UNIVERSITI PUTRA MALAYSIA

EXPRESSION OF CYCLOOXYGENASE-2 (COX-2) AND CYCLIN DEPENDENT KINASE IB (CDKNIB/p27Kipl) IN HUMAN PROSTATE ADENOCARCINOMA

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FPSK(m) 2011 45

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By

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Thesis submitted to School of Graduate studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Master of Science

March 2011

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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Chairman: **Norhafizah Mohtarrudin, MBBS, M. Path, AM**

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Prostate adenocarcinoma is one of the most common forms of malignancy occurring in the Malaysian male population. Inflammation has been identified in many studies to play key roles in the process of carcinogenesis. Inflammation is responsible for prompting angiogenesis, enhancing cellular motility and increasing resistance to apoptosis. Cyclooxygenase-2 (COX-2) is an enzyme that converts arachidonic acid into pro-inflammatory prostaglandins and other eicosanoids. In addition, COX-2 is also highly expressed in a wide number of human cancers including prostate adenocarcinoma. Moreover, loss of Cyclin dependent kinase inhibitor 1B (p27^{Kip1}) expression has been implicated in the malignant development in many human cancers. p27^{Kip1} is a gene that encodes protein for cell cycle regulation generally, and controls the cell cycle progression at G_1 phase specifically. Low expression of p27^{Kip1} has been associated with

a poor prognosis in malignant tumours, including breast, gastric and prostate carcinoma. The objectives of this study were to determine the expression level of COX-2 and $p27^{Kip1}$ in different types of prostate tissue and to relate the association with the clinicopathological parameters. $p27^{Kip1}$ (V109G) polymorphism frequency in prostate adenocarcinoma was also determined to assess its relationship with advance prostate cancer susceptibility. Paraffin-embedded prostatic tissue (n = 263) was obtained from the Pathology Department of Hospital Kuala Lumpur. The mean age of the patients is 64.54 ±10.79 years. The tissue retrieved consisted of 63 normal prostate tissue samples, as well as 100 each for benign prostatic hyperplasia (BPH) and prostate adenocarcinoma (PCa). COX-2 expression was performed using standard immunohistochemistry methods. Anti-human COX-2 monoclonal mouse primary antibody was used in a 1:100 dilution, whereas the anti-human $p27^{Kip1}$ monoclonal mouse primary antibody was used in a dilution of 1:50. For each sample, the extent and intensity of staining with COX-2 antibody was graded on a scale from 0 to 4+. Staining was classified as 0 (no expression), 1+ (weak expression), 2+ (moderate expression), 3+ (strong expression) and 4+ (very strong expression). The results showed that, 56/100 PCa samples showed strong COX-2 expression (P=0.000), in comparison 16/100 samples of BPH (P=0.001), while weak COX-2 expression was observed in all 63 normal samples. Very strong expression for $p27^{Kip1}$ was seen in 62/63 normal prostate tissue samples and 77/100 BPH samples (P=0.000), while 39/100 PCa samples exhibited weak $p27^{Kip1}$ expression and 25 of the rest had no expression (P=0.000). Next, to confirm the accuracy of staining, we further analysed selected samples by semiquatitative reverse transcriptase PCR (RT-PCR) method on COX-2 and $p27^{Kip1}$ genes. This RT-PCR analysis, COX-2 expression was detected in high Gleason scores of 8 and 9, which were 2.01 and 2.17-fold

