plays a central role in binding and trafficking bile acids in ileal epithelium cells to catalyse the cholesterol, but also can bind and transport fatty acids (Furuhashi & Hotamisligil 2008; Smathers & Petersen 2011). It has been suggested that when FABP6 exhibit high affinity for bile acids, it regulates bile acids homeostasis in the intestine (Chiang 2002; Grober et al. 1999).

Introduction

As previously reported that bile acids have the ability to induce colon carcinogenesis, inflammation and apoptosis in colonic epithelial cells (Nagengast, Grubben & van Munster 1995; Zhu et al. 2016). Overexpression of FABP6 has been identified to be correlated with colorectal cancer and adenomas when compared to normal epithelium, suggesting that FABP6 may play a promoting role in early colorectal carcinogenesis (Ohmachi et al. 2006). It has been shown a positive correlation between the expression of FABP6 and the renal cell carcinoma and bladder cancer (Aaboe et al. 2005; Schrödter et al. 2016). FABP6 has not been previously reported to be related to progression of prostate cancer. Therefore, any factors that may affect the expression of FABP6 may significantly contribute to the pathogenesis of different diseases.

1.6.6.7 FABP7

FABP7 is mainly expressed in radial glia cells in brain tissue, it is not known yet whether it can be expressed in tissues other than brain (Mita et al. 2007; Sharifi et al. 2011). FABP7 can be distinguished from other FABP family members by its strong affinity with n-3 polyunsaturated fatty acids which is an essential nutrient for central nervous system (Balendiran et al. 2000). Additionally, FABP7 does not bind retinoic acid or palmitic acid, but affinities for arachidonic acid and oleic acid are almost 0.25 μ M and 4 μ M, respectively (Smathers & Petersen 2011).

Overexpression of FABP7 is associated with different pathological conditions such as Down's syndrome, schizophrenia and cancer (Sánchez-Font et al. 2003; Shimamoto et al. 2014). Increased expression of FABP7 has been recognized in malignant glioma cells and associated with the development and invasion of brain tumour (De Rosa et al. 2012). In addition, expression of FABP7 has been linked to poor prognosis of breast cancer and

Introduction

renal carcinoma (Alshareeda et al. 2012; Teratani et al. 2007). A research study has revealed that MAPK/ERK and PKC pathways in melanoma regulate FABP7, suggesting that FABP7 is correlated with tumour progression of melanoma (Slipicevic et al. 2008).

1.6.6.8 FABP8

FABP8 is highly expressed in peripheral nervous system myelin, but also expressed in central nervous system myelin (Smathers & Petersen 2011; Storch & Thumser 2010). In addition to its traditional function of binding to long chain fatty acids, FABP8 stabilise myelin membrane and involves in membrane biogenesis. Apart from these, the function of FABP8 is largely unknown. An animal study has revealed that cholesterol is a likely ligand for FABP8 (Majava et al. 2010). FABP8 shares high sequence similarities of almost 67% to FABP4 (Furuhashi et al. 2014).

1.6.6.9 FABP9

FABP9 is highly expressed in the testis and has several important physiological roles in sperm development; including attachment of the acrosome to the sperm nucleus during fertilization and spermatogenesis in mammalian testis (Kido & Namiki 2000; Liu, Li & Godbout 2008; Oko & Morales 1994; Smathers & Petersen 2011). It has been suggested that FABP9 protect sperm fatty acids from oxidation to be able to fertilise oocytes. More studies are needed to investigate whether the level of immunoreactive FABP9 can be used as diagnostic or prognostic biomarker for any type of cancer or other diseases. FABP9 is a poorly understood protein and no previous study has addressed the role of FABP9 in human prostate cancer (Al Fayi et al. 2016).

Introduction

1.6.6.10 FABP12

FABP12 is highly expressed in ganglion cells in adult retina, suggesting that it may play a key role in the regulation of genes involved in adult retina (Liu, Li & Godbout 2008). It was believed that FABP9 is the only FABP family member expressed in testis; but several studies have shown the expression of FABP12 in testicular germ cells. FABP12 is poorly understood because it has only been discovered recently, and no reports or articles on this protein function have been published.

1.6.7 Therapeutic target of FABPs

FABPs are promising targets for treatment of cancer, atherosclerosis, obesity, diabetes and metabolic syndrome (Furuhashi et al. 2007; Hoo et al. 2013; Miao et al. 2015; Rao et al. 2015). Several studies have been reported in developing novel specific and efficient FABPs inhibitors which can be potential drugs for different diseases including prostate cancer (Wang et al. 2014). For instance, FABP4 inhibitors were used to treat ovarian cancer or suppressing invasion and migration of this cancer (Nieman et al. 2011). Several studies created pharmacological agents to modify FABP4 function that may offer a new class of therapeutic agents for obesity, insulin resistance and atherosclerosis (Furuhashi et al. 2015; Furuhashi et al. 2014). Our group now is focusing on FABP5 to be used a novel target for therapy in castration-resistance prostate cancer. Novel FABP5 inhibitors have been used to establish an experimental treatment for castration resistant prostate cancer in nude mice by suppressing the biological activity of FABP5.

Introduction

1.7 Hypothesis

After FABP5 was proven to be a prognostic marker and potential treatment target (Bao et al. 2013; Forootan et al. 2016; Forootan et al. 2014; Morgan et al. 2008), a frequently asked question was whether any other FABP family proteins can also be used as diagnostic or prognostic markers for prostate cancer.

In the first part of this study, investigations were performed to investigate this issue by addressing following questions:

- Are any other FABPs overexpressed in a wide range of prostate cancer cells and tissues?
- Are there any correlations between the increased levels of the possibly overexpressed FABPs and the combined GS of patients?
- Are there any correlations between the increased levels of the overexpressed FABPs and AR index or PSA levels of prostate cancer patients?
- Is there any correlation between the increased levels of the overexpressed FABPs and survival period of the patients after diagnosis?
- What is the biological significance of the elevated expression of these FABPs in prostate cancer?

1.8 Aim and scope

- Assessment of potentially differential expression levels of all FABPs in six different benign and malignant cell lines by RT-quantitative PCR (qPCR) in mRNA level.
- Comparison of differential expression levels of all FABP proteins in six different benign and malignant cell lines by Western blot in protein level.

Introduction

- Detection of differential expression levels of candidate FABPs in benign and carcinoma prostate tissues using immunohistological staining.
- Assessing the correlation of the increased expression levels of candidate FABPs in prostate carcinoma tissues with the degree of malignancy, as measured by Gleason score (degree of malignancy), PSA level, AR index and patient's survival time using various statistical analyses.
- Assessing the correlation of survival time of the prostate cancer patients with the combined Gleason scores (degree of malignancy), PSA level, AR index using different statistical analyses.
- Suppressing the highly expressed candidate FABPs in highly malignant prostate cancer cells, transiently and stably, using RNAi techniques.
- Evaluation of the suppressing effects of candidate FABPs on growth rate, invasiveness, migration and anchorage-independent growth of prostate cancer cell, *in vitro*, using proliferation assay, invasion assay, wound healing assay and soft agar assay, respectively.

**CHAPTER TWO:
MATERIALS AND METHODS**

2. Materials and Methods

2.1 Cell culture

2.1.1 Initiate cell culture from frozen stock

For long term storage, seven different cell lines had been frozen down in liquid nitrogen. To start new cell culture, cryovials were collected from liquid nitrogen and placed on ice. Before seeding the cells to cell culture dishes, vials were carefully cleaned with 70% ethanol from outside. Because dimethyl sulphoxide (DMSO) in frozen cryopreserved cells was used to induce differentiation, we removed the DMSO by diluting thawed cells in 20ml of fresh medium and centrifuged at $91 \times g$ for 3 minutes, the supernatant was decanted gently, and the cell pellet was re-suspended in 10 ml fresh medium in a 25 cm² flask and maintained in humidified incubator with 5% CO₂ at 37°C.

2.1.2 Cell lines and culture condition

The following seven cell lines were used in this study: MCF7 breast cancer cell lines (the positive control for FABP6), benign prostate epithelial cell line PNT-2, weakly malignant cell line LNCaP, moderately malignant cell line 22RV-1 and highly malignant cell lines Du145, PC-3 and PC3-M. Rat testis extract (Santa Cruz Biotechnology, Germany) was used as a positive control for FABP9. The routine cell culture took place in tissue culture hood in the Tissue Culture Laboratory. All cell lines were grown and maintained as monolayer cultures in RPMI 1640 nutrient medium (Invitrogen, Paisly, UK) in 75 cm² cell culture flask then kept in a humidifier incubator at 37°C with 5%

Materials and Methods

CO₂. RPMI 1640 nutrient medium was supplemented with 10% (vol/vol) foetal calf serum, penicillin (100 U/ml) (Bioser, East Sussex, UK), streptomycin (100ug/ml) and L-glutamine (20mM) (Invitrogen). For LNCaP cells sodium pyruvate (100ug/ml) (Sigma, Grillingham, UK) was also added into the culture medium. Cells were fed every 3-4 days with fresh medium. When cells reached 70-80% confluency, medium was aspirated off, and the cells were washed once with phosphate buffer saline (PBS) and passaged in 1/10 fold. Cells were detached by adding Trypsin 2.5% (v/v) in Versene (T/V) (3ml/75 cm² flask or 2ml/25 cm² flask) and incubated for 3 minutes in humidifier incubator at 37°C with 5% CO₂. For deactivation of T/V, double volume of culture medium was added. Cells with medium were centrifuged (91 ×g for 3 minutes) to remove T/V, cell pellets were suspended in fresh medium and divided to different flasks.

2.1.3 Transfected cells culture

For transfected cells, Zeocin 100µg/ml (Invitrogen, CA, USA) was added to routine selective medium (RSM). All cell culture protocols were same as non-transfected culture except using RSM.

2.1.4 Cryopreservation of cells

Healthy cells were detached from tissue culture vessel by Trypsin/Versene, re-suspended in PBS, counted and centrifuged at 91 ×g for 3 minutes. Cells was re-susp×ended in medium containing 7.5% DMSO (1-1.5×10⁶/ml) to prevent ice crystal formation. Then, 1ml of cells was pipetted into several cryovial tubes. Cryovials were placed in cryobox,

Materials and Methods

containing isopropanol to decrease the temperature approximately 1°C/min, and stored at -80°C overnight before transferring to liquid nitrogen for long term storage.

2.2 Evaluating mRNA transcription in cell lines using qPCR

2.2.1 RNA isolation

Total RNAs of each cell line were isolated using the RNeasy Mini Kit. When cells reached 70-80% confluence, they were harvested and washed with PBS which was removed by centrifuging for 3 minutes, 91 ×g as described in section 2.1.1. Then, 350µl buffer RLT (containing β-Mercaptoethanol) was added to lyse the cells followed by homogenization by centrifuging through PrepEase filter for 1 minute at 1008 ×g and precipitated in 70% ethanol. The mixture (700µl) was placed in RNeasy mini column sitting in a 2ml collection tube and centrifuged for 15 seconds in ≥8,000 ×g. The column was washed with 700µl buffer RW1 followed by 2 washes with 500µl RPE buffer and centrifuged at ≥8,000 ×g for 15 seconds to eliminate any carryover buffer. Then, 30µl of RNase free water was added and incubated at room temperature for 1 minute. Finally, the total RNA was eluted by centrifuging the column for 1 min at 1008 ×g.

2.2.2 Calculation of RNA concentrations

The total RNA yield and purity was determined by NanoDrop ND-1000 spectrophotometer (Labtech, UK). One µl of sample was loaded on the lower measurement pedestal. The RNA sample concentration was shown on the screen in

Materials and Methods

ng/ μ l according to the absorbance at 260 nm the modified Beer-Lambert equation. The purity of the sample was also assessed using ratio of absorbance at 260nm and 280nm: a ratio of ~ 1.8 was generally accepted as pure for DNA; a ratio of ~ 2.0 was generally accepted as pure for RNA.

2.2.3 Integrity and quality of total RNAs

The integrity and quality of total RNAs were determined by an Agilent 2100 bioanalyzer using RNA 6000 Nano kit (Agilent Technologies, Germany). First, the electrode was decontaminated using electrode cleaner with 350 μ l RNaseZAP. Then, one of the wells of another electrode cleaner was filled with 350 μ l RNase-free water and placed in the Agilent 2100 bioanalyzer. Before preparing the gel, all reagents were allowed to equilibrate to room temperature for 30 minutes. After that, 550 μ l of Agilent RNA 6000 Nano gel matrix was placed on the top of receptacle of the spin filter. Spin filter was placed in the microcentrifuge at 1792 \times g for 10 minutes. Filtered gel (65 μ l) was aliquoted into 0.5 ml RNase-free microfuge tubes and stored as aliquots at 4°C. RNA 6000 Nano dye concentrate was vortexed and span down for 10 seconds. One μ l of RNA 6000 Nano dye concentrate was added to 65 μ l aliquot of filtered gel and vortexed thoroughly. Spin tube was centrifuged at 13,000 g for 10 minutes at room temperature. Before loading the gel-dye mix, it was allowed to equilibrate to room temperature for 30 minutes. New RNA Nano chip was placed on the chip priming station. Then, 9 μ l of the gel-dye mix was loaded into the bottom of the well and the chip priming station was closed for 30 seconds. RNA Nano marker (5 μ l) was loaded into the ladder well and

Materials and Methods

each well for all samples. One μl of the RNA ladder was pipetted into the well specified for ladder and 1 μl of each sample into sample wells, and vortexed gently for 1 minute at 645 $\times\text{g}$. Within 5 minutes, the chip was carefully placed the receptacle in the Agilent 2100 bioanalyzer. The 2100 expert software confirmed that chip had been inserted and the lid closed by displaying an icon on the screen to start the chip run. The minimum RIN accepted in this study was 9.0.

2.2.4 Polymerase chain reaction (PCR)

PCR is the most common technique used in molecular biology to detect RNA expression levels by qualitatively detection of gene expression through creation of complementary DNA (cDNA) transcripts from mRNA. Then qPCR is used to quantitatively measure the amplification of DNA using fluorescent probes. The gene expression level of all FABPs (1-12) in 6 prostate cell lines were investigated. In this study, it was applied to show the levels of all FABPs gene expressions in prostate cell lines.

2.2.4.1 PCR primer design

All *FABP* primers were designed using the PerlPrimer program (perlprimer.sourceforge.net/) (**Table 2.1**). Wherever possible all primers were selected according to the following parameters: melting temperature between 57°C and 63°C, the G+C content between 30-80%, length between 18-22 bp, less than 2°C difference in melting temperature (T_m) between the forward and backward primers. All primers including those for β -actin were purchased from Life Technologies Ltd. (Paisly, UK).

Materials and Methods

Table 2. 1: PCR forward and reverse primer sequences for all *FABP* genes.

Name and accession no.	Forward	Tm	Reverse	Tm
FABP1 (P07148)	CACTTCAAGTTCACCATCACC	61.49	GAACCACTGTCTTGACTTTCTC	61.15
FABP2 (P12104)	AATATTGCGTCCATGTTGCT	60.71	CTACTCAGAAGCCTGAGGC	61.73
FABP3 (P05413)	AAGCCTACCACAATCATCGA	61.30	GTGTCACAATGGACTTGACC	61.20
FABP4 (P15090)	GAAAGAAGTAGGAGTGGGCT	61.23	GTGACTTCGTCAAATTCCTGG	61.54
FABP5 (Q01469)	AAGGAGCTAGGAGTGGGA	61.24	GTAAAGTTGCAGACAGTCTGAG	61.21
FABP6 (P51161)	CATAAAGGAAAGCCTCCCAG	60.42	GTGACGATCTTGAAGTTGCG	61.93
FABP7 (O15540)	TCAAGAAGGAGACAAAGTGG T	61.32	CAGGCTAACAACAGACTTACAG	60.96
FABP8 (Q9I8L5)	ATAGAAAGACCAAGAGCATC GT	61.58	AGACCTTCTCATAGATTCTGGTG	61.32
FABP9 (Q0Z7S8)	AGGGTTAGTGAAACCGACAG	61.80	GTTATGGTGCTCTTTACTTTCCG	61.85
FABP12 (A6NFH5)	GGAAGGAAATAGCAACAGTG G	61.29	CTACACGCTCACCATATAAGC	60.66

2.1.1.1 Blast

The specificities of all FABPs were confirmed by aligning the working sequence with that stored in NCBI data bank, using the nucleotide Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

Materials and Methods

2.1.1.2 First strand cDNA synthesis

First strand cDNA was synthesized using total RNA isolated from 6 different prostate cell lines as described in section 2.2.1. Total RNA (1 µg) was mixed with 1µl of 50µM Oligo (dT)₂₀ primer and 2µl of dNTP mixture (10mM each dCTP, dATP, dTTP dGTP, at neutral pH). After the volume was adjusted to 13µl with nuclease-free water, the mixture was incubated at 65°C for 5 mins and chilled on ice for 1 minute. Then, the mixture was mixed with 1µl of 0.1M DTT, 4µl of 5× first strand buffer, 1µl of SuperScriptIII reverse transcriptase (200units/µl) and 1µl of RNaseOUT (40units/µl). The reaction mixture was incubated at 50°C for 1 hour, followed by incubation at 70°C for 15mins to inactivate the reaction. The First strand cDNA was used as a template for qPCR.

2.1.1.3 Relative real-time PCR

Total RNAs of each cell line using RNAeasy Mini Kit as described 2.2.1 and reverse transcribed 1µg of total RNA to cDNA as described in section 2.2.4.3. The real time PCR mixtures of β-actin and all FABPs were prepared with 5µl of 2× Brilliant SYBR Green qPCR master mix (containing SureStart Taq DNA polymerase, dNTPs mixture, MgCL₂ and optimized buffer), 1µl of forward primer, 1µl of reverse primer, 1µl of cDNA generated by reverse transcription and 2µl of nuclease-free water. After gentle mix, the reactions were centrifuged briefly and placed in the real-time PCR thermocycler. The real-time PCR program is listed in **Table 2.2**.

Materials and Methods

Table 2. 2: Real-time PCR program for amplification of short target DNAs

	Temperature	Time
Step 1 Denaturation	95°C	15 minutes
Step 2 Denaturation	94°C	14 seconds
Step 3 Annealing	60°C	30 seconds
Step 4 Extension	72°C	30 seconds
Step 5 Plate reading	57°C	15 seconds
Step 6 Go back to step 2 and repeat 38 cycles		
Step 7 Final extension	72°C	10 minutes
Step 8 Melting curve	65-95°C	1°C increment for 10 minutes

2.1.1.4 Relative quantitation analysis

Melting curve analysis was carried out to detect the presence of nonspecific products and the primer dimers. The relative fold differences of all FABPs mRNA were obtained using the formula listed below:

$$\text{Relative fold difference} = 2^{-\Delta\Delta Ct}$$

- ΔCt was calculated as the average Ct for the gene of interest minus the average Ct for the house keeping gene, β actin.
- $\Delta\Delta Ct$ was calculated as the ΔCt of the test sample minus the ΔCt of the calibrator sample.

2.2 Evaluating protein expression in cell lines using Western blotting

2.2.1 Isolation of protein extract from cell culture

When cells reached to 80% confluence, they were trypsinized as described in section (2.1.1). Cell suspension was centrifuged at $91 \times g$ for 3 minutes and the supernatant was discarded. The cells were lysed with CeLLytic-M reagent (with protease inhibitor) ($100\mu\text{l}/5 \times 10^6$ cells) and incubated on a roller mixer for 15 minutes at room temperature. Then mixture was centrifuged at $11,200 \times g$ for 20 minutes to pellet the cellular debris. The supernatant was harvested into fresh microcentrifuge tubes.

2.2.2 Bradford assay

The concentration of protein extract was quantified using Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munchen, Germany). A standard curve was established by measuring the absorbance of a serial dilutions of BSA standards (0.2 – 1.5 mg/ml). Protein samples were diluted with PBS and incubated the 1ml $1 \times$ dye reagent, which filtered through Whatman paper, for 15 minutes. Then, the absorbance was measured at 595nm using MultiSkan plate reader (BioTeck Instruments, USA). The protein concentration was calculated using the established standard curve.

Materials and Methods

2.2.3 Sodium dodecyl sulphate polyacrylamide protein gel electrophoresis (SDS-PAGE)

Quantified proteins at concentration of 20µg/10µl were mixed with 10µl of 2× sample loading buffer. Then, the mixture was heated at 95°C on hot plat (Techne, Ori-Block, USA) for 10 minutes then chilled on ice for 5 minutes before loaded into the SDS gel. Proteins were loaded in 12.5 % Next Gel (Next Gel™ 12.5%, Amresco, USA) which was prepared by adding 60µl of 10% ammonium persulphate (APS) and 6µl of TEMED to 10ml of 12.5% Next Gel. Electrophoresis was performed in a chamber filled with 500ml of 1× running buffer using Bio-Rad miniprotein system and ran at 150V for 60 minutes.

2.2.4 Transfer proteins from SDS gel to PVDF membrane

After electrophoresis, proteins were electrophoretically transferred from SDS-PAGE gel to PolyVinylidene DiFluoride membrane (PVDF membrane) (Immobilon-P, Transfer Membrane, Millipore, USA) using Bio-Rad miniprotein system. PVDF membrane and six pieces of Whatman 3MM filter paper cut to the size of 8×9 cm. PVDF membrane was soaked in methanol then washed with distilled water and equilibrated in cold 1× transfer buffer for 10 minutes. The transfer sandwich was made as follows: the cassette was placed into a tray with the black side down, then pre-wet fiber pad, 3 sheets of filter paper, gel, membrane, 3 sheets of filter paper and fiber pad were placed on the black side of the cassette. After air bubbles were gently removed using glassy roller, the cassette was firmly closed. The transfer was performed in the transfer apparatus filled with 4°C 1× transfer buffer at 100V for 1 hour. After transfer, the gel and the membrane

Materials and Methods

were stained with Coomassie blue and 10% Ponceau S, respectively, to assess the efficiency of the transfer.

2.2.5 Immunodetection

To block non-specific bindings, the membrane was incubated with 10ml of 5% blocking reagent (TBS-T milk) for 1 hour on a shaker at RT. Then membranes were incubated with primary antibody, which was either anti-FABP6 antibody (1:500 Rabbit polyclonal FABP6 antibody, Abcam, UK) or anti-FABP9 antibody (1:500 sheep polyclonal FABP9 antibody, R&D Company) for 1 hour at 4°C, followed by 3 times wash with 1× TBS-T, for 5 minutes each time at room temperature. Then membranes were incubated with appropriate secondary antibody conjugated with horseradish peroxidase for 1 hour at RT. The secondary antibody was either rabbit anti-mouse antibody (1:10000) for FABP6 or mouse anti-sheep antibody (1:10000) for FABP9 (Dako, Cambridge, UK). Afterwards, membranes were washed 3 times with 1× TBS-T, for 5 minutes each time. Then, membranes were incubated with ECL reagents (GE Healthcare, UK) for 5 minutes at RT. Finally, protein-antibody complex was visualized by exposure to Kodak XAR-5 film at room temperature. The intensity of the peak areas of the bands was measured using an Alpha Imager 2000 densitometer (Alpha Innotech, Cannock, UK). To correct for possible loading discrepancies, the membrane was incubated with β -actin mouse-antibody (1:50000) for 30 minutes in room temperature, followed by washes as previously described. Then membranes were incubated with the secondary antibody rabbit anti-mouse (1:10000) for checking β -actin expression. Protein-antibody complex was visualized as previously described.

2.3 Evaluating Protein expression in tissues using immunohistochemistry

2.3.1 Tissue samples and patient data

Human prostate cancer tissues used in this work were obtained from an archival set of prostate cancer cases that were held in the archives of the original Pathology Department and our Molecular Pathology Laboratory, as described previously (Forootan et al. 2006; Forootan et al. 2007; Jing et al. 2002; Zhang et al. 2007). Those patients who were originally diagnosed with prostate cancer but died from other causes were excluded from this study. All samples were taken from patients with an average age of 67 and 73 years who had undergone trans-urethral resection of the prostate (TURP) in the Royal Liverpool University Hospital between 1995 and 2001. This study, approved by the National Science Ethics Committee, was carried out in accordance with the Medical Research Council guidelines (project reference number: Ke; 02/019). All 35 BPH samples and 97 adenocarcinomas had been preserved in 10% (v/v) formalin and embedded in paraffin wax (Foster, Gosden & Ke 2006). Cases were re-examined independently by two qualified pathologists and the carcinomas were classified into weakly, moderately and highly malignant tissues according to their combined Gleason scores (GS) (Gleason & Mellinger 1974). PSA level were classified into 2 groups low (<10ng/ml) and high (\geq 10ng/ml) according to the initial diagnosis obtained through telepath system (Ito et al. 2003).

