PROTEOMIC ANALYSIS OF PROSTATE CARCINOGENESIS INDUCED BY HETEROCYCLIC AMINE 2-AMINO-1-METHYL-6-PHENYLIMIDAZO[4,5-*b*]PYRIDINE (PhIP) USING *IN VITRO* MODEL

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by

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TABLE OF CONTENT

CHAPTER 3 - MATERIALS AND METHODOLOGY

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

2D-DIGE	2D-differential gel electrophoresis
ADT	Androgen deprivation therapy
AIA	Amino imidazo azoarenes
ANXA2	Annexin A2
AR	Androgen receptor
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
CCT	Cytosolic chaperonin contaning t-complex polypeptide 1
CID	Collision-induced fragmentation
CK	Cytokeratin
CTA	Cell transformation assay
CYP	Cytochrome P450
DAVID	Database for Annotation, Visualization and Integrated Discovery
DDI	Distilled, deionized
DHT	5α-dihydrotestosterone
DMEM	Dulbecco's Modified Eagle'smedium
DMSO	Dimethyl sulfoxide
D-PBS	Dulbecco’s Phosphate-buffered saline
DRE	Digital rectal examination
DTT	Dithiothreitol
ECM	Extracellular matrix
ER	Endoplasmic reticulum
ESI	Electrospray ionization
ETF	Eukaryotic translation factors
FBS	Fetal bovine serum
FDR	False discovery rate
FT	Fourier Transform
GO	Gene ontology
GSTs	Glutathione S-transferases

HCA	Heterocyclic amine
HG-PIN	High grade prostatic intraepithelial neoplasia
HMT	Histone-lysine N-methyltransferases
HPLC	High performance liquid chromatography
HPO	Human Phenotype Ontology
HPV-18	Human papillomavirus 18
HRPC	Hormone-refractory prostate carcinoma
HSP	Heat shock protein
IAA	Iodoacetamide
IARC	International Agency for Research on Cancer
IL	Interleukin
IQ	Imidazoquinoline
IQx	Imidazoquinoxaline
KEGG	Kyoto Encyclopedia of Genes and Genomes
KRT	Keratin
KSFM	Keratinocyte-serum free medium
LC-MS/MS	Liquid chromatography-Mass spectrometry/ Mass spectrometry
LDH	Lactate dehydrogenase
LG-PIN	Low grade prostatic intraepithelial neoplasia
M1	Mortality stage 1
M2	Mortality stage 2
MALDI	Matrix-assisted laser desorption ionization
MEM	Minimum essential medium
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MS	Mass spectrometry
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NATs	Arylamine N-acetyltransferases
N-OH-PhIP	N-hydroxy-PhIP
NRS	NADPH-regenerating system

OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate-buffered saline
PCSC	Prostate cancer stem-like cells
PDI	Protein disulphide isomerase
PG	Phosphoglycerates
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PK	Pyruvate kinase
PMS	Phenazine methosulfate
POTE	Prostate, ovary, testis-expressed proteins
PRK	Protein kinase
PSA	Prostate specific antigen
PSM	Peptide-spectrum matches
PTM	Post-translational modifications
RB	Running buffer
RPMI-1640	Roselle's Park Memorial Institute-1640
S.E.M.	Standard error of mean
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SULTs	Sulfotranferases
SV40	Simian virus 40
TERT	Telomerase reverse transcriptase
TGS	Tris/glycine/SDS
TIC	Total ion chromatogram
TNM	Tumour/node/metastases
TOF	Time-of-flight
UGTs	Uridine diphosphate-glucuronosyltranferases

ANALISA PROTEOMIK KE ATAS PROSES PEMBENTUKAN KANSER PROSTAT TERARUH OLEH HETEROSIKLIK AMIN 2-AMINO-1-METIL-6-FENILIMIDAZO[4,5-*b*]PIRIDIN (PhIP) MENGGUNAKAN MODEL IN VITRO

ABSTRAK

2-amino-1-metil-6-fenilimidazo[4-5-*b*]piridin (PhIP) merupakan penyebab kanser daripada kumpulan heterosiklik amin yang paling banyak ditemui dalam daging yang dimasak. Objektif kajian ini adalah untuk menyiasat kesan PhIP ke atas sel normal prostat manusia, RWPE-1, dalam proses karsinogenesis prostat menggunakan dos yang releven kepada manusia, pada peringkat sel dan protein. Sel prostat RWPE-1 didedahkan dengan kepekatan PhIP 10^{-7}, 10^{-8}, 10^{-9} atau 10^{-10} M, yang dilarut dalam pelarut DMSO dengan kepekatan 0.003%, 0.025% atau 0.25% dengan kehadiran/tanpa sistem pengaktifan metabolik. Kaedah-kaedah yang digunakan dalam kajian ini adalah asai MTS untuk ujian kesitotoksikan dan proliferasi sel, Alamar Biru untuk ujian proliferasi sel, asai transformasi dan invasi sel, dan proteomik 'shotgun'. Keputusan yang diperoleh daripada asai kesitotoksikan dan proliferasi sel menunjukkan peningkatan dalam kebolehhidupan dan proliferasi sel RWPE-1 yang dirawat dengan semua kepekatan PhIP dalam semua kepekatan DMSO dengan kehadiran sistem pengaktifan metabolik, dimana semakin rendah kepekatan DMSO, semakin tinggi tindakbalas yang berlaku. Dalam asai transformasi sel menggunakan parameter yang sama dengan asai sebelumnya, keputusan menunjukkan semua kumpulan rawatan PhIP dengan kehadiran sistem pengaktifan metabolik mengalami perubahan neoplastik dimana sel-sel tersebut menunjukkan pertumbuhan pengankoran tak bersandar iaitu keupayaan untuk membiak di atas

