

**EFFECTS OF PADDY WASTE PRODUCTS IN
PROSTATE DISEASE USING IN VITRO AND IN
VIVO MODELS**

by

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TABLE OF CONTENTS

ACKNOWLEDGEMENT	ii
TABLE OF CONTENTS	iii
LIST OF TABLES	xii
LIST OF FIGURES	xiv
LIST OF SYMBOLS AND ABBREVIATIONS	xxi
ABSTRAK	xxiv
ABSTRACT	xxvi

CHAPTER ONE : INTRODUCTION

1.1	Research background	1
1.2	Relevance of the study	3
1.3	Hypothesis and objectives of the study	5
1.4	Flow chart of the study	6

CHAPTER TWO : LITERATURE REVIEW

2.1	Prostate gland	
2.1.1	Development of prostate gland	7
2.1.2	Structure of prostate gland	8
2.1.3	Function of prostate gland	10
2.1.4	Clinical significance of prostate	10
2.2	Prostate Hyperplasia	13
2.2.1	Origin and development of prostate hyperplasia	13
2.2.2	Risk factors of prostate hyperplasia	14

2.9.2	Paddy waste	39
2.9.2(a)	Paddy husk and its chemoprevention potential	39
2.9.2(b)	Paddy straw and its chemoprevention potential	40

CHAPTER THREE : MATERIALS AND METHODS

3.1	List of reagents	41
3.2	Paddy waste (husk and straw) extraction	42
3.2.1	Water extracts	42
3.2.2	Aqueous methanol extracts	43
3.3	Cell culture	43
3.3.1	Cell lines	43
3.3.1(a)	Human prostatic adenocarcinoma (DU145) cells	43
3.3.1(b)	Mouse skin fibroblast cell line (L929)	44
3.3.2	Maintaining the growth of cells	44
3.3.2(a)	Resuscitation of frozen cells and subculture	44
3.3.2(b)	Cryopreservation of cells	45
3.3.3	Determination of inhibitory concentration (IC ₅₀)	45
3.3.3(a)	Calculation of paddy husk and straw extracts concentration	45
3.3.3(b)	Treatment of DU145 cells with paddy waste compound in different concentrations	46
3.3.3(c)	Trypan blue exclusion assay (TBEA)	46
3.3.3(d)	Microscopic observation of morphological changes	47
3.3.4	Cell proliferation assay	47

3.3.5	Cell cytotoxicity assay	47
3.3.6	Apoptosis analysis with flow cytometer	
	3.3.6(a) Preparation for treatment samples	48
	3.3.6(b) Preparation of controls for flow cytometer setup	49
3.3.7	Cell cycle analysis with flow cytometer	
	3.3.7(a) Preparation of treatment samples	49
	3.3.7(b) Preparation of DNA QC Particles for flow cytometer calibration	50
3.3.8	DNA fragmentation analysis	51
3.3.9	Hoechst 33342 stain analysis	51
3.3.10	Scanning electron microscopy analysis	51
3.3.11	Protein Extraction	
	3.3.11(a) Preparation of total cell lysates	52
	3.3.11(b) Determination of protein concentration	53
	3.3.11(c) Preparation of BSA standards	53
	3.3.11(d) Preparation of protein samples	54
	3.3.11(e) Preparation of dye reagent	55
	3.3.11(f) Measuring protein concentration with spectrophotometer	55
3.3.12	Western Blot Analysis	
	3.3.12(a) Preparation of protein samples	56
	3.3.12(b) SDS-PAGE	56
	3.3.12(c) Semi-dry Blotting of protein to nitrocellulose membrane	57
	3.3.12(d) Blocking of nitrocellulose membrane	58

	3.3.12(e) Immunoblotting of primary and secondary antibody	58
	3.3.12(f) Imaging of membrane	59
3.4	Animal study	
3.4.1	Preparation of extracts and dose calculation	59
3.4.2	Animals	60
3.4.3	Experimental design and sample collection	61
3.4.4	Measurement of blood pressure	62
3.4.5	Tissue analysis	
	3.4.5(a) Haematoxylin and eosin staining analysis	63
	3.4.5(b) Immunohistochemistry analysis	
	3.4.5(b)(i) Androgen receptor (AR) and proliferation marker (Ki67)	65
	3.4.5(b)(ii) Apoptosis TUNEL assay	66
3.4.6	Protein Extraction	
	3.4.6(a) Preparation of tissue lysates	67
	3.4.6(b) Determination of Protein concentration	67
	3.4.6(c) Preparation of BSA standards	67
	3.4.6(d) Preparation of protein samples	67
	3.4.6(e) Preparation of dye reagent	67
	3.4.6(f) Measuring protein concentration with spectrophotometer	67
3.4.7	Western Blot Analysis	68
3.4.8	Gene expression analysis with real time PCR	
	3.4.8(a) RNA isolation	68

3.4.8(b) cDNA synthesis	69
3.4.8(c) Standard PCR and agarose gel electrophoresis	70
3.4.8(d) Quantitative real time PCR	72
3.5 Statistical analysis	73

CHAPTER FOUR : RESULTS

4.1 Extraction yield	74
4.2 Effect of paddy waste extracts on androgen-independent prostate cancer cell line (DU145)	75
4.2.1 Half maximal inhibitory concentration (IC ₅₀) of paddy waste extracts on DU 145 cells	75
4.2.2 Cell proliferative analysis of paddy waste product on DU145 cells	80
4.2.3 Cytotoxicity test of paddy waste water and aqueous methanol extracts on mouse skin fibroblast cell line (L929)	82
4.2.4 Apoptotic effect of paddy waste extracts on DU 145 cells	86
4.2.5 Effect of paddy waster extracts on cell cycle phase of DU145 cells.	88
4.2.6 DNA fragmentation analysis	90
4.2.7 Observation on the morphological changes of DU145 cells	91
4.2.8 Effect of paddy waste extracts on protein expression in apoptosis pathways of DU 145 cells	95
4.3 Effect of paddy waste aqueous methanol extracts on prostate hyperplasia by using Spontaneously Hypertensive Rats (SHR)	100

4.3.1	The effect of paddy waste aqueous methanol extracts on SHR and WKY body weight	100
4.3.2	The effect of paddy waste aqueous methanol extracts on SHR and WKY water intake	101
4.3.3	The effect of paddy waste aqueous methanol extracts on SHR and WKY food intake	102
4.3.4	Blood pressure in SHR and WKY rats after treated with paddy waste aqueous methanol extracts.	103
4.3.5	Haematology and biochemistry analysis	106
4.3.6	Relative organ weight analysis	108
4.3.7	Histology analysis	109
	4.3.7(a) Histomorphometric analysis in ventral prostate of SHR rats.	109
4.3.8	Immunohistochemistry analysis in ventral prostate of SHR and WKY rats	116
	4.3.8(a) Androgen receptor and Ki 67 expression	116
	4.3.8(b) Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End Labeling (TUNEL) assay analysis	120
4.3.9	Effect of paddy waste extracts on protein expression in ventral prostate of SHR rats.	122
4.3.10	Effect of paddy waste extracts on gene expression in ventral prostate of SHR rats.	126

CHAPTER FIVE : DISCUSSION

5.1	Synergistic effect of paddy waste product.	128
5.2	In vitro studies of paddy waste product against androgen-independent prostate cancer cell line (DU145)	130
5.2.1	Extraction yield	130
5.2.2	Inhibitory and cytotoxic activities of paddy waste product on androgen-independent prostate cancer cell line (DU145)	131
5.2.3	Paddy waste product inhibits proliferation and induces apoptosis in DU145 cell line	133
5.3	In vivo studies of paddy waste product against Spontaneously Hypertensive Rat (SHR)	137
5.3.1	Toxicity analysis of paddy waste product consumption on body weight, dietary intake and vital organs.	137
5.3.2	Effect of paddy waste aqueous methanol extracts on haematology and biochemical parameters of SHR.	138
5.3.3	Effect of paddy waste aqueous methanol extracts on blood pressure of SHR.	139
5.3.4	Effect of paddy waste aqueous methanol extracts on relative organ weight of SHR	140
5.3.5	Effect of paddy waste aqueous methanol extracts on proliferation of rat hyperplasia of SHR.	141
5.3.6	Effect of paddy waste aqueous methanol extracts on androgen receptor-induced proliferation of ventral prostate	143
5.4	Strength, limitations and recommendation for future research	145

CHAPTER SIX : CONCLUSION	147
REFERENCES	149
APPENDICES	
LIST OF PUBLICATION	

LIST OF TABLES

	Page
Table 2.1 Staging of BPH	15
Table 2.2 Androgen receptor status in different forms of prostate cancer.	27
Table 3.1 Preparation for BSA standard solutions using 2mg/ml BSA	54
Table 3.2 Preparation of protein samples	54
Table 3.3 Primer sequence of gene of interest and housekeeping gene	69
Table 3.4 Genomic DNA elimination reaction components	69
Table 3.5 Reverse transcription master mix preparation	70
Table 3.6 Standard PCR master mix preparation	71
Table 3.7 Pre-optimized cycling protocol	71
Table 3.8 Real time PCR reaction mix	72
Table 3.9 Thermal cycle profile	73
Table 4.1 Extraction yield of paddy husk and straw extracts	74
Table 4.2 Paddy husk (water extract) treated DU145 cell number and viability	76
Table 4.3 Paddy straw (water extract) treated DU145 cell number and cell viability	77
Table 4.4 Paddy husk (aqueous methanol) treated DU145 cell number and viability	78
Table 4.5 Paddy straw (aqueous methanol) treated DU145 cell number and viability	79
Table 4.6 Cells proportions (%) distribution of Annexin V/ PI staining	87
Table 4.7 Cell cycle phase distribution (%) of untreated DU 145 cells and paddy waste extracts treated DU145 cells.	89