Materials and Methods

2.3.2 Tissue sections

Formalin-fixed paraffin-embedded tissues were pre-cooled in a box filled with ice for at least 1 hour. Tissue blocks were sectioned at 4µm thickness using microtome (MICROM, Oxford, UK). Tissues were placed on labelled Superior Adhesive Slides (Apex, Leica, UK), then placed in 37°C oven for overnight to dry out.

2.3.3 Immunohistochemistry

Immunohistochemistry is a widely used technique to diagnose abnormal cells found in malignant lesion (Ramos-Vara 2005). The principle of this technique is to detect targeted antigen (or protein) in a cell of tissue section by applying a specific antibody to the antigen in biological tissue. In this work, it was used to evaluate the expression of different FABP proteins in prostate tissues.

The resulting 4µm slide-mounted tissues were deparaffinised in 2 changes of xylene for 5 minutes, rehydrated in 2 changes of 100% ethanol for 5 minutes each. To block endogenous peroxidase, slides were transferred to 3% hydrogen peroxide (H₂O₂) in methanol for 12 minutes. Then sections were rinsed in running tap water for 1 minute. Antigen retrieval was applied to the sections by incubating all slides in ethylene diamine tetra acetic acid (EDTA) solution in the microwave for 15 minutes for FABP9. For FABP6, antigen retrieval was performed by microwaving tissues slides in 10 mM Sodium Citrate buffer for 15 minutes. Slides were allowed to cool down for 15 minutes, then rinsed in running tap water and TBS-T. Slides were transferred to sequenza cassette in order to apply the primary antibodies. They were incubated with 100µl primary anti-FABP9 antibody (1:500 sheep polyclonal FABP9 antibody, R&D Company) or with

Materials and Methods

100µl anti-FAB6 antibody (1:50 rabbit polyclonal FABP6 antibody, Sigma-Aldrich, UK) at room temperature for 1 hour and washed with TBS-T 3 times (**Table 2.3**). Then incubated with 100µl rabbit anti-sheep IgG linker or 100µl mouse anti-rabbit IgG linker (Vector Laboratories, Burlingame, CA, USA) for 30 minutes followed by 3 washes with TBS-T. To detect bound antibodies, 200µl of EnVision™ FLEX/HRP (DakoCytomation, Ely, UK) was applied to the slides and incubated for 1 hour followed by 3 washes with TBS-T solution. Sections were incubated with 100µl Envision™ FLEX DAB+ chromogen mixed with Envision™ FLEX substrate (1 drop/ml) (Dakocytomation, Ely, UK) to visualize and stained each section for 15minutes and rinsed in distilled water for 5 minutes. All sections were counterstained with hematoxylin (BWR international, London, UK) for 2 minutes, followed by rinsing in 1% acid-alcohol and blue Scott water. Finally, slides were rinsed in 3 changes of xylene and mounted with cover slip using DPX mountant (Sigma-Aldrich LTD, UK).

Table 2. 3: Primary antibodies used for immunohistochemistry staining.

Targeted protein	Primary antibody
FABP6	1:50 rabbit polyclonal FABP6 antibody, Sigma-Aldrich, UK
FABP9	1:500 sheep polyclonal FABP9 antibody, R&D Company

Materials and Methods

2.3.4 Controls for immunohistochemistry

According to the literature and manufacturer recommendations normal kidney tissue and breast cancer tissue were used as positive controls for FABP9 and FABP6, respectively. For negative control, all staining steps were applied to the same sample except the step of adding primary antibody to confirm the efficiency of immunohistochemistry staining and the specificity of the antibodies. Isotype controls were not included as all primary antibodies used were polyclonal antibodies.

2.3.5 Scoring immunoreactivity

A standard light microscope ($\times 400$) was used to evaluate cytoplasmic and nuclear expression of FABP6 and FABP9. Immunoreactivity was examined independently by two qualified observers. In case of different scoring between 2 observers, a joint evaluation needed to re-examine the slides that had different result by using multiple-headed microscope (Nikon). The slides were scanned with ScanScope (Aperio Technologies) image scanner. The intensity of cytoplasmic staining was assessed by scoring 10 fields at $40\times$ magnification and was classified into 4 categories: unstained, weakly, moderately and strongly stained which were expressed as 0 (-), 1 (+), 2 (++) and 3 (+++), respectively. Nuclear staining was assessed by the intensity of staining which was expressed as 0 (-), 1 (+), 2 (++) and 3 (+++). The differences in scoring categories between 2 observers were $<5\%$.

2.4 Molecular Biology

2.4.1 RNA Interference:

RNA interference (RNAi) is a natural process that can knock down the expression of targeted gene with high specificity. Small or short interfering RNA (siRNA) is the most commonly used RNAi tool which is a class of double-stranded RNA molecule, 20-25 base pairs in length. siRNA is a synthetic RNA interfering with the expression of specific genes with complementary nucleotide sequence for validating gene function. siRNA tool can also be used for drug targeting in the post-genomic era. Gene knockdown by transfection of exogenous siRNA is often unsatisfactory due to that the effect of transfection is only transient. Therefore, siRNA sequence is modified introducing a short loop between two strands to create a short hairpin RNA (shRNA), which can be processed into a functional siRNA by dicer in its usual fashion.

2.4.2 Designing siRNA sequences

Bioinformatics & Research Computing software (Whitehead siRNA selection program) was used to choose three different target sequences as shown in **Table 2.4**. Specificity of these sequences to FABP9 was confirmed by Blast search. Sequences were ordered from Ambion (by Life technologies, USA).

Materials and Methods

Table 2. 4: FABP9 target and siRNA sequences. Highlighted regions are targeted by siRNAs against FABP9 gene.

Position	FABP9 siRNA probes
Sequence 1 (173-195)	Sense 5' CAGGACACUAAGAUCUCCUUU mRNA TCCAGGACACTAAGATCTCCTC Antisense 5' AGGAGAUCUUAGUGUCCUGUU
Sequence 2 (102-124)	Sense 5' GGUUAGUGAAACCGACAGUUU mRNA A GGGTTAGTGAAACCGACAGTAA Antisense 5' ACUGUCGGUUUCACUAACCUU
Sequence 3 (268-290)	Sense 5' CUCAAUGAUUCACGUCCAAUU mRNA GGCTCAATGATTCACGTCCAAA Antisense 5' UUGGACGUGAAUCAUUGAGUU

2.4.3 Transient Transfection

First, 1×10^5 of PC3-M cells was plated into each well in 6-well plates 24 hours prior to transfection to reach 30 to 60% confluency. Medium was changed on the day of transfection. X-tremeGENE siRNA transfection reagent (Roche, Germany) was diluted with serum-free Opti-MEM I Medium (Gibco, Invitrogen, Paisley, UK) (without antibiotics or fungicides) and the optimal dilution of transfection reagent per ml was 47.5ul of Opti-MEM medium plus 2.5ul X-TremeGENE transfection reagent (total 50ul/ml) added directly into the medium without allowing contact with the walls of plastic tubes. Mix cautiously by pipetting up and down. Also in a separate tube, 2µg/well of siRNA was diluted with Opti-MEM I medium to a final volume of 100µl. After 15 minutes' incubation in room temperature, diluted siRNA should be combined with X-tremeGENE siRNA Transfection Reagent within 5 minutes and mix cautiously

Materials and Methods

by pipetting up and down. Silencer® Negative Control siRNA (Ambion, Inc., USA) was also diluted to use as scrambled RNA (control). The transfection reagent and siRNA complex was incubated for 15 minutes at 25 °C. The complex was then added to certain wells. The complex was overlaid drop-wise to the cells, and swirl the wells cautiously to ensure distribution over the entire surface. Dishes were incubated in normal cell culture conditions for 48 hours. Proteins from cells were extracted and the concentration was measured by Bradford dye assay in different time points and expression of FABP9 was assessed in extracts. The most efficient siRNA was used to design shRNA for stable transfection in the most appropriate incubation time.

2.4.4 Designing short hairpin RNA (shRNA) sequences

siRNA Wizard™ Software (InvivoGen, USA) was used to choose the highest suppression level for FABP9 (sequence 3). Specificity of these sequences to FABP9 was confirmed by Blast searching. Sequences were ordered from Ambion (by Life technologies, USA) as shown in **Table 2.5**. The restriction enzymes that were used are HINDIII (AAGCTT) and Acc651 (GGTACC).

Materials and Methods

Table 2. 5: Sequences of shRNAs targeting FABP9 and the negative control. shRNAs are capable of DNA integration and consist of two complementary 19–22 bp RNA sequences linked by a short loop of 4–11 nt (highlighted) similar to the hairpin found in naturally occurring miRNA.

shRNA Sequences (oligos)		
FABP9 Sequence 2 (102-124)	Sense:	5' GTACCTC GGTTAGTGAAACCGACAGT TCAAG AGACTGTCGGTTTCACTAACCTTTTTGGAAA 3'
	Antisense	5' AGCTTTTCCAAAAA GGTTAGTGAAACCGACA GTCTCTTGA ACTGTCGGTTTCACTAACCG GAG 3'
Scramble	Sense	5' GTACCTC GGTCAATTACGGGCTAGAAT TCAAG AGTTCTAGCCCGTAATTGACCTTTTTGGAAA 3'
	Antisense	5' AGCTTTTCCAAAAA GGTCAATTACGGGCTAG AACTCTTGATTCTAGCCCGTAATTGACCGAG 3'
FABP9 Sequence 3 (268-290)	Sense	5' GTACCTC CTCAATGATTCACGTCCAAT TCAAG AGTTGGACGTGAATCATTGAGTTTTTGGAAA 3'
	Antisense	5' AGCTTTTCCAAAAA CTCAATGATTCACGTCC AACTCTTGATTGGACGTGAATCATTGAGGAG 3'
Scramble	Sense	5' GTACCTC GCAGACCTTCCCATATAAT TCAAG AGATTATATGGGAAGGTCTGCTTTTTGGAAA 3'
	Antisense	5' AGCTTTTCCAAAAA GCAGACCTTCCCATATAA TCTCTTGA ATTATATGGGAAGGTCTG CAG 3'

2.4.5 Molecular cloning

2.4.5.1 Cloning silencing sequences into vector

psiRNA is specifically designed for cloning (Figure 2.1). Therefore, psiRNA-h7SKGFPzeo plasmid (InvivoGen, USA) was chosen to perform the vector based approach of shRNA.

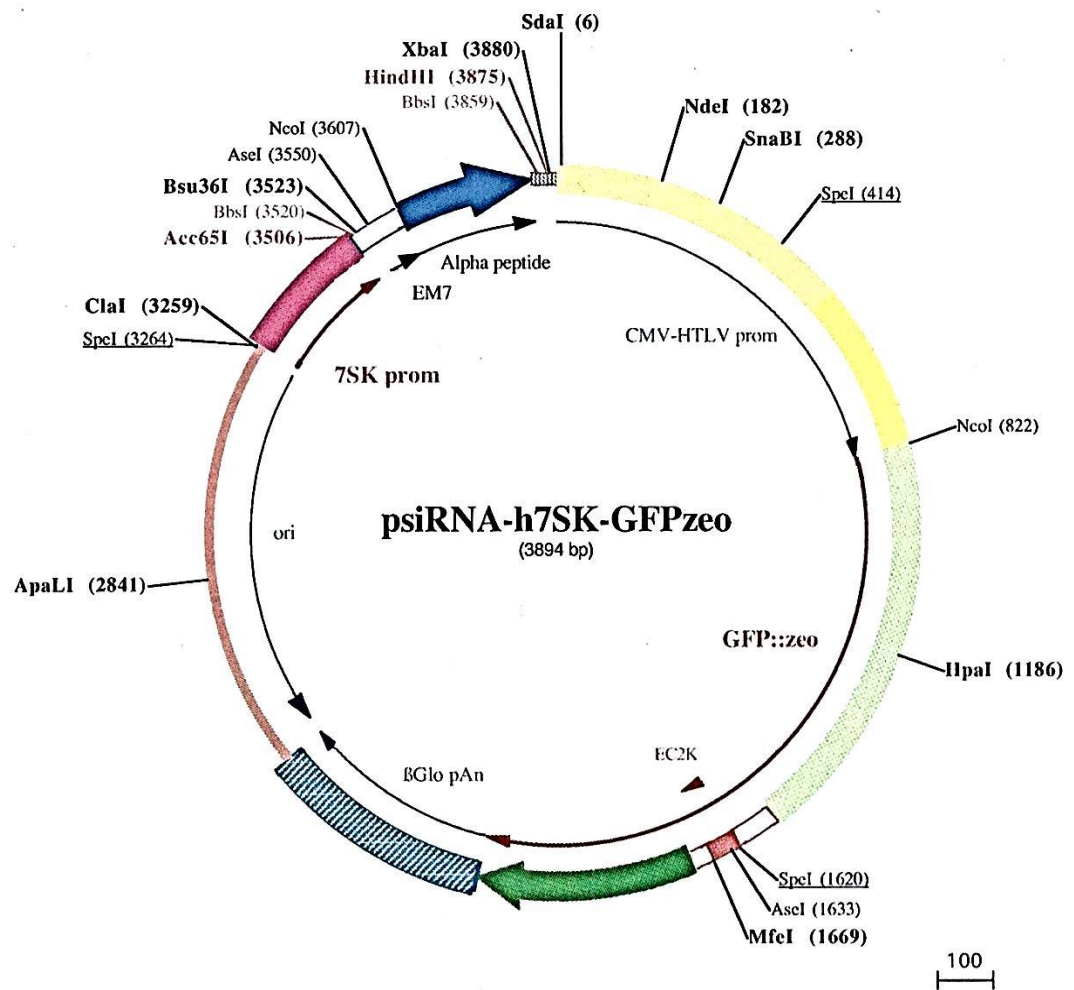


Figure 2. 1: psiRNA-h7SK-GFPzeo vector map.

Materials and Methods

2.4.5.2 Annealing sense and anti-sense oligos

Forward and reverse oligonucleotides were dissolved at a concentration of 100 μ M (stock concentration), and were more diluted to 25 μ M (working concentration). Reaction was prepared by mixing the components shown in **Table 2.6**. The prepared reaction was incubated for 2 minutes at 80°C then stopped the heating and maintained in water bath till the temperature reached to 35°C. Annealed inserts were stored at -20°C.

Table 2. 6: Annealing reaction.

Forward oligonucleotide (25 μ M)	2 μ l
Reverse oligonucleotide (25 μ M)	2 μ l
NaCl (0.5M)	6 μ l
H ₂ O to a final volume of	30 μ l

2.4.5.3 Competent bacteria cells preparation

The most compatible strain with hairpin harbouring plasmids is *E. coli* GT116 strain. Therefore, in this preparation *E. coli* GT116 was used for transformation. First *E. coli* GT116 was kept on ice for 5 minutes, then 1ml of re-constitutive solution was added and kept on ice for 5 minutes. A stock solution of *E. coli* GT116 was prepared by incubating 25ml of LB medium (without antibiotic) with 25-100 μ l of re-constitutive cells. Overnight culture was aliquot in 20% glycerol, flash freezing in LN and stored in -80°C. *E. coli* GT116 glycerol stock was streaked on a LB-agar plate and incubated at 37°C. Then, a single colony was inoculated into 10ml of LB broth and incubated overnight at

Materials and Methods

37°C with shaking at 6 ×g. On the following day, 1ml of overnight grown bacteria was transferred into 100ml SOB medium containing 1ml 2M magnesium salt and incubated for 90 minutes at 37°C with shaking at 6 ×g until the OD₅₅₀ reached 0.4. After that, the culture solution was divided into 8 universals (12.5ml/universal) and placed on ice to cool down for 10 minutes, then centrifuged at 4°C in 2000 ×g for 10 minutes. The supernatants were discarded and the bacterial cell pellets were re-suspended in 8.25ml of pre-cooled RF1 buffer (100mM KCL, 50mM MgCL₂. 4H₂O and 15% v/v glycerol, PH6.8) and placed on ice for 10 minutes, then centrifuged at 2500×g for 10 minutes. The bacterial cell pellets were re-suspended in 2ml of RF2 (10mM MOPS, 10mM KCL, 75mM CaCl₂.2H₂O and 15% v/v glycerol, PH6.8). Finally, *E. coli* GT116 bacteria solution was dispensed into 1ml cryovials, flash frozen in liquid nitrogen and stored at -80°C.

2.4.5.4 Double digestion of plasmid DNA

To prepare double digestion of plasmid, psiRNA plasmid was thawed on ice, the following components was mixed in order to prepare digestion reaction (**Table 2.7**):

Table 2. 7: Double digestion reaction.

psiRNA-h7SK-GFPzeo plasmid (1µg)	2µl
Enzyme buffer	2µl
HindIII, restriction enzyme	1µl
Acc651, restriction enzyme	1µl
H ₂ O to final volume of	20µl

Materials and Methods

The digestion reaction was incubated for 1 hour at 37°C water bath then transferred to 68°C for 10 minutes to deactivate enzymes. Agarose gel analysis (0.8%) was used to validate enzyme digestion. Wizard SV Gel and PCR Clean-Up system (Promega, WI, USA) was used to purify the digested plasmid. After running DNA samples on the gel, the digested plasmid band was cut with sterile blade and transferred to sterile microcentrifuge tube and Membrane Binding Solution (10µl/10µg of gel) was added. Reaction was mixed by vortex and incubated for 10 minutes at 50-56°C. Mixed reaction was loaded in Wizard SV Mini columns and centrifuged for 1 minute in 10,000×g followed by two steps washing with 700 and 500µl of Membrane Wash Solution, which was removed by 5 minutes centrifuging at 10,000×g. Nuclease-free water (30µl) was added directly to the centre of column followed by 1-minute centrifugation at 21952 ×g . Finally, eluted DNA (plasmid) was used for ligation and kept at -20°C.

2.4.5.5 Ligation shRNA insert into psiRNA

Ligation reaction was prepared by mixing digested psiRNA-h7SK-GFPzeo plasmid, annealed shRNA (FABP9 and negative control), T₄ DNA ligase, 10× ligation buffer and H₂O according to volume shown in **Table 2.8**. The reaction was incubated at 16°C overnight.

Materials and Methods

Table 2. 8: Ligation reaction.

Digested psiRNA-h7SK-GFPzeo plasmid	1µl (100ng)
Annealed shRNA	1µl
T ₄ DNA ligase	1µl
10× ligation buffer	2µl
H ₂ O to a final volume of	20µl

2.4.5.6 Transformation

A cryovial of *E. coli* GT116 competent cells was thawed on ice. No more than 50ng of plasmid DNA (10µl) of ligation mixture was added to 200µl of competent cells, mixed gently and placed on ice for 30 minutes. Another tube containing competent cells only was used as control. The reaction was incubated at 42°C water bath for 90 seconds then placed on ice for further 2 minutes. Then 800µl of SOC (2% w/v bactotryptone, 0.5% w/v yeast extract, 10mM NaCl₂, 10mM MgCl₂, 10mM MgSO₄, 2.5mM KCl, 20mM glucose) was added and incubated for 1 hour at 37°C in a shaking incubator at 225×g. Transformed bacteria (200µl) was plated onto agar-plate containing 100µg/ml Zeocin and incubated at 37°C overnight.

2.4.5.7 Transformation efficiency

Transformation efficiency is calculated by dividing the number of successful colonies by the amount of DNA (per μg) used during transformation preparation. Transformation efficiency is affected by many factors such as procedure of transformation, plasmid size, cell genotype etc.

2.4.5.8 Plasmid DNA isolation

To isolate DNA containing shRNA sequence, colonies were picked from the plate and grown in LB broth containing Zeocin overnight at 37°C shaker. Plasmids were isolated and purified using QIAGEN plasmid preparation kits. Mini-preparation kit (QIAGEN, USA) was used for preparation of up to 20 μg plasmid DNA. Preparation of more than 20 μg , QIAGEN plasmid midi-preparation kit (QIAGEN, USA) was used.

2.4.5.8.1 Mini-preparation DNA extraction

The overnight-cultured bacteria (5ml) in LB medium containing Zeocin were harvested by centrifuging at 6800 $\times\text{g}$ for 3 minutes at room temperature. The cell pellets were re-suspended in 250 μl buffer P1 (50mM Tris-HCl PH8.0, 10mM EDTA and 100 $\mu\text{g}/\text{ml}$ RNaseA) in 1.5ml micro-centrifuge tubes and mixed by vortex. Then, 250 μl of cell lysis buffer P2 (200mM NaOH and 1% w/v SDS) and was mixed by inverting several time followed by adding 350 μl of neutralization buffer N3 (4.2M Gu-HCl, 0.9M Potassium acetate PH4.8) and mixed gently by inverting tubes several times. The mixture was centrifuged at 13,000 $\times\text{g}$ for 10 minutes and the supernatant which contained DNA was loaded into the mini-prep spin column. The column was centrifuged at 13,000 $\times\text{g}$ for 1

Materials and Methods

minute and washed by buffer PB (500µl) followed by centrifugation for 1 minute and the flow through was discarded. Then, the column washed by adding 750µl of buffer PE followed by centrifugation at 13,000×g for 1 minute and the flow through was discarded. To remove residual wash buffer, the column was centrifuged for an additional 1 minute. The column was placed in clean micro-centrifuge tube and plasmid DNA was eluted by adding 30µl of buffer EB and incubated for 1 minute at room temperature followed by 1-minute centrifugation at full speed (18928 ×g).

2.4.5.8.2 Midi-preparation DNA extraction

The overnight-cultured bacteria (200ml) were centrifuged for 15 minutes at 6800×g at 4°C and the supernatant was discarded. The pellets were re-suspended in 4ml buffer P1 in PP bottles by using vortex. The bacteria cells were lysed by adding 4ml of buffer P2 and mixed gently by inverting the bottles 4-6 times and incubated for 5 minutes at room temperature. Then, buffer P3 (4ml) was added and mixed gently by inverting several time and placed on ice for 5 minutes. The mixture was centrifuged at 16,000×g for 30 minutes at 11°C. After transferring to clean tube, plasmid DNA was further centrifuged for 30 minutes at 20,000×g, 11°C. The supernatant was loaded into a Qiagen-tip 100 and equilibrated by adding 4ml of equilibration buffer QBT and allowed to empty by gravity flow. The column was washed twice with 10ml of buffer QC and plasmid DNA eluted by adding 5ml of elution buffer QF. Then, 3ml of isopropanol was added to the eluted DNA and mixed followed by 30 minutes' centrifugation at 11,000×g, 4°C. The supernatant was carefully removed and DNA pellet was washed by adding 2ml of 70% alcohol and centrifuged at 20,000×g for 15 minutes. The DNA pellet was air-dried for

Materials and Methods

10 minutes then dissolved in 200µl of nuclease-free water. The quality and concentration of DNA was assessed by NanoDrop spectrophotometer and the isolated plasmid DNA was kept at -20°C.

2.4.5.8.3 Agarose gel analysis

After DNA isolation, the presence of shRNA inserts in the plasmid is assessed by few techniques. One is to digest the plasmid and apply the digested DNA to agarose gel electrophoresis and visualized the presence of insertion. Here, the plasmid DNA was double digested (2.5.1.5.4). The agarose concentration of the gel was 0.8% according to DNA size. Then a 0.8% agarose gel was prepared in 200ml of 0.5× TBE buffer using microwave to dissolve the agarose until the temperature reached to 50°C. When the temperature reached to 50°C, safe view (Nucleic Acid Stain) (NBS Biological Ltd., Cambridgeshire, UK) was added to agarose mixture for visualization, then poured in cassette and placed at 4°C to solidify. Samples were loaded in the gel and undigested plasmid was used as a control. The gel was run at 80v in 0.5×TBE buffer for 1 hour and UV light was used for visualization digested bands.