lapisan agar dan membentuk koloni. Pembiakan semula koloni-koloni sel RWPE-1 ini menunjukkan perubahan tambahan dalam tingkahlaku pertumbuhan sel bagi kumpulan yang dirawat dengan PhIP pada kepekatan 10^{-7}M dalam larutan DMSO 0.003% yang mempamerkan keupayaan untuk membiak yang paling tinggi berbanding kumpulan lain. Penilaian lanjut dalam asai invasi sel menunjukkan sel-sel kumpulan ini tidak dapat bergerak menembusi lapisan *matrigel*, menunjukkan kemungkinan sel-sel ini masih berada diperingkat awal transformasi. Keputusan ujian pengecaman protein menunjukkan sebanyak 41 protein dikenalpasti dalam kumpulan kawalan dan 81 protein dalam kumpulan rawatan. Analisis famili protein menunjukkan terdapat 11 famili berada dalam kedua-dua sampel manakala 24 famili hanya terdapat didalam kumpulan rawatan. Pengiraan peptida menggunakan intrumentasi aplikasi pengiraan tanpa label PEAKS gagal memberikan sebarang keputusan. Namun, kajian ilmiah yang meluas menunjukkan semua protein yang dikenalpasti dalam kumpulan rawatan telah dilaporkan mempunyai kaitan dengan kanser prostat. Analisis anotasi protein menunjukkan anotasi yang lebih banyak bagi kumpulan rawatan dalam semua kategori; persetempatan sel, proses biologi dan fungsi molekul. Analisis pengayaan laluan telah mengenalpasti pengayaan beberapa laluan yang diketahui terlibat dalam proses kanser. Kesimpulannya, semua keputusan menunjukkan sel RWPE-1 yang dirawat dengan PhIP dalam kehadiran sistem pengaktifan metabolik pada dos yang releven bagi manusia, terutamanya dos 10^{-7}M dalam 0.003% DMSO, mempamerkan peringkat awal transformasi neoplastik pada peringkat sel dan protein dalam karsinogenesis prostat, manakala keputusan analisa proteomik menunjukkan semua protein yang dikenalpasti dalam kumpulan rawatan mempunyai potensi untuk kajian seterusnya bagi menentukan peranan dan sumbangan protein-protein ini dalam kanser prostat.

PROTEOMIC ANALYSIS OF PROSTATE CARCINOGENESIS INDUCED BY HETEROCYCLIC AMINE 2-AMINO-1-METHYL-6-PHENYLIMIDAZO[4,5-b]PYRIDINE (PhIP) USING IN VITRO MODEL

ABSTRACT

2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most abundant type of carcinogenic heterocyclic amine produced in cooked meat. This study aims to investigate the carcinogenic effect of human physiologically relevant concentration of PhIP on human prostate epithelial cell line, RWPE-1, at cellular and protein levels in prostate carcinogenesis. RWPE-1 cells were exposed to 10^{-7}, 10^{-8}, 10^{-9} or 10^{-10} M PhIP, diluted in 0.003%, 0.025% or 0.25% DMSO, with or without metabolic activation system. The methodologies used in this study were MTS assay for cytotoxicity and cell proliferation tests, Alamar Blue assay for cell proliferation test, cell transformation assay, invasion assay and shotgun proteomic. In cytotoxicity and cell proliferation assays, results showed that the viability and proliferation of RWPE-1 cells treated with PhIP at all doses in all DMSO concentrations in the presence of metabolic activation system were increased depending on the vehicle concentration, where the lower the concentration of the vehicle used, the higher the cellular responses observed. In the cell transformation assay conducted using the same experimental settings as the previous assays, the results showed that all PhIP-treated groups with metabolic activation system exhibited neoplastic transformation characteristic as these transformed cells showed anchorage-independent growth growing above the agar layer and formed colonies in soft agar. Subsequent re-plating of these colonies showed that only RWPE-1 cells treated with 10^{-7}M PhIP in 0.003% DMSO showed additional changes in growth behaviour exhibiting the highest

proliferative activity compared to other groups. Further evaluation of this group in cell invasion assay revealed that the cells were unable to migrate through the matrigel barrier into secondary sites signifying a high possibility that the cells were in the early stage of transformation. Protein identification results showed 43 and 81 proteins identified in control and PhIP-treated samples, respectively. Analyses of the protein families' abundance for both samples revealed 11 protein families in both samples whereas 24 protein families were exclusively identified in PhIP-treated RWPE-1 cells. Peptide quantification using PEAKS label-free quantification tool failed to produce any result. However, an extensive literature search revealed that all proteins identified in the PhIP-treated RWPE-1 cells have been reported to be associated with prostate cancer. Protein annotation analysis presented more annotations for PhIP-treated sample in all classifications; the cellular localization, biological process and molecular functions. Pathway enrichment analysis identified overrepresentation of several pathways which were known to be involved in the cancer process. In conclusion, all results obtained indicated that in the presence of metabolic activation system, RWPE-1 cells treated with physiologically relevant concentration of PhIP, in particular at the dose of 10^{-7}M in 0.003% DMSO vehicle concentration, exhibited a considerable degree of early neoplastic transformation at both the cellular and protein levels in prostate carcinogenesis, whereas the results obtained from the proteomic analysis showed that all proteins identified in the PhIP-treated RWPE-1 cells can be potential candidates for further evaluation of their roles and contributions in prostate cancer.

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

Cancer exacts an overwhelming price on the society as a whole since the burden and the devastating effects of this disease affect both the patients and their families especially due to the long term curative and palliative treatment regimes which can be fairly costly as well as the consequential loss because of morbidity and mortality. For decades, both scientists and physicians alike have put tremendous efforts in investigating the means to identify various forms of cancer at early stage especially well before they become malignant and thus, incurable or fatal. Elucidating and understanding the etiology of cancer and the mechanisms involved in the induction and progression of this disease at each genetic, molecular and cellular level can substantially aid in devising a strategy for early detection, prevention, intervention and therapy.

According to the International Agency for Research on Cancer, prostate cancer was reported to be the second most common cancer among men in the world in 2012 (Ferlay *et al*., 2013). About one third (30-35%) of all cancers are caused by dietary habits, followed by tobacco smoking (20-30%) and genetic defects (5-10%), while the rest are caused by environmental factors and lifestyle (Anand *et al*., 2008). Many epidemiological studies have shown the association of diet and cancer which is mainly due to consumption of animal products, in particular cooked meat. Various genotoxic and mutagenic compounds have been identified in both processed and cooked food and

one of them is the heterocyclic amines (HCA). Cooking of muscle meats and fish will result in the production of many different HCA compounds where most of them has been associated with the increase of cancer risk as they possess high mutagenic and carcinogenic potentials (Sugimura *et al*., 2004). In general, the HCAs can be divided into two groups, polar and non-polar compounds. The former includes imidazoquinoline (e.g. IQ), imidazoquinoxaline (IQx), and imidazopyridine types, whereas the latter have a common pyridoindole or dipyridoimidazole group. The most abundant HCAs formed is the polar type 2-amino-1-methyl-6-phenylimidazole [4,5-*b*] pyridine (PhIP). The action of PhIP was reported to be organ-specific as it was found to induce tumour specifically in the breast, colon, liver and prostate, which are considered to be diet-related target sites for cancer (Murkovic, 2007; Knize *et al*., 2002; Sugimura, 2000). The IARC (1993) has classified PhIP as Group 2B carcinogen, which is possibly carcinogenic to humans.

