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EXPRESSION OF NOTCH SIGNALING GENES IN PUTATIVE BLADDER CANCER STEM CELLS

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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EXPRESSION OF NOTCH SIGNALING GENES IN PUTATIVE BLADDER CANCER STEM CELLS

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Chair: **Chau De Ming, PhD**
Faculty: **Medicine and Health Sciences**

Notch signalling is a canonical pathway that is involved in the regulation of stem cell self-renewal and proliferation. This pathway is also involved in the regulation of other cellular processes such as differentiation and apoptosis. Hence, aberrant activation of this pathway is often linked to tumourigenesis. However, the role it plays varies in different cancers. Emerging data has shown that *NOTCH1* receptor mutation has a tumour suppressive role in bladder cancer. However, a recent study has shown that not all Notch receptors play a tumour suppressive role. For example, *NOTCH2* receptor mutation has an oncogenic role in bladder cancer. Nevertheless, the role of Notch signalling in putative bladder cancer stem cells (CSCs) remains poorly understood. Hence, the main aim of this study was to characterise the expression of Notch signalling associated genes in putative bladder CSCs. To achieve this objective, the putative bladder CSCs were selectively grown as spheroids from four different bladder cancer cell lines (RT112 and SW780 - minimally invasive bladder cancer cells; EJ28 and 5637 - highly invasive bladder cancer cells) in three dimensional (3D)-culture conditions using ultra-low attachment plates. The culture media was optimised for each cell line to allow for spheroid formation. The selectively grown spheroid cells expressed higher level of most commonly studied genes for characterisation of CSCs' stemness property such as *SOX2*, *NANOG* and *OCT4* which is in support of the potential stem-like properties of these cells. Analysis of surface markers that are associated with stem cells (CD44, CD49f and CD133) via flow cytometry showed that the expression levels of these markers in the monolayer and spheroid cells were variable, suggesting heterogeneity in the expression signatures of these cells. The expression of Notch signalling associated genes such as *NOTCH1-4*, *HES1*, and *DUSP1* were also analysed in the spheroid cells using QRT-PCR. All the six Notch signalling genes analysed are genes which has either an oncogenic or tumour suppressive role in cancer cells besides having role in proliferation of CSCs from different cancer types. The expression level of *NOTCH2* and *HES1* gene was significantly higher in the spheroid cells suggesting that these could be the candidate genes which can be

further explored to study putative bladder CSCs. Finally the effect of a gamma-secretase inhibitor, GSI-34, on spheroid cell formation was investigated. GSI-34 is a small molecule inhibitor which had been shown to inhibit cleavage of the gamma-secretase enzyme thus preventing activation of the Notch signalling pathway. In this study, GSI-34 was able to inhibit and reduce the formation and growth of spheroid; albeit there was variation in response across the different spheroids. This study sets the stage for future studies to discover the exact role and mechanism on how Notch pathway regulates the proliferation of bladder cancer stem cells.