2.4.5.8.4 Sequencing analysis

Plasmid psiRNA-h7SK-GFPzeo sequences were sent to Beckman Coulter Genomics, UK for sequencing. Plasmid primers that were used are: [(forward):5'CGATAAGTAACTTGACCTAAGTG3']; (reverse):5'GCGTTACTATGGGAACATAC3']. Specificity of these sequences was confirmed by Genbank Blast search.

2.4.6 Stable Transfection

1×10^5 of PC3-M cells were seeded per well in 6-well plates 24 hours prior to transfection to allow them to reach the confluency of 30-60%. PC3-M was stably transfected with vectors containing different FABP9 shRNAs using X-tremeGENE HP DNA Transfection Reagent (Roche, Germany). 1 μ l X-tremeGENE HP DNA Transfection Reagent was diluted in 99 μ l of Opti-MEM I medium (Gibco, Invitrogen, Paisley, UK) to a final volume of 100 μ l. 4 μ g of psiRNA with inserted shRNA was added to the diluted Opti-MEM I medium and mixed gently then incubated at room temperature for 15 minutes. In a separate transfection, a vector containing scrambled RNA was used as a control. The mixture was added dropwise to the wells of 6-well plates and distributed by rocking the plate back and forth. The 6-well plates were incubated in standard growth conditions for 48 hours. Then, the medium was replaced with those containing ZeocinTM (100 μ g/ml) (Invitrogen, by Life technology, USA) and changed every three days until control transfection cells died out and healthy cell clones had formed in the plate (3-4 weeks). The surviving transfected cells were distributed into 9cm cell culture plates with selective medium ZeocinTM for two weeks.

2.4.6.1 Ring cloning of transfected cells

Ring cloning technique was used to isolate five single colonies to establish FABP9-suppressed PC3-M transfectants and the level of FABP9 in these transfectants was measured by Western blot analysis as shown in section (2.3). Single colonies were selected and isolated by using sterilized 8 \times 8 mm cloning rings (Sigma-Aldrich LTD, UK), forceps and Dow Corning[®] high-vacuum silicone (Sigma-Aldrich LTD, UK).

Materials and Methods

Using forceps, cloning rings were dipped in silicon grease and placed in over the colony. The small amount of grease used was to form a watertight seal around the colony and to stick to the plate. Then, 100µl of 2.5% trypsin/versine (v/v) was added to the colony and incubated for 4 minutes to detach the cells. Equal amount of routine cell culture medium was added to deactivate the trypsin and centrifuged for 3 minutes at 91 ×g. The pellets were re-suspended in 1ml selective medium and grown in 24-well plate in normal cell culture conditions.

2.5 In-vitro assays

2.5.1 MTT cell proliferation assay

The increased proliferation is one of cancer cell characteristics which can predict the potential of tumorigenicity and metastasis. Therefore, MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) cell proliferation assay was used to measure the cell proliferation rate. The linear relationship between cell number and signal production was established for each cell line to allow an accurate quantification of changes in the rate of cell proliferation.

2.5.1.1 Growth curve preparation

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is a yellow chemical reagent has the ability to enter the mitochondria of cells and converted into blue dye by oxidative enzymes forming so-called formazan crystal in cell cytoplasm. Formazan crystals are dissolved by adding DMSO and the intensity of blue solution is directly proportional to the number of the cells. Optical density (OD) of dye will be measured at 570nm.

A standard curve was established for each cell line by preparing a serial dilution for both parental cells and transfectants at a density of: 6.25×10^3 /ml, 1.25×10^4 /ml, 2.5×10^4 /ml, 5×10^4 /ml, 1×10^5 /ml, 2.5×10^5 /ml, 5×10^5 /ml cells per well in 96-well round bottom plate in triplicate. Then, cell suspension containing 6.25×10^4 /ml was prepared and 1.25×10^4 (200 μ l) and incubated in 96-well plate in triplicate to measure proliferation activity of each cell line. After that, 50 μ l of MTT solution was added to each well and incubated at

Materials and Methods

37°C, 5% CO₂ and 95% humidity. For the OD of dye will be measured at 570nm. A standard curve was established between the optical density of formazan and the number of cells. From this standard curve the cell viability of each cell line can determined.

2.5.1.2 Cell number determination

Parental cell and transfectants were seeded in 96-well plate in culture medium in triplicate at 5,000 cells per well at day zero. A MTT solution was prepared by a concentration of 5mg/ml in PBS. On days 1-6, 50µl of MTT solution was added to each well and incubated for 4 hours at 37°C, 5% CO₂ and 95% humidity. Then, 200µl of medium was removed from each well and 200µl of DMSO was added and incubated for 10 minutes. The optical density of the cell proliferation colour was measured by Multiscan plate reader at 570nm every day for 6 days. The cell viability was calculated corresponding to the standard curve.

2.5.2 Invasion Assay

Tumor invasion and metastasis are hallmarks of tumour malignancy. The Boyden chamber system has been used to assess different invasion aspects of cancer cells. The principal is that cancer cells migrate along a chemoattractant gradient from an upper compartment into the lower compartment. Many factors can affect the results such as cells number, the duration of the assay and measurement methods. The BD BioCoat™ Growth Factor Reduced (GFR) Matrigel™ Invasion Chamber (BD Biosciences, USA) is

Materials and Methods

a solubilized tissue basement membrane preparation, contains collagen type IV, laminin, growth factor, heparin sulphate proteoglycan and entactin.

PC3-M cells and transfectants were cultured until 70-80% confluent. Twenty-four hours prior to performing the assay, cells were starved (grow in serum free medium) at 5×10^4 cells/ml. In the meantime, the invasion chambers were brought out and allowed to come to room temperature and dehydrated by adding 0.5ml of warm medium and incubated in a humidified incubator at 37°C , 5% CO_2 for 2 hours. Then, medium was removed carefully without disturbing the GFR Matrigel layer. Cells (2.5×10^4) were seeded in the upper compartment of chambers in 500 μl of growth medium while 1ml of routine medium was loaded in lower compartments. As a negative control, serum free medium was placed in the lower compartments. All cell lines were set as triplicate and assays was incubated at 37°C , 5% CO_2 . After 24 hours, cotton swabs were used to remove the remained cells in upper compartments and washed twice with PBS. Finally, cells migrated to the lower side were fixed, stained with 2% crystal violet for 10 minutes and washed with water several times and left to dry at 37°C . The number of invading cells was counted using a light microscope (Leitz, Labovet, Luton, UK) at 124 \times magnification. Olympus digital camera (Olympus C-4040) was used to take photographs.

Materials and Methods

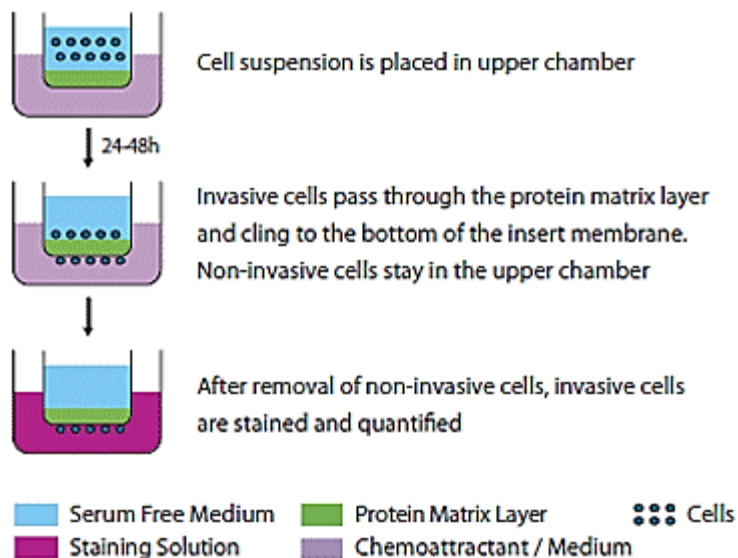


Figure 2. 2: Principle of Invasion assay performed in chamber with 8µm pore size which coated with Matrigel matrix.

2.5.3 Soft Agar Assay

Anchorage-independent growth is considered to be a hallmark of cell transformation and carcinogenesis. Soft agar colony formation assay is one of the most common *in vitro* techniques for malignant transformation in cells to monitor anchorage-independent growth, which measure proliferation in semisolid matrices. Therefore, this assay was designed in order to examine the tumorigenicity of each cell line in anchorage-independent environment.

To perform the assay, 6-well plates were pre-coated with 2ml of 2% (w/v) low melting agarose gel in routine and selective culture medium with 10% FCS and the mixture solidified in refrigerator at 4°C for 20 minutes. Parental PC3-M, PC3M-FABP9-siM and PC3M-FABP9-siH cells were routinely grown to 70-80% confluency, detached,

Materials and Methods

harvested and adjusted with routine medium to 5000 cells/well. Then, 1ml of cells suspension of each cell line (5000 cells/ml) was mixed with 1ml of 1% low melting point agarose gel and placed on top of the pre-coated wells. Complexes were placed in 4°C for 10 minutes for solidification. Then, the 6-well plates were placed in the incubator at 37 °C, 5% CO₂ for 4 weeks. In the meantime, cells were feed with routine culture medium 200ul/well once a week. At the end of assay, colonies were stained by adding 0.5ml of 2% MTT (5mg/ml) (3-(4, 5-dimethylthiazl-2-yl)-2, 5-diphenylterazolium bromide) then incubated at 37°C, 5% CO₂ for 4 hours. Colonies larger than 150um were counted using Gel Count (Oxford Optronix, UK).

2.5.4 Wound healing assay

Wound healing assay is one of the most common in *vitro* techniques to study cell migration. This method is based on the observation that so-called scratch on a confluent cell monolayer. After creating a scratch, the cells on the edge of the artificial gap will migrate toward the gap to close the scratch until cell-cell contacts are observed. To determine the rate of cell migration, images are captured at the beginning and regular intervals during cell migration. Captured images are compared using Image J to determine migration rate.

Parental PC3-M cells, PC3M-FABP9-siM and PC3M-FABP9-siH were seeded in 6-well plate and cultured until 75-80% confluent. Then, yellow pipette tip was used to make a straight scratch, creating a wound. The pipette tip was kept under an angle of around 30 degrees to keep the scratch width limited. The positions were selected to take photos and 3 images were taken per well at 0 hour. After that, the migration was monitored by taking images at 6 hours, 12 hours and 24 hours.

2.6 Statistical analysis

In all statistical analysis, if the p value is less than 0.05 (Two-sided test) the results were regarded as significant.

2.6.1 Statistical Package for Social Sciences (SPSS)

The analysis was performed using the Statistical Package for Social Sciences (SPSS) version 20 (SPSS Inc., Chicago, IL., USA).

2.6.2 Chi-square (χ^2)

Chi-square (χ^2) test was used to assess whether the differences between the expected frequencies and the observed frequencies is significant or not. Correlation between FABP6 and FABP9 expression and the benign and malignant prostate tissues were assessed by χ^2 test.

2.6.3 Survival analysis

2.6.3.1 Kaplan-Meier curve

Kaplan Meier plots were used to separate survival times of patients with differentially stained samples for individual factors. In this study Kaplan Meier plots were used to assess the correlation between survival and expression of FABP6, FABP9, Gleason score, PSA level, AR level.

2.6.3.2 Log Rank test

The significance association between survival time and expression of FABP6 or FABP9 was assessed by Log Rank test. It is a nonparametric test and appropriate to use when the data are right skewed and censored (technically, the censoring must be non-informative).

2.6.3.3 Mann-Whitney U test

Man-Whitney U test is a non-parametric test that is used to compare two sample mean ranks, when the variable is either ordinal or continuous, but not normally distributed. The correlation between the expression of FABP9 and different markers was assessed using Box plot analysis and Mann-Whitney U test.

2.6.3.4 Student t-test

Student's t-test was used to evaluate the significant difference in average between two groups and is most commonly applied when the test statistic would follow a normal distribution. In this study, Student's t-test was used to compare any observed difference between experimental and control groups in Western blot, proliferation assay, invasion assay, soft agar assay. A value of $p \leq 0.05$ was used to define the statistical significance.

CHAPTER THREE: RESULTS

3. Results

3.1 Measurements of levels of FABP mRNAs in prostate cell lines

3.1.1 The quality and integrity of the total RNAs

The relative levels of FABPs levels in six different cell lines were measured by quantitative RT-PCR. Total RNAs for each cell line were isolated and purified by RNAeasy Mini column. The total RNA yield and purity was determined by NanoDrop ND-1000 spectrophotometer. The purity of the sample was also assessed using ratio of absorbance at 260/280nm. The optimal rang of ratio were:

-260/280 ratio: 1.8-2.0 was generally accepted as pure DNA.

-260/280 ratio: 2.0-2.1 was generally accepted as pure RNA.

The quality and integrity of the mRNA were assessed using RNA 6000 Nano LabChip kit on Agilent 2100 Bio-analyzer because poor quality RNA can affect subsequent reactions leading to erroneous results in the expression results. The 2100 Expert Software and visual output was used to analyze the results to confirm that the total RNA was in high quality by showing two clear bands representing both rRNA 18S and 28S sub-units as shown in **Figure 3.1**. The high RNA integrity number (RIN) of each isolated RNA (at least 9.0 out of 10) showed that the total RNA was intact and no degradation was observed (**Figure 3.2**).

Results

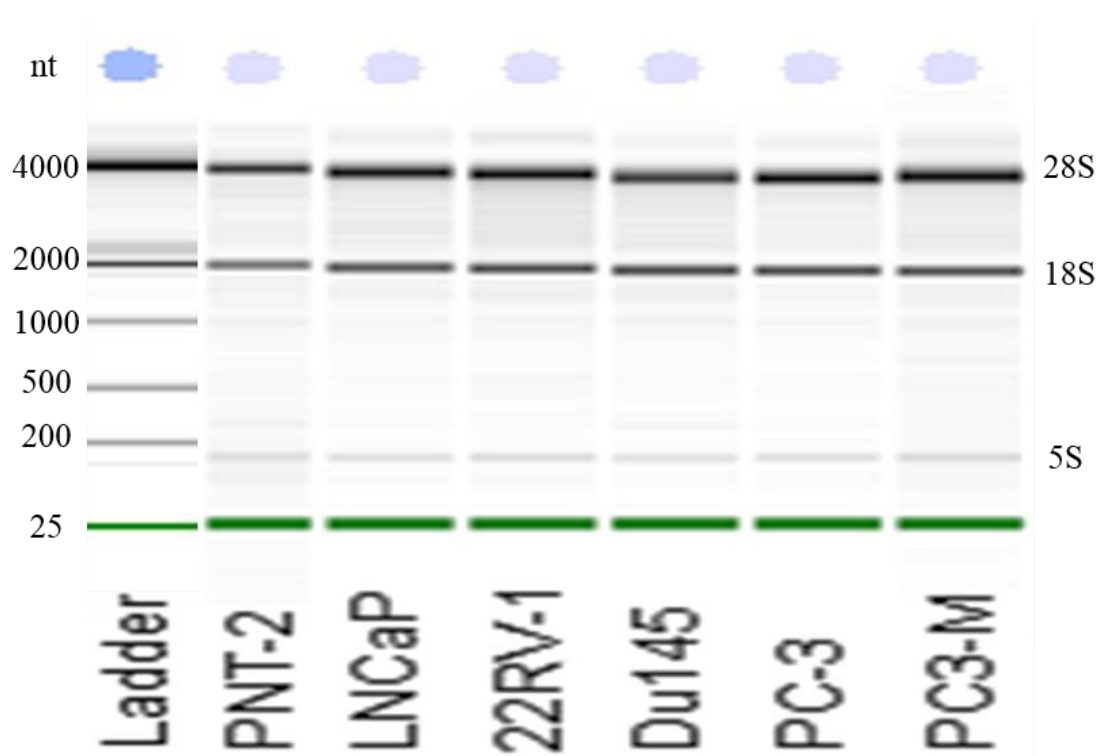


Figure 3. 1: The quality and quantification of total RNA were assessed using the RNA 6000 Nano LabChip on Agilent 2100 bioanalyzer.

The electropherograms of the gel-like images of mRNAs from 6 cell lines and from the RNA ladder (marker). The gel electropherogram images for different benign and malignant cell lines were shown in this figure.

Results

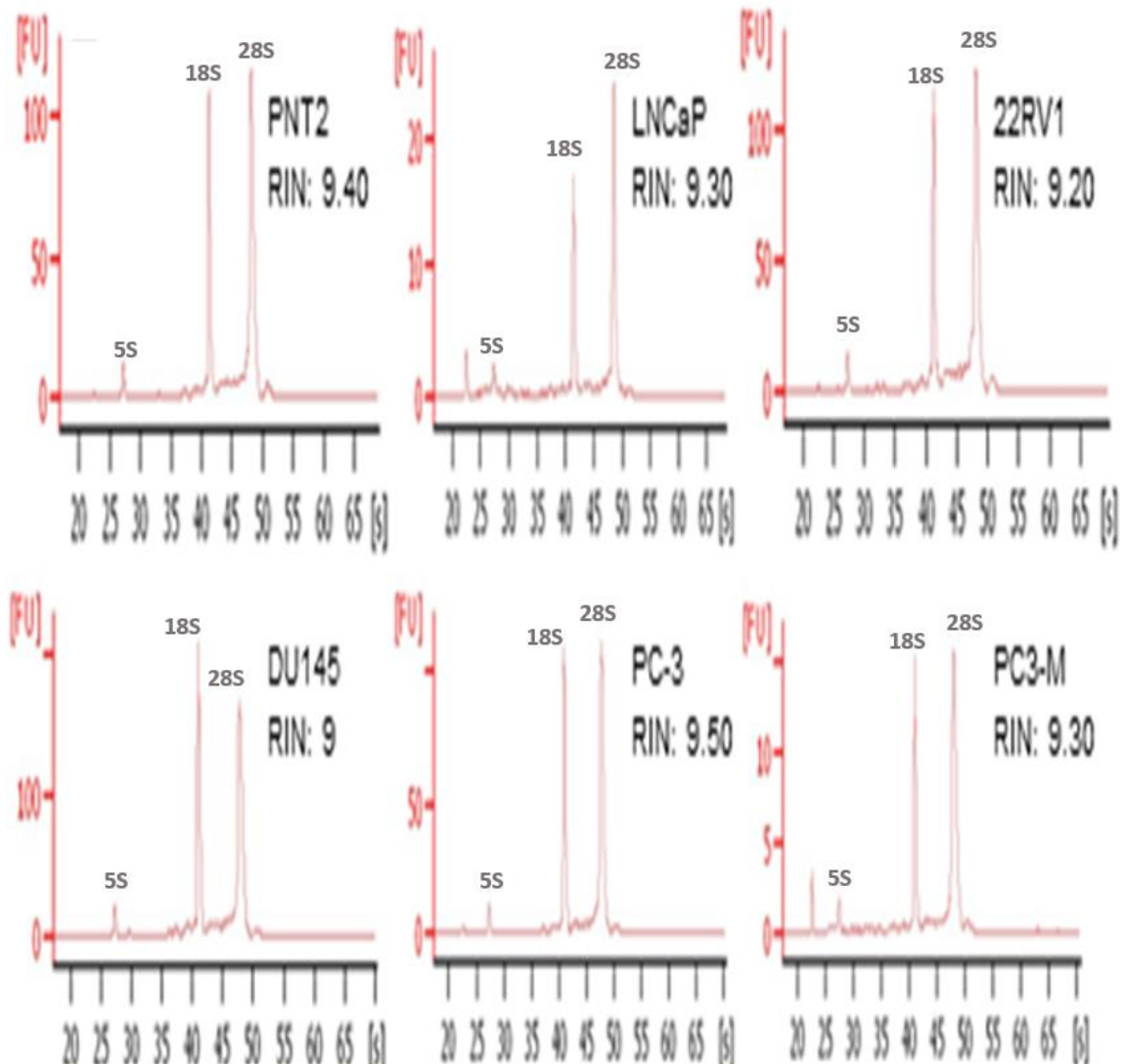


Figure 3. 2: The quality and quantification of total RNA were assessed using the RNA 6000 Nano LabChip on Agilent 2100 bioanalyzer.

The double peaks representing both rRNA 18S and 28S sub-unites and the RNA integrity numbers (RIN) of the samples from different cell lines. The fluorescence plots with two peaks of 18S and 28S ribosomal RNAs of each sample were shown in panel B. The qualities of RNAs were presented by RINs which were calculated from the plots.

3.1.2 Relative levels of mRNAs of all *FABPs* in prostate cells

Messenger RNAs of 10 different FABPs were isolated, their relative levels were analysed by quantitative RT-PCR between the benign PNT-2 cells, and compared and the 5 malignant prostate cell lines (LNCaP, 22RV-1, Du145, PC-3 and PC3-M) and the results are shown in **Figure 3.3**.

3.1.2.1 Expression Pattern of FABP1, FABP2, FABP3, FABP7 and FABP8

Assessing transcription profiles between the benign and malignant cells showed that FABP1, FABP2, FABP3, FABP7 and FABP8 exhibited no unified patterns in mRNA levels between benign and malignant cell lines. Thus, no clear differences in their mRNA levels were identified when compared with those in the benign cells. Therefore, FABP1, 2, 3, 7 and 8 were excluded from any further studies.

3.1.2.2 FABP4

Assessing transcription profiles between the benign and malignant cells showed that FABP4 mRNA exhibited clearly higher level in 2 of the 5 malignant prostate cells PC-3 and PC3-M cells, but its expression in other prostate cells including benign PNT-2 cells was not detectable. The expression of FABP4 was one of the predominant FABP family members in prostate cells when compared with all of the other FABPs.

Results

3.1.2.3 FABP5

Expression level of FABP5 was elevated significantly in all of prostate cancer cell lines LNCaP, 22RV-1, PC-3 and PC3-M when compared with the benign PNT-2 cells. PC-3 and PC3-M, examples of highly aggressive prostate cancer cells, showed higher levels of FABP5 when compared with the other prostate cancer cell lines as shown in our group previous work (Jing et al. 2000).

3.1.2.4 FABP6

RT-PCR analysis of 6 different prostate cell lines showed a marked level of FABP6 mRNA expression in malignant cells compared with no expression in the benign cells PNT-2. Notably, FABP6 expression was significantly higher in prostate cancer cells than those detected in the benign cells PNT-2. In contrast, the expression of FABP6 in metastatic to brain was lower than the level obtained with other malignant cells. Therefore, FABP6 was found to be another predominant FABP family members in prostate cells when compared with all of the other FABPs at mRNA level.

3.1.2.5 FABP9

Gene expression of FABP9 in malignant cell lines was 5-47 fold higher than that detected in the benign PNT-2 cells. PC-3 and PC3-M, examples of highly aggressive prostate cancer cells, showed higher levels of FABP9 when compared with the other prostate cancer cell lines. Thus, FABP9 was found to be one of the highly expressed FABP members in prostate carcinoma cell lines.

Results

3.1.2.6 FABP12

FABP12 mRNA level in prostate cancer cells was 3-105 fold higher than that in the benign PNT-2 cells. Notably, benign PNT-2 cells showed the lowest expression of FABP12 amongst prostate cell lines.