Most of the previous *in vitro* and/or *in vivo* experiments on PhIP were fairly unsuitable to investigate the mechanisms of carcinogenesis in human or to identify proteins implicated in this process. Although numerous *in vitro* studies using commercial human cell lines have been reported, most of them do not fully reflect the intricate activation and detoxification processes of PhIP in humans. In *in vivo* studies, extrapolating the results of such experiments to humans is not entirely appropriate as laboratory animals have different metabolic pathways and capacities than human. In addition, the doses investigated in these studies were tens to hundreds times higher than the human exposure level. Thus, the question whether humans are exposed to levels of PhIP in their diet is sufficient to contribute to the induction of prostate cancer is yet to be answered. Moreover, in prostate carcinogenesis, the anatomical location regarding

where the carcinogenic process take place in both species is still a debate among the scientists. Thus, experimental protocols and models that can somewhat reflect the carcinogenic process of PhIP in human are required in order to detect the biomarkers/proteins relevant for humans. It is hoped that through this study, the carcinogenic effect of PhIP in the prostate carcinogenesis can be understood and investigated.

1.2 GENERAL OBJECTIVE

The general objective of this study is to investigate the carcinogenic effect of human physiologically relevant concentration of PhIP on normal human prostate epithelial cell line at cellular and protein levels in prostate carcinogenesis.

1.3 SPECIFIC OBJECTIVES

1.3.1 To determine the cytotoxic effect of PhIP on human prostate epithelial cells.

1.3.2 To determine the effects of PhIP on proliferative activity of human prostate epithelial cells.

1.3.3 To determine the neoplastic characteristics acquired by human prostate cells when treated with PhIP.

1.3.4 To identify the proteins present in human prostate epithelial cells treated with PhIP that can potentially be used as PhIP-induced prostate cancer biomarkers.

1.3.5 To determine the effect of PhIP on protein expression of human prostate epithelial cell line.

1.4 HYPOTHESIS

Human exposure level of PhIP exerts carcinogenic effects on human prostate epithelial cell line at the cellular and protein level.

CHAPTER 2

LITERATURE REVIEW

2.1 CANCER

2.1.1 Incidences

Due to the resemblance of appendage-like projections of tumour blood vessels to a crab's body and legs, Hippocrates used the Greek word *karkinoma*, meaning crab, to describe tumours (Doyle & Shaw, 2011; Virshup & McCance, 2008). Today, cancer represents a major burden of disease globally and poses a great challenge both to the physicians and researchers, yet the etiology and the exact mechanisms of this disease remain obscured. Worldwide, International Agency for Research on Cancer (IARC) reported 14.1 million new cancer cases, 8.2 million cancer mortality and 32.6 million people were living with cancer in 2012 (Ferlay *et al*., 2013). The Surveillance Epidemiology and End Results (SEER) program database by National Cancer Institute estimated that in 2017 over 1.6 million will be diagnosed with cancer and 600 920 will die from it in United States (Howlader *et al*., 2018). According to the 5-year report by the Malaysian National Cancer Registry, which covers all cancer cases registered by the state registries in Malaysia from 2007 to 2011, a total number of 103,507 new cancer cases were reported in Malaysia during this period with a total of 64,275 medically certified and non-medically certified cancer deaths (Azizah *et al*., 2016).

2.1.2 Carcinogenesis

Normal cellular physiology is a process which is strictly regulated to ensure balance in the cellular multiplication and growth with positive and negative feedback loops. It determines the action of a cell whether to differentiate, divide, adapt to its microenvironment or commit suicide via apoptosis when necessary (Kyprianou, 2012). In adult, this process is generally restricted to the replacement of lost cells and these cells normally respect their own place and space in the body's society of cells. When mutations occur within the genes responsible for controlling cell division and growth, a normal cell may transform into a pre-neoplastic/neoplastic one (Sherwood, 2001). Eradication or inactivation of tumour suppressor proteins and/or the activation of oncoproteins will cause the cells to reproduce excessively and/or unable to commit suicide. Further division and proliferation of the cells will cause the mutations to accumulate. These cells will remain impaired and cancer-related genes may be activated when the genes required to repair the damage are disabled (Jain, 2014). This will result in imbalances of the regulation of normal physiology causing the cells to become oblivious to the control mechanisms normally limiting their growth and subsequently giving rise to an abnormal mass of proliferating cells called neoplasm (Kumar *et al*., 2013; Sherwood, 2001). These neoplastic cells will occupy the space in the surrounding normal tissue, an event called local invasion, resulting in infiltration of local tissue, blood vessels and the lymph system (Doyle & Shaw, 2011).

If the mass is slow growing, persists in its original location, consists of cells that remain compacted and encapsulated, does not invade the surrounding tissue/area and seldom kill the host if removed before it compresses the vital organ, it is considered

benign neoplasm. In contrast, malignant neoplasms consist of transformed cells which may multiply rapidly and relentlessly forming a non-encapsulated invasive mass that lacks the altruistic behaviour characteristic of normal cells. These malignant cells, collectively referred to as cancer, usually resemble immature cells, invade their surrounding rather than pushing them aside, do not adhere well to the neighbouring normal cells which enable them to escape from the primary mass and migrate via blood or lymph to distant organs/sites where they form secondary cancer masses. This aptitude to travel, spread and invade other parts of the body is called metastasis (King & Robins, 2006; Sherwood, 2001; Marieb, 2001). Clinically, cancer is regarded as a group of diseases that vary in the age of onset, growth rate, state of cellular differentiation, invasiveness and metastatic potential, diagnostic detectability, response to treatment, and prognosis (Ruddon, 1995).

Carcinogenesis, or cancer formation, is a multistep process resulting from accumulation of errors in vital regulatory pathways at both phenotypic and genetic levels that collectively give rise to the transformed phenotype (Marieb, 2001; King & Robins, 2006). Although genetic damage lies at the heart of cancer formation, the actual causes of cellular alterations that produce a cancer is somewhat elusive. It is well known that both internal (e.g. genetic predisposition, defective immune system) and external factors (e.g. tobacco, radiation, chemical/medical/occupational carcinogens, viruses, physical mutagens, microbial agents and environmental influences) can act as carcinogens (Kumar *et al*., 2013; Doyle & Shaw, 2011; Allen *et al*., 2005; Campbell and Reece, 2002; Ruddon, 1995). What these factors have in common is that all of them can cause mutations (Marieb, 2001). Formation of tumour does not occur immediately following exposure to a carcinogen. This disease usually develops after a long latent period and

there are three major stages involved in this process, termed tumour initiation, promotion and progression (Figure 2.1) (Grant, 2013; Greenwald, 2008).

