THE INVESTIGATION OF CYCLIN-D1 GENE EXPRESSION USING SYBR GREEN 1 BASED REAL- TIME PCR IN BLADDER CANCER

By

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CERTIFICATE

This is to certify that the dissertation entitled 'INVERSTIGATION OF CYCLIN-D1 USING SYBR GREEN 1 BASED REAL- TIME PCR IN BLADDER CANCER' is the bonafide record of research work done by MRS AISYA BINTI ZULKIFLI during the period from July 2009 to October 2009 under my supervision.

29 October 2009

CONTENT

ABSTRACT

Bladder cancer is one of the most common malignancies in developed countries while transitional cell carcinomas (TCC) are the origin of more than 90% of diagnosed bladder cancers. Patients diagnosed with bladder cancer basically belong to two clinically distinct groups, namely non-muscle invasive (which are papillary pattern) and muscle invasive (which are solid). These carcinomas pose the greatest clinical problems due to the high recurrence of non-muscle invasive tumors even after transurethral resection of the tumors. At present, there are no clinically useful markers available for identifying bladder cancer patients with a high risk of disease recurrence or progression.

Initial aim of this study was to investigate by using gene expression level of Cyclin-D1. The experiment was carried out by using SBYR Green 1 based Real-Time PCR analysis. However, the result was not achieved as expected. This is due to some undetermined and invalid result few factors such as contamination, mispairing of the primer and primer dimer. Therefore the investigation of the factor that contributes to invalidation was investigated by using previous journals.

ABSTRAK

Kanser pundi kencing merupakan kanser yang paling kerap berlaku di negara membangun. 90% neoplasma adalah dari jenis 'transitional cell carcinoma' (TCC). Secara klinikal, TCC terbahagi kepada dua kumpulan iaitu yang dipanggil sebagai 'non-muscle invasive' dan 'muscle invasive'. Karsinoma tersebut memberikan masalah klinikal yang tinggi kerana biasanya pesakit dengan kanser 'non-muscle invasive' mengalami masalah serangan ulangan yang kerap walaupun setelah dilakukan prosedur 'transurethral resection'. Buat masa ini, masih tiada penanda klinikal yang berpotensi untuk mengenalpasti pesakit kanser pundi kencing terutamanya yang mempunyai risiko serangan ulangan atau berpotensi untuk berkembang keperingkat yang lebih teruk.

Di dalam kajian ini, aras ekspresi gen Cyclin-D1 di dalam pundi kencing telah dikaii dengan menggunakan kaedah analisis SBYR Green I berdasarkan Real Time PCR. Walaubagaimanapun, keputusan yang diperolehi tidak seperti yang dijangkakan disebabkan beberapa factor seperti kontaminasi, primer yang salah berpasangan dan primer dimer. Oleh tiu, kajian telah dijalankan keatas faktor yang menyebabkan masalah tersebut berlaku berdasarkan jurnal-jurnal yang terdahulu.

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In the Name of Allah, Most Gracious, Most Merciful

"Over the knowledgeable, is Allah the most knowledgeable"

All praises and gratitude is due to Allah, the Lord to whom every single creature in the heaven and the earth belongs to. Thank you Allah for giving me the strength and patient during this trying times. May peace and blessings be on the leader of all creation, the prophet Muhammad S.A.W, his family and companions.

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List of Abbreviations

Symbol	Meaning
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
Cdk4	Cyclin-dependent kinase 4
Cdk6	Cyclin-dependent kinase 6
Cdk7	Cyclin-dependent kinase 7
Cdk8	Cyclin-dependent kinase 8
Cis	Carcinoma *in situ*
Cp	Ceruloplasmin
Ct	Crossing threshold
CCND1	Cyclin D1
PCR	polymerase chain reaction
DEPC	Diethypyrocarbonate
dNTPs	Dioxynucleotide triphosphate
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetate
g	gram
h	hour
H & E	Hematoxylin and eosin
HKB	Hospital Kota Bharu
L	Liter
LOH	Loss of heterozygosity