Results

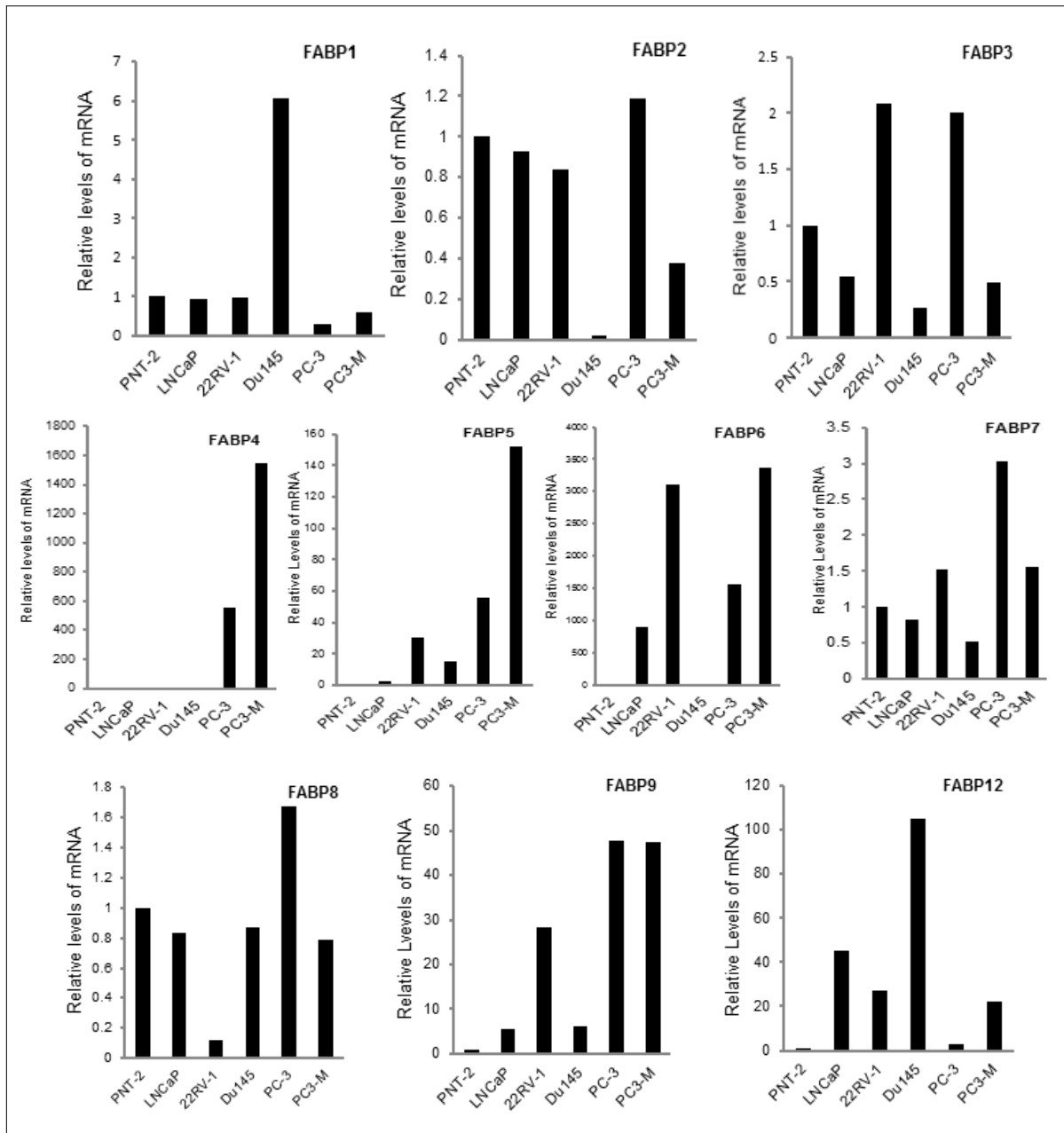


Figure 3.3 : Quantitative PCR analysis of relative levels of FABP mRNAs in benign and malignant prostate epithelial cells.

Benign cell line PNT-2, weakly malignant cell line LNCaP, moderately malignant cell line 22RV-1 and highly malignant cell lines Du145, PC-3 and PC3-M were cultured to 80% confluence and harvested for mRNA extraction.

3.2 Expression of FABP6 and FABP9 in prostate cells at the protein level

Amongst the differentially expressed *FABPs*, FABP4 was released from adipocytes, implicated in obesity (Hotamisligil et al. 1996) and its possible involvement in the malignant progression of prostate cancer has been investigated previously (Uehara et al. 2014). The crucial role of FABP5 in promoting tumorigenicity and metastasis of prostate cancer have been characterized by our group. FABP12 is the most recent member, it may play an important role in prostate cancer and thus a separate investigation has been conducted in another project to study FABP12 by a member of our team. Therefore, the main work was focused on the role of FABP6 and FABP9 in prostate cancer.

3.2.1 Expression of FABP6 protein

The Western result analysis of FABP6 in prostate cell lines is shown in **Figure 3.4**. A single FABP6 band of 14 kDa was detected in positive control breast cancer MCF7 cell line, benign cell line PNT-2, weakly malignant cell line LNCaP, moderately malignant cell line 22RV-1 and highly malignant cell lines Du145, PC-3 and PC3M (**Figure 3.4A**). When the densitometric level of FABP6 in PNT-2 is set at 1, the levels in weakly malignant LNCaP, moderate malignant 22RV-1 and highly malignant Du145, PC-3 and PC3-M were 0.86 ± 0.15 , 0.82 ± 0.30 , 2.15 ± 0.04 , 0.63 ± 0.01 and 0.49 ± 0.28 , respectively. Quantitative analysis showed that the level of FABP6 in highly malignant Du145 cells was more than 2 times than that in the benign PNT-2 cells (Student's t-test, $p = 0.01$). The levels of FABP6 in low malignant LNCaP cells, moderately malignant 22RV-1 cells, highly malignant PC-3 and PC3-M cells were lower than that in PNT-2 cells (**Figure 3.4B**) but the differences were not significant (Student's t-test, $p > 0.05$).

Results

Therefore, FABP6 appear to be overexpressed in at least one of the highly malignant cells lines.

3.2.2 Expression level of FABP9 at protein level

Western blots analysis showed that FABP9 protein was highly expressed in 2 (PC-3 and PC3-M) of the 5 malignant cell lines, but its expression in other prostate cells including benign PNT-2 cells was not detectable (**Figure 3.5A**). When the level of FABP9 was set at 1 in PC-3, the level in the most aggressive cell line PC3-M was 1.2 ± 0.04 . Thus the level of FABP9 in the highly malignant cells PC3-M was 20% significantly higher than that in PC-3 (Student's t-test, $p = 0.006$) (**Figure 3.5B**).

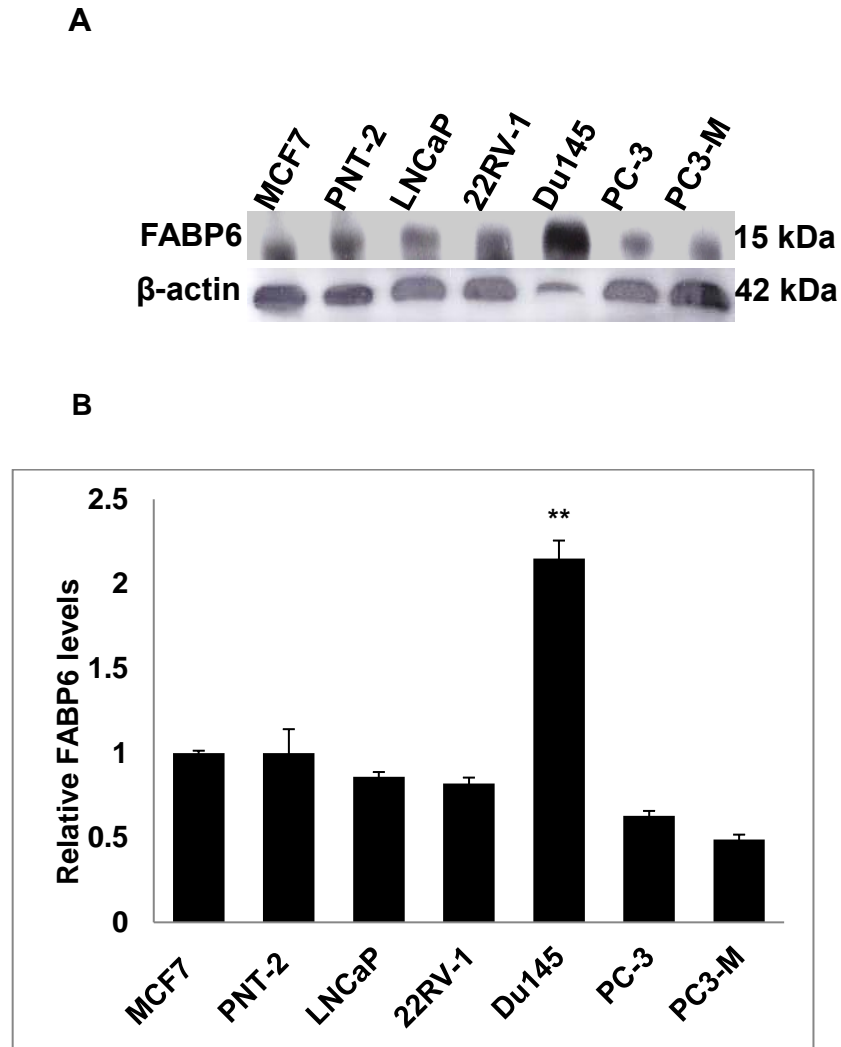


Figure 3. 4: Expression of FABP6 in benign and malignant prostate cells.

A), Western blot analysis of FABP6 in benign and malignant prostate cells. B), relative levels of FABP6 in different cell lines. The level of FABP6 in benign PNT-2 cells was set at 1.0; levels in other cell lines were obtained by relating to that in PNT-2. The results were obtained from 3 separate experiments (mean \pm SD).

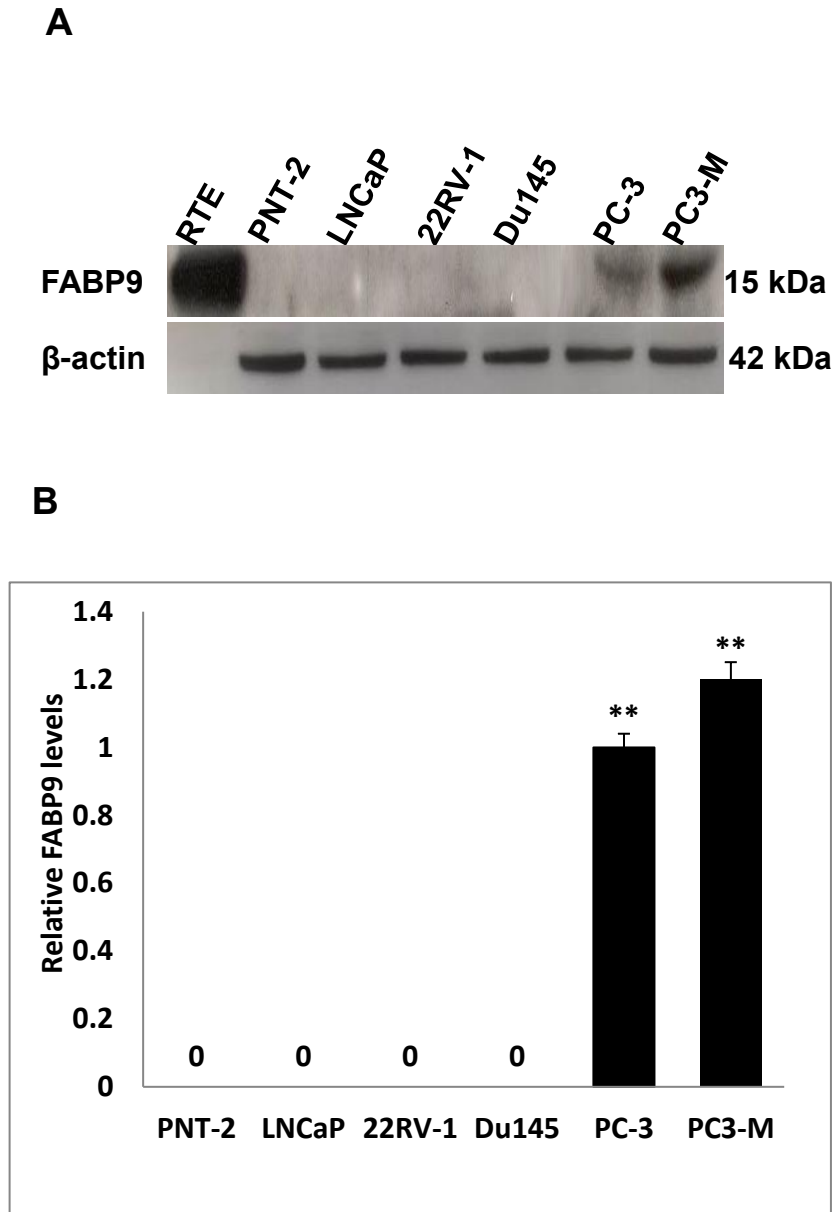


Figure 3. 5: Expression of FABP9 in benign and malignant prostate cells.

A), Western blot analysis of FABP9 in benign and malignant prostate cells. B), relative levels of FABP9 in different cell lines. Since the expression in PNT-2, LNCaP, 22RV-1 and Du145 is not detectable, their levels were set as “0”. When the level in PC-3 cells was set at 1.0, the level in PC3-M cells was obtained by relating to that in PC-3. The results were obtained from 3 separate experiments (mean \pm SD).

3.3 Expression of FABP6 and FABP9 in prostate tissues

The expressions status of FABP6 or FABP9 in 133 prostate tissue sections, including 36 BPH and 97 carcinoma tissues, was detected by immunohistological staining and representative staining patterns are shown in **Figure 3.7** and **Figure 3.8**. Some cases were excluded from the study because of technical or other reasons, and the exact numbers of cases that were used in this study presented in **Table 3.1** and **Table 3.2** is summarised in **Figure 3.6**.

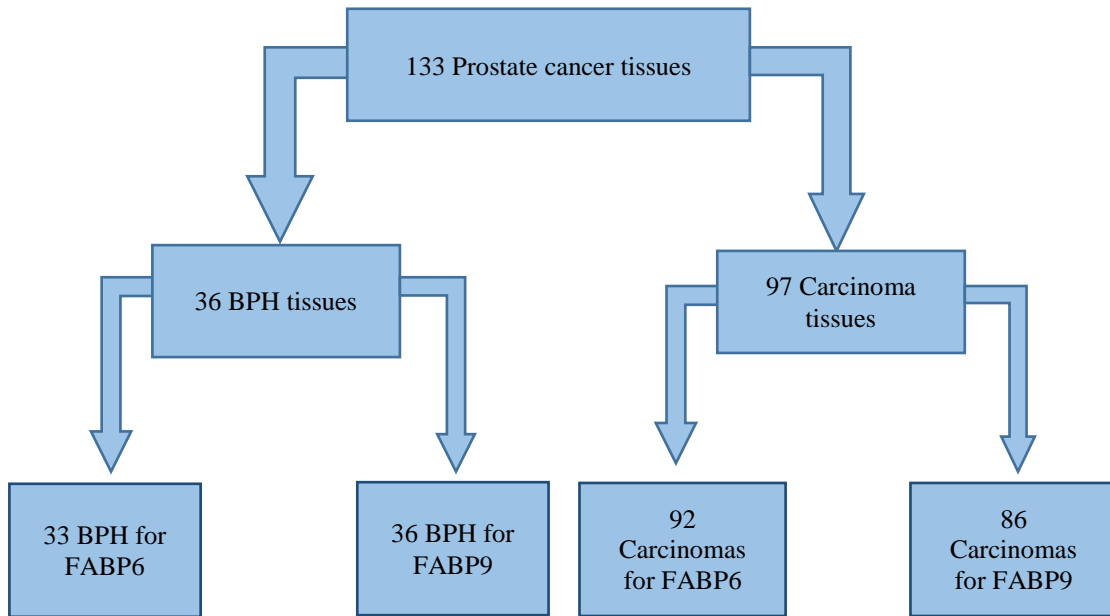


Figure 3. 6: Summary of patient tissue numbers used in this study for both FABP6 and FABP9.

3.3.1 Expression of FABP6 in prostate tissues

For anti-FABP6, staining was observed only in the nucleus of the cells (**Table 3.1**) and no cytoplasmic staining was seen. Nuclear staining was observed in all 33 BPH tissues (100%); 3 tissues (9%) stained weakly, 25 tissues (76%) stained moderately, and 5 tissues (15%) stained strongly (**Figure 3.7**). Among the 92 adenocarcinomas, 28 tissues (30%) were unstained, 11 tissues (12%) stained weakly, 23 tissues (25%) stained moderately and 30 tissues (33%) stained strongly. Although FABP6 was detected in the nucleus of the cells, the staining intensities between BPH and carcinoma tissues were not significantly different (χ^2 test, $p > 0.05$).

3.3.2 Expression of FABP9 in prostate tissues

The expression of FABP9 in both BPH and carcinoma tissues was detected in the cytoplasm (**Figure 3.8**) and no nuclear staining was seen, the results are shown in **Table 3.2**. Among 36 BPH cases 25 (70%) were unstained and 11 (30%) stained weakly. Among 86 adenocarcinomas, staining was weak in 31 (36%), moderate in 39 (45%) and strong in 16 (19%) cases (**Table 3.2**). No nuclear staining was observed in any BPH and carcinoma samples. Comparing the FABP9 expression between BPH and carcinomas, the staining intensity in carcinoma tissues was significantly stronger (χ^2 test, $p < 0.0001$).

Results

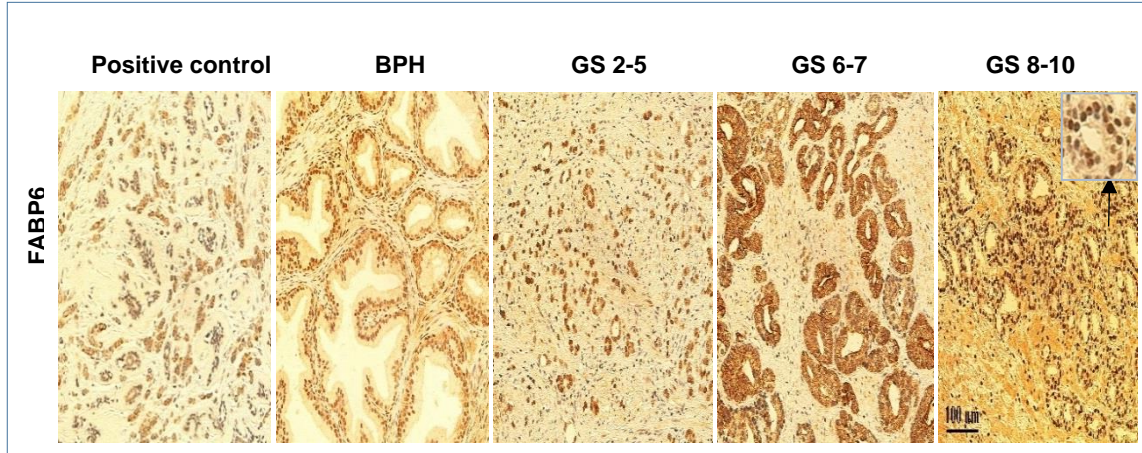


Figure 3. 7: Examples of immunohistochemical detection of FABP6 in benign and malignant prostate tissues.

Following the manufacturer instruction, breast cancer tissue was used as positive controls for FABP6 (x100). Carcinomas were divided into three categories according to their combined Gleason scores (GS): weakly malignant (or $GS \leq 5$), moderately (or GS 6-7) and high malignant (or GS 8-10) tissues.

Results

Table 3. 1: Immunohistological stains of tissues with antibody against FABP6.

FABP6	Nuclear stain intensities ^a				No. of cases
	0	+	++	+++	
Tissues					
BPH	0	3	25	5	33 ^b
Carcinomas (Total)	28	11	23	30	92 ^b
GS^c ≤5	14	0	6	3	23
GS^c 6-7	10	6	10	10	35
GS^c 8-10	5	5	7	17	34

^a Staining was observed only in the nucleus of the cells. ^b Total BPH were 36 and total carcinomas were 97, but some cases were excluded because of technical reasons. ^c Combined Gleason scores.

Results

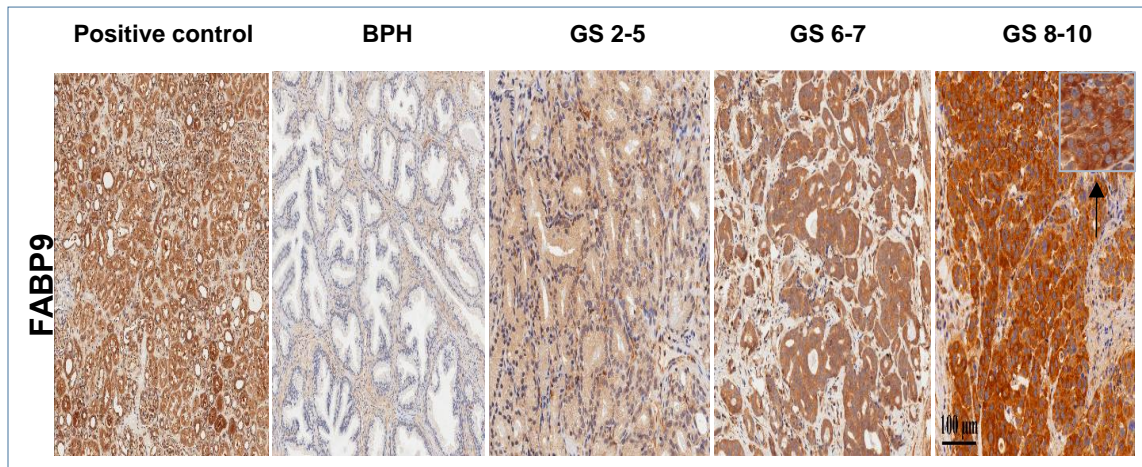


Figure 3. 8: Examples of immunohistological detection of FABP9 in benign and malignant prostate tissues.

Following the manufacturer instruction, normal kidney tissue was used as positive controls for FABP9 (x100). Carcinomas were divided into three categories according to their combined Gleason scores (GS): weakly malignant (or $GS \leq 5$), moderately (or GS 6-7) and high malignant (or GS 8-10) tissues.

Results

Table 3. 2: Expression status of FABP9 in benign and malignant prostate tissues.

FABP9	Cytoplasmic stain intensities ^a				No. of cases
	0	+	++	+++	
Tissues					
BPH	25	11	0	0	36
Carcinomas (Total)	0	31	39	16	86 ^b
GS ^c ≤5	0	22	6	0	28
GS ^c 6-7	0	7	13	5	25
GS ^c 8-10	0	2	20	11	33

^a Staining was observed only in the cytoplasm of the cells. ^b Total carcinomas were 97, but some cases were excluded because of technical reasons. ^c Combined Gleason scores.

3.4 Correlation of FABP6 and FABP9 expression to Gleason Scores

According to their GS, carcinomas were divided into 3 groups: low (GS ≤ 5), moderate (GS 6-7) and high malignant (GS 8-10), as shown in **Table 3.1** and **Table 3.2**.

Of the 23 weakly malignant carcinoma tissues, 14 (60%) no staining was observed, none of them stained weakly, 6 (26%) stained moderately, 3 (14%) stained strongly. Among the 35 moderately malignant cases with GS 6-7, 10 (28%) no staining was observed, 6 (16%) stained weakly, 10 (28%) stained moderately and 10 (28%) stained strongly. Of the 33 highly malignant cases with GS 8-10, 4 (13%) no staining was observed, 5 (15%) stained weakly, 7 (21%) stained moderately and 17 (51%) stained strongly. The intensity of FABP6 staining in highly malignant cases was significantly higher than that in low malignant cases (χ^2 test, $p < 0.00001$). A significant difference was also observed on staining intensities between low malignant cases and moderately malignant cases (χ^2 test, $P = 0.02$). When the staining intensities between the moderately malignant and the highly malignant cases was compared, the difference was not statistically significant (χ^2 test, $p > 0.05$).

Of the 28 weakly malignant carcinoma tissues, 22 (78%) stained weakly, 6 (22%) stained moderately, none of them stained strongly. Among the 25 moderately malignant cases with GS 6-7, 7 (27%) stained weakly, 13 (53%) stained moderately and 5 (19%) stained strongly. Of the 33 highly malignant cases with GS 8-10, 2 (6%) stained weakly, 20 (63%) stained moderately and 11 (31%) stained strongly. The intensity of FABP9 staining in moderately malignant cases was significantly higher than that in low malignant cases (χ^2 test, $p < 0.00001$). Highly significant difference was also observed on

Results

staining intensities between low malignant cases and highly malignant cases (χ^2 test, $P < 0.00001$). When the staining intensities between the moderately malignant and the highly malignant cases were compared, the difference was not statistically significant (χ^2 test, $p = 0.09$).

3.5 Correlations with patient survival

The level of FABP9 (**Figure 3.9**), AR index (**Figure 3.10**), PSA (**Figure 3.11**) or GS (**Figure 3.12**) and the duration of patients' overall survival time (the length of survival time from initial diagnosis until death, or until data were censored) was plotted using Kaplan-Meier survival curves and the significance of the differences was assessed by Log Rank test as follows:

3.5.1 Relationship between FABP9 and patient survival

When the correlation between patient survival time and the FABP9 staining intensity was assessed (**Figure 3.9**), the median survival time for patients with weak staining for FABP9 was 60 months which was significantly longer than those patients with moderate (24 months) and strong (18 months) staining for FABP9, respectively. Overall, the increased FABP9 staining intensity was significantly correlated with reduced patient survival time (log-rank test, $p = 0.02$).

