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IMMUNOHISTOCHEMICAL ANALYSIS OF HOMEOBOX PROTEIN EXPRESSION IN UROTHELIAL CARCINOMA OF BLADDER

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By

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Thesis submitted to the School of Graduate Studies Universiti Putra Malaysia, in fulfilment of the requirement for the Degree of Master of Science

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December 2016

Chair: Huzlinda Binti Hussin, Mpath
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Urothelial carcinoma of the bladder is a common malignant neoplasm that has a poor prognosis and high grade. In order to prevent the tumour from recurring or becoming invasive, constant disease surveillance with periodic and long term cystoscopy examination is necessary. However, the monitoring and therapy regimen is expensive and causes a massive burden to patients and the government. Therefore, development of specific biomarkers for urothelial carcinoma for detection of early stage tumours as well as prediction of tumour recurrence becomes top priority. Homeobox genes are a family of genes that are involved in tumourigenesis. They might be potential prognostic markers for urothelial carcinoma of the bladder. The objectives of our study were to describe the expression of homeobox genes (NANOG, ISL1 and LHX5) and identify their cellular localisation in urothelial carcinoma. The correlation expressions between these three proteins were also carried out. Lastly, we correlated the expression of these genes with demographic factors and clinicopathological parameters.

The expression of NANOG, ISL1 and LHX5 in 100 formalin-fixed paraffin-embedded urothelial carcinoma of the bladder tissues that were collected from Hospital Kuala Lumpur were determined by immunohistochemistry. Immunohistochemical staining results showed that the localization of NANOG, ISL1 and LHX5 antibodies were detected in the cytoplasm, nuclei and nuclear membrane of urothelial carcinoma of the urothelial cells. Positive expression of NANOG, ISL1 and LHX5 was detected in 100%, 94% and 98% of specimens respectively. The immunohistochemical expression of NANOG, ISL1 and LHX5 were not significantly associated with pathological stage (p= 0.127, 0.846 and 0.681 respectively) and grade (p=0.580, 0.588 and 0.099 respectively). There was also no significant correlation between NANOG expression with ISL1 and LHX5 expression. The immunohistochemical expression of NANOG, ISL1 and LHX5 were also not significantly associated with demographic factors such as gender (p=0.469, p=0.637 and p=0.910 respectively), race (p=0.718, p=0.858 and p=0.285 respectively) and age (p=0.067, p=0.803 and p=0.203 respectively) as well as with

clinicopathological parameters such as lymph node metastasis (p=0.208, 0.621 and 0.586 respectively) and distant metastasis (p=0.240, p=0.170 and p=0.303 respectively). Interestingly, NANOG expression showed significant correlation with tumour invasion whereby the p-value is p=0.01. However, there was no significant association between tumour invasion and the expression of ISL1 and LHX5 in urothelial carcinoma of the bladder.

In conclusion, NANOG and ISL1 are potential biomarkers for urothelial carcinoma of the bladder. NANOG is also a potential prognostic marker for urothelial carcinoma of the bladder invasion. Their role in urothelial carcinoma might be better understood with more functional studies that elucidate the phenotype-genotype correlations.

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

ANALISIS IMMUNOHISTOKIMA TERHADAP EXPRESI HOMEOBOX PROTEIN DI DALAM KANSER UROTELIA PUNDI KENCING

Oleh

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Kanser urotelial pundi kencing adalah satu neoplasma malignan yang kerap berlaku. Ia mempunyai kadar prognosis yang rendah dang red yang tinggi. Untuk mengelakkan tumor daripada berulang atau menjadi invasif, pengawasan melalui sistokopi secara berkala dan konsisten dalam jangka masa yang panjang perlu dijalankan. Walaubagaimanapun, pemantauan dan rawatan adalah mahal dan membebankan pesakit serta kerajaan. Di samping itu, prosedur sistokopi yang invasif juga tidak menyenangkan pesakit. Oleh itu, pembangunan penanda biologi khusus untuk mengesan tumor pada peringkat awal dan juga untuk meramal kadar berulangnya tumor menjadi keutamaan. Gen homeobox adalah salah satu keluarga gen yang terlibat dengan pembentukan tumor terutama kepada tumor di dalam badan manusia. Ia berpotensi sebagai penanda ramalan yang berpotensi untuk karsinoma urotelia pundi kencing. Objektif kajian dibuat adalah untuk mengenal pasti kedudukan selular serta melihat corak ekspresi protein pada gen homeobox (NANOG, ISL1 dan LHX5) di dalam tisu kanserurotelialpundi kencing. Kajian ini juga dijalankan untuk menyiasat hubung-kait antara ketiga-tiga corak ekspresi protein pada gen homeobox berkenaan. Seterusnya menghubung kaitkan ekspresi protein pada semua gen homeobox berkenaan dengan faktor-faktor demografik dan klinikal pesakit.