Table 4.8	Systolic blood pressure of SHR and WKY rats after 16 weeks of treatment	104
Table 4.9	Hematology and biochemistry analysis of control and treated SHR group	107
Table 4.10	Relative organ weight of ventral prostate, liver and kidney of SHR rats.	108
Table 4.11	Histomorphometric analysis of ventral prostate of treated SHR rats with control SHR rats	110
Table 4.12	Effects of paddy waste product on the androgen receptor and Ki-67 labelling index in ventral prostate of SHR and WKY	117
Table 4.13	Effects of paddy waste product on the apoptotic index through TUNEL assay in ventral prostate of SHR and WKY.	120

LIST OF FIGURES

	Page
Figure 2.1 Schematic representation of normal prostate.	8
Figure 2.2 Multistage carcinogenesis model which divided into three main stages: initiation, promotion and progression.	16
Figure 2.3 Prostate cancer: Incidence, Mortality and Prevalence Worldwide in 2012 (age-standardised rates per 100,000)	20
Figure 2.4 Prostate: Age-specific incidence rate, Malaysia, 2007-2011	21
Figure 2.5 Prostate: International comparisons – Age-standardised rates	21
Figure 2.6 Extrinsic and intrinsic pathway of apoptosis.	29
Figure 2.7 Chemo preventive agents act within the process of carcinogenesis preferably in the initiation and promotion process.	32
Figure 3.1 The arrangement of the transblot sandwich.	58
Figure 3.2 Experimental design	61
Figure 3.3 Pictures from Image J software (E and F) been added with formulation of relative epithelial area to acinar area. Magnification of all photomicrographs is 100X	64
Figure 4.1 Cell viability of DU 145 cells treated with paddy husk water extract. Values are represented in $\pm$ SD manner of triplicates. White marker represents the IC ₅₀ value. *p<0.05 compared to untreated cells by one-way ANOVA with post hoc Tukey test.	76
Figure 4.2 Cell viability of DU 145 cells treated with paddy straw water extract. Values are represented in $\pm$ SD manner of triplicates. White marker represents the IC ₅₀ value. *p<0.05 compared to	77

untreated by one-way ANOVA with post hoc Tukey test.

- Figure 4.3 Cell viability of DU145 cells treated with paddy husk aqueous 78
methanol extract. Values are represented in $\pm$ SD manner of
triplicates. White marker represents the IC₅₀ value. *p<0.05
compared to untreated by one-way ANOVA with post hoc
Tukey test.
- Figure 4.4 Cell viability of DU 145 cells treated with paddy straw 79
aqueous methanol extract. Values are represented in $\pm$ SD
manner of triplicates. White marker represents the IC₅₀ value.
*p<0.05 compared to untreated by one-way ANOVA with post
hoc Tukey test.
- Figure 4.5 The viable cell numbers determined by using Trypan Blue 81
Exclusion Assay (TBEA) demonstrated that proliferation of
DU145 cells was significantly suppressed by IC₅₀ doses of all
four extracts after 8 days treatment; paddy husk water extract,
paddy straw water extract, paddy husk aqueous methanol
extract and paddy straw aqueous methanol extract. Values are
represented in $\pm$ mean SD manner of triplicates.
- Figure 4.6 The cytotoxic effect of paddy waste water extract on L929 cell 83
line (a) paddy husk water extract; (b) paddy straw water
extract.
- Figure 4.7 The cytotoxic effect of paddy waste water extract on L929 cell 84
line (c) paddy husk aqueous methanol extract; (d) paddy straw
aqueous methanol extract.
- Figure 4.8 Morphology of L929 cells before and after treatment with 85

paddy waste extracts which indicated no cytotoxicity effects occurred.

- Figure 4.9 Agarose gel electrophoresis for DNA fragmentation assay 90
(apoptotic ladder) showing Lane 1 (DNA ladder 100bp), Lane 2 (negative control), Lane 3 (DNA isolated from untreated cells), Lane 4 (HW- paddy husk water extract), Lane 5 (SW- paddy straw water extract), Lane 6 (HM- paddy husk aqueous methanol extract), Lane 7 (SM- paddy straw aqueous methanol extract) and Lane 8 (DNA ladder 100bp). Lane 4 to Lane 7 indicated there are fragmentation (apoptotic ladder) (**white box**) after treatment with paddy waste extracts for 72 hours.
- Figure 4.10 Cell morphology of DU 145 cells with and without paddy 93
waste extracts treatment. There are few apoptotic morphological changes seen (400x) such as cell shrinkage (**C**), membrane blebbing (**M**) and apoptotic bodies (**A**).
- Figure 4.11 Hoechst 33342 staining carried out with control cells were 94
seen with uniformly light blue nuclei while treated DU 145 cells showed nuclear shrinkage (**NS**) and nuclear fragmentation (**NF**). SEM analysis revealed membrane ruffles (**MR**) more in control untreated, membrane blebs (apoptosis feature) (**black arrow**) and membrane protrusion (**Cyt**) scattered on the treated cancer cell surface.
- Figure 4.12 Effect of paddy waste aqueous methanol extracts on protein 97
expression involved in caspase 3 mediated intrinsic apoptosis pathway of DU 145 cells. Membrane images captured with

Molecular Imager VersaDoc™ MP 4000 System software, the average fold changes of triplicates were below the bands.

- Figure 4.13 Effect of paddy waste treatment on protein expression involve 98
caspase 3 mediated intrinsic apoptosis pathway of DU 145
cells. Analysis of fold change data was done by using Image J
software. Values are represented in $\pm$ SD manner of triplicates.
* $p < 0.05$, ** $p < 0.01$ as compared to control untreated by using
one-way ANOVA with post hoc Tukey test.
- Figure 4.14 Schematic representation of the molecular pathway of caspase 99
3 mediated intrinsic apoptotic effects. This diagram has
showed how crucial the protein markers expression (black
box) induced by the paddy husk aqueous methanol extract on
effect on intrinsic apoptosis pathway (Adapted from Fiandalo
and Kyprianou., 2012).
- Figure 4.15 SHR rats mean weekly body weights during 17 weeks of 100
dietary intervention (mean $\pm$ SD), differences between groups
did not reach statistical significance.
- Figure 4.16 SHR rats mean weekly water intake during 17 weeks of dietary 101
intervention (mean $\pm$ SD), differences between groups did not
reach statistical significance.
- Figure 4.17 SHR rats mean weekly food intake during 17 weeks of dietary 102
intervention (mean $\pm$ SD), differences between groups did not
reach statistical significance.
- Figure 4.18 Systolic blood pressure decreased with addition of paddy 105
waste aqueous methanol extracts (husk and straw) in drinking

water compared to control group. Graphs show mean $\pm$ SEM.

* $p < 0.05$; ** $p < 0.01$ hypertensive vs. Control by using one-way ANOVA with post hoc Tukey test.

Figure 4.19 Photomicrograph showed the differences of number of acini in ventral prostate lobe of A (control SHR group), B (normotensive WKY group), C (paddy husk group) and D (paddy straw group). Magnification of all photomicrographs is 40X. 111

Figure 4.20 Photomicrograph showed the differences of epithelial and luminal area in ventral prostate lobe of A (control SHR group), B (normotensive WKY group), C (paddy husk group) and D (paddy straw group). Pictures from Image J software (E and F) been added with formulation of relative epithelial area to acinar area. Magnification of all photomicrographs is 100X. 112

Figure 4.21 Photomicrograph showed the differences of epithelial height in ventral prostate lobe of A (control SHR group), B (normotensive WKY group), C (paddy husk group) and D (paddy straw group). Magnification of all photomicrographs is 200X. 113

Figure 4.22 Histology analysis on dorsolateral prostate and seminal vesicle showed there were no significance difference between the lobes of dorsolateral prostate and epithelial cells lining the seminal vesicle. Magnification of all photomicrographs is 100X. 114

Figure 4.23 Histology analysis on dorsolateral liver and kidney of all 115

groups showed there were no significance difference of histology structure. Magnification of all photomicrographs is 100X.

Figure 4.24 Immunohistochemical staining for androgen receptor (AR) in ventral prostate of control SHR (A), WKY (B), paddy husk methanol extract group (C) and paddy straw methanol extract group (D). A, AR expression was found higher in cell nucleus (black arrows) B, AR staining less intense in ventral prostate of WKY tissue, C and D, downregulated AR expression was found in cell nucleus (white arrows). Magnification×1000 for all images. 118

Figure 4.25 Immunohistochemical staining for Ki-67 in ventral prostate of control SHR (A), WKY (B), paddy husk methanol extract (C) and paddy straw methanol extract (D). A, Ki-67 expression was found in cell nucleus of ventral prostate tissue and the arrow indicates the positive area in the tissues (black arrows). B, Ki-67 staining less intense in ventral prostate of WKY tissue, C and D, downregulated AR expression was found in cell nucleus (white arrows). Magnification×200 for all images. 119

Figure 4.26 TUNEL assay analysis in ventral prostate of control SHR (A), WKY (B), paddy husk methanol extract (C) and paddy straw methanol extract (D). The arrows indicate typical positively stained apoptotic epithelial cells that were used to determine the TUNEL index. Magnification 200X for all images. 121

Figure 4.27 Effect of paddy waste methanol extracts on protein expression 123

that involved in proliferation and androgen dependent cell cycle of ventral prostate of SHRs. Membrane images captured with Molecular Imager VersaDoc™ MP 4000 System software, the average fold changes of triplicates were underneath the bands.