Results

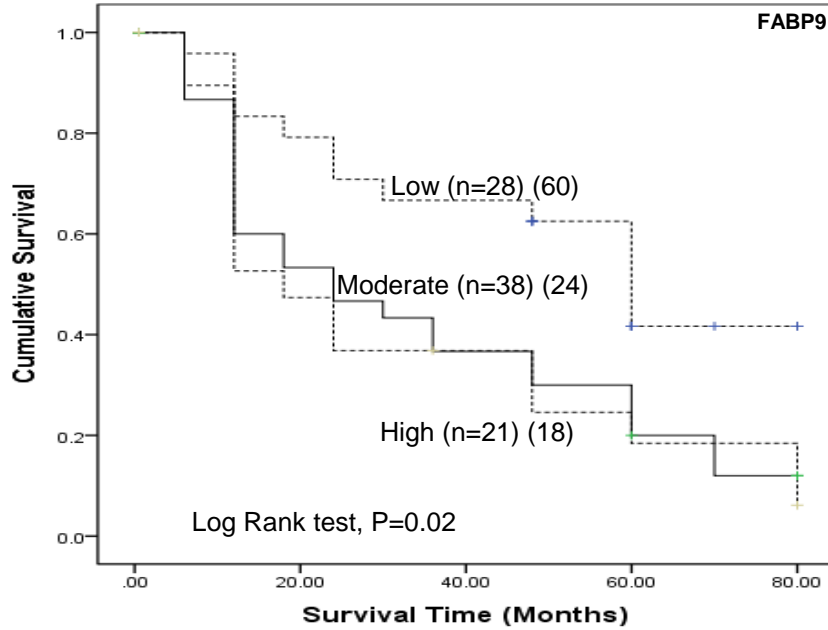


Figure 3. 9: Kaplan-Meier survival curves of patients with prostate cancer.

The cumulative survival of patients was plotted against the time in month for FABP expression. Different levels of FABP9 stains: group 1, weakly positive (n=28); group 2, moderately positive (n=38); group 3, strongly positive (n=21).

3.5.2 Relationship between AR index and patient survival

When the correlation between the patient survival time and AR index level was assessed (**Figure 3.10**), the median survival for patients with weak, moderate and strong staining for FABP9 was 60, 24 and 24 months respectively. The overall increase for the three groups in intensity of AR staining was not (but close to) significantly correlated with reduced patient survival time (log-rank test, $p = 0.052$). Although, the overall increase in intensity of AR staining was of borderline significance in correlation with reduced patient survival time, the median survival for patients with weak was significantly longer

Results

than patients with strong staining for FABP9 (log-rank test, $p = 0.031$). Also, significant difference was observed between the median survival for patients with weak was significantly longer than patients with moderately staining for FABP9 (log-rank test, $p = 0.041$). However, the difference between patients with moderately and strong staining for FABP9 was not statistically significant (log-rank test, $p > 0.05$).

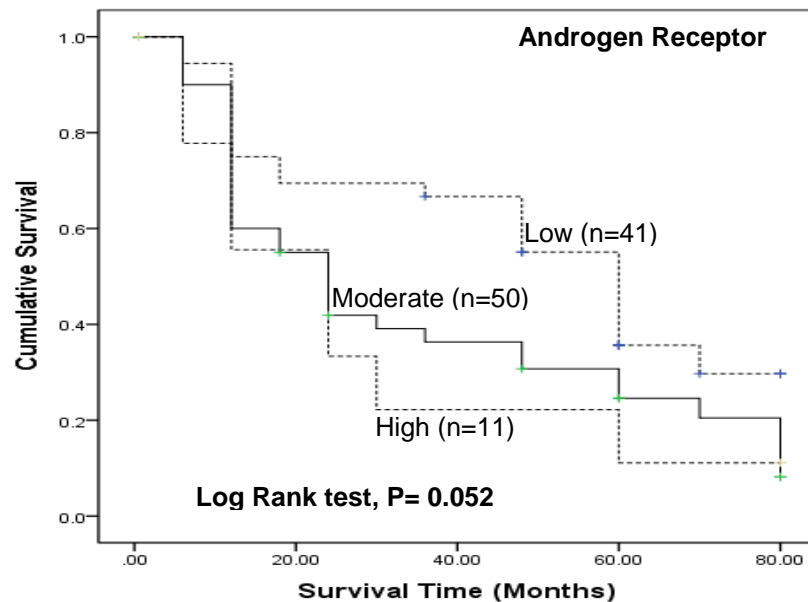


Figure 3. 10: Kaplan-Meier survival curves of patients with prostate cancer.

The cumulative survival of patients was plotted against the time in month for AR index. Different AR indices: low group, <4 ($n=41$); moderate group, $4-6$ ($n=50$); high group, $6-9$ ($n=11$).

3.5.3 Relationship between PSA and patient survival

When the correlation between patient survival time and the blood level of PSA was assessed (**Figure 3.11**), the median survival time for patients with low (<10ng/ml) and high (\geq 10ng/ml) PSA levels were 48 and 18 months, respectively. The difference was not statistically significant (log-rank test, $p = 0.246$). The cut off used in this study was 10ng/ml (PHE 2016b; Roddam et al. 2005).

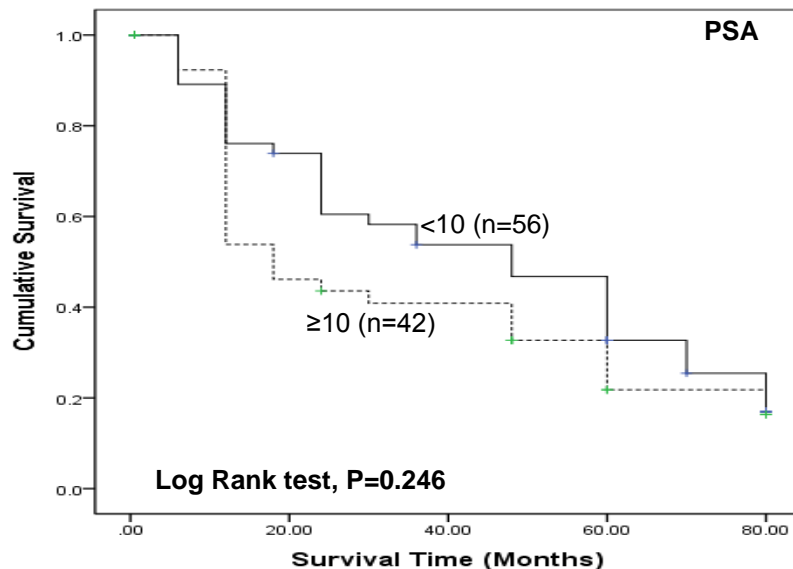


Figure 3. 11: Kaplan-Meier survival curves of patients with prostate cancer.

The cumulative survival of patients was plotted against the time in month for PSA levels. Different levels of PSA: group 1, PSA <10 ng/ml (n=38) and group 2, PSA \geq 10 ng/ml (n=64).

3.5.4 Relationship between GS and patient survival

When the relationship between patient survival time and GS was assessed (**Figure 3.12**), the median survival time for patients with low GS (not reached) was significantly longer than for those patients with moderate (60 months) and high GS (12months), respectively. The increased GS was significantly correlated with the reduced patient's survival time (log-rank test, $p < 0.0001$).

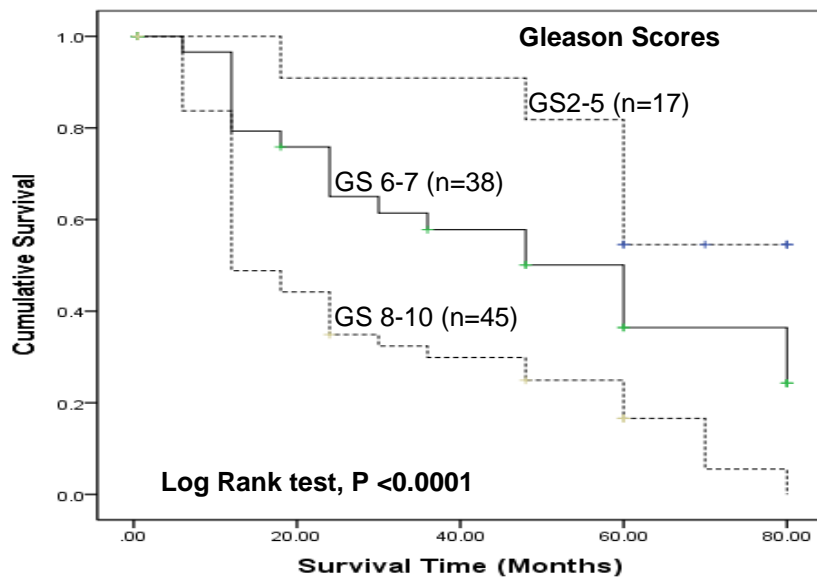


Figure 3. 12: Kaplan-Meier survival curves of patients with prostate cancer.

The cumulative survival of patients was plotted against the time in month for GS.

Different GS: group 1, GS ≤ 5 (n=17); group 2, GS 6 to 7 (n=38); group 3, GS 8 to 10 (n=45).

3.6 Correlation of FABP9 expression with levels of GS, AR index and PSA

Assessment of correlation between the intensity of staining for FABP9 and AR index (Figure 3. 13) and PSA level (Figure 3. 14).

3.6.1 Correlation of FABP9 and AR index

Box plot analysis of the correlation between staining intensity for FABP9 and AR index showed that the AR index level was significantly higher in cases with strong staining for FABP9 than those cases with weak staining for FABP9 (Figure 3.13) (Mann-Whitney U test, $p < 0.03$). However, the differences in AR index levels either between cases with moderate and weak FABP9 satins (Mann-Whitney U test, $p = 0.11$) or between cases with strong and weak stains for FABP9 (Mann-Whitney U test, $p = 0.084$) were not statistically significant.

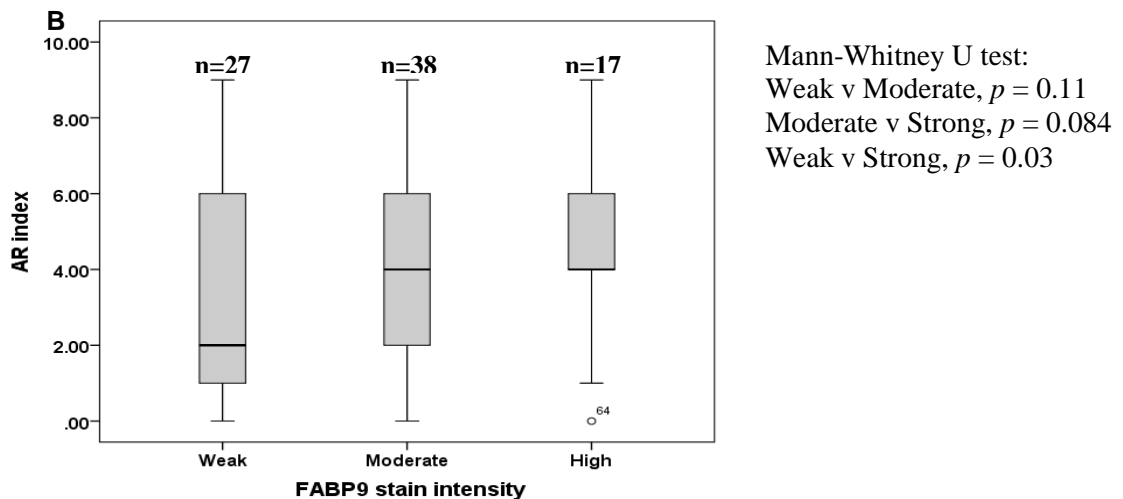


Figure 3. 13: Box plot of correlation between stain intensity for FABP9 and androgen receptor (AR) index in 3 groups of patients with prostate cancer: weak, moderate and strong staining for FABP9.

3.6.2 Correlation of FABP9 and PSA level

For the correlation between the staining intensity of FABP9 and PSA level (**Figure 3.14**), box plot analysis showed that the differences in PSA levels between cases with strong and weak stains for FABP9 (Mann-Whitney U test, $p = 0.142$), between strong and moderate stains (Mann-Whitney U test, $p = 0.098$) and between moderate and weak stains for FABP9 (Mann-Whitney U test, $p = 0.812$) were not significantly different.

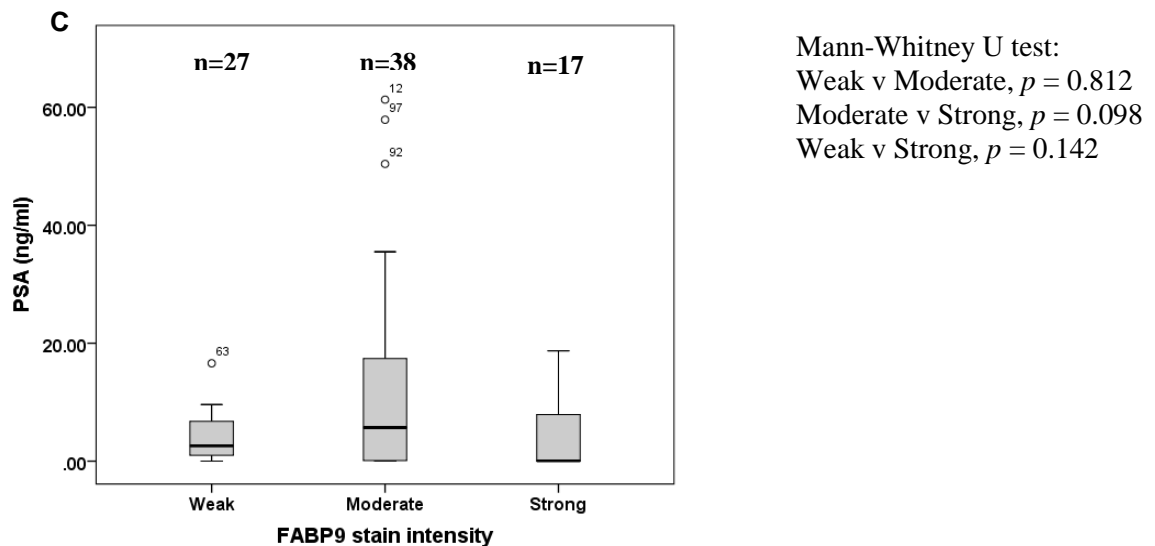


Figure 3. 14: Box plot of correlation between stain intensity for FABP9 and prostatic specific antigen (PSA) level in 3 groups of patients with prostate cancer: weak, moderate and strong staining for FABP9.

3.7 Suppression of FABP9 expression by siRNA

PC3-M cells, highly malignant prostate cancer cells, express high level of FABP9. FABP9 mRNA in PC3-M was transiently knocked down by RNAi technique. The most efficient siRNA sequence was used to design double-stranded shRNA for stable transfection. In stable transfection, shRNAs were transfected into PC3-M cells to knockdown the *FABP9* mRNA to assess whether suppression of FABP9 expression can inhibit the tumorigenicity of prostate cancer cells.

3.7.1 Selection of most efficient siRNA for FABP9 suppression

Three different target sequences within *FABP9* cDNA were designed to form ds-siRNA constructs. Highly-expressing PC3-M cells were transiently transfected for 48 hours with 3 different siRNAs. Western blots showed that levels of FABP9 expression after transfection were reduced (**Figure 3.15A**). Quantitative analysis showed that the levels of FABP9 in scramble control and PC3-M treated with transfection reagent alone were similar to that in the parental PC3-M cells. The relative levels in other three PC3-M transfectants with siRNA 1, 2 and 3 were 0.86 ± 0.14 , 0.75 ± 0.35 and 0.45 ± 0.30 , respectively (**Figure 3.15B**). The most efficient suppression was achieved by siRNA-3 (up to 60%) (Student's t-test, $p < 0.0001$) in combination with 2.5 μ l/ml of X-tremeGENE siRNA Transfection reagent in 24-hours incubation. Therefore, siRNA-3 was identified as the most effective suppresser and its sequence was used to design double-stranded shRNA for stable transfection.

Results

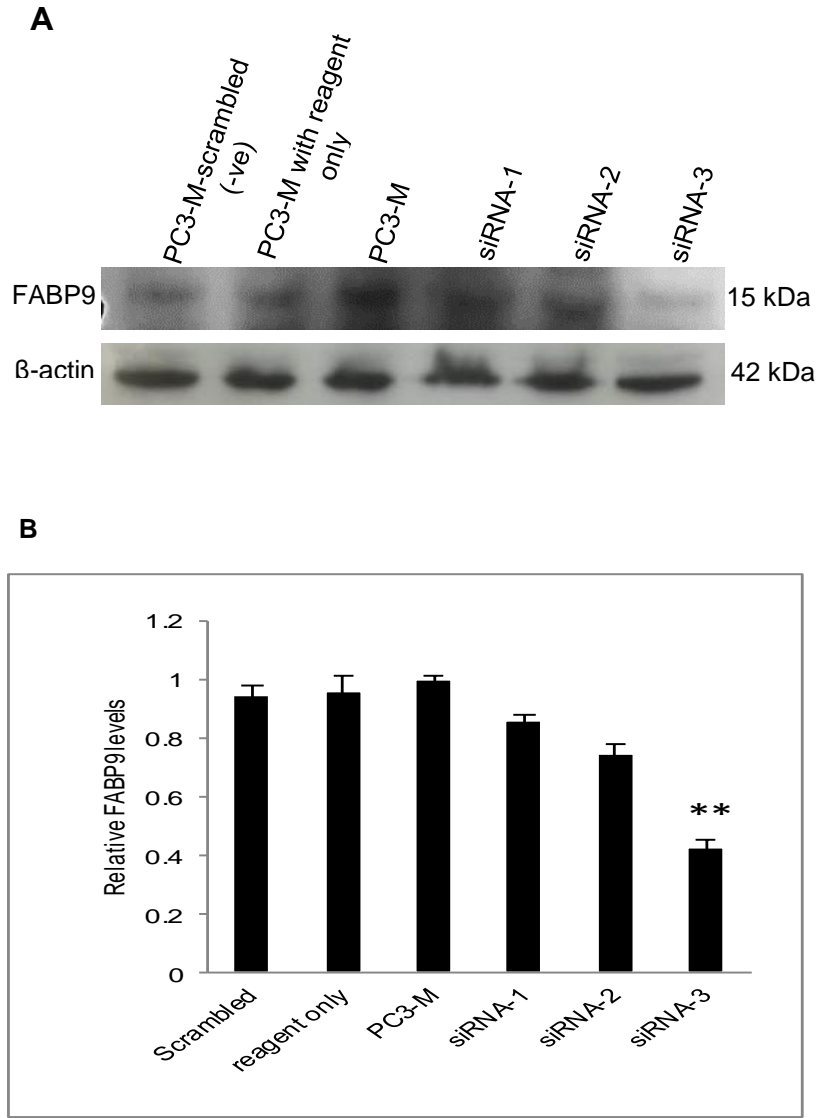


Figure 3. 15: Knockdown of FABP9 mRNA in highly malignant prostate cancer cells.

A), the effect of mRNA Knockdown on FABP9 expression in PC3-M cells. Western blot analysis of FABP9 expressed in PC3-M cells and cells transiently transfected with 3 different siRNA molecules. As loading control, β -actin was detected on the same blot.

B), relative levels of FABP9 in PC3-M after transient transfection. The level of FABP9 in parental PC3-M was set at 1.0 and levels expressed in transiently transfectant cells were related to that in parental cells. Results were obtained from three different measurements (mean \pm SD).

Results

3.7.2 Establishment of stably FABP9-suppressed PC3-M cell lines

To establish stably suppressed FABP9 with highly malignant PC3-M cells, shRNA was designed using siRNA-3 as template. psiRNA-h7SKGFPzeo plasmid was double digested with two restriction enzymes HindIII and Acc651. Electrophoresis in low-temperature melting agarose (1%) gel was performed to confirm the successful double cleavages (**Figure 3.16**). The gel containing the appropriate band was cut out, the DNA was extracted from the gel and ligated with a short shRNA molecule formed by annealing a forward and a reverse oligonucleotide. Ligation product was transformed into *E.coli* GT116 competent cells, plated onto a LB-Zeocin agar plate, and incubated overnight in a 37°C incubator. The control and shRNA plasmid transformation efficiencies were 3800 and 1500 cfu, respectively.

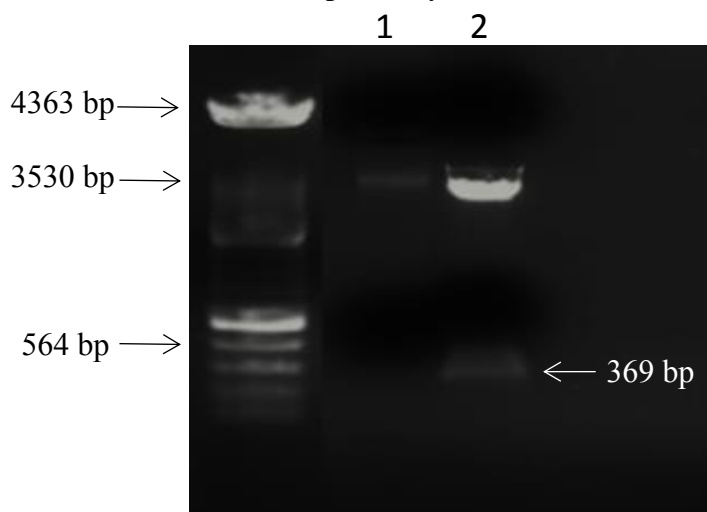


Figure 3. 16: Digestion of psiRNA-h7SKGFPzeo plasmid.

Electrophoresis was performed in Low-melting temperature agarose gel (1%) to confirm the successful cleavage of the plasmid DNA. Undigested psiRNA-h7SKGFPzeo plasmid was loaded in well 1 and digested one in well 2. The extra small band (arrow) was the 369 bp fragment of plasmid that was replaced by shRNA in ligation process.

Results

Three single clones of transformed bacteria with FABP9-suppressed and 1 clone from scramble shRNAs were picked up and grown in LB medium containing 100µg/ml Zeocin. Then, QIAGEN plasmid mini-preparation kit was used to extract and purify plasmid DNAs from bacteria cells. Plasmids were double digested with Hind III and Acc651 restriction enzymes. Electrophoresis in low-melting temperature agarose (1%) gel was performed to confirm that the shRNA fragments were inserted into the vector (identified by the arrows) (**Figure 3.17**). The undigested psiRNA-h7SKGFPzeo plasmid in parallel with the digested sample was used as control as shown in **Figure 3.17**. After ascertain the shRNA was inserted, plasmids from three different clones (2-4) were sent to Beckman Coulter Genomics, UK for sequencing analysis. Sequencing analysis result confirmed the correct size, sequence and orientation of inserts as shown in **Table 3.3**. Plasmid from clone 4 was chosen for the subsequent stable transfection. Specificity of these sequences was confirmed by Genbank Blast search.

Results

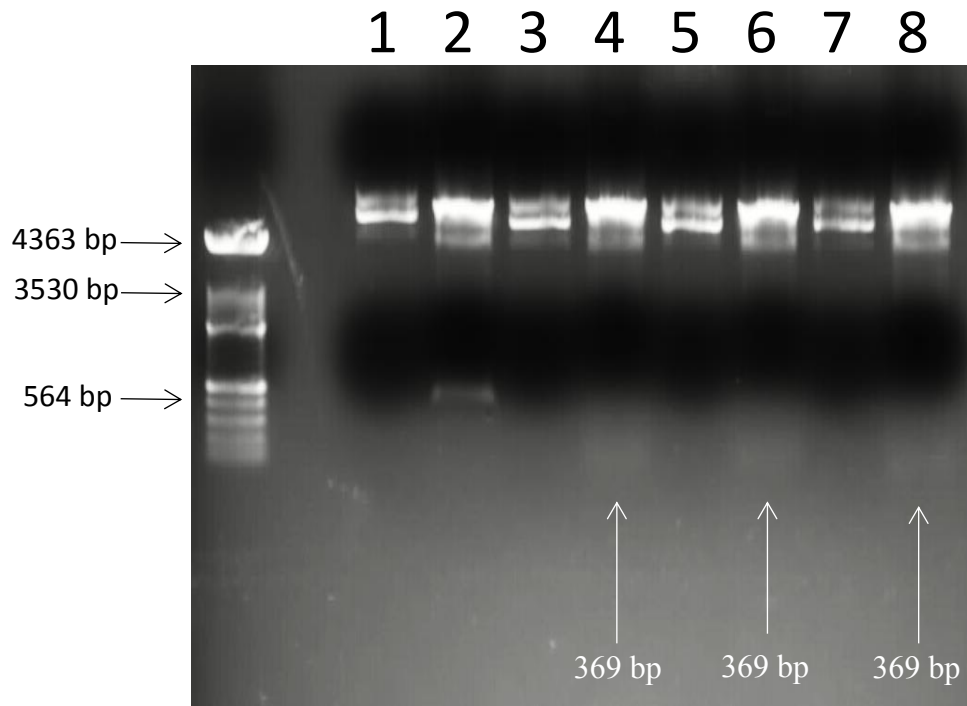


Figure 3. 17: Confirmation of the correct DNA insertion.