M	molar
min	minutes
mg	milligram
mL	milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
NaOH	Sodium hydrogen
ng	nanogram
nM	Nanometer
NTC	Non-template control
Rb	Retinoblastoma
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Second
SDS	Sodium dodecyl sulfate
ssDNA	Single stranded deoxyribonucleic acid
TCC	Transitional cell carcinoma
TNM	Tumor, Node , Metastasis
TUR	Transurethral resection
TURBT	Transurethral resection of bladder tumor
TSGs	Tumor suppressor genes
U	Unit
UV	Ultraviolet
V	volt
WHO	World Health Organization
Wnt	Wingless type

x g	Gravity
ºC	degree celcius
µg	Microgram
µL	Microliter
µM	Micromolar

CHAPTER 1

INTRODUCTION

Bladder cancer is one of the most serious cancers occurs throughout the world. The number of new cases has risen since 1960s with 70,980 of new cases and 14,330 of death cases has been reported in the United State in 2009 (National Cancer Institute United Stated, 2009). Men are approximately three times more likely than women to develop bladder cancer. Constant pattern also has been shown in Malaysian statistic (Malaysia cancer stastistic, 2006). Cigarette smoking and occupational exposure are the two major recognized risk factors for bladder cancer (Jung and Messing, 2000, Negri and La Vecchia, 2001). Bladder cancer is a disease of middle-age or elderly person with the median age of diagnosis is being 67 years in males and 71 years in female (Lynch and Cohen, 1995).

Most neoplasms are TCC, accounting for more than 90% of all bladder cancer that are either papillary non-muscle invasive or muscle invasive tumors at the time of initial diagnosis. Human bladder carcinomas shows the greatest problems because it can cause high recurrence of non-invasive tumors (70%) after transurethral resection (TUR) and progression into potentially life-threatening muscle invasive (15-20%) cancer after conservative surgical treatment (Jung and Messing, 2000), making bladder cancer one of the most prevalent cancers worldwide (Dyrskjot, 2003). Patients with superficial tumors are under continued surveillance by routine cystoscopy examination for early detection of new tumor developments. This is due to the lack of clinically useful markers to identify the patients with a very high or very low risk of disease recurrence or progression.

Consequently, identification of diagnostic and prognostic markers that identify high and low risk bladder cancer patients would be of tremendous benefit to both patients and the healthcare system. Therefore, to identify patients with high risk of progressing to this life-threatening stage, reliable genetic markers are needed in the management of these problems.

Like any other cancers, the development and progression of bladder cancer is driven by over expression of oncogenes or inactivation of tumor suppressor genes (TSGs). The cyclin D1 gene (*CCND1*) located at chromosome 11q13 is frequently detected in TCCs of the bladder cancer (Hunter *et al.*, 1994). Over expression of cyclin D1 plays an important role in the human cancer progression. Furthermore, over expression of *CCND1* frequently occurs in TCCs of the bladder and may be associated with growth of low-grade papillary tumors. Previous study has demonstrated that *CCND1* is a significant proto-oncogene in genesis and progression of TCC (Wang et al., 2004).

The main goal of the experiment is to determine the expression level of Cyclin D1 in different stage of bladder cancer. Preliminary data collected during this project is very important to further investigate whether this gene can be further develop as a genetic marker in differentiating superficial stage of bladder cancer from muscle invasive bladder cancer. Information obtained can be used for future screening, early diagnosis and surveillance of bladder cancer.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of bladder cancer

Cancer of the urinary bladder is a major epidemiological problem and it also one of the most serious cancers worldwide. According to the American Cancer Society 2005, an average of 260,000 new cases of urinary bladder cancer is diagnosed worldwide every year, with an estimated 63,210 new cases in 2005 in the United States alone, leading to 13,180 deaths.

Bladder cancer occurs about three times more often in men than women. It's also more likely to strike older adults. Environmental factors such as tobacco and industrial chemicals can increase the risk of bladder cancer as well. Chemicals such as industrial solvents, paints and paint thinners have been linked to a higher rate of bladder cancer and the risk is even higher for smokers exposed to these chemicals (Dyrskjot, 2003). However, number of bladder cancer diagnosed annually in the United States increased 33%, at roughly the same rate in both sexes from 1985 to 2000 (Greenlee *et al.*, 2000). The number of new cases has risen since 1960s when women worked outside the home and have changed habits, exposing them to both industrial and environmental carcinogens such as cigarette smoking from which they have previously been excluded. In Malaysia, based on the report released by National Cancer Registry (National Cancer Registry Report, 2006), 570 of bladder cancer cases were reported with the same trend where men were more affected than women. The Malay has the highest risk of developing bladder

cancer among the ethnic groups. Age specific incidence for bladder cancer followed an exponential fashion with a steps rise from the age of 40 years.

The bladder is an organ which is located at the lower part of the abdomen (Figure 2.1). Function of the bladder is to stores urine until it is passed out of the body. There are three types of bladder cancer that begin in cells in the lining of the bladder. These cancers are named for the type of cells that become malignant (cancerous):

- Transitional cell carcinoma (TCC): Cancer that begins in cells in the inner most tissue layer of the bladder. These cells are able to stretch when the bladder is full and shrink when it is emptied. Most bladder cancers begin in the transitional cells.
- Squamous cell carcinoma: Cancer that begins in squamous cells, which are thin, flat cells that may form in the bladder after long-term infection or irritation.
- Adenocarcinoma: Cancer that begins in glandular (secretory) cells that may form in the bladder after long-term irritation and inflammation.