Figure 4.28 Effect of paddy waste treatment on protein expression of proliferation and androgen dependent cell cycle of ventral prostate of SHRs. Analysis of fold change data done by using Image J software. Values were represented in $\pm$ SD manner of triplicates. * $p < 0.05$, ** $p < 0.01$ as compared to control untreated by using one-way ANOVA with post hoc Tukey test. 124

Figure 4.29 Schematic representation of the molecular pathway of androgen dependent cell cycle. This diagram has showed how crucial the protein markers expression (**black box**) induced by the paddy husk aqueous methanol extract on effect on androgen dependent cell cycle (Won *et al.*, 2012). 125

Figure 4.30 Verification of expression differences in candidate gene (Androgen receptor) in ventral prostate of SHR using relative quantification (RQ). Control group indicate gene expression verification of untreated SHR ventral prostate while husk and straw groups indicate gene expression verification of SHR ventral prostate after being treated with paddy husk and straw aqueous methanol extracts. * $p < 0.05$, ** $p < 0.01$ as compared to control untreated by using one-way ANOVA with post hoc Tukey test. 127

LIST OF SYMBOLS AND ABBREVIATIONS

Apaf-1	apoptotic protein activating factor-1
PC-3	human prostate adenocarcinoma cell line
BPH	benign prostatic hyperplasia
%	percentage
µm	micrometer
ADT	androgen deprivation therapy
ALP	alkaline phosphatase
ALT	alanine transferase
AR	androgen receptor
ARASC	Animal Research and Service Center
ARC	Animal Research Complex
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
CAPE	caffeic acid phenethyl ester
CDKs	cyclin-dependent kinases
DBD	DNA binding domain
DHT	5α -dihydrotestosterone
DRE	digital rectal examination
DU145	androgen-independent prostate cancer cell line
g	gram
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Hb	haemoglobin
HCT	haematocrit

HG-PIN	High-grade prostatic intraepithelial neoplasia
HG-PIN	High-grade prostatic intraepithelial neoplasia
HMDS	hexamethyldisilazane
hPAP	Human Prostatic Acid Phosphatase
L929	Mouse skin fibroblast
LBD	C-terminal ligand binding domain
LH	luteinizing hormone
LNCaP	androgen sensitive prostate cancer cell line
MITs	minimally invasive treatments
ml	millilitre
mm ³	cubic millimetre
nm	nanometer
NTD	N-terminal domain
°C	Celsius
PBS	phosphate buffer saline
PCNA	proliferating cell nuclear antigen
PSA	prostate-specific antigen
RB	retinoblastoma
RBC	red blood cell
RQ	relative quantification
SHR	spontaneously hypertensive rats
TAE	Tris-acetate-EDTA buffer
TBEA	trypan blue exclusion assay
TNM	tumour, node and metastasis
TRAMP	transgenic adenocarcinoma of the mouse prostate

TSG	Tumour suppressor genes
TUNEL	Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End Labeling
TURP	transurethral resection of the prostate
UGS	urogenital sinus
WBC	white blood cell
WKY	Wistar Kyoto rat

KESAN PRODUK BUANGAN PADI KE ATAS PENYAKIT PROSTAT

MENGGUNAKAN MODEL IN VITRO DAN IN VIVO

ABSTRAK

Hiperplasia prostat benigna (BPH) merupakan penyakit yang biasa dihadapi oleh warga emas lelaki dan kanser prostat ialah penyebab kedua utama kematian dalam kalangan lelaki. Sebelum ini, sisa padi (kedua-dua sekam dan jerami) dilaporkan mempunyai potensi mencegah penyakit terutamanya kanser melalui agen kimia (kemocegah). Tujuan utama kajian ini adalah untuk meneroka sifat kemocegah sisa padi menentang penyakit prostat. Kajian ini menentukan aktiviti perencatan dan anti-proliferatif produk sisa padi masing-masing dalam titisan sel kanser prostat yang bebas androgen, DU145 dan tikus hipertensi spontan (SHR); kesan produk sisa padi terhadap proliferasi dan apoptosis ekspresi gen dan protein yang berkaitan dalam DU145; kesan ekstrak metanol akueus terhadap histologi, PCNA dan ekspresi reseptor androgen dalam SHR. Terdapat dua jenis pelarut yang digunakan untuk ekstrak sekam dan jerami padi dalam kajian ini: air dan metanol akueus. Eksperimen kultur sel memperlihatkan bahawa ekstrak metanol akueus mempunyai kesan perencatan yang lebih baik daripada ekstrak air bagi DU145 tanpa ketoksikan. Analisis apoptosis memperlihatkan bahawa ekstrak metanol akueus mempamerkan kesan mengaruh apoptosis bagi DU145 dan dakwaan ini disokong dengan analisis DNA (kehadiran jalur yang menunjukkan degradasi DNA) dan analisis mikroskopi (pengecutan sel, pelepuhan membran dan jasad apoptotik). Analisis blot barat memperlihatkan bahawa apoptosis diaruh melalui laluan intrinsik berperantara kaspase 3. Dua protein pro-apoptotik, Bax dan Bad menunjukkan lebih tinggi ($p < 0.05$) apoptosis dan protein anti-apoptotik, Bel-2 mengawal atur secara menurun

($p < 0.01$) selepas perawatan. Keadaan ini meningkatkan ekspresi kaspase 9 dan seterusnya mengaktifkan kaspase 3 dan 7 ($p < 0.01$) yang menyebabkan sel mengalami apoptosis. Ekstrak metanol akueus dipilih berbanding ekstrak air bagi kajian in vivo bagi model SHR. SHR yang diberi air minuman yang dicampurkan dengan ekstrak metanol akueus selama 17 minggu menunjukkan tiada kesan ketoksikan pada pengambilan pemakanan, berat badan dan organ vital (hati dan ginjal). Air minuman ini dapat mengurangkan tekanan darah [72.0 mm Hg ($p < 0.01$)] dan saiz prostat ventral kepada lebih kurang 0.05% ($p < 0.01$). Analisis histologi memperlihatkan tanda-tanda anti-proliferatif seperti pengurangan bilangan asinus [(7.50) ($p < 0.01$)], ketinggian epitelium [(10.55 μ m) ($p < 0.001$)] dan keluasan asinus epitelium [(18.17% ($p < 0.01$))]. Oleh sebab cerakin TUNEL menunjukkan keputusan yang tidak bererti yang mengecualikan apoptosis, ekstrak metanol akueus telah memberhentikan kitaran sel dengan mengawal atur secara menurun ($p < 0.01$) penanda proliferasi, Ki-67 dan PCNA. Pertumbuhan sel prostat diberhentikan melalui pengawalan secara menurun AR yang merencatkan transkripsi mRNA AR (analisis RTPCR) dan mengaruh pemberhentian kitaran sel pada fasa S melalui p27 dan siklin E2 (analisis blot barat). Sekam padi ialah agen kemocegah yang lebih baik daripada jerami padi dalam menentang penyakit prostat.

EFFECTS OF PADDY WASTE PRODUCTS IN PROSTATE DISEASE

USING IN VITRO AND IN VIVO MODELS

ABSTRACT

Benign prostate hyperplasia (BPH) is common in elderly men and prostate cancer is the second leading cause of death among men. Previously, paddy waste (both husk and straw) reportedly have chemopreventive potential. The main aim of this study was to explore the chemopreventive property of paddy waste against prostate disease. This study determines the inhibitory and anti-proliferative activity of the paddy waste product in androgen-independent prostate cancer cell line, DU145 and spontaneously hypertensive rat (SHR) respectively; the effects of paddy waste products on proliferation and apoptosis related genes and protein expression in DU145; the effect of aqueous methanol extracts on histology, PCNA and androgen receptor expressions in SHR. There were two types solvent used for paddy husk and straw extracts in this study; water and aqueous methanol. Cell culture experiments revealed that the aqueous methanol extracts have better inhibitory effect than water extracts on DU145 without toxicity. Apoptosis analysis revealed that aqueous methanol extracts exhibit apoptosis inducing effect on DU145 and this claim was supported with DNA analysis (presence of bands which indicate DNA degradation) and microscopy analysis (cell shrinkage, membrane blebbing and apoptotic bodies). Western blot analysis reveals that apoptosis induced through caspase 3-mediated intrinsic pathway. Two pro-apoptotic proteins, Bax and Bad expressed higher ($p<0.05$) and anti-apoptotic protein, Bcl-2 downregulated ($p<0.01$) after treatment. This leads to increase ($p<0.01$) of caspase 9 expression which in turn activate caspase 3 and 7 ($p<0.01$) which lead cells to apoptosis. Aqueous methanol extracts

were chosen over water extracts for the *in vivo* study on the SHR model. SHRs were given drinking water mixed with aqueous methanol extracts for 17 weeks showed no toxicity effect on dietary intake, body weight and vital organ (liver and kidney). It managed to reduce blood pressure [72.0mmHg ($p<0.01$)] and the size of ventral prostate to around 0.05% ($p<0.01$). Histological analysis revealed anti-proliferative signs such as reduction in number of acini [(7.50) ($p<0.01$)], epithelial height [(10.55 μ m) ($p<0.01$)] and epithelial acinar area [(18.17%) ($p<0.01$)]. Since TUNEL assay showed non-significant outcome which ruled out apoptosis, aqueous methanol extracts have arrested cell cycle by downregulate ($p<0.01$) proliferative marker, Ki-67 and PCNA. Prostate cell growth is arrested by downregulation of AR which inhibited AR mRNA transcription (RTPCR analysis) and induced cell cycle arrest at S phase through p27 and cyclin E2 (western blot analysis). Paddy husk is a better chemopreventive agent than paddy straw against prostate disease.