Mini-preparation of DNA was made from 2 colonies harbouring FABP9-suppressed DNA constructs (lane 6 and 8) and 1 colony harbouring scramble-shRNA plasmids (lane 4). DNA from each clone was double digested using HindIII and Acc651 restriction enzymes (lane 2, 4, 6 and 8) and an undigested plasmid DNA sample was loaded in parallel with the digested sample as control (lane 1, 3, 5 and 7). The smaller bands (identified by the arrows) represent shRNA fragments (369 bp). The bigger bands represent digested psiRNA-h7SKGFPzeo plasmid.

Results

Forward

```
5'NNNNANTNNTNNGTATNAGCTTGTGCGCCGCTGGGTACCTCGGTTAGTGAAACCGACAG
TTCAAGAGACTGTCGGTTTCACTAACCTTTTTGGAAAAGCTTCTAGACTTAATTAACCTGCAG
GCGTTACATAACTTACGGTAAATGGCCCGCTGGCTGACCGCCAACGACCCCGCCATTG
ACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATG
GGTGGAGTATTTACGGTAAACTGCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTA
CGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCTGGCATTATGCCCAGTACATGACC
TTATGGGACTTTCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGATGATG
CGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCT
CCACCCCATTTGACGTCAATGGGAGTTTGTGTTTACTAGTAAATCAACGGGACTTTCCAAAATG
TCGTAACAACCTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATA
TAAGCAGAGCTCGTTTAGTGAACCGTCAGATCAGCTTCGAGGGGGCTCGCATCTCTCCTTCACG
CGCCCGCCGCCCTACCTGAGGCCGCCATCCACGCCGTTGAGTCGCGTCTGCCGCTCCCGC
CTGTGGTGCCTCCTGAACTGCNNCCGCCGTCTAGGTAAGTTTAAAGCTCAGGTTCGAGACCGG
GCCTTTGTCCGGCGCTCCCTTGGAGCCTAGCTAGACTCAGCCGGCTCTCCACGCTTTGCCTGA
CCCTGCTTGCTCAACTCTACGTCTTTGATTCCGTNATCTGTTCTGCGCCGTTACAGATTCCAAG
CCACCATGGGTTTCTAACGGGAGAAGAACTNNTAACTGGCAGTTGTCCCATTCNNGGTTG
ACCNTGANNGNTGATGTGAATGGCCNNNAATTCTCTGTGTGCNNNGNNTAATATGGAAGG
NAANTGCAANCCTCATGGAANTCT 3'
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Reverse

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5'TTANNGGNCGGNNTCGTTGGNCGGTCAGCCAGGCGGGCCATTTACCGTAAGTTATGTAACG
CCTGCAGGTTAATTAAGTCTAGAAGCTTTTCCAAAAGGTTAGTGAAACCGACAGTCTCTTG
AACTGTCGGTTTCACTAACCGAGGTACCCAGGCGGCGCACAAAGCTATATAAACCTGAAGGAA
ATCTCAACTTTACTTAGGTCAAGTTACTTATCGTACTAGAGCTTCAGCAGGAAATTTAACT
AAAATCTAATTTAACCAGCATAGCAAATATCATTTATCCCAAATGCTAAAGTTTGAGATA
AACGGACTTGATTTCCGGCTGTTTTGACACTATCCAGAATGCCTTGCAGATGGGTGGGGCAT
GCTAAATACTGCAGCACTAGTATCGATTAAGAATGACCAAATCCCTTAACGTGAGTTTTT
CGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTT
TGCGCGTAATCTGCTGCTTGCAAACAAAAAACCCAGCTACCAGCGGTGGTTTGTGTTGCCG
GATCAAGAGCTACCAACTTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAAA
TACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCCGCTAC
ATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTAC
CGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCCGGGCTGAACGGGGGGT
TCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGA
GCTATGAGAAAGCGCCACGCTTCCCGAANGGAGAAAGGCGGACAGGTATCCGGTAAGCGGC
AGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGAAACGCCTGGTATCTTTATA
GTCTGTCCGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGN
CGGANCTATGGNAAAANCAGCAACGCTGGCTTTTTNACGGGTTCTGGNCCTTTNGCT
GGNCNTTTNGCTCACATGCATAAAAAANCCNNCAAANCGGGNTTTNANNTTAAAANTTA
AGCCATACCAANCC 3'
```

Table 3. 3: A part of psiRNA-h7SKGFPzeo plasmid sequence map.

To confirm the correct insertion of shRNA (highlighted in red), plasmid from clone 4 was sequenced using internal plasmid primers by Beckman Coulter Genomics, UK.

Sequence alignment was performed using BioEdit for searching Genbank and the results proved that the insertion was correct.

Results

3.7.3 Stable transfection

For stable transfection, shRNAs from clone 4 were cloned into the psiRNA-h7SKGFPzeo plasmid and were transfected into PC3-M cell line using X-tremeGENE HP DNA Transfection Reagent (2.5µl/ml) to knockdown *FABP9* mRNA. After 48 hours, culture medium was replaced with fresh media containing ZeocinTM (100µg/ml). Then, transfectant cells were transferred into 9cm cell culture plates (petri dish) until clones can be visualized with naked eye. Five single clones of FABP9-suppressed transfectants and 2 scramble transfectants were isolated using ring cloning method described in section 2.5.6.1 and grown in a 96-well plate. The clones were grown in normal cell culture condition for one week.

When Western blot was used to measure the level of FABP9 from different individual colonies of transfectants, a single 15 kDa FABP9 band was detected and levels of FABP9 in scramble control PC3-M, parental PC3-M and 5 single transfectant clones are shown in **Figure 3.18A**. Further quantitative analysis showed that the level of FABP9 in scramble control was similar to that in the parental PC3-M cells. The relative levels of FABP9 in 1-5 single colonies were reduced by 15%, 25%, 43%, 50% and 91%, respectively (**Figure 3.18B**). Thus sub-lines established from clone 4 and clone 5 were selected as moderately (PC3M-FABP9-siM) and highly (PC3M-FABP9-siH) suppressed transfectant lines, respectively. To confirm that the suppression was not via FABP5, Western blot analysis was used to assess the effect of FABP9 mRNA knockdown on FABP5 expression in PC3-M cells (**Figure 3.19A**). Quantitative analysis showed that the levels of FABP5 in PC3M-FABP9-siM and PC3M-FABP9-siH were similar to that in the parental PC3-M cells (**Figure 3.19B**).

Results

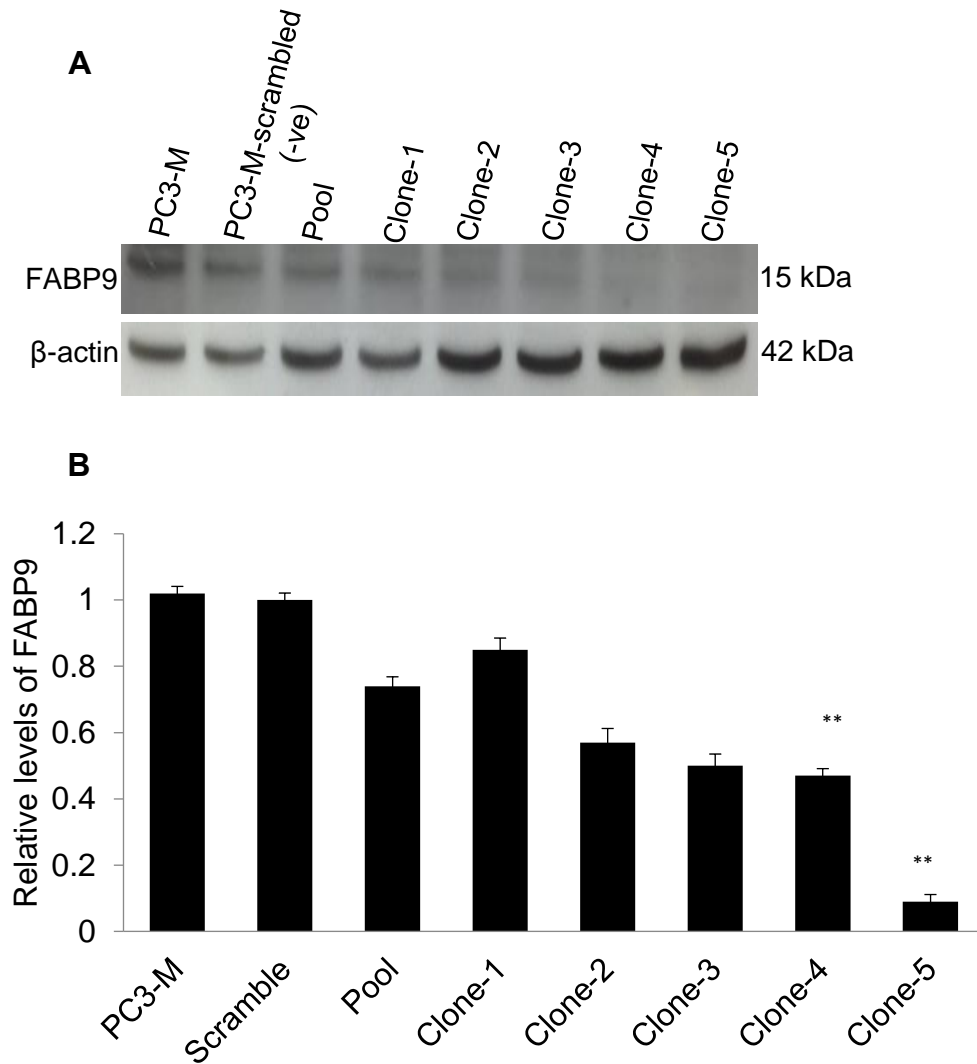


Figure 3. 18: Knockdown of FABP9 mRNA in highly malignant prostate cancer cells.

A) Western blot analysis of FABP9 in control PC3-M cells and 5 different clones generated by transfection with the shRNA based on siRNA-3. As loading control, β -actin was detected on the same blot. B) the relative levels of FABP9 in PC3-M transfected with scramble shRNA control was set at 1.0 and levels expressed in other transfected cell lines were obtained by comparing with the control. Results were obtained from three different measurements (mean \pm SD).

Results

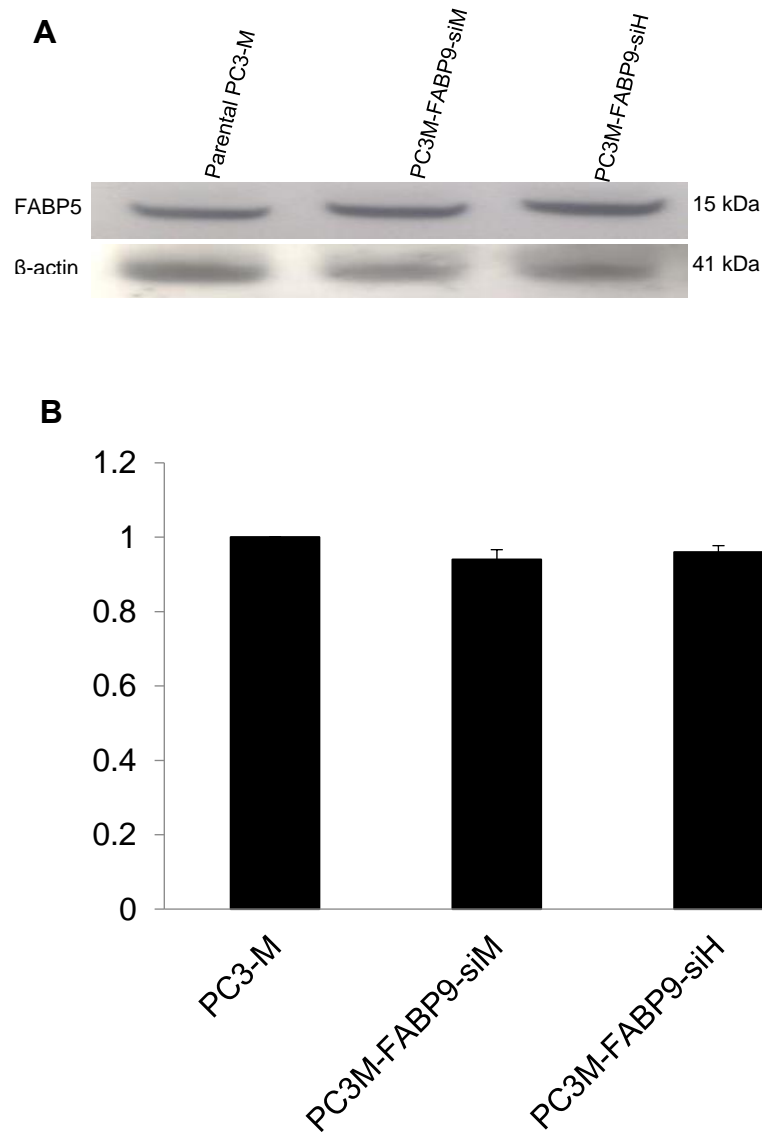


Figure 3. 19: A) Western blot analysis of FBP5 in transfectants expressing reduced levels of FABP9. B) The relative levels of FBP5 in PC3M-FABP9-siM and PC3M-FABP9-siH were similar to that in the parental PC3-M cells.

3.8 Effect of *FABP9*-suppression on tumour cells *in vitro*

3.8.1 Effect of *FABP9*-suppression on cellular proliferation

To identify the cell number in MTT assay, standard curve of each cell line was generated to correlate optical density of formazan with cell number as shown in **Figure 3.20**. Proliferation assay was performed triplicate on controls and transfectants over 6 days to test whether suppression of FABP9 can affect the growth rate of PC3-M cells. The result was shown in **Figure 3.21**. MTT proliferation assay was performed to assess the effect of reduced suppressed-FABP9 on the proliferation ability of PC3-M cells. The density of culture cells was determined by extrapolating standard curve, and proliferation rates of different transfectants were measured by MTT assay. Overall, growth rate of parental cells and transfectants exhibited a very similar growth pattern. On 1st and 2nd day, no significant difference was detected between parental control and PC3M-FABP9-siM and PC3M-FABP9-siH (Student's test, $p > 0.05$). Although at day 3, there was a reduction in both PC3M-FABP9-siM (15566 ± 2016) and PC3M-FABP9-siH (18441 ± 2820) cells compare to parental control (21709 ± 4901), but not statistically significant (Student's test, $p > 0.05$) (**Table 3.4**). On the 4th, 5th and 6th day, the number of PC3M-FABP9-siM and PC3M-FABP9-siH cells growth rates were not significantly reduced in both PC3M-FABP9-siM and PC3M-FABP9-siH cells compared to parental PC3-M cells (Student's test, $p > 0.05$). Therefore, cell proliferation of PC3M-FABP9-siM cells and PC3M-FABP9-siH cells were not significantly different compare to control cells.

Results

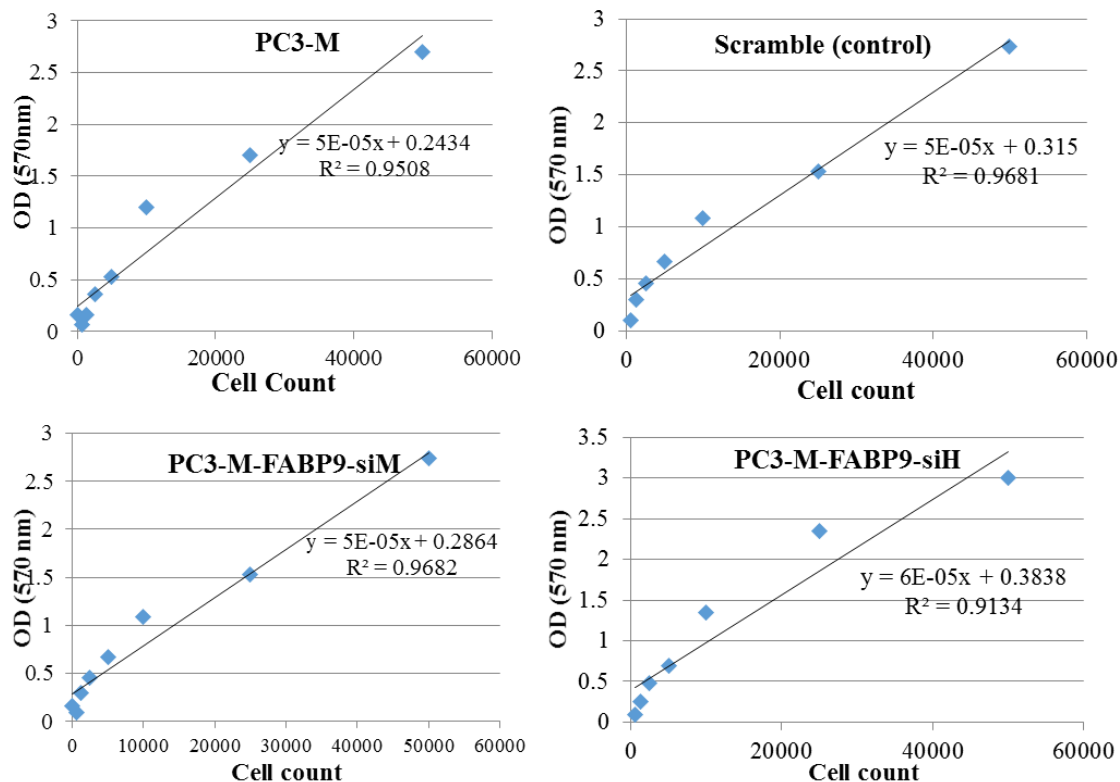


Figure 3. 20: Standard curves of parental PC3-M and different transfectant cell lines. Using linear regression analysis, Standard curves were established for each cell line by plotting absorbance (OD at 570 nm) (Y axis) against the cell numbers (X axis). The curve equation and regression of each standard curve is presented in diagram.

Table 3. 4: Cell counts of parental and transfectant PC3-M cells at the 3rd day of proliferation assay in 3 separate experiments. Using Student's t-test, *p* values were obtained by comparing data from test groups to parental group.

Cell Line	Mean No. of Cells \pm SD	P Value
PC3-M	21709 \pm 4901
PC3-M-FABP9-siM	15566 \pm 2016	= 0.115
PC3-M-FABP9-siH	18441 \pm 2820	= 0.373

Results

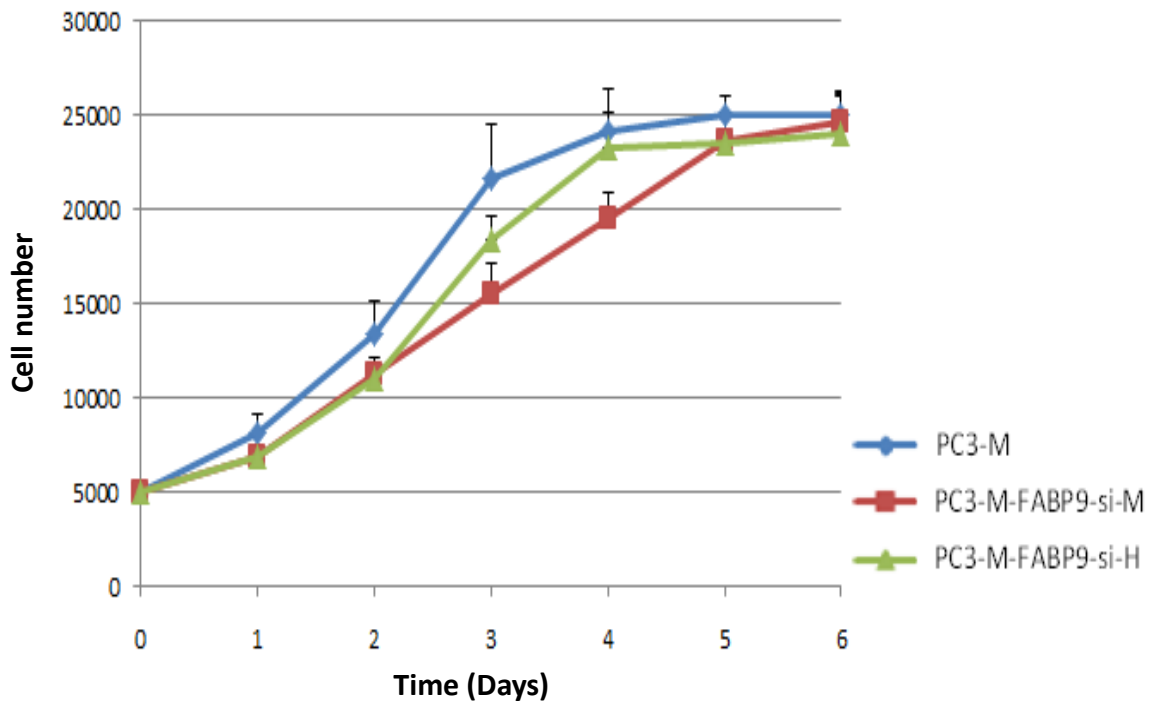


Figure 3. 21: The impact of FABP9 suppression on the proliferation rate of transfectant cells.

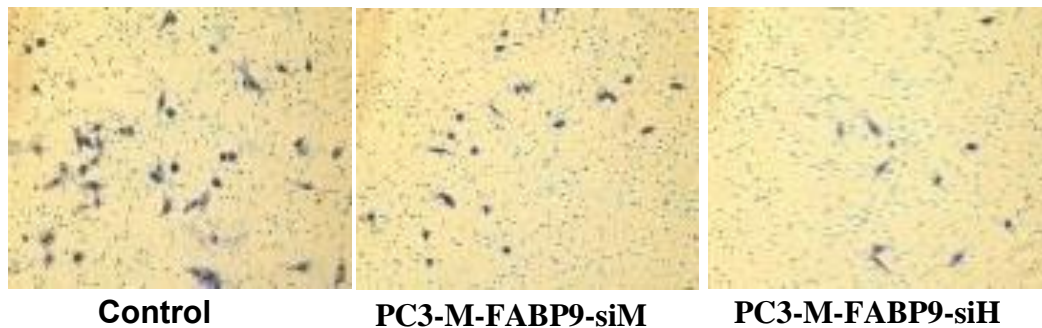
The growth rates of parental, PC3M-FABP9-siM and PC3M-FABP9-siH cells during a 6- day experimental period. Cell count of each sample was calculated by comparing to serially diluted standard from standard curve. Data expressed as means + SD of three independent experiments.

3.8.2 Effect of FABP9 suppression on invasiveness of prostate cancer cells

The effect of FABP9-suppression on invasiveness of prostate cancer cells was evaluated by an invasion assay using BD Matrigel coated invasion chambers as shown in **Figure 3.22**. The invasiveness of the transfectants was assessed in a BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences, USA). After 24-hour starvation, PC3-M and transfectant cells at a density of 2.5×10^4 cells/ml were loaded into every upper compartment of chambers. Routine medium was loaded in the lower compartment and serum-free medium was used as negative control for each cell line. The chambers were maintained in a humidified tissue culture incubator at 37°C , 5% CO_2 (v/v). All cell lines were set as triplicate and assay was run in a humidified tissue culture incubator for 24 hours. After 24-hour cells in the upper compartment were removed and washed with PBS. Then, the invaded cells in the lower part were fixed and stained with 2% (v/v) crystal violet for 10 minutes. The number of invaded cells was counted under a light microscope. The invaded cells were counted in nine random fields using light microscope at $125 \times$ magnification (**Figure 3.22A**). The number of invaded cells (mean \pm SD) from scramble control, PC3M-FABP9-siM and PC3M-FABP9-siH were 43 ± 7 , 26 ± 4 and 15 ± 5 , respectively (**Figure 3.22B**). PC3M-FABP9-siM and PC3M-FABP9-siH were representing significant reductions by 39.5% (Student's t-test, $p = 0.008$) and 65.1% (Student's t-test, $p = 0.001$), respectively as tested in BD Matrigel coated invasion chambers (**Table 3.5**). Invasiveness of PC3M-FABP9-siH was significantly reduced when compared with the invasiveness of PC3M-FABP9-siM cells (Student's t-test, $p = 0.021$).