In the initiation stage, a normal cell is converted to an initiated cell in response to DNA damaging agents which, if not repaired before the next cell division, would lead to erroneous DNA replication resulting in fixation of mutations within the genome of individual cells. There are three factors that determine the likelihood of tumour initiation: the rate of pro-carcinogen activation, the efficiency and fidelity of DNA repair and the capacity for cell proliferation. Although initiation is irreversible, not all initiated cells will advance towards becoming a tumour as many of them may die through apoptosis. Further proliferation-enhancing signals are also required for the evolvement (Grant 2013; Greenwald, 2008).

The promotion stage is characterized by the transformation of the initiated cells into a population of pre-neoplastic cells resulted from alterations in gene expression. This phenomenon can be prompted by various tumour-promoting agents that tend to be non-genotoxic in their own right. The promoters are highly likely to be able to promote clonal expansion of initiated cells resulting in the survival and proliferation of pre-neoplastic cells and the formation of benign lesions. Although most of these lesions may regress spontaneously, a few cells may advance into a malignant neoplasm after acquiring additional mutations (Grant 2013; Greenwald, 2008; Franks & Knowles, 2005).

Tumour progression is the stage whereby the pre-neoplastic cells are converted into neoplastic cell populations as a result of further genetic alteration, either spontaneously or following additional exposure to carcinogens. It is thought that endogenous elements such as hormones, growth factors, nutrients, cell growth, and other common cellular

processes advance the promoted cancerous cell further (Grant 2013; Greenwald, 2008; Franks & Knowles, 2005). Once these cells are promoted into neoplastic cells, they can be characterized by ten fundamental functional capabilities that collectively, dictate malignant phenotype: genome instability and mutation, resisting cell death, deregulating cellular energetics, sustaining proliferative signalling, evading growth suppressors and immune destruction, enabling replicative immortality, tumour-promoting inflammation, activating invasion and metastasis, and inducing angiogenesis (Hanahan, 2014).

Cancer causes mortality in most cases for several interrelated reasons. The cancer cells crowd out normal cells by robustly contending for space and nutrients with them, yet are unable to perform the functions of the cells they are destroying. Cancer cells typically remain immature and do not become specialized and as such lack the ability to execute the specialized functions of the normal cell type from which they mutated. The impacted organs will gradually become disrupted until they are no longer able to perform their life-sustaining functions, and death results (Sherwood, 2001).

2.2 PROSTATE GLAND

2.2.1 Anatomy, Histology and Physiology

The prostate gland is a single doughnut-shaped gland measuring 4 cm in diameter and weighing approximately 20 g (Figure 2.2). Adjacent to the rectum, it encircles the part of the urethra just inferior to the bladder. Enclosed by a thick fibroelastic capsule, it is made up of 20-30 compound tubuloalveolar glands embedded in a rich fibromuscular stroma of smooth muscle and dense connective tissue.

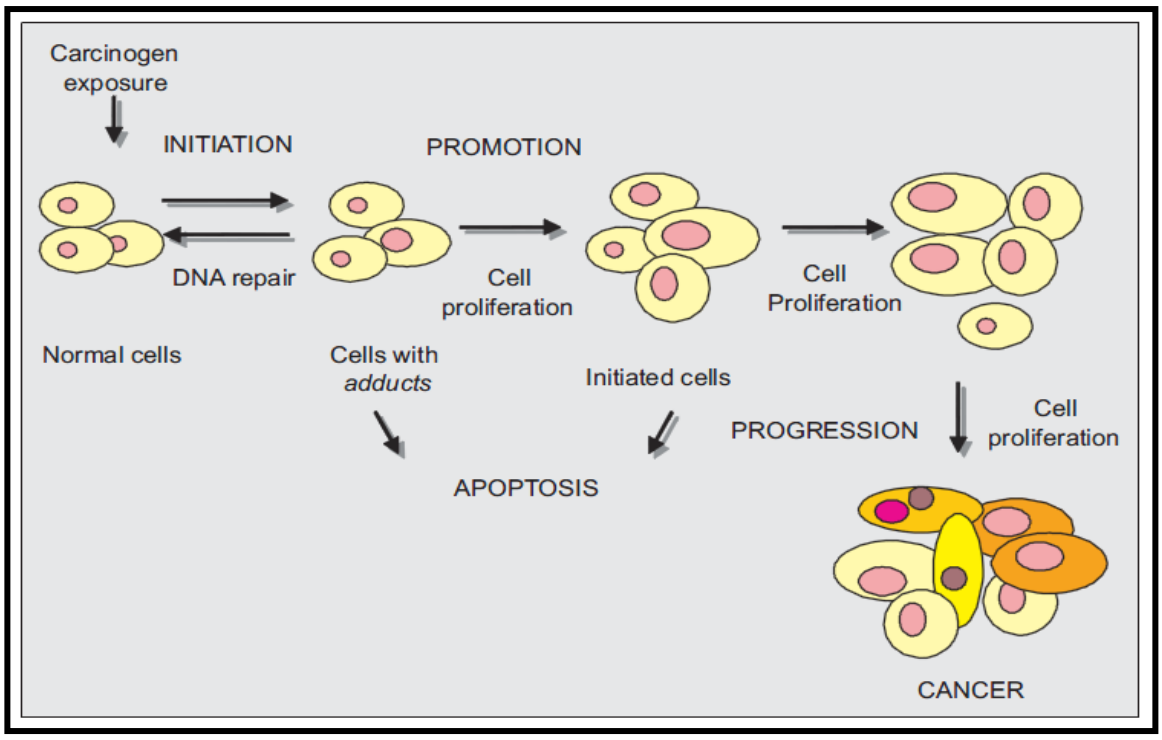

Figure 2.1: Sequential model of carcinogenic process divided into three stages; tumour initiation, tumour promotion, and tumour progression (extracted from Oliveira *et al*., 2007).

Septa from this capsule penetrate the gland and divide it into lobes that are indistinct in adult men. The prostate has three distinct zones. 25% of the gland is formed by the central zone which contains the ejaculatory ducts. The peripheral zone takes up 70% of the gland, which occupies the posterolateral region of the prostate whereas the transition zone sits near the urethra. The peripheral zone is the major site of prostatic adenocarcinomas is found to be most susceptible to neoplastic transformation while most benign prostatic hyperplasia originates from the transition zone. Generally, carcinoma of the latter zone have been attributed to good prognosis, i.e., low Gleason grade, low rates of capsule penetration and margin positivity (True & Vakar-Lopez, 2011; Nelson & De Marzo, 2007; Marieb, 2001).