CHAPTER ONE

INTRODUCTION

1.1 Research background

Prostate disease is common in men, particularly for those over 55 years of age. There are two major types of prostate disease; benign prostatic hyperplasia (BPH) and prostate cancer. BPH is a noncancerous enlargement of the prostate gland which develop among aging men (Lee and Peehl., 2004). Patients with BPH will undergo a careful medical history and physical examination such as digital rectal examination (DRE). Generally, alpha blockers and 5-alpha reductase inhibitors are among the medical treatments used to treat BPH. For the intermediate and long term effect to relieve the symptoms of BPH, transurethral resection of the prostate (TURP), minimally invasive treatments (MITs), laser surgery, and traditional surgery can be an option. On the contrary, all the procedures can develop serious complications such as blood loss, erectile dysfunction, and urinary incontinence (Fitzpatrick *et al.*, 2007, Kirby *et al.*, 2007, Roehrborn and McConnell., 2007, Laurencio *et al.*, 2008 and Rich *et al.*, 2008).

Meanwhile, prostate cancer is the second most common malignancy in men which accounts for nearly 20% of new cancer cases recorded in men worldwide. Prostate cancer incidence is influenced by the introduction of prostate-specific antigen (PSA) testing, which later leads to surge in the detection of asymptomatic prostate cancer (Ferlay *et al.*, 2015 and Siegel *et al.*, 2017). Since almost all the

prostate cancer cases starts in an androgen-dependent state, most popular treatment for prostate cancer is androgen deprivation therapy (ADT) followed by prostatectomy and chemotherapy (Saraon *et al.*, 2012).

Although ADT improved clinical prognosis, as time goes, some cancerous cells can survive and grow during the treatment, resulting in dangerous androgen-independent prostate cancer which has no effective targeted therapies available. Other treatment options for organ confined prostate cancer range from active monitoring of low grade cancers to prostatectomy and chemotherapy for higher grades, but it comes with side effects such as blood disorder, cognitive disruption, reproductive issue, osteopenia and erectile dysfunction (Alibhai *et al.*, 2006 and Johansson *et al.*, 2011).

Since drug therapy of prostate diseases are expensive and comes with side effects that influences the daily life of patients, an alternative therapy is needed to discuss the dilemmas. Natural products can play the key role in offering the alternative therapy for treatment of prostate diseases. Dietary consumption of food and herbal medicines is a convenient method of administering potentially chemo preventive phytochemicals in a cost-effective manner. As time goes, new discoveries of bioactive compounds which have medicinal properties from natural products or by-products were promoted as a treatment for prostate disease. Natural products and their compounds may act as a mediator either as raw or combination of separate entities which has been proven to have clinical synergistic benefits for prostate diseases (HemaIswarya *et al.*, 2006, Minami *et al.*, 2010, Toshiya *et al.*, 2012, Shin

et al., 2012, Wu *et al.*, 2013, Yang *et al.*, 2013, Bagul *et al.*, 2015, Medjakovic *et al.*, 2016 and Mbaka *et al.*, 2017).

1.2 Relevance of the study

Most of the prostate disease cases are preventable via dietary modification because 90-95% of cases are due to the environment and lifestyle factors (Willett, 2002, Anand *et al.*, 2008, Tantamango-Bartley *et al.*, 2012 and Espinosa *et al.*, 2013). Previous research has shown that there are varieties of dietary and botanical natural products that act against prostate diseases such as green tea (Henning *et al.*, 2011), soy, curcumin (Horie., 2012), sweet potatoes (Karna *et al.*, 2011) and resveratrol from grape (Seeni *et al.*, 2008). Another natural product that has proven to be a chemo preventive agent against prostate disease is paddy or *Oryza sativa* (Henderson *et al.*, 2012). Rice, *Oryza sativa*, is a staple food crop for more than half of the world's population. The paddy milling process produced milled rice and several paddy by-products such as husk, straw, germ, bran layer and broken kernels (Zhang *et al.*, 2010). A few decades ago, paddy by-products were considered as waste and often discarded to animals as a source of roughage or as building materials. Since the chemoprevention potential of paddy bran was discovered, it is the most studied paddy by-products which has many active compounds exhibiting anticancer activities (Hudson *et al.*, 2000, Chen *et al.*, 2012 and Henderson *et al.*, 2012).

Apart from paddy bran, global agriculture produces tens of millions of tons of paddy waste product every year. Paddy husk is inedible and contains a high proportion of organic substances which are used as an agricultural waste product (Kalapathy *et al.*, 2003). Previous studies reported that rice husk has high amounts of

phytochemicals such as tocopherol, tocotrienols, phenolic acids, flavonoid and momilactones which are known for its antitumor, pro-apoptotic and antiangiogenic properties (Jeon *et al.*, 2004, Kim *et al.*, 2007, Butsat *et al.*, 2010, Huang *et al.*, 2011, Carocho *et al.*, 2013 and Baek *et al.*, 2015). In tropical countries, ruminants depend on year-round grazing on natural pastures or the animals are fed with cut grass and crop residues. Most of these areas face seasonal dry periods in which the availability of pasture decreases and its quality by a reduction in the content of digestible energy and nitrogen. Since in these areas, the rice straw is abundantly available from cultivating rice, farmers offer rice straw as the main roughage source for their animals (Sarnklong *et al.*, 2010). Although seen as roughage source, earlier research reported that paddy straw has several phytochemicals such as phenolics (Seal *et al.*, 2004) and flavonoids (Macias *et al.*, 2006) which exhibit anti-cancer properties (Kato-Naguchi *et al.*, 2005).

Since both paddy husk and straw have potential to become chemo preventive agent, the aim of this work was to elucidate its effect on prostate disease; BPH and prostate cancer, by using *in vitro* and *in vivo* models. This work seeks to figure out the role and mechanism of apoptosis and cell cycle in chemoprevention studies of paddy waste product based on *in vitro* model: with androgen-independent prostate cancer cell line (DU145). Another part of work was to describe work that focuses on the biological changes associated with benign prostate hyperplasia in spontaneously hypertensive rats (SHR) and intervention with paddy waste products with the aim of preventing it. The work encompasses evaluation of physical and metabolic parameters, histopathology analysis to investigate the morphological

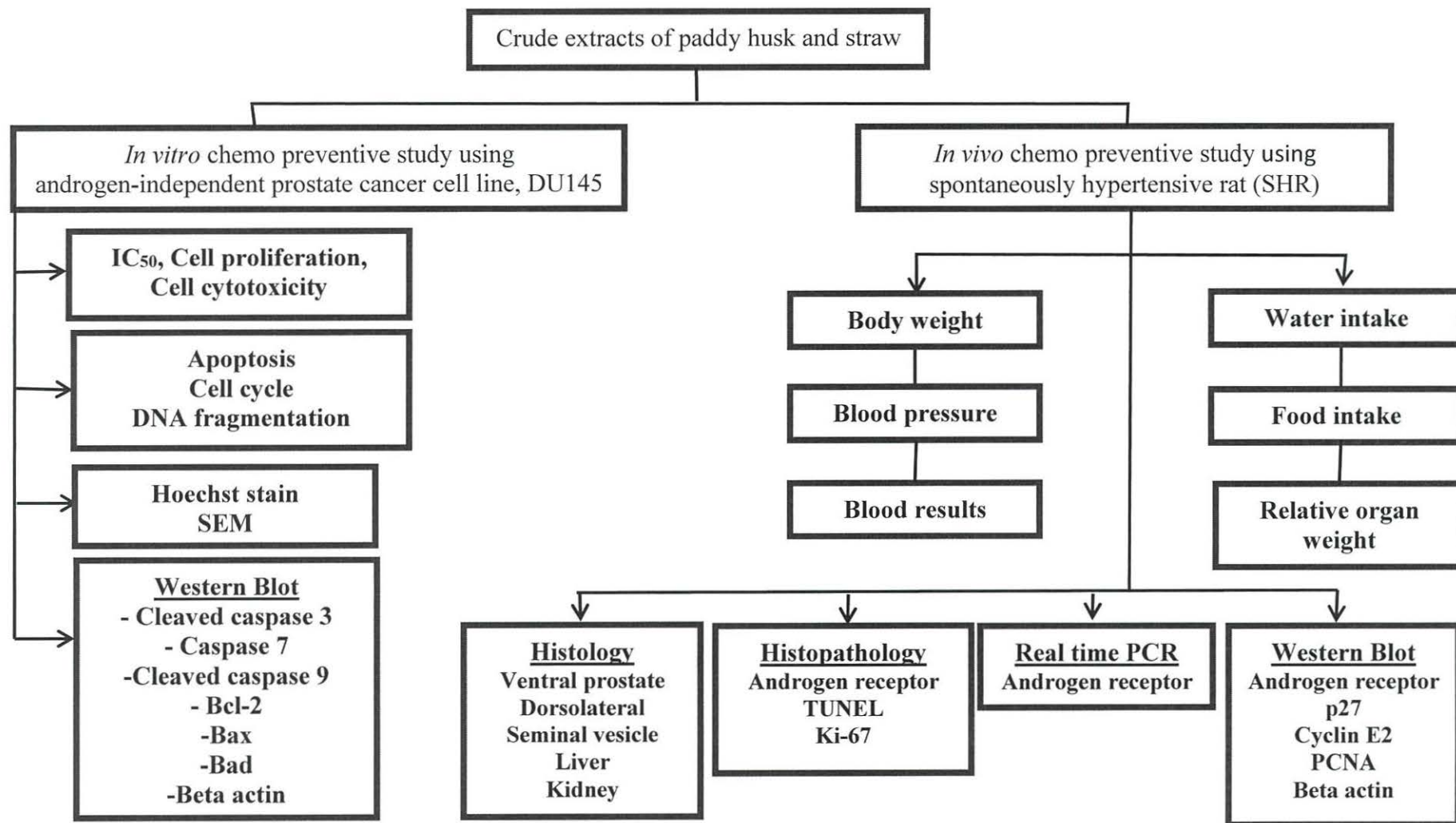
changes and distribution patterns of selected markers, proteomic and genomic assessment to analyse the mechanism of paddy waste product on BPH.