The bladder itself is made up of four layers that determined how deeply the tumor has invaded and the ultimate stage of the cancer (Figure 2.2). Epithelium that lines the bladder is referred as transitional epithelium or urothelium. Most bladder cancers originate from the cells of this transitional epithelium. Lamina propria located under the epithelium comprises of connective tissue and blood vessels. Within the lamina propria is a thin and often discontinuous layer of smooth muscle called the muscularis mucosae. The third layer called muscularis propria or detrusor muscle. This deep muscle layer consists of thick smooth muscle bundles that form the wall of the bladder. For purposes of staging bladder cancer, the muscularis propria has been divided into a superficial (inner) half and a deep (outer) half. The last layer called perivesical soft tissue, this outermost layer consists of fat,

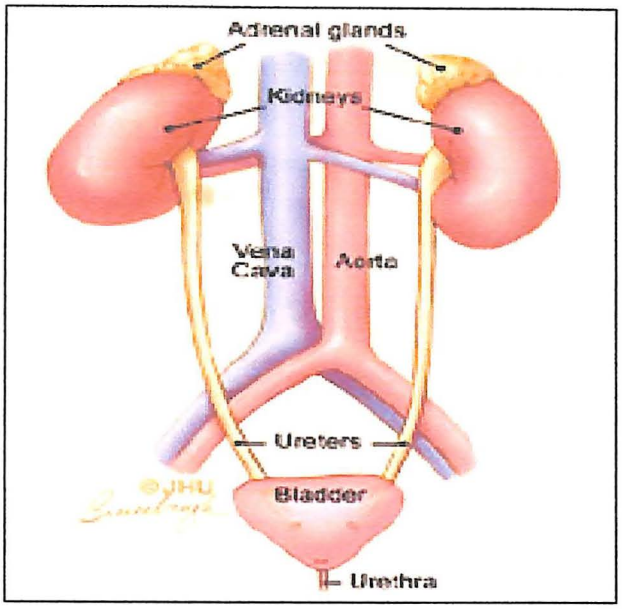

Figure 2.1: **Anatomy of urinary bladder** (Johns Hopkin University, 2009)

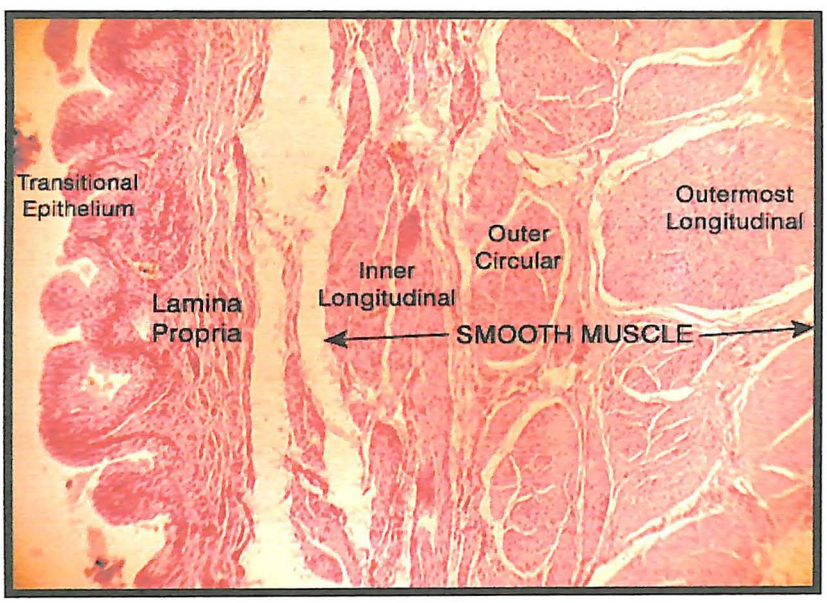

Figure 2.2 **Histology of human bladder**

(Source: http:///www.asb.aecom.yu.edu/histology/labs/images/slides/B52_Bladder_4X.jpg)

. fibrous tissue and blood vessels. When the tumor reaches this layer, it is considered out of the bladder (Prema, 2005).