Prostate tissues contain an epithelium, the source of prostatic secretions, and a stroma, occupied by fibroblasts, smooth muscle cells, nerves, and blood vessels. The prostate epithelium is composed of basal cells, some of which are believed to serve as the multipotent epithelial stem cells, luminal/columnar secretory cells, terminally differentiated to produce ejaculate secretions, and rare neuroendocrine cells, which are found scattered throughout the prostate epithelium in both basal and luminal compartment (Nelson & De Marzo, 2007).

Normal growth and development of the prostate require both androgenic steroids and a functioning androgen receptor (AR). Testosterone, produced by Leydig cells in the testes, is the major circulating androgenic hormone. An enzyme, 5α-reductase converts testosterone to 5α-dihydrotestosterone (DHT), a more potent androgen that can bind ARs and promote dissociation of the receptors from chaperone protein (Nelson & De Marzo, 2007). The prostatic gland secretion is a thin, milky substance that contains citrate (a nutrient source) and several enzymes (fibrinolysin, hyaluronidase, acid

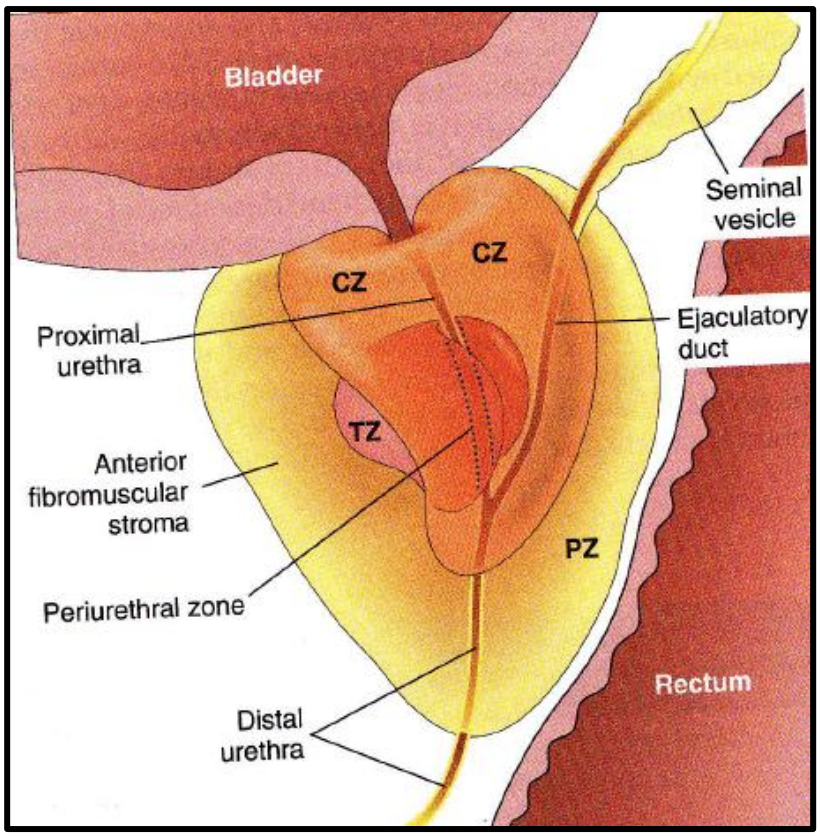

Figure 2.2: Anatomy of the adult normal prostate containing several distinct regions, including a central zone (CZ), a peripheral zone (PZ), a transitional zone (TZ), and a periurethral zone (extracted from Kumar *et al*., 2013).

phosphatase), and prostate-specific antigen (PSA). This secretion accounts for up to one-third of the semen volume and has an alkaline pH that neutralizes the acidic vaginal secretion which helps the sperm to survive in the female reproductive tract as sperm are more viable in a slightly alkaline environment (Deneris & Huether, 2008; Nelson & De Marzo, 2007; Marieb, 2001; Sherwood, 2001).

2.2.2 Pathophysiology

There are three major disorders of the prostate gland, namely, prostatitis, hyperplastic lesions, and neoplastic disorders, of which prostate cancer is by far the most important clinically. Prostatitis is an inflammation of the prostate and typically involves some of the gland's excretory ducts only. Inflammatory processes are manifested by infiltration of inflammatory cell in prostate tissue which are either of acute (neutrophilic cell-rich) or chronic (mononuclear cell-rich) type. Prostatitis is divided into four categories: i) acute bacterial prostatitis, caused by the organisms that are also implicated in other urinary tract infections; ii) chronic bacterial prostatitis also caused by common uropathogens; iii) chronic nonbacterial prostatitis, in which no uropathogen is identified despite the presence of local symptom; and iv) asymptomatic inflammatory prostatitis, related with incidental identification of leukocytes in prostatic secretions without uropathogens (Epstein, 2013; True & Vakar-Lopez, 2011; Deneris & Huether, 2008).

Benign prostatic hyperplasia (BPH), also called benign prostatic hypertrophy, is the enlargement of the prostate gland. This condition is a result of an increase in stromal:epithelial cell ratio and is typically limited to the transition zone. This may be due to proliferation of both stromal and epithelial elements or decreased programmed cell death, either which can lead to cellular accumulation resulting in the enlargement of

the gland and, in some cases, urinary obstruction. DHT was identified to be the major hormonal stimulus for the excessive proliferation. The affected prostate typically weighs between 60 to 100 g and contains many well-circumscribed nodules that may appear solid or contain cystic spaces, the latter corresponding to dilated glandular elements. These nodules compress the periurethral zone and chronic obstruction may result in recurrent urinary tract infections. Clinical signs and symptoms include increased urgency to urinate, hesitancy during urination, decreased force of urinary stream and nocturia (Vuichoud & Loughlin, 2015; Epstein, 2013; True & Vakar-Lopez, 2011; Deneris & Huether, 2008).

2.3 PROSTATE CANCER

2.3.1 Incidences

Prostate cancer occurs predominantly in men older than 50 years of age (Epstein, 2013). The incidence intensifies with advancing age where more than 75% of all prostate cancer cases are commonly diagnosed in men older than 65 years (Azizah *et al*., 2016; Deneris & Huether, 2008). According to IARC, prostate cancer is the second most common cancer and the fifth-leading cause of cancer-related mortality among men in the world in 2012 (Ferlay *et al*., 2013). In Malaysia, it was listed as the fifth most frequent cancer among men during the period of 2007 to 2011 and more than half of the patients were diagnosed at a late stage (stage 3 and 4) as shown in Figure 2.3 (Azizah *et al*., 2016).