1.3 Hypothesis and objectives of the study

The hypothesis of the study is that paddy waste product would show inhibitory and anti-proliferative effect against androgen-independent prostate cancer cell (DU145) and in prostate hyperplasia using spontaneously hypertensive model (SHR) model respectively. The specific objectives of this study were:

1. To measure the inhibitory activity of the paddy waste product in androgen-independent prostate cancer cell line, DU145.
2. To determine the anti-proliferative activity of the paddy waste product in spontaneously hypertensive rat (SHR).
3. To compare the effects of paddy waste products on proliferation and apoptosis related genes and protein expression in DU145 cell line.
4. To determine the effect of aqueous methanol extracts on histology in SHR model.
5. To measure the difference in PCNA and androgen receptor expressions with aqueous methanol extracts treatment in SHR model.

1.4 Flow chart of the study



CHAPTER TWO

LITERATURE REVIEW

2.1 Prostate gland

2.1.1 Development of prostate gland

In human, prostate development occurs from second to third trimester and completed by the time of birth. Prostate gland originates from the urogenital sinus (UGS) and endodermal in origin unlike other male accessory sex glands which are mesodermal. The prostate development process is continuous and can be categorized into five distinct stages; determination, budding, branching morphogenesis, differentiation and pubertal maturation. Prostate development is entirely dependent upon the amount of androgen produced by the fetal testes. The determination and budding stages start with UGS epithelial buds that penetrate the surrounding mesenchyme. Branching morphogenesis begins when UGS epithelial buds are elongated to reach prostate mesenchymal pads. Phenotype development continues with UGS epithelial cells differentiating into surrounding ventral, dorsal and lateral area. Once the further branch points are established, the final growth of prostate maturation occurs when androgen level increases (Prins *et al.*, 2008 and Burnstein., 2013).

2.1.2 Structure of prostate gland

■ Prostate zones

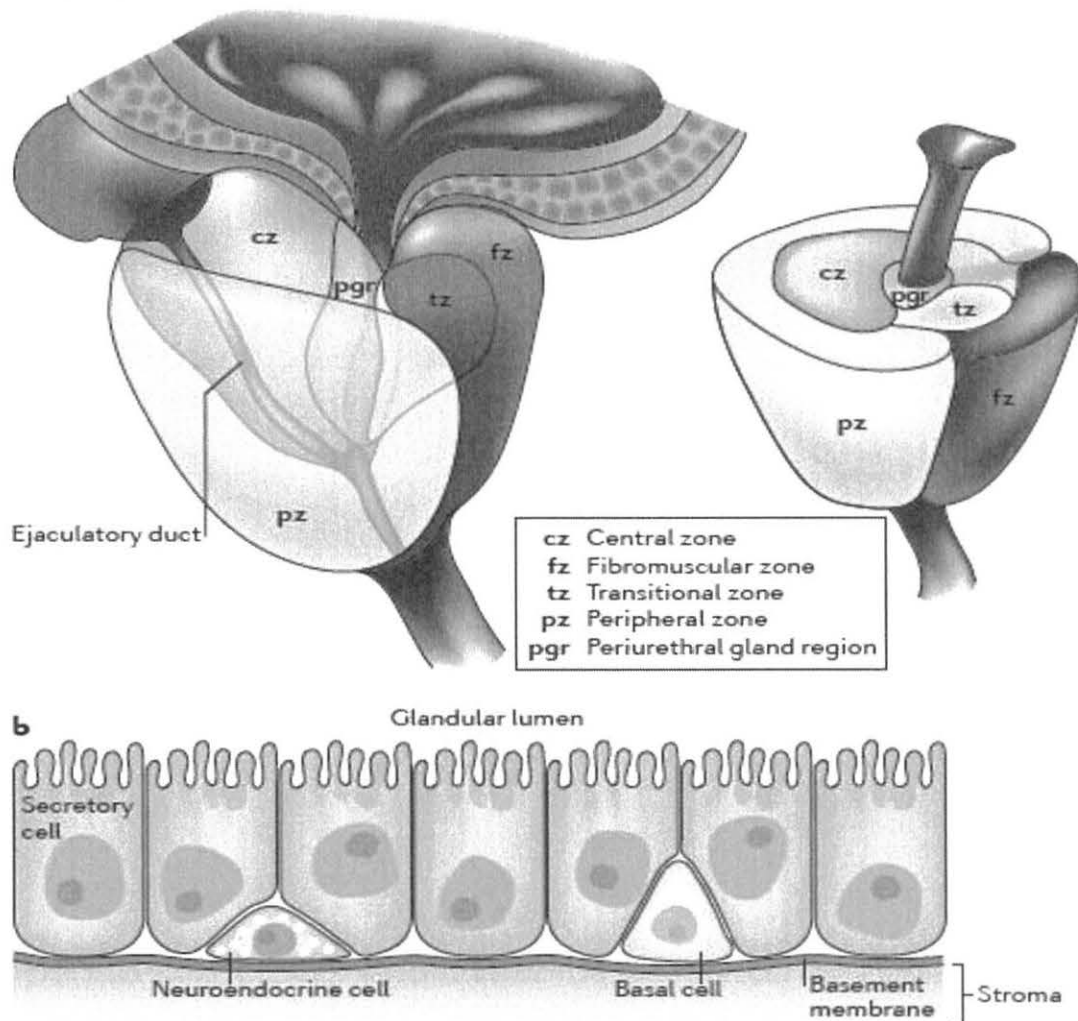


Figure 2.1 Schematic representation of normal prostate. **(a)** Normal prostate has few regions, including a central zone (cz), fibromuscular zone (fz), peripheral zone (pz), a transitional zone (tz), and a periurethral zone (pgr). **(b)** Prostate epithelium is a double histologically distinct cell layers; a flat basal cell layer and columnar secretory cell layer on top of basal cell layer surrounded by prostatic stroma (Verze *et al.*, 2016).

The prostate is the main accessory gland of the male reproductive system. The prostate is a fibromuscular gland around the size of a walnut and pyramidal in

shape. It is about 2 cm in anteroposterior, 3 cm in its vertical diameters. It is about 8 g in weight for youth, enlarges continuously throughout life with the development of benign prostate hyperplasia (BPH) can cause enlargement around 40 - 150 g. The prostate gland can be divided into four distinct zones, peripheral, central, transitional and fibromuscular as in Figure 2.1a. The peripheral zone is cup-shaped prostatic glandular tissue representing 70% of the prostate gland. It covers the gland's posterior, lateral and encloses the transitional zone. The central zone is a conical shaped zone which constitutes 20–25% of the glandular tissue of the prostate that surrounds the ejaculatory ducts to the base of the bladder. Transitional zone accounts for 5–10% of the glandular tissue of the prostate and lies around the proximity of central zone and ejaculatory ducts as well as distal part of the preprostatic urethra. Remaining space between anterior of peripheral zone towards preprostatic urethra is filled with non-glandular tissue (fibromuscular zone) (Kumar *et al.*, 2013 and Verze *et al.*, 2016).

The prostate gland consists of glandular epithelium that ingrained with fibromuscular stroma. Prostate epithelium exists in the form of a basal cell layer and a columnar secretory cell layer on top of it surrounded by prostatic stroma as depicted in Figure 2.1b. Basal cell layer consists of cuboidal epithelial cells and neuroendocrine cells lined with a basement membrane which act as barriers with the stroma. Secretory luminal layer on top of basal cell layer is made up of columnar cells that handle the production of prostate specific antigen (PSA), prostatic acid phosphatase and human kallikrein-2 as part of the seminal fluid (Van De Graaff, 2001, Standring *et al.*, 2008 and Tortora *et al.*, 2011,).

2.1.3 Function of prostate gland

The prostate gland will slowly increase in size from birth until puberty before rapidly expanding until age 30. Since the prostate gland consists of stromal and epithelial layers, both components complement each other to function properly. Stromal components ensure the suitable microenvironment for the epithelial components and produce many supportive signals important for gland homeostasis. Meanwhile, the main function of prostate epithelial components is to secrete the prostatic fluid that constitutes 20% of the volume of entire ejaculate. Prostatic fluid, a milky, slightly acidic fluid, is an important contributor to male fertility especially the ejaculation process. It is important for the maturation and motility of sperm, liquefaction of semen and clotting cycle. There are few substances from prostatic fluid such as citric acid (used by sperm for ATP production), prostate-specific antigen (PSA), acid phosphatase, pepsinogen, lysozyme, amylase, and hyaluronidase which breaks down the clotting proteins from the seminal vesicles. Prostatic epithelial cells are the only healthy human cells that produce energy by glycolysis (a hallmark of proliferating cancer cells) rather than the Krebs cycle (Verze *et al.*, 2016). Secretions of the prostate enter the prostatic urethra through many prostatic ducts (Tortora *et al.*, 2011). Extensive bands of smooth muscle course throughout the prostate to form a meshwork that supports the glandular tissue. Contraction of the smooth muscle expels the contents from the gland and offers part of the propulsive force needed to ejaculate the semen (Van De Graaff., 2001).