respectively higher compared to normal tissue. BPH displayed only 1.04-fold higher COX-2 expression than normal tissue. Significant down-regulation of $p27^{Kip1}$ was observed in Gleason scores 7, 8 and 9 which were less than 0.5-fold in changes compared to normal prostatic tissue. No significant $p27^{Kip1}$down-regulation was observed in BPH samples compared to normal samples. PCR-RFLP was used to investigate $p27^{Kip1}$ polymorphism using Bg*l*I restriction enzyme. PCR–RFLP analysis showed that distribution of genotypes were not statistically significant between PCa and normal prostate, whereby the genotypes VV and VG were observed more frequently in PCa and normal prostate, while GG genotype was not found in any PCa or normal samples. The results of this study, suggest that COX-2 overexpression and $p27^{Kip1}$ down-regulation may play a role in the progression of prostate adenocarcinoma. Therefore, expression of COX-2 and $p27^{Kip1}$ as potential therapeutic targets for prostate cancer should be evaluated further.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGEKSPRESAN CYCLOOXYGENASE-2 DAN CYCLIN DEPENDENT KINASE 1B (CDKN1B/p27^{Kip1}) DALAM ADENOKARSINOMA PROSTAT MANUSIA

Oleh

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Adenokarsinoma prostat adalah salah satu bentuk tumor malignan yang biasa berlaku dalam populasi lelaki di Malaysia. Inflamasi telah dikenalpasti dalam banyak kajian, memainkan peranan penting dalam proses karsinogenesis. Inflamasi bertanggungjawab dalam proses pembentukan saluran darah, meningkatkan pergerakan sel, dan menambah kerintangan sel terhadap apoptosis. Cyclooxygenase-2 (COX-2) adalah enzim yang menukarkan asid arakidonik kepada prostaglandin pro-inflamasi dan eikosanoid yang lain. Selain itu, pengekspresan COX-2 yang tinggi juga dijumpai dalam banyak kes kanser termasuk kanser prostat. Sementara itu, ketiadaan pengekpresan protein Cyclin dependent kinase inhibitor 1B (p27^{Kip1}) telah dikaitkan dengan pembentukan banyak kanser malignan manusia. p27^{Kip1} adalah gen yang mengekod protein perencat untuk kitaran sel secara amnya, dan mengawalatur perjalanan kitaran sel pada peringkat G_1 secara spesifiknya. Pengekspresan p27^{Kip1} yang rendah telah dikaitkan dengan prognosis yang teruk dalam tumor malignan, temasuk tumor malignan payudara, perut, dan prostat.

Tujuan kajian ini adalah untuk menentukan tahap pengekspresan COX-2 dan $p27^{Kip1}$ di dalam tisu prostat yang berbeza dan mengenalpasti hubungkait antara pengekpresan dengan parameter patologiklinikal. Selain itu juga frekuensi polimorfisma $p27^{Kip1}$ (V109G) juga dilihat bagi menentukan genotip yang manakah, lebih cenderung untuk membentuk adenokarsinoma prostat yang lebih teruk. Sebanyak 263 blok parafin tisu prostat dikumpul daripada Jabatan Patologi Hospital Kuala Lumpur. Purata umur sampel – sampel dari pesakit adalah 64.54±10.79 tahun. Tisu-tisu terdiri daripada 63 tisu postat normal, 100 tisu benign prostatik hyperplasia (BPH) dan 100 tisu prostat malignan (PCa). Pengekspresan COX-2 dan $p27^{Kip1}$ dijalankan dengan menggunakan kaedah piawai pewarnaan imunohistokimia. Antibodi primer monoklonal anti-manusia telah digunakan dengan nisbah pencairan 1:100, manakala antibodi primer monoklonal anti-manusia $p27^{Kip1}$ pula dicairkan dengan nisbah 1:50. Hasil dinilai berdasarkan keamatan dan peringkat perwarnaan dengan antibodi masing-masing dengan skala 0 hingga 4+. Perwarnaan diklasifikasikan sebagai 0 (tiada pengekspresan), 1+ (pengekpresan lemah), 2+ (pengekspresan sederhana), 3+ (pengekspresan kuat) dan 4+ (pengekspresan sangat kuat). Hasil kajian menunjukkan, sebanyak 56/100 tisu PCa menunjukkan pengekspresan COX-2 yang kuat (P=0.000), berbanding tisu BPH iaitu sebanyak 16/100 (P=0.001). Pengekspresan COX-2 dilihat lemah dalam kesemua 63 tisu prostat normal. Manakala pengekpresan $p27^{Kip1}$ yang sangat kuat dilihat dalam 62/63 tisu normal dan 77/100 tisu BPH (P=0.000) manakala 39/100 tisu PCa menunjukkan pengekspresan $p27^{Kip1}$ yang lemah dan 25 tisu PCa selebihnya tidak menunjukkan sebarang pengekpresan (P=0.000). Seterusnya, untuk mengesahkan ketepatan hasil perwarnaan, kaedah PCR transkriptase berbalik (RT-PCR) secara semikuantitaitf menggunakan gen COX-2 dan $p27^{Kip1}$ telah dijalankan ke atas sampel-sampel terpilih. Analisis RT-PCR