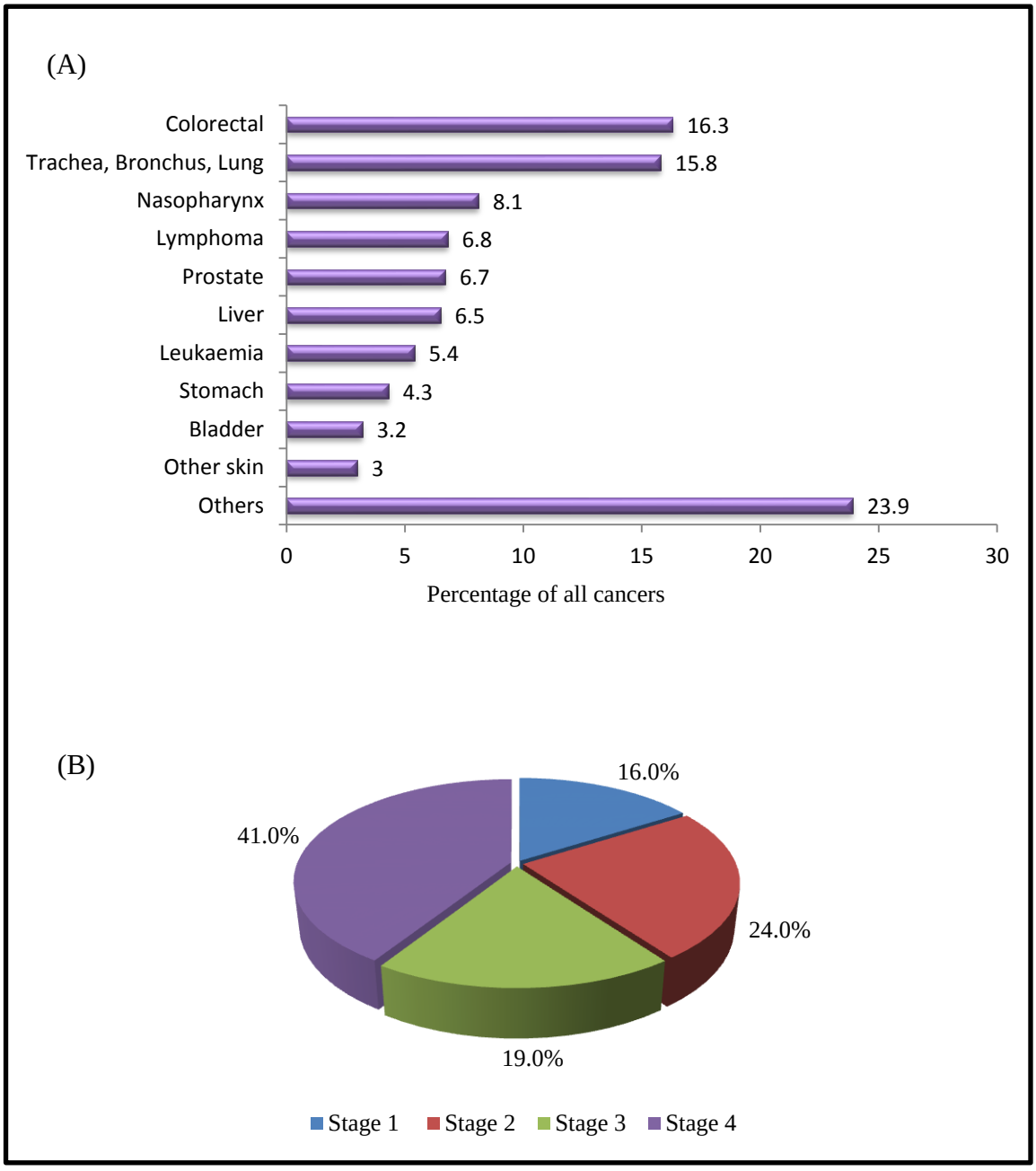

Figure 2.3: (A) Ten most frequent cancers being diagnosed among male population in Malaysia for the period of 2007 to 2011 and (B) stages of prostate cancer at diagnosis in Malaysia during the same period (Azizah *et al*., 2016).

2.3.2 Histopathology

Prostate cancer may originate from tumours derived from either the epithelial component or the non-epithelial/stromal component. The former can be divided into acinar or non-acinar type based on their morphological appearances. The most common appearance of epithelial-derived tumours is the acinar type comprising 90% of tumours. Some variants under this type include signet ring, atrophic, microacinar and pseudohyperplastic. The non-acinar type consists of urothelial carcinoma, ductal and sarcomatoid. The non-epithelial/stromal tumours which are the rarer type of prostate cancer comprise solitary fibrous tumours and leiomyosarcomas. Squamous cell carcinoma and small cell carcinoma are among the less common histological variants occurring in prostate cancer. Both of them are associated with aggressive disease, hormonal therapy resistance and poor prognosis (Oxley, 2014; Humphrey, 2012).

Different cell types of origin can undergo oncogenic transformation creating distinct subtypes of tumours with specific histopathological and/or molecular features that can influence patient responsiveness to treatment and its outcome (Lee & Shen, 2015). Acinar-type adenocarcinoma is the predominant histological subtype of prostate cancer characterized by rare neuroendocrine cells, luminal secretory cells and an absence of basal cells (Stoyanova *et al*., 2013; Humphrey, 2012). The luminal phenotypic feature of prostate adenocarcinoma may imply that either this corresponds to a luminal cell of origin or as a result of basal-to-luminal differentiation from basal cell of origin (Lee & Shen, 2015; Kwon *et al*., 2014; Stoyanova *et al*., 2013). The less common prostate cancer variants such as small-cell carcinoma has features such as loss of p53 expression and proliferating neuroendocrine cells (Chen *et al*., 2012); whereas squamous cancers,

characterized by basal cells, can occur as squamous cell carcinoma or in the context of adenocarcinoma (Stoyanova *et al*., 2013; Humphrey, 2012; Parwani *et al*., 2004).

Prostate cancer is generally a latent disease as it usually develops slowly and dysplastic lesions may take years or even decades before becoming cancer (WCRF/AICR, 2014). Some histopathological conditions of the prostate have been suggested to give rise to pre-neoplastic lesions. Prostatic intraepithelial neoplasia (PIN) is a lesion in which neoplastic prostate epithelial cells appear to proliferate within the confines of otherwise normal glandular structure and can be divided into low-grade (LG-PIN grade 1) and high-grade PIN (HG-PIN grades 2 and 3) (True & Vakar-Lopez, 2011; Nelson & De Marzo, 2007). However, only the latter is reported in current practice. In LG-PIN, the epithelial cells grow in multiple layers projecting into the lumen of the gland with intact basal layer and no increase of serum PSA is noted in this lesion. In contrast, this basal layer has been described as incontinuous in HG-PIN. It is associated with invasive adenocarcinoma due to its resemblance in the cytological features and similarities in many genetic abnormalities. The location of the HG-PIN is similar to that of prostate cancer as both reside in the peripheral zone of the prostate (Tuomela & Harkonen, 2014; Oxley, 2014; True & Vakar-Lopez, 2011).