In most cases, blood in the urine (hematuria) is the first warning signal of bladder cancer. Blood in the urine does not mean the existing of have bladder cancer. Much more often it is caused by other factors such as infection, benign tumors, stones in the kidney or bladder, or other benign kidney diseases. Blood may be present one day and absent the next with the urine remaining clear for weeks or months. With bladder cancer, blood eventually reappears. Usually, the early stages of bladder cancer cause bleeding but little or no pain. Having to urinate more often than usual can also be a symptom of bladder cancer. Irritative symptoms, such as dysuria (burning during urination) and urgency (feeling as need to go but not being able to) can also be symptoms of bladder cancer. However, these symptoms are also more likely to be caused by a benign condition such as infection, benign tumors, bladder stones, an overactive bladder or an enlarged prostate.

If bladder cancer is suspected, a cystoscopy will be performed by a trained urologist. During cystoscopy, bladder washings may be done for to look for cancer cells. Washings are taken by placing a salt solution into the bladder (through a tube) and then removing the solution for microscopic testing. The urine is examined under a microscope to determine the presence of cancer or pre-cancer cells. Cytology is also done on any bladder washings taken during cystoscopy procedure. Urine culture can be used to diagnose bladder cancer to role-out infections that may cause similar symptoms. Biopsy also can used to diagnose bladder cancer. Bladder biopsy samples are most often obtained during cystoscopy. This allows the doctor to be precise in terms what tissue is removed. A biopsy can show if cancer is present and what type of cancer it is (transitional cell carcinoma, squamous cell carcinoma, adenocarcinoma, etc.). It can also show how

deeply the cancer has penetrated into the bladder wall, which is very important in deciding treatment.

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The stage of a cancer tells the doctor how big the cancer is and whether it has spread. There are different ways of staging cancers. The most common is the TNM system and is used for all cancers. TNM stands for 'tumor, node, metastasis'. This staging system takes into account how deeply the tumor has grown into the bladder. If the cancer has spread it is called metastasis or metastatic bladder cancer. The graded also can show how well the cells developed under the microscope.

- Grade 1 cancers have cells that look very like normal cells : they are called 'low grade' or 'well differentiated' and tend to grow slowly and are not likely to spread
- Grade 2 cancers have cells that look more abnormal : they are called 'medium grade' or 'moderately differentiated' and may grow or spread more quickly than low grade

- Grade 3 cancers have cells that look very abnormal : they are called 'high grade' or 'poorly differentiated' and are more quickly growing and more likely to spread

If early bladder cancer, grade is one thing that doctor may take into account when deciding treatment. If the cells are high grade, treatment to stop the cancer coming back after specialist has removed it is needed (recurrent). Carcinoma in situ tumors of the bladder are high grade.

In 2004 the World Health Organisation (WHO) developed a new grading system for early bladder cancer, which is increasingly being used. This system divides bladder cancers into the following groups

- Urothelial papilloma – non cancerous (benign) tumour
- Papillary urothelial neoplasm of low malignant potential (PUNLMP) – slow growing and unlikely to spread
- Low grade papillary urothelial carcinoma – slow growing and unlikely to spread
- High grade papillary urothelial carcinoma – more quickly growing and more likely to spread

The 'T' part of TNM explained how far into the bladder the cancer cells have grown. Doctors find the T stage by a combination of looking at the grade of the cancer cells after a biopsy, examination of the bladder under anaesthetic, and a CT or MRI scan.

- CIS – very early, high grade, cancer cells are detected only in the innermost layer of the bladder lining
- Ta – the cancer is just in the innermost layer of the bladder lining

- T1 – the cancer has started to grow into the connective tissue beneath the bladder lining
- T2 – the cancer has grown through the connective tissue into the muscle
- T2a – the cancer has grown into the superficial muscle
- T2b – the cancer has grown into the deeper muscle
- T3 – the cancer has grown through the muscle into the fat layer
- T3a – the cancer in the fat layer can only be seen under a microscope (microscopic invasion)
- T3b – the cancer in the fat layer can be seen on tests, or felt by your doctor during an examination under anaesthetic (macroscopic invasion)
- T4 – the cancer has spread outside the bladder
- T4a – the cancer has spread to the prostate, womb or vagina
- T4b – the cancer has spread to the wall of the pelvis or abdomen