pula menunjukkan, pengekspresan COX-2 dilihat dalam PCa skor Gleason 8 dan 9 iaitu 2.01 dan 2.17 kali masing–masing lebih tinggi berbanding tisu prostat normal. Tisu BPH hanya menunjukkan 1.04 kali lebih tinggi dari tisu prostat normal. Penurunan pengekspresan $p27^{Kip1}$ yang signifikan dilihat menerusi tisu PCa skor Gleason 7, 8, dan 9 di mana masing-masing menunjukkan perubahan lebih kecil daripada 0.5 kali terhadap tisu prostat normal. Tiada penurunan pengekspresan $p27^{Kip1}$ yang signifikan dilihat dalam tisu BPH berbanding tisu prostat normal. Analisis PCR-RFLP juga dijalankan ke atas sampel adenokarsinoma prostat untuk melihat polimorfisma $p27^{Kip1}$ dengan menggunakan enzim pembatasan Bg*l*1. Analisis PCR – RFLP menunjukkan taburan genotip yang tidak signifikan antara tisu PCa dan prostat normal, di mana genotip VV dan VG dilihat lebih kerap diperhatikan dalam tisu PCa dan prostat normal, sementara genotip GG tidak dijumpai langsung dalam kedua-dua jenis tisu PCa mahupun BPH. Berdasarkan hasil kajian, adalah dicadangkan pengekspresan COX-2 yang tinggi dan rendah pada $p27^{Kip1}$ memungkinkan, kedua-dua enzim ini memainkan peranan dalam perubahan kanser prostat untuk menjadi lebih malignan. Oleh itu, pengekspresan COX-2 dan $p27^{Kip1}$ berguna untuk dinilai lebih lanjut sebagai agen terapeutik yang berpotensi untuk kanser prostat.

ACKNOWLEDGEMENTS

First and foremost, I would like to dedicate my deepest thanks to Allah S.W.T for blessing me with the patience and determination to complete this study successfully.

I would like to express my deepest gratitude to my supervisor, Dr. Norhafizah Mohtarrudin, for her valuable ideas, advice, guidance and support throughout this whole project. Without her help and support, this project would not have been completed at a time.

I would also like to sincerely thank my co-supervisors, Dr. Chong Pei Pei, Dr. Malina Osman and Dr. Noraini Mohd Dusa for their ideas, help and advice. Sincere appreciation is extended to all my seniors in the laboratory for their immeasurable support, assistance and encouragement.

My utmost appreciation also to the staff of the Histopathology Laboratory, Biomedical Sciences Laboratory, and Department of Pathology in Hospital Kuala Lumpur, who have been involved directly or indirectly in this study. Their generosity, help and sacrifice of valuable time is truly appreciated. My deepest gratitude also goes to my family and coursemates for their moral support and encouragement throughout my study.

Last but not least, sincere thanks to Jabatan Perkhidmatan Awam (JPA) for the scholarship awarded to me during my study, as well as to Fundamental Research Grant Scheme (FRGS) from the Ministry of Higher Education for their support of this project.

I certify that a Thesis Examination Committee has met on 29 March 2011 to conduct the final examination of Mohd Rohaizad bin Md. Roduan on his thesis entitled "Expression of Cyclooxygenase-2 (COX-2) and Cyclin Dependent Kinase 1B ($p27^{Kip1}$) in Human Prostate Adenocarcinoma" in accordance with the Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Master of Science.