Proliferative inflammatory atrophy (PIA) is another lesion that was suggested as a precursor lesion to prostate cancer. Inflammatory processes in the prostate tend to cause collateral damage to the surrounding tissue resulting in this specific lesion. PIA lesions are characterized by luminal epithelial cells which are reduced in size and not fully differentiated into columnar secretory cells. PIA refers to focal atrophy lesions with vigorously proliferating epithelial cells that are associated with inflammation whereas atrophic lesions that are not associated with obvious inflammatory infiltrates are termed

proliferative atrophy (PA). However, both lesions have been proposed as precursors to both PIN and prostate cancer (True & Vakar-Lopez, 2011; Nelson & De Marzo, 2007).

The prevalent form of prostatic neoplasms is adenocarcinoma. The degree of differentiation contributes largely to the aggressiveness of this disease rather than the size of the tumour (Kyprianou, 2014; Deneris & Huether, 2008). In the initial stage of prostate cancer, this disease is found to be relatively indolent with no clinical significance and essentially curable when confined to the prostatic capsule, such that most mortality of patients diagnosed with prostate cancer are due to other causes (Kumar *et al*., 2013; Abate-Shen & Shen, 2000). However, if this disease is not detected early and in an already aggressive form, prostate cancer will pose a significant threat to life or health, advancing from a locally invasive carcinoma to a metastatic stage resulting in mortality. The transition from localized to metastatic state is normally accompanied by a switch from androgen dependence to androgen independence condition (Abate-Shen & Shen, 2000).

DHT, a potent intra-prostatic androgen, binds to the AR to induce activation of various target genes accountable for cell differentiation, proliferation and survival (Datta *et al*., 2011; Deneris & Huether, 2008). Although androgens are required for normal prostate development, they act as strong tumour promoters in prostate carcinogenesis through AR-mediated mechanisms (Deneris & Huether, 2008). AR is a 110 kDa phosphoprotein which belongs to the nuclear receptor superfamily (Kyprianou, 2012). The majority of AR molecules reside in the cytoplasm in an inactive form. The liganded AR translocates to the nucleus and binds to the promoter/enhancer region of the target genes leading to transcription and downstream effects. AR regulates gene expressions that stimulate cellular differentiation in prostate luminal epithelial cells, which is the

principal step of malignant transformation to prostate adenocarcinoma (Kyprianou, 2012; Datta *et al*., 2011; Deneris & Huether, 2008). Androgen independence stage of prostate cancer may be caused by changes in AR, AR target genes, or AR signals (Held-Warmkessel, 2007). The prostate cancer cells possess the ability to adapt to their extracellular microenvironment through alterations of i) epithelial-stromal interactions; ii) pathophysiologic cellular stress responses; iii) growth factor-receptor pathways; and/or iv) the inflammatory response. This ability confers the cells the aptitude to survive in almost any microenvironments and, eventually, giving rise to aggressive phenotypes (Kyprianou, 2012).

Prostate cancer cells have to travel via neurovascular bundles that lie adjacent to the prostate, as shown in Figure 2.4, in order to reach various metastatic sites, and must be able to adapt, survive and grow in varied microenvironments (Nelson & De Marzo, 2007; Kirby & Brawer, 2004). Locally advanced prostate cancers often infiltrate the seminal vesicles and periurethral zones of the prostate and may subsequently invade the adjacent soft tissues, the wall of the urinary bladder, or, less commonly, the rectum. Distant metastases of prostate cancer are commonly occurring in the liver, lymph nodes, lungs, adrenals and bones; whereas in cases of bone metastasis, the pelvis, ribs, femur, thoracic spine, lumbar spine, humerus and skull are the most common targets (Kumar *et al*, 2013; Deneris & Huether, 2008; Held-Warmkessel, 2007). Prostate cancer may metastasize and grow well in the bone because of the unique bone microenvironment enabling the cells to advance their aggressive phenotype by undermining the coordinated behaviour of osteoblasts and osteoclasts, thus producing destructive bony lesions, and, subsequently, spread malignant growth (Kyprianou, 2012; Nelson & De Marzo, 2007).

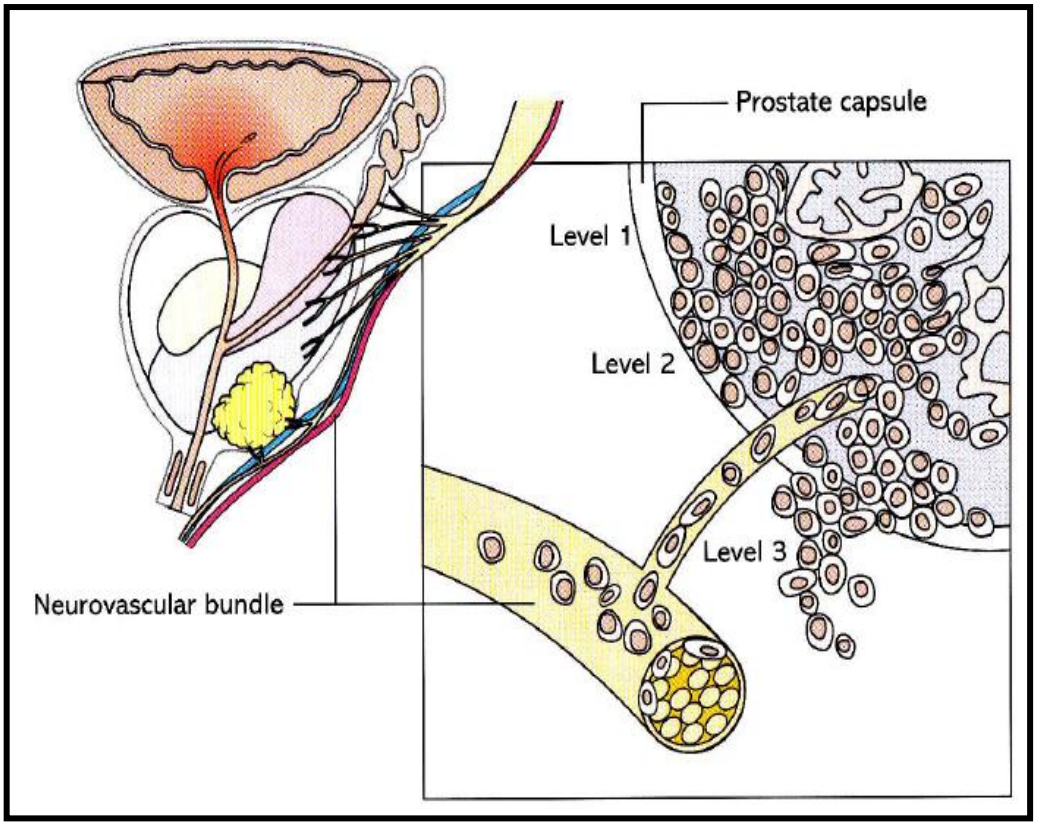

Figure 2.4: Human prostate cancer progression. Level 1 is the PIN stage, Level 2 is invasive carcinoma and Level 3 is metastatic stage (adapted from Kirby & Brawer, 2004).