2.1.4 Clinical significance of prostate

There are three main types of prostate disease which are prostatitis, benign prostate hyperplasia and prostate cancer. Each region of prostate exhibits different

types of proliferative lesions such as hyperplasia lesions always arise in the transition zone while most of prostate carcinoma arises in peripheral zones (Kumar *et al.*, 2013).

Prostatitis is an inflammation of the prostate gland caused by bacteria infection. Prostatitis is divided into two categories; acute and chronic prostatitis. Acute prostatitis is caused by uropathogen that is associated with urinary tract infections and usually the symptoms will last for a few days. Chronic prostatitis is an infection of the prostate that can last for a long time – at least three months. The prostate is usually not biopsied in men with symptoms of acute or chronic prostatitis, since the findings are usually non-specific and are not helpful in managing patients (Kumar *et al.*, 2013).

Benign prostatic hyperplasia (BPH) is a common abnormality that normally present in men by the age of 40. BPH is a non-malignant condition in which the prostate gland becomes enlarged (Rich *et al.*, 2008). This enlargement is a normal consequence of aging and is rarely life threatening but may produce distressing symptoms. The prostate is a composite organ that forms three distinct zones; the peripheral zone, central zone and transition zone. The transition zone is the smallest and the only zone where BPH develops. BPH is characterized by the proliferation of both stromal and epithelium from preexisting ducts. BPH will cause the prostate to increase in size because the transition zone slowly expands and compresses the peripheral zone (Lee *et al.*, 2004 and Roehrborn., 2005). BPH is most probably caused by excessive androgen-dependent growth of stromal and glandular elements. Symptoms of BPH include an urgency to urinate, a hesitation before urine flow starts

despite the urgency to urinate, straining when urinating, weak or intermittent urinary stream, a sense that the bladder has not emptied completely, a frequent nocturnal urination and inability to void. There are various methods to diagnose BPH such as using uroflometry to measure the peak urinary flow and residual volume by ultrasound or by catheterization (Rich *et al.*, 2008). Patients can undergo optional testing such as a serum PSA assay testing, imaging studies, and (rarely) cystourethroscopy (Roehrborn., 2005, Wasserman., 2006, Standring *et al.*, 2008 and Kumar *et al.*, 2013).

Prostate cancer is the most common malignancy in men and occurs mainly in men older than 50 years of age. The incidence number between populations with and without PSA screening differ but still accounted about 1–2% of deaths in men. There is greater prevalence in the west because of lifestyle and environmental risk factor. Further advance in technology for prostate cancer treatment has been made and better understanding of the underlying biology of the disease. Most of the prostate cancer starts androgen dependent initially and needs an androgen receptor (AR) signalling for survival. When the cancer progresses to androgen-independence, androgen receptor signalling is altered and enhance mechanisms such as AR gene amplifications, AR gene mutations, changes in expression of AR proteins and changes in expression of steroid-generating enzymes. Any of these changes can lead to formation of androgen-independent prostate cancer cells (Saraon *et al.*, 2012 and Kumar *et al.*, 2013).

2.2 Prostate Hyperplasia

2.2.1 Origin and development of prostate hyperplasia

A successful treatment for BPH needs a therapy to prevent or reverse the progression of BPH. To develop therapeutic approaches, knowledge about the origin and development of BPH is needed. BPH, one of the common diseases in man, is a condition where prostate undergoes progressive enlargement. This is because of non-malignant proliferation of epithelial glandular and fibroblast/myofibroblast elements near the urethra in the transition zone of the prostate gland. Development of BPH exhibits larger proportion of glandular hyperplasia than stromal nodules.

The BPH rose from the centrally located peri urethral tissue area that lies along the proximal urethra called as the transition zone (makes up 2% of the mass of the prostate gland). In the early stages of BPH development, microscopic enlargement occurs where fibroblastic tissue nodules form glandular buds that induce the proliferation of epithelial cells into new glandular branches (acini) in the transition zone. In the next stage of development, microscopic glandular nodules undergo continuous hyperplastic changes which increase the size of nodules. Budding and branching process continues within nodule and changes the anatomy of the prostate gland. This process generally occurs in men older than 60 years old. The final stage in the histogenesis of BPH is the development of clinical symptoms that is caused by an enlarged prostate gland. Since BPH nodules arise from transition zone, further growth of nodules will compress the urethra and cause obstruction of the lower urinary tract. Endocrine hormone plays an important role in BPH, where androgen stimulation is crucial for prostate growth and development. Although the androgen level in the BPH and normal tissues are not significantly different, presence

of functioning testes and changing the ratio of androgens to oestrogens in aging men has been speculated to play an important role in the pathogenesis of BPH (Isaacs *et al.*, 1989, Oesterling., 1991, Bushman., 2009 and Foo., 2015,)

2.2.2 Risk factors of prostate hyperplasia

BPH is an age-related process where androgen is necessary for the development of BPH. There are two categories of risk factors for BPH which are non-modifiable (age and genetics) and modifiable (sex steroid hormones, obesity, diet, and inflammation). The prevalence of BPH tends to rise with age, so older age can be a risk factor. Prostate volume increases naturally with age with a rate of enlargement is varied individually. Normally patients who had larger baseline volumes tend to experience more rapid enlargement (Taylor *et al.*, 2006 and Bushman., 2009). Genetic inheritance plays an important part, where men with an autosomal dominant pattern of inherited forms of BPH has six-fold increase in risks of BPH. They also tend to have a larger volume prostates and earlier age of onset of clinical symptoms than men with sporadic BPH (Pearson *et al.*, 2003).

Sex steroid hormones such as testosterone, influences BPH where the hormone 5-alpha reductase converts testosterone to dihydrotestosterone (DHT), a potent stimulator of prostate growth which is crucial in BPH histogenesis (Parsons *et al.*, 2010 and Kaplan *et al.*, 2013). Obesity will increase the risk of BPH where increase deposition of adipose tissue is correlated with prostate volume (Parsons *et al.*, 2013). Dietary consumption may affect the risk of BPH, with high intake of red meat and dairy products will increase the symptomatic BPH while vegetables, linoleic acid, vitamin A and vitamin D will decrease the risk. For micronutrients,

higher intake of lycopene, selenium, carotene and vitamin E will reduce the BPH risk (Tavani *et al.*, 2006, Parsons *et al.*, 2007, Kristal *et al.*, 2008 and Maserejian *et al.*, 2011).

2.2.3 Treatment of prostate hyperplasia and its complications

BPH is a slow progressive disease in nature. Normally for BPH treatment, patients undergo a careful history and a physical examination including a digital rectal examination (DRE). The severity of BPH can be classified according to Table 2.1. Stage I patients were recommended on proper fluid intake and healthy lifestyle. Stage II patients can be treated with medication while Stage III patients would be recommended for more aggressive treatment with surgery as a choice. Stage IV patients would generally need surgical treatment.

Table 2.1 Staging of BPH (Foo *et al.*, 2015)

Stage	Significant obstruction	Bothersome symptoms	Treatment
I	Absent	Absent	Watch and counsel
II	Absent	Present	Medical treatment
III	Present	Irrespective	Surgical options
IV	Complication of BPH		Surgery

Alpha blockers and 5-alpha reductase inhibitors are among the medical therapies that are used to treat BPH. The alpha blockers relieve symptoms by blocking the sympathetic nerve endings at the bladder neck. They have not been shown to prevent progression of the disease (Roehrborn *et al.*, 2007, Fitzpatrick *et al.*, 2007, Kirby *et al.*, 2007, Laurencio *et al.*, 2008 and Rich *et al.*, 2008).

For the intermediate and long-term effect to relieve the symptoms of BPH, minimally invasive treatments (MITs), laser surgery, and traditional surgery are being used. Surgery would be the ideal treatment to restore prostate anatomy. The most effective surgical procedure, transurethral resection of the prostate (TURP) is also the most invasive. Incomplete removal of residual adenoma always leads to recurrent symptoms and obstruction. This will eventually cause serious complications, including blood loss, erectile dysfunction, and urinary incontinence (Roehrborn *et al.*, 2007, Fitzpatrick *et al.*, 2007, Kirby *et al.*, 2007, Laurencio *et al.*, 2008, Rich *et al.*, 2008 and Foo *et al.*, 2015).