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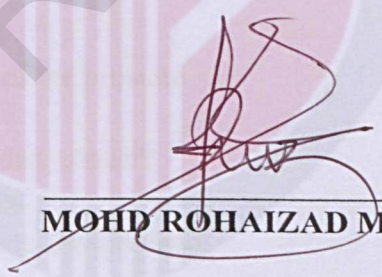
DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.



MOHD ROHAIZAD MD RODUAN

Date: 29 March 2011

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%	Percentage
µ	Micro
µg	Microgram
µl	Microliter
µm	Micrometer
^{0}C	Degree of Celcius
5-HT	5-hydroxy-triptamin
bp	Base pairs
BPH	Benign prostatic hyperplasia
CDI	Cdk inhibitors
Cdk	Cyclin dependent kinases
CDKNIB	Cyclin dependent kinase inhibitor 1B
CI	Confidence intervals
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DAB	3, 3'-Diaminobenzidine
ddH_2O	Double distilled water
DHT	Dihydrotestosterone
DRE	Digital rectal examination
FA	Formaldehyde Agarose gel
FPC	Familial prostate cancer
g	Gram
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase

H & E	Hematoxylin and Eosin
hK3	Human kallikrein-3
HoLEP	Holmium Laser Enucleation of Prostate
HPC	Hereditary prostate cancer
IHC	Immunohistochemistry
Il-10	Interleukin-10
kDa	Kilo Dalton
L	Liter
LHRH	Luteinizing Hormone-Releasing Hormone
M	Molar
MMLV	Moloney murine leukemia virus
NSAID	Non-steroidal anti inflammatory drugs
OR	Odd ratio
P	Significant alpha value
$p27^{Kip1}$	Cyclin dependent kinase inhibitor 1B
PBS	Phosphate buffer saline
PCa	Prostate carcinoma
PG	Prostaglandin
PGG2	Prostaglandin endoperoxidase synthase-2
PGH2	Prostaglandin H2
PIN	Prostatic Intraepithelial Neoplasia
PSA	Prostate specific antigen
PSAP	Prostate specific acid phosphatase
PSMA	Prostate specific membrane antigen
PTGS	Prostaglandin-endoperoxide synthase
RFLP	Restriction Fragment Length Polymorphisms
RT-PCR	Reverse Transcription-Polymerase Chain Reaction

SNP	Single nucleotide polymorphism
SPC	Sporadic prostate cancer
TGF	Transforming growth factor
TRUS	Transrectal ultrasonography
TUNA	Transurethral Radio Frequency Needle Ablation of the Prostate
TURP	Transurethral resection of the prostate
v	Volume
VEGF	Vascular endothelial growth factor
w	Weight
α	Alpha
β	Beta

CHAPTER 1

INTRODUCTION

1.1 Background

Prostate cancer is one of the most frequently diagnosed cancers in Malaysian males. A report from National Cancer Registry 2005 on incidence of cancer in Malaysia revealed that prostate cancer is the fourth most frequent cancer in males and accounted for 7.3% of the total cancer cases. Majority of morphologically reported cases were adenocarcinoma, which accounted for 96% of the total cases (National Cancer Registry, 2005). In developed countries such as USA, more than 230 000 prostate cancers have been diagnosed in 2005 and caused more than 30 000 death (Jemal *et al.,* 2005). According to the American National Cancer Institute, an estimated that 186 320 new cases and 28 660 deaths from prostate cancer were reported in the United States in 2008 (Jemal *et al.,* 2008).

Cancer is a disorder characterized by transformation of normal cells to abnormal cells that grow and multiply uncontrollably. In prostate cancer, there is proliferation of malignant glandular epithelium, which forms prostate adenocarcinoma. The molecular pathogenesis of prostate cancer has been linked with alteration of genes

and proteins. Most of the abnormal cells have undergone mutation involving several types of genes. In the past few years, inflammation and cell cycle regulator protein have been linked to prostate carcinogenesis.