Results

A



B

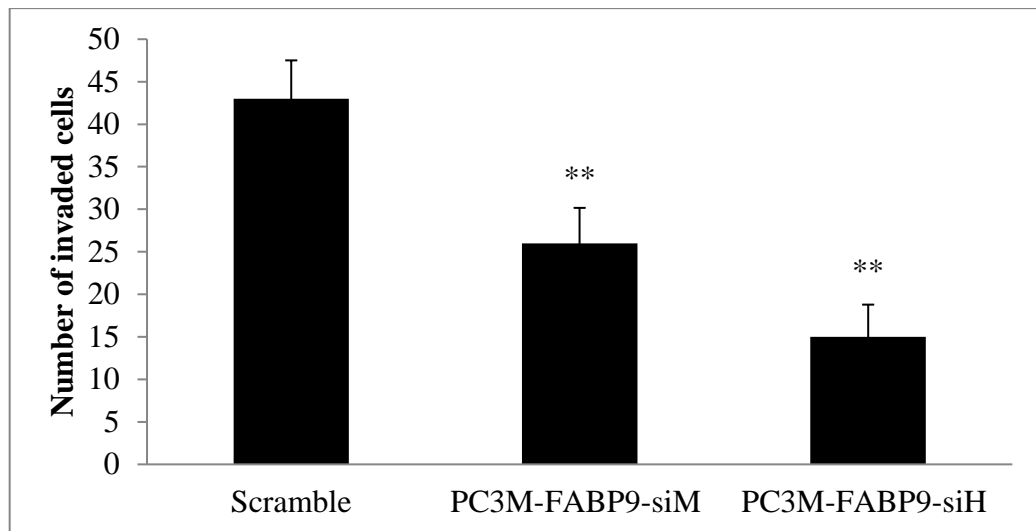


Figure 3. 22: The effect of FABP9 suppression on invasiveness of transfectant cells.

A) 3 panels represent the invasiveness of the control, moderately suppressed (PC3-M-FABP9-siM) and highly suppressed (PC3-M-FABP9-siH) cells respectively. B) Number of invaded cells per field in invasion assay; cells were incubated in BD Matrigel coated invasion chambers with serum-free medium for 24 hours. The results (mean \pm SD) were obtained from 3 separate experiments.

Results

Table 3. 5: Number of invaded cells (mean \pm SD) produced in three separate experiments. Differences of invasiveness between different groups were assessed by Student's t-test. *p* values were obtained by comparing data in test groups to control group.

Cell Line	Mean No. of Colonies \pm SD	P Value
PC3-M	43 \pm 7
PC3-M-FABP9-siM	26 \pm 4	0.008
PC3-M-FABP9-siH	15 \pm 5	0.001

3.8.3 Effect of *FABP9* suppression on anchorage-independent growth of prostate cells

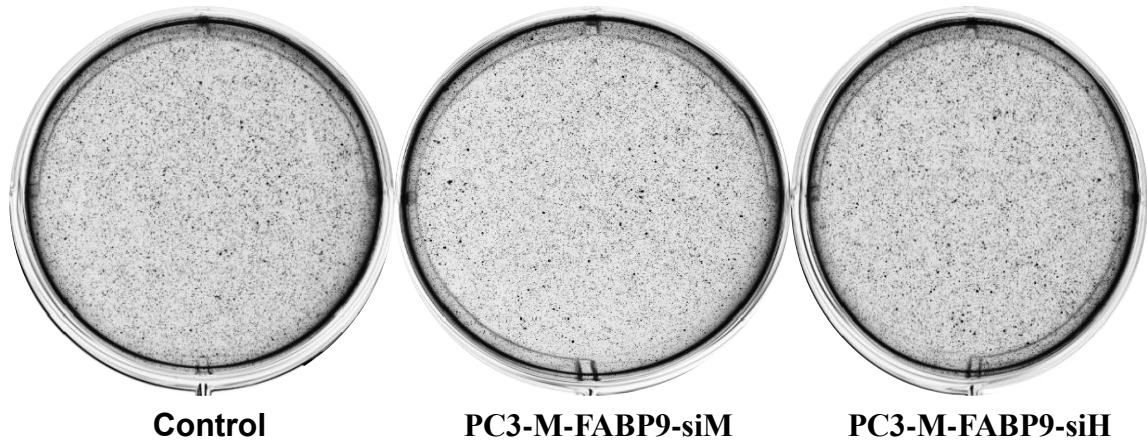
To assess anchorage-independent growth of *FABP9*-shRNA transfectants, soft agar assay was performed (**Figure 3.23**). The assay was set in triplicate and scramble cells were used as control. Control cells and transfectants were grown for 4 weeks. During the 4-week incubation, cells were feed with 200ul/well once a week. At the end of the assay, colonies were stained by adding 0.5ml of 2% MTT (5mg/ml). Colonies larger than 150µm were counted using Gel Count. The result of the anchorage-independent growth capability of the cells were shown in **Figure 3.23A**. The average number of colonies produced by scramble, PC3M-*FABP9*-siM and PC3M-*FABP9*-siH cells were 188±6, 138±10 and 161±9, respectively (**Figure 3.23B**). Colony formation of PC3M-*FABP9*-siM (Student's t-test, $p = 0.3$) and PC3M-*FABP9*-siH cells (Student's t-test, $p = 0.5$) were not significantly different from control cells as shown in **Table 3.6**.

Table 3. 6: Colony counts of transfected cells in at 4th week of triplicate soft agar assay. Differences of number of colonies between different groups were assessed by Student's t-test. p values were obtained by comparing sample groups to control group data.

Cell Line	Mean No. of Colonies ± SD	P Value
PC3-M	188 ± 6
PC3-M- <i>FABP9</i> -siM	138 ±10	0.3
PC3-M- <i>FABP9</i> -siH	161 ±9	0.5

Results

A



B

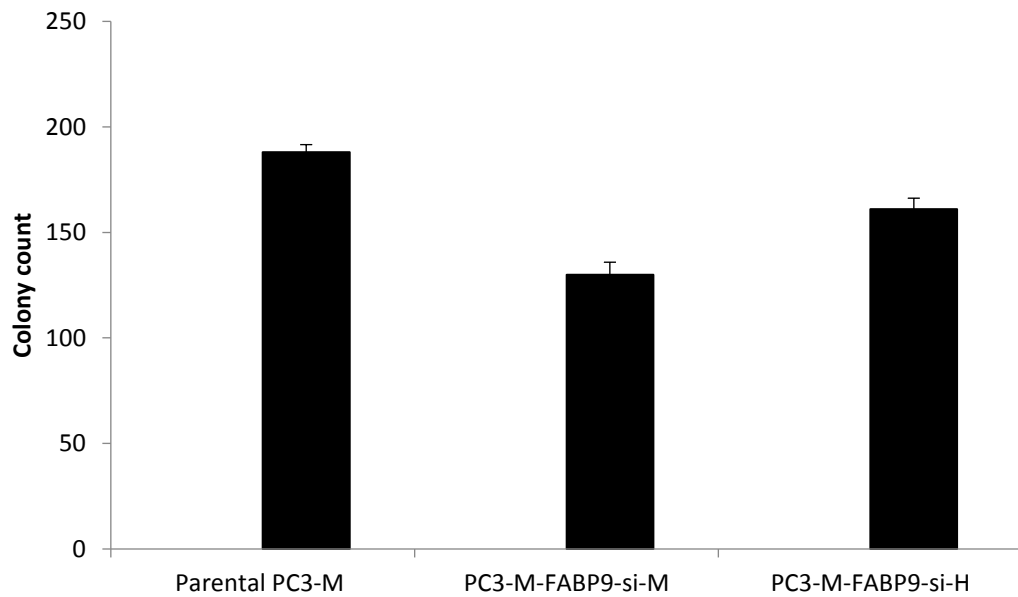


Figure 3. 23: The impact of FABP9 silencing on the anchorage-independent growth of transfectant cells.

A) Representative plates of soft agar colony formation with different transfectants. B) Colony counts of different transfectants; Results were obtained from three separate experiments (mean \pm SE).

3.8.4 Effect of *FABP9* suppression on cell migration

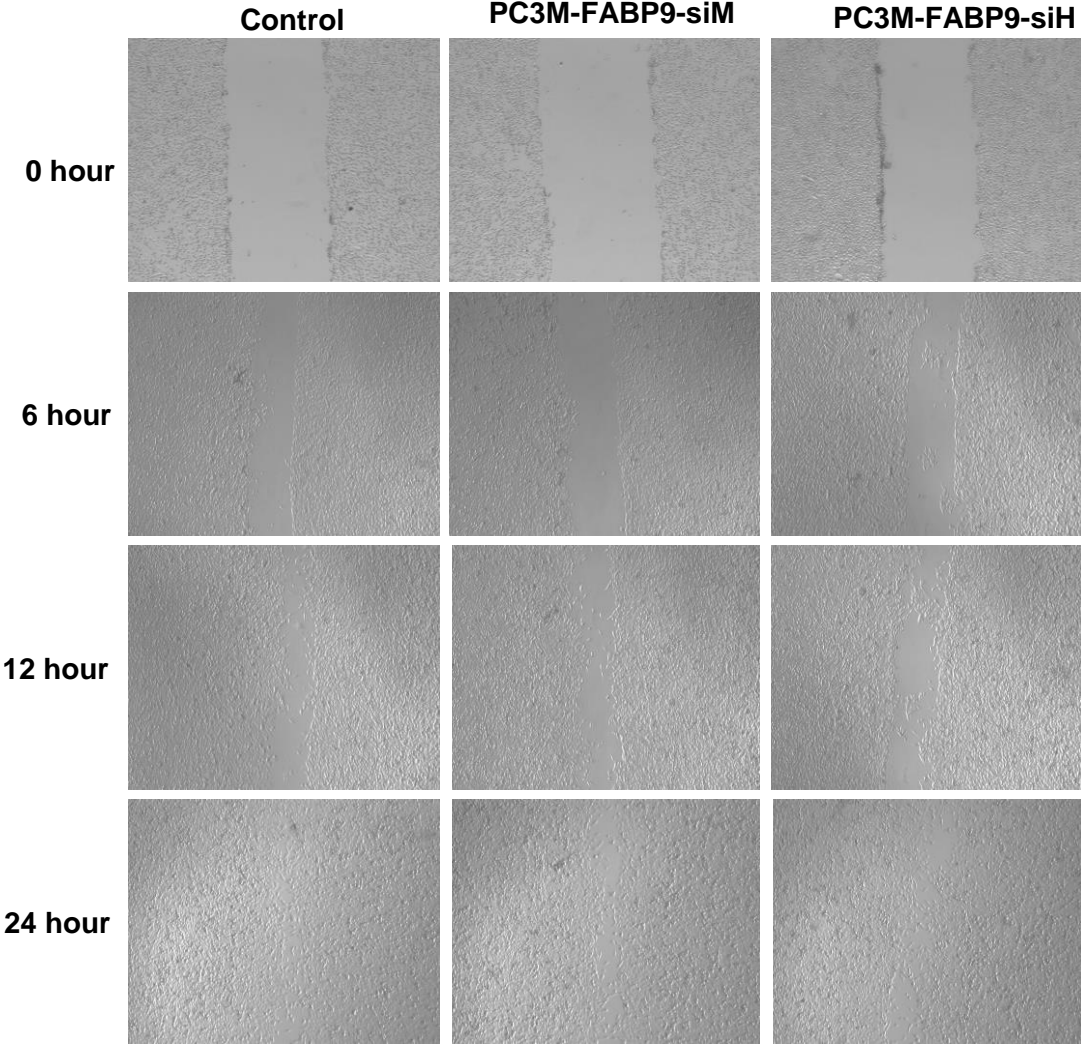
To assess the functional effect of FABP9 knockdown on the migratory ability of cells with the transfectants, wound healing assay was performed and the results are shown in **Figure 3.24**. The assay was set in triplicate and scramble cells were used as control. Control cells and transfectant cells (PC3M-FABP9-siM and PC3M-FABP9-siH) cells were grown in 6-well plate until reached 75-80% confluency. Then, a wound was created by making a scratch, the cells on the edge of the artificial gap migrate to close the scratch were monitored until cell-cell contacts are observed (**Figure 3.24A**). At different time intervals, the migration rate (as reflected by width of the scratch) of PC3M-FABP9-siM and PC3M-FABP9-siH cells were not significantly different from control cells at wound closure (Student's t-test, $p > 0.05$) as shown in **Table 3.7**. Images were captured at the beginning and at regular intervals to determine the cell migration rate. The result showed that transfectants with either PC3M-FABP9-siM or PC3M-FABP9-siH did not exhibit any significant difference in suppressing cell migration ability when compared to the control in 24 hours (**Figure 3.24B**).

Table 3. 7: Migration rates of transfected cells at 24 hours in wound healing assay.

Time intervals	PC3-M	PC3M-FABP9-siM	PC3M-FABP9-siH
0 Hour
6 Hours	<i>P</i> value > 0.05	<i>P</i> value > 0.05
12 Hours	<i>P</i> value > 0.05	<i>P</i> value > 0.05
24 Hours	<i>P</i> value > 0.05	<i>P</i> value > 0.05

Results

A



B

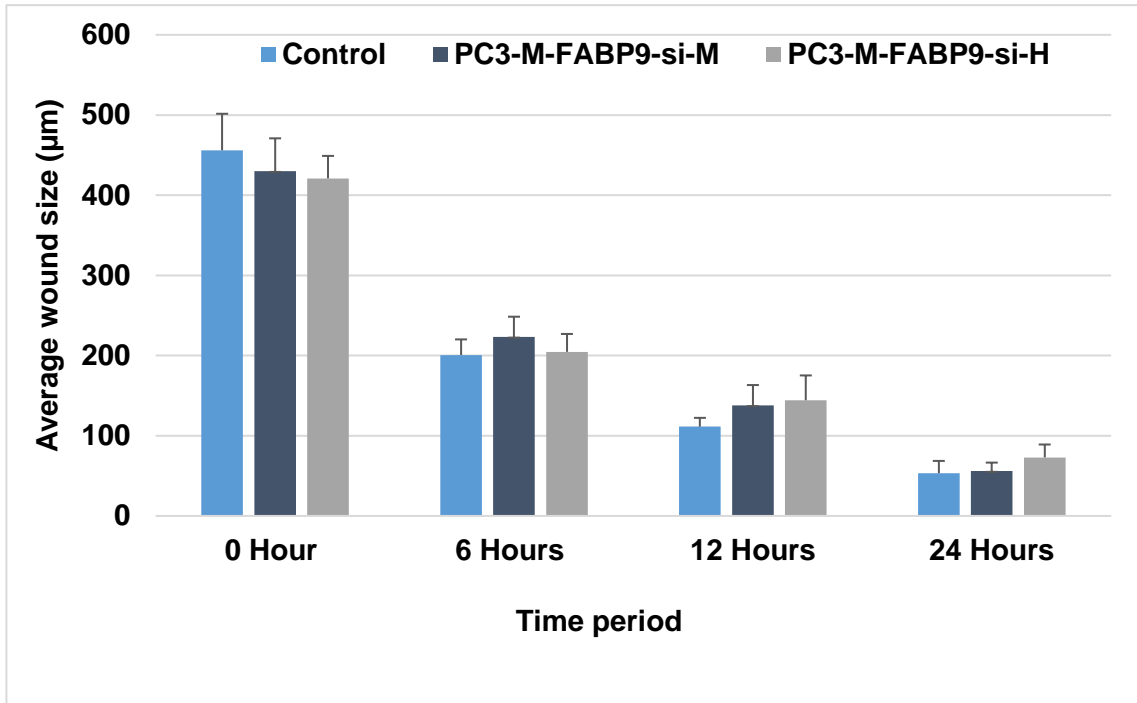


Figure 3. 24: The impact of FABP9 silencing on migration rate of transfected cells.

A) Representative images of wound healing with control and different transfecteds (PC3M-FABP9-siM or PC3M-FABP9-siH). B) Migration rate of different transfecteds; Results were obtained from three separate experiments (mean \pm SE).

CHAPTER FOUR: DISCUSSION

4 Discussion

Prostate cancer is the most common male cancer in the developed countries and a worldwide male health threat (CRUK 2013). Previous studies showed that some FABPs were implicated in progression of several cancer types including prostate cancer and could be used as tumour biomarkers (Boiteux et al. 2009; Hammamieh et al. 2005; Ohmachi et al. 2006). FABPs are intracellular lipid-binding proteins that bind intracellular hydrophobic ligands such as saturated or unsaturated medium- or long-chain fatty acids and consist of at least 12 members. Amongst the 12 members of FABP family proteins, FABP10 and FABP11 are restricted to fish only and not expressed in *Homo sapiens* (Agulleiro et al. 2007; Sharma et al. 2006; Venkatachalam et al. 2009). FABPs have been suggested to be involved in intracellular transport of lipids to subcellular-organelles (Furuhashi & Hotamisligil 2008). FABPs have also been suggested to control cancer growth through co-ordination with other fatty acid transporters and carcinogens (Schachtrup et al. 2004), steroids (Gao et al. 2010), hormones (Pinthus et al. 2007) and their derivatives (Chmurzynska 2006). FABP5 played a crucial promoting role in tumorigenicity and metastasis of prostate cancer (Jing et al. 2001; Jing et al. 2000). Increased FABP5 expression in archival prostate cancer tissues is found to be significantly associated with a reduced patient survival time (Myers, von Lersner & Sang 2016), suggesting that it is a valuable prognostic factor. It was also involved in malignant progression of pancreatic cancer (Sinha et al. 1999). It was reported that the FABP5 and FABP6 were overexpressed and might play promoting roles in colorectal cancer (Kawaguchi et al. 2016b; Ohmachi et al. 2006). FABP1 was shown to play a key role in breast cancer cells and has the potential to serve as a

Discussion

diagnostic marker (Li et al. 2007). Several studies have linked FABP3 with different cancer types such as embryonic cancer, breast cancer and NSCLC. It has been suggested that FABP4 is a useful prognostic marker for urinary bladder cancer and NSCLC. It was also suggested to be a therapeutic target for metastasis of prostate cancer (Uehara et al. 2014). FABP7 was shown to be a specific biomarker for renal cell carcinomas (Teratani et al. 2007). In addition, increased expression of FABP7 was associated with proliferation and invasion of melanoma cells (Slipicevic et al. 2008). Although functional characterisation work and other studies demonstrated that FABP5 and other FABPs may be prognostic markers and treatment targets for different cancer types, the potential involvement of some other FABPs in malignant progression of prostate cancer had not been fully investigated (Al Fayi et al. 2016). The aim of this work is to investigate the expression of FABP proteins in a wide range of samples, including 6 different prostate cell lines and 135 prostate tissues, with the hypothesis that FABP family proteins apart from FABP5 (Bao et al. 2013; Forootan et al. 2016; Forootan et al. 2014; Morgan et al. 2008) might be diagnostic and prognostic markers or potential treatment targets.

4.1 FABP6 and FABP9 were differentially expressed at mRNA level

In this study, we measured mRNA levels of the other 10 different *FABPs* in prostate epithelial cells to assess whether any of them were differentially expressed between benign and malignant phenotypes. The results showed that *FABP4*, *FABP5*, *FABP6*, *FABP9* and *FABP12* exhibited clearly higher levels in all tested malignant cell lines compared to their levels in the benign PNT-2 cells (**Figure 3.3**). Amongst differentially expressed *FABPs*, FABP4 is released from adipocytes, implicated in obesity

Discussion

(Hotamisligil et al. 1996) and its possible involvement in the malignant progression of prostate cancer has been investigated previously (Uehara et al. 2014). The crucial role of FABP5 in promoting tumorigenicity and metastasis of prostate cancer have been characterized by our group. For *FABP6*, a marked level of *FABP6* mRNA expression in malignant cells was observed when compared with no expression in the benign cell line PNT-2. For *FABP9*, its expression in malignant cells was 5-47 folds higher than that in benign cells. For *FABP1*, *FABP2*, *FABP3*, *FABP7* and *FABP8* no clear differences were observed and therefore they are unlikely to be involved in carcinogenesis of prostate cancer. FABP12 is the most recently discovered member of the FABP family, its mRNA level in malignant cells was 3-105 fold higher than that in benign PNT-2 cells (**Figure 3.3**). This result suggested that in addition to FABP4 and FABP5, FABP6, FABP9 and FABP12 may involve in the malignant progression of prostate cancer. For FABP12, it may play an important role in prostate cancer and thus a separate investigation has been conducted in another project to study FABP12 by a member of our group. In contrast to the extensive studies on FABP proteins in different cancer types, the possible involvement of FABP9 in the development of prostate cancer has not previously reported. First, our findings were based on preliminary data suggested overexpression of FABP6 and FABP9 mRNA in prostate cancer cell lines when compared with benign cell line. Our findings suggested that FABP6 and FABP9 may play an important role in progression of prostate cancer because of their correlations with malignant characteristics at mRNA level and were selected for further studies. Notably, the measurement of mRNA of 10 FABPs in cell lines was not repeated. This might cause

Discussion

false negative, although the positive finding was subsequently confirmed by Western blotting. Therefore, the future work should consider this limitation.

4.2 FABP9 was overexpressed in prostate cancer cells and tissues

Although *FABP6* was differentially expressed at the level of its mRNA, the expression of FABP6 in malignant cell lines was not significantly different when compared with benign PNT-2 cells (Student's t-test, $p > 0.05$). Moreover, its nuclear expression at the protein level was not significantly different between the BPH and the carcinoma tissues (**Table 3.1**). These findings suggested that the result on expression of FABP6 in prostate tissues obtained by immunohistological staining was similar to that by Western blot. However, a significant difference was observed in the intensity of FABP6 staining between in highly malignant cases and low malignant cases (χ^2 test, $p < 0.00001$) and between low malignant cases and moderately malignant cases (χ^2 test, $P = 0.02$). The results of the study indicate that the higher expression of FABP6 in human prostate cancer correlated with the GS. Therefore, this study suggest that FABP6 can predict the high stage of prostate cancer and can be used as a potential prognostic marker of disease progression in patients with clinically organ-confined prostate cancer who undergo radical prostatectomy. But to what extent and at what level of accuracy the status of an individual patient can be predicted should be evaluated in further studies. Most FABPs are fatty acid transporters and are localised mainly in cytoplasm (not fixed in a specific site) and move in and out of cell membrane to transport fatty acids. Why FABP6 is localised in nucleus is not known.

Discussion

The expression of FABP9 at the mRNA level (with a size of 365 KB) (Stejskal, Karpisek & Bronsky 2008), in malignant cells was 5-47 fold higher than that in benign cells. When Western blot was used to measure FABP9 protein, its expression was high in highly malignant cell lines PC-3 and PC3-M, whereas its expression in the benign PNT-2 and low-malignant cells was not detectable (**Figure 3.5**). This result demonstrated that elevated levels of FABP9 is correlated with increased malignant characteristics, indicating that FABP9 might play an important role in progression and development of prostate cancer. Using immunohistological staining, it was found that the staining intensity of FABP9 was significantly higher in carcinomas than in BPH. The result also showed that the overall increase in FABP9 was significantly associated with GS (χ^2 test, $p < 0.05$; **Table 3.2**), suggesting that the increased FABP9 stain can reflect the increased degree of malignancy, according to GS. These findings were consistent with PCR and Western blot results which all were in favour of FABP9 overexpression in prostate cancer cells and this is why this work was focused on FABP9.