2.3 Prostate cancer

2.3.1 Principle of carcinogenesis

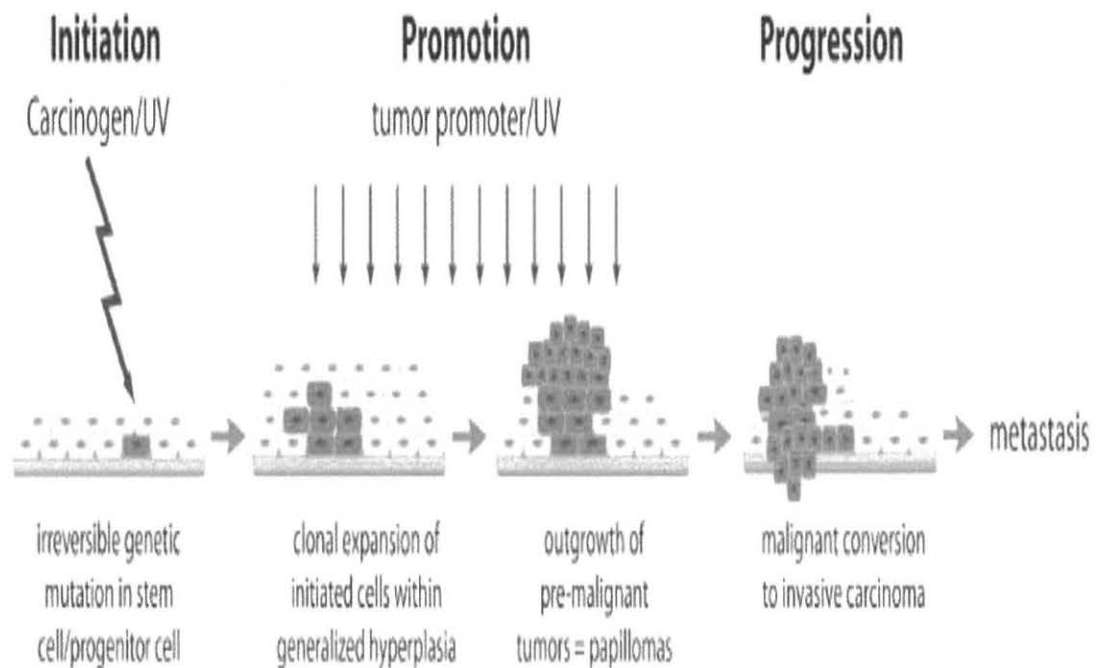


Figure 2.2: Multistage carcinogenesis model which divided into three main stages: initiation, promotion and progression (Rundhaug *et al.*, 2010).

Carcinogenesis is a process of cancer development when a single normal cell undergoes a continuous series of mutations that changes proliferative capacity and invading potential. It is a multistage process which was first proposed by Berenblum and Schubik in 1948 and can be divided into three distinct stages: initiation, promotion and progression as in Figure 2.2.

Initiation is the first step in cancer development which is an irreversible process of the cellular genome undergo DNA transformation after induced by exposure to a carcinogen (initiator) leading to mutation. Initiators are the chemicals that bind to macromolecules or DNA within the cell and able to cause mutations which leading to permanent genotoxic damage. Oncogenes are activated and responsible for the transformation in cellular characteristic and subsequently dysregulation of cell cycle genes. Initiating mutation creates a platform for potential development of transforming cells undergoing division into mutated karyotype. Once a cell affected by an initiator, it is susceptible to promotion stages (Couch., 1996, Crawford., 2003, Devi., 2004, Rundhaug *et al.*, 2010, Pan *et al.*, 2011 and Liu *et al.*, 2015).

Promotion stage, a slow and epigenetic phenomenon, which involves enhanced cell proliferation, behaviour and changes in gene expression. Key components for promotion are the induction of cell proliferation, oxidative stress, chronic inflammation and maintenance of a sustained hyperplasia. The transformed (initiated) cell can still be benign until stimulation by tumour promoter to undergo further proliferation, upsetting the cellular balance. The tumour promoters refer to the compounds that promote the proliferation of the cell into many daughter cells

having the mutation created by the initiator. There are two types of promoters: specific promoters that communicate with known receptors on or in cells and nonspecific promoters that change gene expression without involving known receptors. Promoters bind to specific cell receptors exposed to an initiator to affect intra-cellular pathways that increase cell proliferation and enhance gene transcription. Tumour growth during promotion is dose dependent where initiated cells need repeated exposure of a certain amount of tumour promoters to develop stimuli. Promoters will not directly cause structural genetic changes but will induce formation of oxygen radicals that alter chromosomes and break DNA strand. These secondary effects may accelerate the transition of cells from promotion to progression (Couch., 1996, Crawford., 2003, Devi., 2004, Rundhaug *et al.*, 2010, Pan *et al.*, 2011 and Liu *et al.*, 2015).

Finally, the progression is the irreversible neoplastic conversion of tumour cells (papilloma) to invasive cells (carcinomas) through genetic changes leading to metastatic potential. A hallmark of progression process is karyotypic changes that lead to dynamic cellular heterogeneity. Karyotypic changes such as aneuploidy results in genetic instability that will increase the growth rate, invasiveness and metastasis of cells. Presence of aneuploidy, common features of cancer cells, suggested that mutations in genes that are involved in genomic stability, DNA replication and cell cycle control. Cellular heterogeneity during progression is the consequence of an elevation of gene mutation rate of initiated cells that destabilize the genome. This will produce a vibrant generation of variant cells that are capable to detach from the restrictive proliferation of aberrant cells. As tumour development advances, detached cells enter the circulating blood (angiogenesis) and are

transported to other organs/ tissues away from primary sites to develop secondary tumours (distant metastases) (Couch., 1996, Crawford., 2003, Devi., 2004, Rundhaug *et al.*, 2010, Pan *et al.*, 2011 and Liu *et al.*, 2015).

2.3.2 Epidemiology and risk factors

Prostate cancer is the second most common cancer in men with nearly one million new cases being diagnosed of which about one-third cases are fatal. Prostate cancer is accounted for 10% of all new cancer cases in men worldwide and life time risk of developing prostate cancer is one out of seven (Ferlay *et al.*, 2015 and Siegel *et al.*, 2017). Prostate cancer cases are expected to reach 1.7 million new cases by 2030 due to population aging (Bashir., 2015). Developed countries like the United States and European countries recorded the highest rates of testing with highest reported rates of prostate cancer whereas Asian and African countries with low rates of testing have the lowest incidence (Figure 2.3). This disparity of cases among regions due to widespread practice of PSA testing, that detects even the asymptomatic tumours. Elevated risk of prostate cancer among migrated populations between regions suggested that lifestyle and environmental risk factors decide prostate cancer risk (Tao *et al.*, 2015).

In Malaysia, prostate cancer is fifth in male and eleventh most common cancer in all residents. The incidence of prostate cancer increased with age with 40% of prostate cancer detected at stages I and II (Figure 2.4). The incidence was highest among Chinese followed by Indian and Malay (Figure 2.5) (Zainal Ariffin *et al.*, 2011).

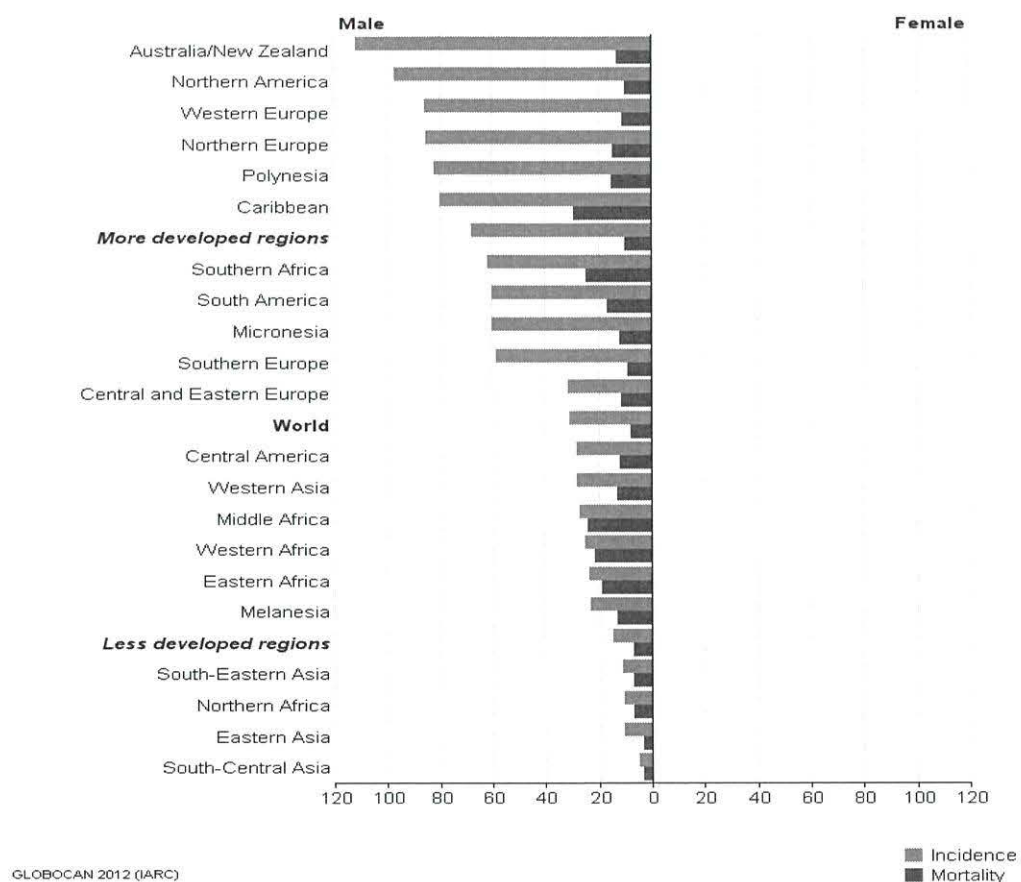


Figure 2.3: Prostate cancer: Incidence, Mortality and Prevalence Worldwide in 2012 (age-standardised rates per 100,000) (Tao *et al.*, 2015)