Epidemiologic studies have suggested a significant association between inflammation and cancer (Vasto *et al.,* 2008). Recent evidences emphasized on inflammation in development of prostate cancer (Richardsen *et al.,* 2010). The study reported, production and action of COX-2, TGF-β and IL-10 were interrelated during prostate carcinogenesis. Inflammation has an important role in carcinogenesis by causing cellular and genomic damage, increasing cellular turnover, enhancing cellular proliferation and inducing angiogenesis. Angiogenesis is important not only for nourishing growing tumour masses, but also for metastasis (Caruso *et al.,* 2004). The link between chronic inflammation and cancer cell proliferation involving pro-inflammatory agents such as cytokines and mediators of inflammatory pathways has been well documented in previous studies (Allavena *et al.,* 2008).

Prostaglandins (PGs) are short-lived compounds that act as local hormones in normal cellular reaction. The PG level has been found to be increased in many pathological conditions, especially inflammation (Caruso *et al.,* 2009). Cyclooygenase (COX) converts arachidonic acid to prostaglandins and thromboxanes. Prostaglandins and thromboxanes maintain physiological regulation in the stomach, kidneys, intestines and platelets. Two isoforms of COX have been identified, namely COX-1 and COX-

2. COX-1 is expressed in many tissues and cell types, whereas COX-2 is inducible by a variety of factors such as tumour promoters, growth factors and cytokines. COX-2 has been shown to be highly expressed in a number of human cancers including prostate cancer (Gupta *et al.,* 2000). Persistent presence and aberrant of COX-2 has been associated with increased resistance to apoptosis, promotion of angiogenesis and increased cellular motility (Tsujii and DuBois, 1995).

Studies have also found that loss of cell cycle regulation control by certain genes may play an important role in carcinogenesis. One of the cell cycle regulators involved in carcinogenesis and tumour progression is $p27^{Kip1}$. $p27^{Kip1}$ is a cyclin dependent kinase 1B (CDKN1B) protein. It is a negative regulator of the cell cycle, and has potential as a tumour suppressor gene, which is contributed by its capacity to inhibit cells upon entry into S-phase from G_1-phase (Slingerland and Pagano, 2000).

$p27^{Kip1}$ is known to be expressed at high concentrations in healthy prostate tissue cells and become lower in most cases of prostatic intraepithelial neoplasia and prostate cancer (De Marzo *et al.,* 1998; Guo *et al.,* 1997). Loss of $p27^{Kip1}$ expression correlates with poor prognosis in prostate cancer (Cote *et al.,* 1998). Previous studies have shown $p27^{Kip1}$ down-regulation in many human malignancies including carcinoma of thyroid (Erickson *et al.,* 1998), breast (Catzavelos *et al.,* 1997), lung (Esposito *et al.,* 1997), gastric (Mori *et al.,* 1997), and colorectal (Loda *et al.,* 1997).

1.2 Problem statement

Previous studies have provided clues on how prostate cancer develops and progresses, but the molecular alterations and cellular mechanism leading to neoplastic transformation and tumour aggressiveness are not well understood. The management of patients with prostate cancer depends on an accurate assessment of the biological potential of the tumour. Unfortunately, current investigative techniques are still inadequate to provide precise and accurate clinical staging and tumour grading.

There is an urgent requirement for better understanding of the molecular abnormalities that define this tumour. It is crucial to identify potential biomarkers for prediction of disease outcome. At the cellular and molecular level, genetic aberration drives the formation and aggressiveness of prostate cancer (Angelo *et al.*, 2003). Every carcinoma is believed to arise from a single cell that accumulates genomic changes affecting regulatory genes.