Immunohistological staining showed that FABP9 protein within prostate cells was localised predominantly in the cytoplasm, as what most of the FABPs do in other cancer cells such as FABP5 in prostate cancer tissue (Morgan et al. 2008). Also, immunostaining for FABP4 in prostate cancer tissue was mainly detected in the cytoplasm (Uehara et al. 2014). However, it has been reported that the localization of FABPs is different amongst different cancer type. In colorectal cancer, FABP1 was localized in both nucleus and cytoplasm (Lawrie et al. 2004). Moreover, FABP7 was expressed in melanomas with a cytoplasmic and/or nuclear localization (Slipicevic et al. 2008).

Discussion

Since the combined GS is the most commonly used parameter to stratify the stage of prostate cancer, the fact that the increased FABP9 level is significantly associated with the increased GS suggests that FABP9 expression may be a useful prognostic factor. FABP9 is poorly understood protein and no previous study has address its role in human disease such as cancer. This work was the first study that directly addressing the involvement of FABP9 in cancer disease.

4.3 Poor patient survival was correlated with increased expression of FABP9 and GS

The lack of reliable biomarkers is a major problem to predict patient survival, the aggressiveness or potential therapeutic response of prostate cancer patients. Further assessment was made on the relationship between AR, PSA, GS or FABP9 expression and the duration of patient survival time. Assessment of the correlation between PSA and the length of patient survival showed no significant difference according to log-rank test and ($p = 0.246$) (**Figure 3.11**). Although PSA is the most common marker for prostate cancer, our finding is in line with the previous studies in which PSA is suggested to be unreliable biomarker.

Results in this work suggested that the increase in staining intensity of AR was of borderline significance in correlation with reduced patient survival time (log-rank test, $p = 0.052$) (**Figure 3.10**). Hence, AR and PSA are not significant prognostic markers in our group of patients, likely because the number of cases was relatively small.

Current results showed that reduced overall patient survival time was significantly associated with increased GS (log-rank test, $p < 0.0001$; **Figure 3.12**). The result also

Discussion

showed that the increase in FABP9 was significantly correlated with reduced overall patient survival time (log-rank test, $p < 0.02$; **Figure 3.9**). The median survival for patients with weak staining for FABP9 was 60 months which was significantly 2.5- and 3.3- times longer than those of the patients with moderate (24 months) and strong (18 months) stains for FABP9, respectively.

These findings suggest that increased level of FABP9 expression is significantly correlated with the poor prognosis in terms of patient's survival time, similar to the prognosis made according to GS. Thus, FABP9 may be an alternative biomarker for reduced cellular differentiation and a reliable prognostic marker to predict patient survival.

Both PSA and AR index (a parameter used to measure the staining intensity of AR (Pertschuk et al. 1995)) are used as biomarkers for prostate cancer. AR plays a key role in carcinogenesis of prostate cancer. Some previous studies showed that AR expression is correlated with the malignant progression of prostate cancer and with the reduced patient survival time. But, there was also evidence suggested that overexpression of AR is not correlated with reduced patient survival (Karantanos, Corn & Thompson 2013; Lee & Chang 2003; Marques et al. 2010). The expression of ARs throughout the progression of the prostate cancer remain controversial which might be because of variable response to endocrine therapy (Lee & Chang 2003). Therefore, AR expression may not always associate with prostate cancer development. The results in this study showed that the AR index is only of borderline significance in its association with reduced patient survival time (log-rank test, $p = 0.052$; **Figure 3.10**).

Discussion

PSA is a glycoprotein belongs to a family so-called kallikrein and produced by the epithelial cells of prostate gland. In prostate cancer, the basal cell layer was disrupted, allowing PSA to leak into blood leading to elevated levels of circulating PSA. Hence, PSA becomes the most commonly used biological marker for prostate cancer. Although the widespread screening for PSA did lead to a degree of decline in mortality rates in some countries, many prostate cancer cases were not picked up by PSA screening in other countries. Several separate studies even suggested that PSA screening had only very limited or even no survival benefits, but it does not have the ability to discriminate between BPH and malignant lesions (Atan & Güzel 2013; Atan et al. 1996). The result in this study agreed with previous studies that correlation between PSA level and patients' survival time was not significantly correlated (log-rank test, $p = 0.246$; **Figure 3.11**). Therefore, neither PSA nor AR index level were reliable prognostic markers for prostate cancer.

4.4 Increased expression of FABP9 was correlated with GS and AR

When the expression of FABP9 was correlated with patient's combined GS, the level of FABP9 was significantly higher in cases with high GS than in those cases with low GS (Mann-Whitney U test, $p < 0.001$). The staining intensity for FABP9 in cases with moderate GS (6 to 7) was significantly higher than that in cases with low GS (Mann-Whitney U test, $p = 0.007$). We suggest that expression status of FABP9 was strongly correlated with GS and the state of tumor differentiation and then the expression of FABP9 can be used to predict patient outcomes and disease relapse.

When the correlation between FABP9 expression and AR index was assessed, the level of the AR index was significantly higher in cases with strong FABP9 expression than in

Discussion

cases with weak expression of FABP9 (Mann-Whitney U test, $p = 0.03$; **Figure 3.12**). In contrast, when FABP9 expression and PSA level was assessed, the differences in patient PSA level were neither significant between strong and weak FABP9 staining (Mann-Whitney U test, $p = 0.142$) nor between strong and moderate FABP9 staining (Mann-Whitney U test, $p = 0.098$) (**Figure 3.14**). This result showed that there was no correlation between FABP9 levels and PSA levels in blood, and suggested that PSA did not reflect the degree of malignancy of the carcinomas.

Like some other previous studies, this work raised questions as to the real value of using PSA as a biomarker for prostate cancer. The real benefit of the widespread use of screening for PSA is still a matter of debate (Kim & Andriole 2015; Schröder et al. 2009; Stavridis et al. 2010). It is confirmed that only 30% of patients with an abnormal PSA value were finally diagnosed with prostate cancer, leading to both an over-biopsy for diagnosis and an over-treatment of low-risk patients (Schröder et al. 2009). Conversely, low serum PSA levels have been detected in patients with advanced metastatic prostate cancer (Lee et al. 2010).

Identification of novel biomarkers that can distinguish between the benign and the malignant nature of each case and can predict the severity of malignancy may provide a new way of disease stratification for prostate cancer and hence reduce unnecessary biopsies (a very invasive procedure) and nonessential treatments. The results in this study suggest that the nature of the patient case and the degree of malignancy are reflected by the level of FABP9 in the primary tumors. The results also suggest that FABP9 is a valuable prognostic marker to predict the outcomes of prostate cancer patients. Male-specific FABP9 protein, because only expressed in the testis, has several

Discussion

important physiological roles in sperm development; including attachment of the acrosome to the sperm nucleus during fertilization and spermatogenesis (Kido & Namiki 2000; Oko & Morales 1994; Smathers & Petersen 2011).

4.5 Suppression of *FABP9* reduced proliferation and invasiveness of prostate cancer cells

As an initial step towards understanding the biological role of FABP9 in prostate cancer events, we assessed whether FABP9 regulates the proliferation, tumorigenicity, invasion and migration of prostate cancer cells. This part of study was performed to test the effect of suppressing expression of FABP9 protein on these cellular characteristics using highly malignant cell line. Knockdown of FABP9 mRNA was performed via RNAi in highly-expressing PC3-M cells to establish highly- and moderately-suppressed transfectant cell lines, named PC3M-FABP9-siH and PC3M-FABP9-siM, respectively. For PC3M-FABP9-siH cells, whose level of FABP9 was reduced by 91%, was reduced by 2.8- fold. In contrast that from PC3M-FABP9-siM, whose level of FABP9 was reduced by only 50%, was reduced by 1.6-fold. This reduction is not achieved by changing the level of FABP5 (**Figure 3.19**); as it has been reported by our group that FABP5 regulates the proliferation, tumorigenicity, invasion and migration of prostate cancer cells (Bao et al. 2013). The effect of FABP9-suppression on growth rate, colony counts, invasiveness and migration rate was evaluated by proliferation assay, soft agar assay invasion assay and cell migration assay, respectively. Soft agar assay showed no significant colony counts (**Figure 3.23**) in transfectants compared to parental control. There was a clear reduction on growth rate of transfectants compared to parental control

Discussion

at day 3 as shown in **Figure 3.21**, but not statistically significant. Knockdown of FABP9 significantly reduced the number of cells invading through the Matrigel-coated membrane (**Figure 3.22**). FABP9 knockdown did not have significant effect on the migratory abilities of prostate cancer cells. The cell migration assay only assesses the simple migratory abilities of cancer cells because it lacks the extracellular matrix barrier. Whereas, invasion assay assesses the abilities to invade and degrade a Matrigel matrix that mimics the *in vivo* matrix barrier. Thus the reduced level of expression of FABP9 is closely related to the reduced invasive ability of the highly-malignant prostate cancer cells. This result suggests that FABP9 may play an important role in malignant progression of prostate cancer cells by promoting cellular invasion.

5 Conclusion

Based on the results obtained from this work, we can make several conclusions which can be summarised as following:

- 1- FABP4, FABP5, FABP6, FABP9 and FABP12 exhibited clearly higher levels in all tested malignant cell lines compared to their levels in the benign PNT-2 cells. For the rest of the FABPs, no clear differences were observed and therefore they are unlikely to be involved in carcinogenesis of prostate cancer.
- 2- The expression of FABP6 at protein level between benign and malignant cell lines and tissues was not significantly different, it is unlikely to be related to prostate cancer.
- 3- Overexpression of FABP9 in highly malignant prostate cancer cell lines, suggesting that FABP9 is correlated with increased malignant characteristics,

Discussion

indicating that FABP9 might play an important role in progression and development of prostate cancer.

- 4- Increased FABP9 staining in prostate tissues was significantly correlated with the increased degree of malignancy, as reflected by the increased GS.
- 5- Increased level of FABP9 expression is significantly correlated with the poor prognosis in terms of patient's survival time, similar to the prognosis made according to GS. Thus, FABP9 may be an alternative biomarker for reduced cellular differentiation and a reliable prognostic marker to predict patient survival.
- 6- The level of the AR index was significantly higher in cases with strong FABP9 staining than in cases with weak expression of FABP9.
- 7- The reduced level of expression of FABP9 is closely related to the reduced invasive ability of the highly-malignant prostate cancer cells. This result suggests that FABP9 may play an important role in malignant progression of prostate cancer cells by promoting cellular invasion.

Thus, FABP9 is a more reliable prognostic marker than PSA to predict the outcome of prostate cancer patients and it may play an important role in the invasion of prostate cancer cells.

6 Future work

To fully establish this study following further studies are needed:

- ❖ FABP6 and FABP9 have the potential to be used as prognostic or diagnostic markers for prostate cancer and are promising due to its specificity for the disease in tissue. However, tissue is unsuitable as substrate for biomarker testing because of its invasiveness and expensiveness. Therefore, testing of cancer-related biomarkers in body fluids such as serum, urine or semen that can be obtained in a non-invasive manner seems a suitable alternative tool. Because of the ease of collection, and the fact that prostate cells are directly released into the urethra through prostatic ducts after DRE, urine has become the future for non-invasive biomarker testing.
- ❖ Investigating the relationship between mRNA and protein expressions of FABP9 and other candidate FABPs in patient tissues using qPCR-based test to find out if the former can replace the latter as a predictive biomarker in prostate cancer.
- ❖ Investigating the interrelationship among FABP4, FABP5 and FABP9 (may be FABP12) in predicting clinical outcomes of prostate cancer, to find out whether these FABPs are dependent or independent on each other to predict patient survival time.
- ❖ More in-vivo studies are needed to assess the ability of FABP9 to promote the proliferation and invasion abilities of prostate cancer cells and the possibility of using it as a treatment target.

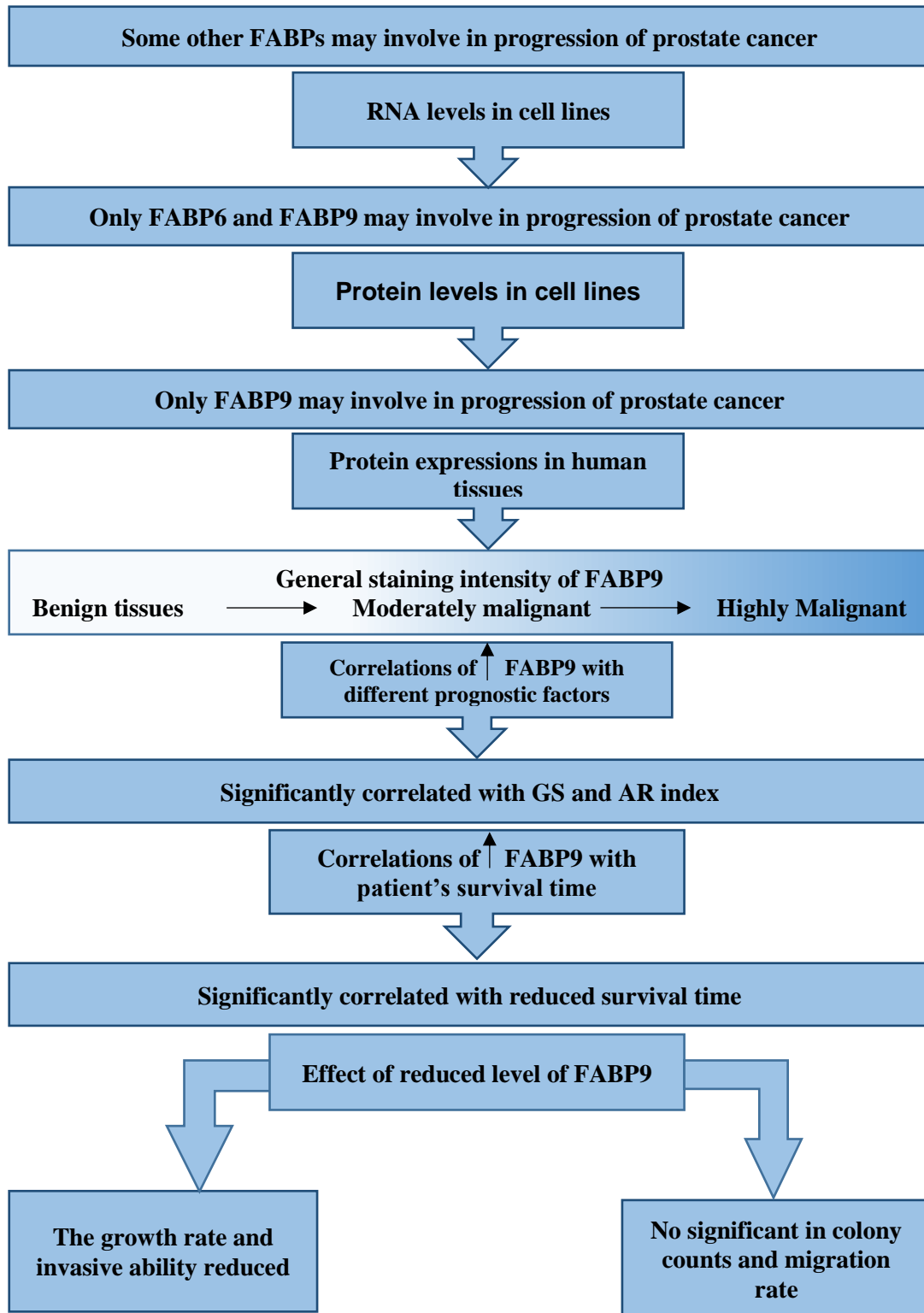


Figure 4. 1: Project summary and findings.

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APPENDIX

Appendix

8 Appendix A:

8.1 Equipment

BD BioCoat™ Growth Factor Reduced (GFR) Matrigel™ 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

.....

Cryobox DNA

Nalgene, UK

.....

Appendix

Falcon 2059 tube

Becton Dickinson, USA

.....

Gel electrophoresis rig

Bio-Rad, Hemel, UK

.....

Gel drier

Flowgen, Nottingham, 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

Appendix

.....
Light microscope

Leitz Labovert, Luton, UK
.....

Microtome HM355

Microm, Oxford, UK
.....

Magnificent stirrer

SLS Ltd., Nottingham, UK
.....

Microtubes

Starlab, Milton Keynes, UK
.....

Microslide

Surgipath, UK
.....

Multiskan MS (plate reader)

Labsystem, Finland
.....

Microcentrifuge

Beckman coulter, UK
.....

Appendix

Needle

BD Microlance 3, Ireland

.....

NanoDrop spectrophotometer

Labtech International, Ringmer, UK

.....

Portable Lab Scale

AccuWeigh, WA, USA

.....

Pipette tips

Qiagen tip

QIA Shredder spin column

QIAGEN, Crawley, UK

.....

Syringes

BD Microlance 3, Ireland

.....

Sirius Luminometer

Berthold detection system, Germany

.....

Spectrophotometer

BioTec, Brigend, UK

.....

Appendix

Superior Adhesive slide

Leica, Germany

.....

Sequenza slide rack

Shandon, UK

.....

Tissue cassette

Surgipath, UK

.....

Thermal cycler (Peltier Thermal Cycler PTC-200)

GMI, MJ Research, MN, USA

.....

Tissue culture pipettes (5-50 ml)

Generier bio-one, UK

.....

Universal tube

Generier bio-one, UK

.....

Water bath

Grant Instruments, UK

.....

Whatman filter paper

Whatman, England, UK

Appendix

8.2 Reagents Supplier

8.2.1 Reagents for cell culture

DMSO	Sigma-Aldrich, Germany
Foetal calf serum	Biosera, East Sussex, 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	PAA, Pasching, Austria
Sodium pyruvate	Sigma, USA
Trypsin	Gibco, Invitrogen, Paisley, UK
Versene	Gibco, Invitrogen, Paisley, UK
X-tremeGENE HP DNA Transfection Reagent	Roche, Germany
X-tremeGENE siRNA Transfection Reagent	Roche, Germany
Zeocin	Invitrogen, CA, USA

Appendix

8.2.2 Reagents for Western blot

β-mercaptoethanol	Sigma, USA
Ammonium persulfate (APS)	Sigma, USA
Bradford reagent	Sigma, USA
Bromophenol blue	Sigma, USA
CellLytic-M	Sigma, USA
Coomassie brilliant blue	Bio-Rad GmbH, Munchen, Germany
ECL detection kit	GE Healthcare, Buckinghamshire, UK
Glycine	Melford, UK
Kodak Developer	Sigma, USA
Kodak film	GE Healthcare, Buckinghamshire, UK
Kodak Fixer	Sigma, USA
Methanol	Fisher scientific, Loughborough, UK

8.2.3 Reagents for IHC

Acetone	Sigma, USA
DPX	Bios, Lancashire, UK
EDTA	Sigma, USA
EnVision™ FLEX/DAB+	DakoCytomation, Ely, UK
Chromogen	
EnVision™ FLEX/HRP	DakoCytomation, Ely, UK
Ethanol (IMS)	GENTA, Tockwith, UK

Appendix

Formaldehyde	Sigma, USA
Haematoxylin	Sigma, USA
Hydrogen peroxide 30% (w/w)	BDH, England, UK
Linker (Anti-gout IgG) (AI 5000)	Vector, USA
Scott's tap water	Sigma, USA
Sodium citrate	Sigma, USA
Sodium chloride	Melford, UK
Tris base ultrapure	Melford, UK
Tween-20	Sigma-Aldrich, Germany
Xylene	GENTA, Tockwith, UK

8.2.4 Reagents for RT-PCR

dNTPs	Thermo scientific, UK
DTT	Invitrogen, Paisley, UK
Oligo (dT)20	Agilent Technologies, CA, USA
PCR primers	Invitrogen, Paisley, UK
Platinum ®PCR SuperMix High Fidelity	Invitrogen, Paisley, UK

8.2.5 Reagents for general molecular biology

Ampicillin	Sigma, USA
Absolute ethanol	BDH, England, UK

Appendix

Agarose	<i>Genflow</i> , Fradley, UK
DNA marker III	Roche, England, UK
DNA marker XIV	Roche, England, UK
<i>E. coli</i> GT116 cell	InvivoGen, USA
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
MOPS	Sigma, USA
psiRNA-h7SKGFPzeo plasmid	InvivoGen, USA
QIAGEN Plasmid midi-preparation kit	QIAGEN, CA, USA
QIAGEN Plasmid mini-preparation kit	QIAGEN, CA, USA
Restriction enzymes	New England BioLabs, MA, USA
Restriction enzymes buffers	New England BioLabs, MA, USA
Safe View (Nucleic Acid Stain)	NBS Biological, Cambridgeshire, UK
Tryptone	Sigma, USA
Wizard® DNA Clean-Up System	Promega, WI, USA
Yeast extract	Fisher scientific, Loughborough, UK
Zeocin	Invitrogen, CA, USA

Appendix

8.2.6 Reagents for cell proliferation assay

MTT Sigma, USA

8.2.7 Reagents for cell invasion assay

Crystal violet Sigma, USA

8.2.8 Reagents for soft agar assay

Low melting point agarose *Genflow*, Fradley, UK

MTT Sigma, USA

8.3 BUFFERS

8.3.1 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 Zeocin™... (100µg/ml)

Appendix

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

1× Versene..... 100ml

Trypsin.....2.5ml

MTT solution (5mg/ml)

MTT.....50mg

PBS.....10ml

PBS

PBS..... 1 tablet

dH₂O..... 500ml

Autoclaved

8.3.2 Western Blot

1M 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

Appendix

2× SDS-PAGE sample loading buffer (SLB)

1M 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

5× SDS-PAGE sample loading buffer (SLB)

1M Tris-HCl (pH 6.8) ...1.25ml

Glycerol 40% (v/v)15ml

Bromophenol blue 0.5% (w/v) ...2.5ml

SDS 10%5ml

β-mercaptoethanol1.25ml

Transfer buffer (pH 8.3)

Glycine14.4g (192mM)

Methanol 20% (v/v)

Tris base 3.03g (25mM)

dH₂O up to 1Lit

pH adjusted with HCl

10× TBS buffer (pH 7.6)

Sodium chloride 87.66gr (1500mM)

Tris base 60.58gr (500mM)

dH₂O up to 1 Lit

Appendix

pH adjusted with HCl

Autoclaved

1×TBS-Tween 1%

10× TBS buffer 100ml

Tween 20 1ml

dH₂O up to 1 Lit

TBS-T-milk 5% (protoblock)

Dried milk 5gr

1×TBS-T 100ml

8.3.3 Immunohistochemistry

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)

EDTA37.2gr

Sodium hydroxide 3.2gr

dH₂O up to 10 Lit

Appendix

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

8.3.4 Molecular Biology

LB medium

LB broth20gr

dH₂O 1 Lit

Autoclaved

LB agar

LB agar35gr

dH₂O 1 Lit

Appendix

Autoclaved

RF 1 buffer (pH 5.8)

KClb 7.456gr (100mM)

MgCl₂.4H₂O 9.9gr (50mM)

K-acetate 2.94gr (30mM)

CaCl₂ 1.5gr (10mM)

Glycerol (v/v) 150ml (15%)

dH₂O up to 1 Lit

Adjust the pH and sterilized by filtration

RF 2 buffer (pH 6.8)

MOPS 2.1 gr (10mM)

KCl 0.745gr (10mM)

CaCl₂ 11gr (75mM)

Glycerol (v/v) 150ml (15%)

dH₂O up to 1 Lit

Adjust the pH and sterilized by autoclave

Glucose 20%

Glucose 20gr

dH₂O 10ml

Sterilized by filtration

Magnesium salt solution (2M)

MgCl₂ 2.033gr (1M)

MgSO₄ 2.465gr (1M)

Appendix

dH₂O 10ml

Sterilized by filtration

SOB medium (pH 7)

Tryptone 20gr

Yeast extracts 5gr

NaCl..... 0.5gr

KCl 0.186gr

dH₂O up to 1 Lit

Adjust the pH and sterilized by autoclave

SOC medium

SOB medium 4.850ml

Mg₂⁺ salt solution (2M) ... 50µl

Glucose 20% 150µl

Stock medium for bacteria

Glycerol 5ml

LB medium 4ml

Bacteria culture 3ml

10× TBE stock solution

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

Appendix

50× 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

6×DNA loading buffer

Bromophenol blue 0.5% 0.5ml

Xylene cyanol FF0.5ml

Glycerol in sterile dH₂O (60%) ... 1ml

9 Appendix B: Publications, Awards, Conferences