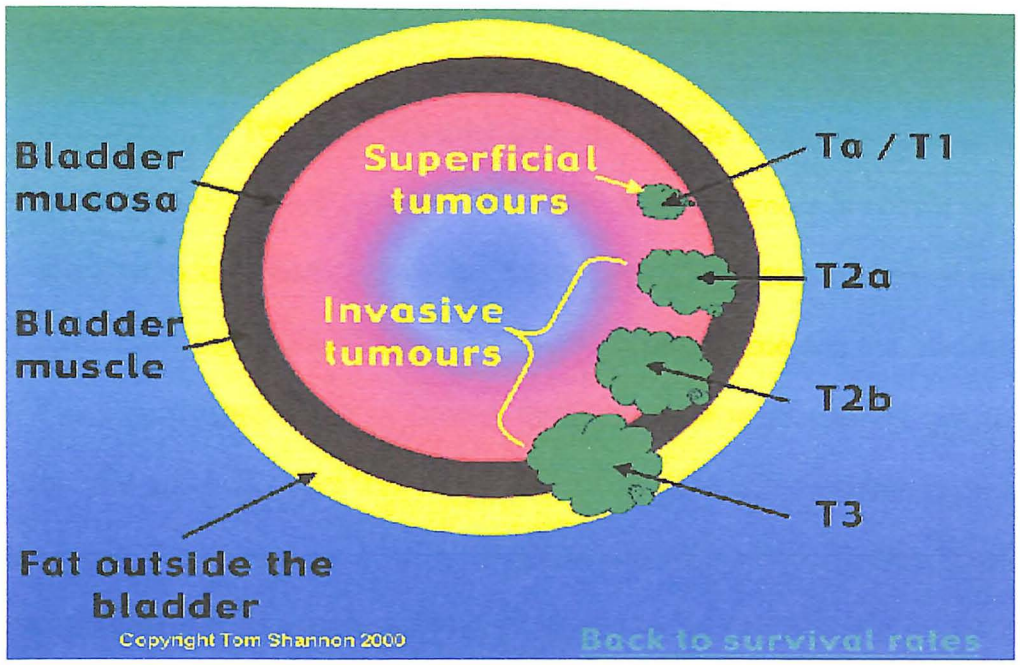

Figure 2.3: **Diagram showing the T stages of bladder cancer**

(Source : http://www.hollywoodurology.com/pics/stage.gif)

2.1.1 Treatment of bladder cancer

The treatment of TCC is different for both non-muscle invasive and muscle invasive bladder cancer. The distinction between these two types of tumors is critical for treatment and only can be made by pathologists via tissue biopsy. The standard initial treatment of non-muscle invasive bladder cancer is cystoscopy (without removing the bladder), usually by TUR. The cystoscope, which is passed through the urethral into the bladder, allows visualization and entire removal of a bladder tumor. Intravesicle therapies such as Bacille Calmette-Guerin (BCG) may be recommended depending on the disease course, number of resected tumors or stage and grade of tumors.

Muscle invasive tumors require cystectomy (partially or complete surgical removal of bladder). It is indicated when bladder cancer is invasive into the muscle wall of the bladder or when patients with superficial tumors have frequent recurrences that are not responsive to the intravesicle therapy. However, when the tumor has spread outside the bladder wall, cystectomy is not usually done. For such advanced stage, radiation and chemotherapy are treatment options.

2.2 Problems in the clinical management of bladder cancer

Non-muscle invasive tumors pose very high rate of recurrence (50-70%) and progression to higher grade or stage occurred in 42% of the patients (Herr *et al.*, 1997). Currently, grading and staging are the most reliable variables for monitoring this phenomenon. Even though most of the conventional methods used today, for instance

dipstick urine analysis is very specific, but the sensitivity is low especially to low grade tumors.

Treatment for superficial bladder tumors, especially high grade papillary non-invasive type is different with that given to low grade papillary non-muscle invasive tumors. The debate about choice of cystectomy, particularly for low grade tumors, has raged for many years. Though complete cure can be achieve, this also result in significant morbidity to the patients. Therefore, the identification of such patients is a priority. Response rates of radiotherapy or chemotherapy given after surgery are not high (Knowles, 2001). Thus, possible identification of the optimum treatment would avoid ineffective, expensive and unpleasant treatment. Cystoscopy that provides specimens for the most important pathological prognostic factors is the gold standard for detection of new recurrent tumors. For those patients with truly benign disease that will not recur, this practice creates unnecessary anxiety and is costly to the health service.

Identification of genetic markers that could identify the presence of distant metastases would greatly help in patient management. Identification of biomarkers may improve the screening and diagnosis of TCC. Thus, identification of biomarkers that are non-invasive, rapid, easy to obtain and interpret, inexpensive and most important specific and sensitive is urgently needed. The target groups of these markers could be high risk patients with history of smoking and patients with symptoms of bladder cancer such as hematuria, irritative voiding symptoms and patients after a bladder cancer diagnosis or treatment. Early identification of patients with poor prognosis would result in better long-term survival for these patients as more aggressive treatment regimens could be used. On the other hand, patient with good prognosis could have fewer routine cystoscopy examination and hence a better quality of life.