2.3.3 Diagnosis, Grading and Staging

Generally, an early detection of prostate cancer may improve many aspects in the management of prostate cancer patients such as the treatment, restricting the progression of cancer and prognosis. The first line of tests performed on patients who are suspected of having prostate cancer are the digital rectal examination (DRE) combined with measurement of serum PSA level (Philippou *et al*., 2014). DRE is a simple, fast and very cost-effective method in detecting prostate cancer. Since it is done by palpating, the tumour must be large enough and posteriorly situated as only the posterior portion of the gland is palpable by physician (Kirby & Brawer, 2004). In PSA blood test, the normal serum level for PSA is $\leq$ 4 ng/ml. However, one limitation of PSA is that while it is prostate-specific, it is not cancer specific (Kumar *et al*., 2013). Approximately 20% of patients were diagnosed with prostate cancer despite having a PSA level lower than 4 ng/ml (van der Poel, 2007; Thompson *et al*., 2004), whereas in some cases, the PSA level may be elevated due to certain conditions such as benign prostatic hyperplasia (BPH), prostatitis, renal insufficiency, prostatic manipulation, irritation and recent ejaculation (Cheetham, 2014). These inadequacies may lead to over diagnosis and over treatment of the disease. As such, patients who have positive findings in digital rectal examination (DRE) and PSA tests is followed by more sophisticated diagnostic techniques, beginning with transrectal ultrasound (TRUS) and guided systemic biopsy, which enable the physician to locate the areas of abnormality (Philippou *et al*., 2014; Cheetham, 2014).

Prostate cancer diagnosis is confirmed through microscopic examination of the tissue biopsy where the grade of the cancer, the number of cores positive for cancer and

percentage of cancer per core are able to be determined (Deneris & Huether, 2008; Ross *et al*., 2007). Other procedures such as computerized tomography (CT), magnetic resonance imaging (MRI), bone scans and lymphography are usually used if metastases are suspected to be in the lymph node, bone or other adjacent tissues (Deneris & Huether, 2008).

The most widely used system for prostate cancer grading is the Gleason system. In this system, the pattern of infiltrating tumour glands is assigned a grade based on their level of aggressiveness from 1 (well differentiated) to 5 (poorly differentiated). As prostatic cancers are often heterogeneous (contain more than one pattern), a primary grade is assigned to the dominant pattern and the secondary grade to the next most frequent pattern. The sum of both values is considered the Gleason score (Table 2.1). This score provides useful prognostic information for the patients where the progressive loss of glandular differentiation is associated with worsening prognosis. Patients who are diagnosed with low Gleason score have an excellent prognosis while those with Gleason scores above 6 are associated with an alarming risk of escalating disease progression/aggressiveness, increased metastatic potential and diminished chance of survival (Epstein, 2015; Kumar *et al*., 2013; Ross *et al*., 2007; Kirby & Brawer, 2004).

Prostate cancer staging is important as it describes the extent of disease spread and strongly correlates with prognosis. The treatment of prostatic cancer is determined primarily by the stage of the disease. The most common system used for prostate cancer staging is the American Joint Committee on Cancer (AJCC) tumour/node/metastases (TNM) system as shown in Table 2.2 (Buyyounouski *et al*., 2017; Ross *et al*., 2007).

Table 2.1: Histological definition of prostate cancer grading system and Gleason score (Epstein, 2015).

Gleason score	**Histological characteristics**
Grade group 1: Gleason score < 6	Only individual discrete well-formed glands
Grade group 2: Gleason score 3+4=7	Predominantly well-formed glands with lesser component of poorly formed / fused / cribriform glands
Grade group 3: Gleason score 4+3=7	Predominantly poorly formed / fused / cribriform glands with lesser component of well-formed glands
Grade group 4: Gleason score 4+4=8; 3+5=8; 5+3=8	Only poorly formed / fused / cribriform glands Predominantly well-formed glands and lesser component lacking glands Predominantly lacking glands and lesser component of well-formed glands
Grade group 5: Gleason scores 9-10	Lack gland formation (or with necrosis) with or without poorly formed / fused / cribriform glands

In TNM system, stages T1-T4 describe the pathological development of the tumour (Figure 2.5). T1 is assigned when the tumour is discovered by PSA testing or after transurethral resection of the prostate, and is not detectable by DRE or ultrasonography. Organ-confined palpable tumour is classified as T2 whereas tumour that palpably exceed beyond the prostate is classified as T3. T4 is assigned when the disease is at an advanced stage, in which the metastatic cancer infiltrates neighbouring organs. The nodal stages (N0-N1) and metastatic stages (M0-M1c) represent the disease clinical progression into the lymph nodes and other organs (Ross *et al*., 2007; Kirby & Brawer, 2004; Buyyounouski *et al*., 2017). For post-prostatectomy patients, a pathological T stage (pT) is often employed, in which there is no pT1 designation (Ross *et al*., 2007; Buyyounouski *et al*., 2017). Patients who are diagnosed at an early stage of prostate cancer at which the disease is still limited and confined to the prostate have better survival than those with disease that has spread beyond the gland (Held-Warmkessel, 2007).

2.3.4 Treatment

Patients with prostate cancer maybe asymptomatic, if at early stage of disease, or may present with a variety of symptoms such as bladder outflow obstruction in local disease or hematuria, dysuria, anuria, hemospermia, impotence, incontinence, renal failure, and/or pain in the perineal, suprapubic, loin, bone and/or low back in locally invasive as well as metastatic disease (Kirby & Brawer, 2004). Thus treatment options of prostate cancer are both curative (eliminating the tumour or preventing cancer-related mortality) and palliative (relieving symptomatic conditions) and depend on several interrelating factors (Held-Warmkessel, 2007; Kirby & Brawer, 2004).