Established conventional prognostic markers like prostate specific antigen (PSA), are not good markers for prostate carcinoma. It has been shown that PSA is tissue specific, but not tumour specific, as BPH and prostatitis also show elevation of serum PSA level (Carter and Isaac, 2004). In addition, immunostaining for PSA also

misses a small but significant percentage of prostate carcinoma, and further additional stains such as prostate specific acid phosphatase (PSAP) and/or prostate specific membrane antigen (PSMA) need to be done to confirm the diagnosis. This will lead to an increase in medical costs (Rodney, 2005).

There is an urgent requirement for new biomarkers of screening and treatment selection, as well as development of new therapies, which may lead to rational strategies for prostate cancer management. More sophisticated analysis of the morphological changes in human prostate cancer is necessary to complement molecular and cellular analysis.

The main treatment options for prostate cancer include radical prostatectomy, radiotherapy, and watchful waiting. Each type of treatment has its own risks and benefits. For examples radical prostatectomy may have side effects such as impotence, incontinence and obstruction in the urethra. Radiation therapy may cause complications related to radiation injury include diarrhea, rectal bleeding, and blood in the urine (Michaelson *et al.,* 2008). The treatment choice is largely based on the patient's preference. A new treatment option that is more effective and has lesser risk than conventional methods is needed. Graham et al., (2005) examined a cohort of patients treated with an NSAID showed Celecoxib was not associated with any increased risk of cardiac events (odds ratio 0.84, CI 95% 0.67-1.04). Selective

inhibitors of COX-2 have potential as a new novel therapy in PCa, as it has been found to be effective in colon cancer prevention and treatment (Xu, 2002).

New advanced treatments like gene therapy should also be given a big attention, as this treatment modality might have potential as an alternative cancer therapy. As reported previously, there is insufficient clinical data on COX-2 as only little attention has been given to COX-2 and its relation to prostate cancer (Steinbach *et al.,* 2000; Kawamori *et al.,* 1998; and Sheng *et al.,* 1997). However, the recent discovery of COX-2 inhibitor as a treatment modality for colon cancer show emphasize that more research be dedicated to the role of COX-2 and prostate cancer, as it might also be more effective in its treatment.

1.3 Hypothesis

1. There are significant differences in the expression of COX-2 and $p27^{Kip1}$ in prostate carcinoma compared to normal prostatic tissue and benign prostatic hyperplasia.

2. There were significant correlations between the expression of COX-2 and $p27^{Kip1}$ with selected clinicopathological parameters (PSA level, Gleason score, tumour amount, and age) observed in prostate carcinoma.

3. There is significant $p27^{Kip1}$/ CDKN1B V109G polymorphism in prostate carcinoma.

1.4 Research objectives

This study embarks on the following general objectives:

1. To determine the expression of COX-2 and $p27^{Kip1}$ in normal prostate tissue, benign prostatic hyperplasia and prostate carcinoma.

2. To determine the frequency of CDKN1B V109G polymorphism in normal prostate and prostate carcinoma tissues.

Specific objectives:

1. To compare the differences between COX-2 and $p27^{Kip1}$ expression in prostate carcinoma with normal prostatic tissue BPH by using immunohistochemical and gene expression approaches.

2. To correlate the expression of COX-2 and $p27^{Kip1}$ with selected clinicopathological parameters (PSA level, Gleason score, tumour amount, and age).

3. To determine the genotype that is more frequently observed in normal prostate and prostate cancer tissues.

1.5 Significance of study

1. The results of this study will contribute towards a better understanding of the roles of COX-2 and $p27^{Kip1}$ in the mechanism of prostate cancer development and progression.

2. COX-2 expression results will be useful as baseline data for future research on prostate cancer treatment involving clinical trials using COX-2 specific inhibitors.

3. $p27^{Kip1}$ expression has potential as a new biomarker, and further evaluation may lead to the discovery and development of a new effective drug for prostate cancer treatment.

4. The results of this study increases scientific information concerning the effects of COX-2 and $p27^{Kip1}$ in the carcinogenesis of prostate cancer.

5. The results of this study will also be useful as reference for further research on the role of COX-2 and $p27^{Kip1}$ in other types of human cancer.

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