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

EKPERESI GEN-GEN ISYARAT LALUAN NOTCH DALAM KANSER STEM SEL PUNDI KENCING

Oleh

ARCANA THIRUMORTHY

April 2017

Pengerusi: **Chau De Ming, PhD**
Fakulti: **Perubatan dan Sains Kesihatan**

Isyarat Notch merupakan laluan kanonik yang terlibat dalam regulasi perbaharuan diri dan perkembangan stem sel. Laluan ini juga terlibat dalam regulasi proses sel yang lain seperti pembezaan dan apoptosis. Oleh itu, pengaktifan aberan laluan ini sering dikaitkan dengan genesis tumour. Walau bagaimanapun, peranannye berbeza dalam jenis kanser yang berbeza. Kajian terkini telah menunjukkan bahawa mutasi reseptor *NOTCH1* mempunyai peranan bersifat menekan tumour dalam kanser pundi kencing. Akan tetapi, satu kajian baru-baru ini telah menunjukkan bahawa bukan semua reseptor Notch memainkan peranan bersifat menekan tumour. Sebagai contoh, mutasi reseptor *NOTCH2* mempunyai peranan onkogenik dalam kanser pundi kencing. Namun begitu, peranan isyarat Notch dalam kanser stem sel (CSCs) pundi kencing masih kurang difahami. Oleh itu, tujuan utama kajian ini adalah untuk mencirikan ekperesi isyarat gen-gen yang dikaitkan dengan *NOTCH* dalam CSCs pundi kencing. Untuk mencapai objektif ini, CSCs pundi kencing telah ditumbuh secara terpilih bagai sferoids daripada empat jenis sel pundi kencing yang berbeza (RT112 dan SW780 – sel pundi kencing yang kurang invasif; EJ28 dan 5637 – sel pundi kencing yang agak invasif) dalam kultur tiga-dimensi (3D) menggunakan plat ultra-rendah lekatan. Kultur media telah dioptimumkan bagi kemua sels untuk membolehkan pembentukan sferoids. Sferoids yang ditumbuh secara terpilih mempunyai expresi yang lebih tinggi bagi gen-gen yang sering dikaji untuk pencirian kestemman CSCs seperti *SOX2*, *NANOG* dan *OCT4* dan ini menyokong potentsi sel-sel itu untuk mempunyai ciri kestemman. Analisis penanda permukaan sel-sel stem seperti CD44, CD49f dan CD133 menggunakan teknik aliran sitometri menunjukan bahawa tahap ekperesi penanda-penanda tersebut dalam sel-sel dalam sel-sel ekalapis dan sferoids adalah tidak tetap menunjukkan kepelbagaian dalam identiti sel-sel ini. Ekperesi gen-gen berkaitan dengan isyarat *NOTCH* turut dianalisis dalam sel-sel sferoid dengan menggunakan teknik QRT-PCR. Kesemua enam gen isyarat Notch yang dianalisis adalah gen yang mempunyai sama ada

peranan yang bersifat onkogenik atau penindas tumor dalam sel-sel kanser di samping mempunyai peranan dalam percambahan CSCs dari jenis kanser yang berbeza. Tahap ekperesi gen *NOTCH2* dan *HES1* adalah lebih tinggi di dalam sel-sel sferoid mengimplikasikan bahawa gen-gen ini boleh dikaji dengan lebih lanjut dalam kajian CSCs pundi kencing. Akhir sekali, impak perencat *gamma-secretase,* GSI-34 turut dikaji. GSI-34 adalah perencat molekul kecil yang telah dibukti menghalang belahan enzim gamma-secretase yang dengan itu menghalang pengaktifan laluan isyarat Notch. Di dalam kajian ini, GSI-34 ternyata mampu menrencat pembentukan dan pertumbuhan sel-sel sferoid walaupun tindak balas tersebut berubah mengikut sferoid yang berlainan. Kajian ini boleh digunakan dalam penyelidikan di masa hadapan untuk mencari peranan dan mekanisme bagaimana laluan Notch mengawal proliferasi CSCs pundi kencing.

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I certify that a Thesis Examination Committee has met on 21st April 2017 to conduct the final examination of Arcana Thirumorthy on her thesis entitled "Expression of Notch signalling genes in putative bladder cancer stem cells" in accordance with the Universities and University Colleges 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. Members of the Thesis Examination Committee were as follows:

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±	plus minus
°C	Celcius
µL	microliter
µm	micrometer
µM	micro molar
3D	three-dimensional
7aad	7-amino-actinomycin D
ABC	ATP-binding cassette
ABCB1	ATP Binding Cassette Subfamily B Member 1
ABCC	ATP Binding Cassette Subfamily C
ABCG2	ATP Binding Cassette Subfamily G Member 2
ALDH	aldehyde dehydrogenase
AML	acute myeloid leukemia
APC	allophycocyanin
BCG	bacille-calmette guerin
b-FGF	basic fibroblast growth factor
BTA	bladder tumour antigen
cDNA	complementary deoxyribonucleic acid
CIS	carcinoma *in situ*
cm	centimeter

cMyc	*Avian* myelocytomatosis virus oncogene cellular homolog
CO_2	carbon dioxide
COX2	cyclooxygenase-2
Cp	crossing point
CSCs	cancer stem cells
DEPC	diethyl pyrocarbonate
DMSO	dimethyl Sulfoxide
DNA	deoxyribonucleic acid
DUSP1	Dual specific phosphatase 1
EDTA	ethylene-diamine-tetra-acetic acid
EGF	epidermal growth factor
EMT	epithelial-to-mesenchymal transition
ErbB2	receptor tyrosine kinase 2
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FGFR3	fibroblast growth factor receptor 3
FITC	fluorescein isothiocyanate
FSC	forward scatter
g	gram
G.M	geometrical mean
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GOI	gene of interest