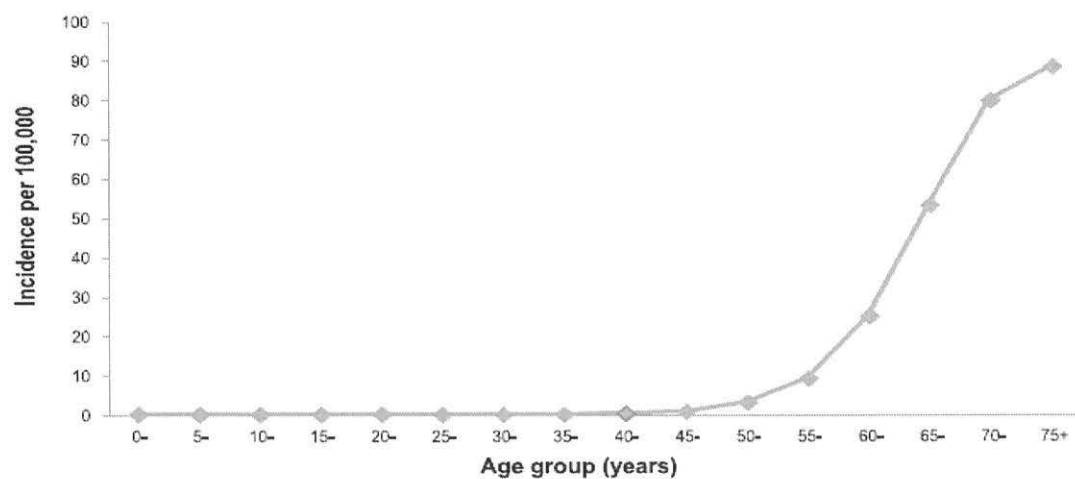


Figure 2.4: Prostate: Age-specific incidence rate, Malaysia, 2007-2011 (Omar *et al.*, 2011).

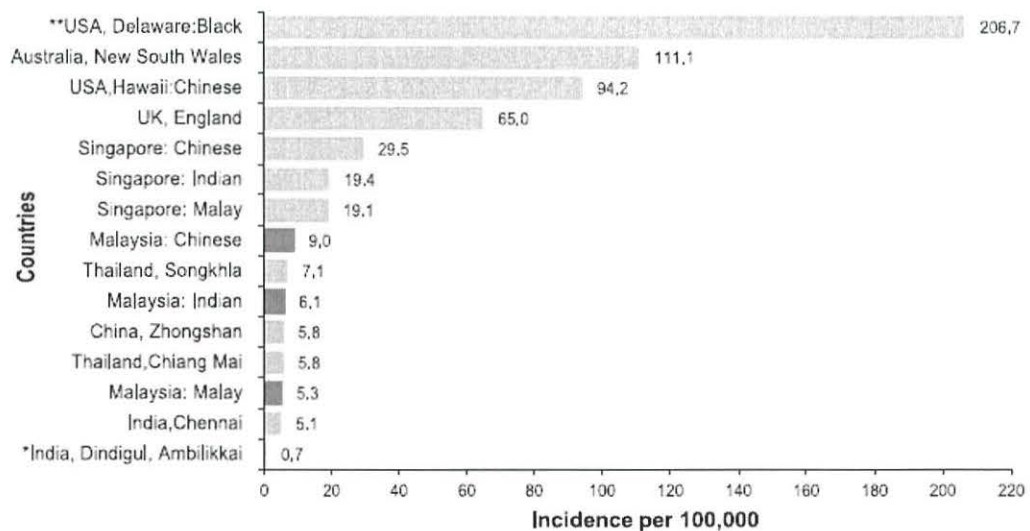


Figure 2.5: Prostate: International comparisons – Age-standardised rates (Zainal Ariffin *et al.*, 2011)

There are several important non-modifiable risk factors, such as age, race/ethnicity, familial history of prostate cancer, genetic susceptibility and modifiable risk factor that offers preventive opportunities such as dietary factor. Prostate cancer is an “old man disease” where 60% of cases are diagnosed at 65 years old or later. The possibility of developing prostate cancer increase after age 50 years and higher risk among older men because of lack of screening at their younger age. Prostate cancer can be considered as hereditary disease where the risk of prostate cancer increased in correlation with the number of first-degree relative suffering from prostate cancer. Variation of number of prostate incidence occurs all over the world with African-Americans having the highest rates of prostate cancer. This variation among ethnics are due to differences in detection practices, treatment and lifestyle factors. Dietary intake plays a major role in influencing the risk of developing prostate cancer. The Western dietary pattern such as fat, red meat and dairy product consumption touted for developing higher prostate cancer risk (Crawford., 2003, Bashir., 2015 and Tao *et al.*, 2015).

2.3.3 Pathology, diagnosis, staging and treatment

The main site of prostate cancer occurrence is the peripheral zone of the prostate. Most of the prostate cancers are adenocarcinomas while remaining cases normally include carcinomas of the epithelial lining on the urethra and glands, neuroendocrine carcinomas and sarcomas (Saraon *et al.*, 2012, Kumar *et al.*, 2013 and Attard *et al.*, 2016).

High-grade prostatic intraepithelial neoplasia (HG-PIN) is regarded as the precursor lesion and offer best predictive value for prostate cancer. The HG-PIN is characterized of being adenocarcinomas (cellular proliferation with similar nuclear enlargement like in prostate cancer) together with the presence of basal membrane. The importance of diagnosing the HG-PIN is based on its similarities with prostate cancer and its presence leads to increased need of needle core biopsy which is crucial for detecting prostate cancer at early stages (Montironi *et al.*, 2011). There are other types of methods used to detect prostate cancer; DRE and PSA determination. DRE is the second most popular means of screening for prostate malignancy but it has low predictive value which makes it unreliable for further tests. Meanwhile, positive predictive value of PSA is not enough to recommend further tests for prostate cancer because PSA is organ specific not disease specific (Velonas *et al.*, 2013).

In 1992, the tumour, node and metastasis (TNM) staging system for prostatic carcinoma has been introduced. The TNM staging system involved the presence, size and invasion extent of the primary tumour (T 0 to 4), the presence or absence of nodules metastasis (N 0 or 1) and the presence and absence of distant metastasis (M 0 or 1). The Gleason score is recognized as a preferred grading system

for prostate cancer with Gleason score together with the value of PSA are being combined into stage grouping. Gleason system is a system involving sums of the two most prominent grades where grades range from 1 to 5; 1 is well differentiated carcinoma with uniform gland pattern and 5 very poorly differentiated carcinomas with no or minimal gland formation. The treatment for prostate cancer patients is decided based on Gleason score and TNM staging (Cheng *et al.*, 2012).

The treatment options for organ confined prostate cancer are normally started with active monitoring for low grade prostate cancer. It will not be a practical option if the cancer is found in the form of foci of well-differentiated malignancy. Then, a repeated biopsy will be recommended for three months. If the cancer is highly graded and poorly differentiated, a prostatectomy or radiotherapy is recommended. Hormone-independent prostate cancer is still incurable to date (Tao *et al.*, 2015).

2.4 Human prostate adenocarcinoma cell line

2.4.1 Androgen-independent prostate cancer cells (DU145) and other type of cell lines.

There are three types of prostate cancer cell lines, androgen-independent prostate cancer cell line (DU145), androgen sensitive prostate cancer cell line (LNCaP) and human prostate adenocarcinoma cell line (PC-3) which are considered as the gold standard of prostate cancer cell culture research. In 1975, Dr Kenneth R. Stone successfully isolated a brain metastatic prostate tumour named DU145 cells which never expressed androgen receptor (AR) or prostate specific antigen (PSA) unlike many human prostate tumours (Stone *et al.*, 1978, Heinlein *et al.*, 2004 and

Saraon *et al.*, 2012). DU145 cell line doubling time is at 33 hours and its karyotype ranges from 46 to 143 chromosomes (modal of 64) (Webber *et al.*, 1997 and Taghizadeh *et al.*, 2009). DU145 is considered as good prostate cancer cell line because it shows independent growth factor via autocrine signaling which is hallmark of cancer (Hanahan *et al.*, 2011).

In 1977, LNCaP cells were first isolated from a human metastatic prostate adenocarcinoma found in a lymph node by Dr Horoszewicz (Horoszewicz *et al.*, 1980). LNCaP cells are an adherent epithelial cell and became an androgen dependent because of the presence of high amount of AR in the cytoplasm of cells. An activated AR will trigger two major prostate epithelium-specific differentiation antigens, PSA and Human Prostatic Acid Phosphatase (hPAP) expression which influence the growth. LNCaP has doubling time of 60 to 72 hours (depending on serum concentration) with hypotetraploid karyotype of 33 to 91 chromosomes (modal of 76–91). LNCaP can be a good model for studying prostatic adenocarcinoma and transcriptional regulation of genes of the prostate (Yang *et al.*, 2008, Dozmorov *et al.*, 2009, Liu *et al.*, 2011 and Wang *et al.*, 2012,)

Since prostate cancer cell line available that time were entirely constituted of carcinoma cells, in 1979, PC3 cells were isolated from a vertebral metastasis of a grade IV prostatic adenocarcinoma from male Caucasian (Kaighn *et al.*, 1979). PC3 cells are similar to DU 145 cells where it is an androgen independent cells that does not express AR and PSA. PC3 cells have more small cell carcinoma characteristics rather than adenocarcinoma. It has doubling time of approximately 33 hours with karyotype has a modal number of 58. This highly aggressive cell line can be used for