GSI	gamma-secretase inhibitor
Hes1	Hes Family BHLH Transcription Factor 1
Hey1	Hes Related Family BHLH Transcription Factor With YRPW Motif 1
HRAS	HRas proto-oncogene
IVP	intravenous pyelogram
Jak	Janus kinase
KLF4	Kruppel Like Factor 4
L	Liter
MAML-1	Mastermind-like 1
MDM2	E3 ubiquitin-protein ligase
MDR	multidrug resistance
mL	milliliter
mm	millimeter
MRI	magnetic resonance imaging
mRNA	messenger RNA
NA	not applicable
ng	nanogram
NICD	Notch intracellular domain
NMP22	nuclear matrix protein 22
NOD	non-obese diabetic
NTC	no template control
OCT4	Octamer-binding transcription factor 4

p21	Cyclin-Dependent Kinase Inhibitor 1A
p53	tumour protein 53
PAH	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PGE_2	prostaglandin E_2
PI	propidium iodide
PSMB2	Proteasome Subunit Beta 2
PTEN	Phosphatase And Tensin Homolog
PUNLMP	papillary neoplasm of low malignant potential
RNA	ribonucleic acid
RPM	rotations per minute
rRNA	ribosomal RNA
QRT-PCR	quantitative real-time PCR
SCID	severe combined immunodeficient
SEM	standard error of the mean
SFM	serum free media
SNAI1/2	Snail Family Transcriptional Repressor 1 or 2
SOX2	Sex Determining Region Y-Box 2
SP	side-population
SSC	side scatter

STAT	Signal Transducer And Activator Of Transcription
TAE	tris-acetate-EDTA
T-ALL	T-cell acute lymphoblastic leukemia
TNM	tumour-node-metastases
TSG	tumour suppresor genes
UPL	universal probe library
UV	ultra-violet
v/v	volume to volume
VEGF	Vascular Endothelial Growth Factor A
ZEB1/2	Zinc Finger E-Box Binding Homeobox 1 or 2

CHAPTER 1

INTRODUCTION

1.1 Background

Annually, around 430,000 patients are diagnosed with bladder cancer and 38% of these cases lead to mortality (Ye *et al.*, 2014). In Malaysia, bladder cancer is the 4th most common cancer in men (Malaysia Oncology Society, 2012). Approximately 90% of these patients are aged 60 years and above (Ye et al., 2014) making aging as one of the top risk factor of this cancer. About three-quarter of bladder cancer cases are comprised of non-muscle invasive papillary tumours while the other quarter is composed of muscle-invasive tumours. The non-muscle papillary tumours have good prognosis and longer survival rates as compared to the muscle-invasive bladder tumours (Van Den Bosch and Witjes, 2011).

Statistically, the 5-year relative survival rates of the more common cancers such as leukemia, breast and prostate cancers have significantly increased in the last 4 decades, however, the 5-year relative survival rate of bladder cancer remains static throughout the same period (National Cancer Institute, Surveillance, Epidemiology and End Results Program (2016). This could be due to the high recurrence risk (60-70%) of the non-muscle invasive bladder cancer in which the cancer often recur after the initial treatment (Aldousari and Kassouf, 2010). Hence, the non-muscle invasive papillary bladder cancer patients who have survived cancer therapy are needed to constantly undergo follow-up and that include frequent cystoscopy for a period of 5 years to avoid risk of cancer recurrence. The need for constant surveillance causes psychological and financial burden amongst patients. It has been postulated that one of the main factors of cancer recurrence is the repopulation of tumour mass by a small population of cells known as cancer stem cells that escaped the initial therapy.

Cancer stem cells (CSCs) are a subset of cells that live within the tumour population and has the ability to self-renew and repopulate an entire tumour mass (Clarke *et al.*, 2006). These cells share a number of common characteristics with normal tissue stem cells:- among which are the self-renewal ability and the ability to resist chemical insults. The serial transplantation technique is one of the most common methods used to characterise the self-renewal ability of the CSCs. To date, CSCs have been identified in various cancers such as breast (Al-Hajj et al., 2003), brain (Singh *et al.*, 2004), bladder (Chan *et al.*, 2009) and melanoma (Quintana *et al.*, 2008). The ability to resist chemical insults aids the CSCs in escaping commercially available chemotherapeutic drugs thus sustaining themselves to continuously self-renew and differentiate. It has been suggested that the increased expression of ATP-binding cassette (ABC) transporters in CSCs aids in the efflux of any chemotherapeutic or cytotoxic agents from the cells, thus contributing to drug-resistance attribute (Schatton et al., 2008). As CSCs have been showed to have increased drug resistance and are highly tumorigenic

cells, it is indeed crucial to understand the biology and signalling pathways regulating these cells as it could provide clues to find precise therapeutics that would target these cells more effectively.

Weissman's group demonstrated the existence of bladder cancer stem cells by using markers that were expressed on the urothelial basal layer ($CD44^{+}CK5^{+}CK20^{-}$) to isolate the tumour initiating cells. Markers expressed on the urothelial basal layer, the niche in which stem cells usually reside were used for isolation of tumour-initiating cells (TICs). Interestingly, as few as 100 TICs was all that was required to initiate tumours in xenografts (Chan et al., 2009). Following this study, putative bladder cancer stem cells (CSCs) have been isolated and studied using many other markers such as CD49f, CD133 and ALDH1A1 (Bentivegna, Conconi, Panzeri, Sala, Bovo, Viganò, Brunelli, Bossi, Tredici and Strada, 2010; Su *et al.*, 2010; Peek *et al.*, 2012a; David R Li *et al.*, 2015).

As CSCs share a number of common characteristics with the normal tissue stem cells, signalling pathways which are often associated with maintenance and proliferation of stem cells such as Wnt, Bmi, Klf4 and Hedgehog are studied to see if these pathways also have a role in maintaining the population of CSCs. The interest of this study is on Notch signalling which is also a pathway involved in the regulation of stem cell self-renewal and proliferation (Koch, Lehal and Radtke, 2013). This pathway has been implicated in many cancers. However, it does not have a similar role in all types of cancers. In both T-cell acute lymphoblastic leukemia (T-ALL) and breast cancer, Notch plays an oncogenic role (Weng *et al.*, 2004; Reedijk *et al.*, 2005) whereas in myeloid leukemia and brain cancer, Notch plays a tumour-suppressive role (Klinakis *et al.*, 2011; Giachino *et al.*, 2015). The role of Notch signalling in the proliferation of CSCs from different cancers such as pancreas (Abel *et al.*, 2014), glioblastoma (Fan *et al.*, 2010) and breast (Suman, Das and Damodaran, 2013) has also been well documented. In bladder cancer, *NOTCH1* receptor gene was shown to play a tumour suppressive role (Rampias *et al.*, 2014). In contrast to that findings, Hayashi et al. (2016) suggested that different Notch receptors have different roles in bladder cancer and showed that *NOTCH2* receptor gene plays an oncogenic role in bladder cancer.

1.2 Problem statement

Despite the current understanding of the role of Notch signalling in bladder cancer and the existence of putative bladder cancer stem cells, whether Notch signalling has a potential role in the development and maintenance of putative bladder CSCs is still relatively unknown.

1.3 General objective

The main objective of this study is to evaluate if Notch signalling has a potential role in the development and maintenance of putative bladder CSCs.

1.4 Specific objective

The specific objectives of this study include:

i. to optimise the culture media to selectively grow bladder cancer spheroids from four different bladder cancer cell lines with varying degree of invasiveness;
ii. to analyse the expression stem cell associated genes and cell surface markers for characterisation of stemness property in the bladder cancer spheroid cells;
iii. to determine the expression of Notch signalling associated genes in the bladder cancer spheroid cells.
iv. to assess the effect of a small molecule gamma-secretase inhibitor, GSI-34 on bladder cancer spheroid forming ability.

1.5 Hypothesis

Putative bladder CSCs have a higher expression of Notch signalling associated genes and the gamma-secretase inhibitor, GSI-34 inhibits the bladder cancer spheroid forming ability.

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