Kaedah pewarnaan immunohistokimia digunakan untuk mengkaji ekspresi NANOG, ISL1 dan LHX5 di dalam 100 sampel tisu kanser urotelia pundi kencing yang diambil dari Hospital Kuala Lumpur. Keputusan pewarnaan immunohistokima adalah seperti berikut: 1) kedudukan selular NANOG, ISL1 dan LHX5 dikesan di sistoplasma, nukleus dan membrane nuklear di dalam tisu karsinoma urotelia pundi kencing. 2) Pewarnaan positif dapat dilihat 100% dengan NANOG antibodi, 94% untuk ISL1 antibodi dan 98% untuk LHX5 antibodi. Ekspresi immunohistokimia daripada NANOG, ISL1 dan LHX5 tidak mempunyai hubungan yang signifikan dengan peringkat patologi dan gred kanser. Nilai p masing-masing adalah p= 0.127, 0.846 dan 0.681 untuk setiap gen bagi peringkat patologi dan untuk gred kanser pula nilai p masing-masing adalah p=0.580, 0.588

and 0.099 untuk setiap gen.Kajian menunjukkan tidak terdapat hubungan yang signifikan diantara ekspresi NANOG dengan ISL1 dan ekspresi LHX5 di dalam tisu karsinoma. 4) Ekspresi immunohistokimia bagi NANOG, ISL1 dan LHX5 juga tidak mempunyai hubungan yang signifikan dengan faktor-faktor demografik seperti jantina (masing-masing p=0.469, p=0.637 dan p=0.910) bangsa (masing-masing p = 0.718 , p = 0.858 dan p = 0.285) dan umur (masing-masing p=0.067, p = 0.803 dan p = 0.203) manakala untuk parameter klinikal, seperti metastasis noda limfa juga tidak menunjukkan hubungan yang signifikan di mana nilai p masing-masing ialah p=0.208, 0.621 and 0.586 dan untuk metastasis yang jauh nilai p masing-masing ialah p=0.240, p=0.170 and p=0.303. Menariknya, ekspresi NANOG menunjukkan hubungan yang signifikan terhadap kadar invasif kanser di mana nilai p ialah p=0.01. Walaubagaimanapun, untuk ekspresi protein ISL1 dan LHX5 tidak menunjukkan hubungan yang signifikan terhadap kadar invasif kanser urotelia pundi kencing.

Kesimpulannya, NANOG dan ISL1 adalah ramalan penanda biologi yang berpotensi untuk karsinoma urotelia pundi kencing. NANOG juga berperanan sebagai penanda prognostik kepada karsinoma urotelia pundi kencing. Peranan mereka dalam karsinoma urotelia pundi kencing mungkin lebih difahami dengan teknik yang lebih maju seperti kajian molekul.

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I certify that a Thesis Examination Committee has met on 1 December 2016 to conduct the final examination of Mohd Khairul Anuar bin Md Akhir on his thesis entitled "Immunohistochemical Analysis of Homeobox Protein Expression in Urothelial Carcinoma of Bladder" 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.

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LIST OF ABBREVIATIONS

ATBF1	AT-binding transcription factor1
BCG	Bacillus Calmette-Geurin
BRCA1	Breast Cancer 1
CIS	Carcinoma In Situ
DAB	3,3'-Diaminobenzidine
DLBCL	Diffuse Large B Cell Lymphoma
DPX	Di-N-Butyl Phthalate in Xylene
DSM	Disease-specific Mortality
EMT	Epithelial-Mesenchymal Transition
EGFR	Epidermal Growth Factor Receptor
ESCs	Embryonic Stem Cells
FFPE	Formalin-Fixed Paraffin-embedded
FGFR3	Fibroblast Growth Factor Receptor 3
G1	Grade 1
G2	Grade 2
G3	Grade 3
H&E	Haematoxylin and eosin
HAND-1	Heart and Neuranal Crest Derivatives Expressed-1
HOXB1	Homeobox-B1
HOXA9	Homeobox A9
HER2	Human Epidermal Growth Factor Receptor 2
IHC	Immunohistochemistry
ISL1	ISL LIM Homeobox-1
ISUP	International Society of Urological Pathologist
LHX5	LIM Homeobox-5
MEIS1	Meis Homeobox 1
MYF-5	Myogenic Factor-5
min	minute
MIBC	Muscle Invasive Bladder Cancer
NANOG	Homeobox protein NANOG
NMIBC	Non-Muscle Invasive Bladder Cancer
OCT3/4	Octamer-binding Transcription Factor 4
OTX1	Orthodenticle Homeobox-1
PAX6	Paired Box-6
PPV	Positive Predictive Value
PUNLMP	Papillary Urothelial Neoplasms of Low Malignant Potential
P53	Tumour Protein p53
SOX2	(Sex Determining Region Y)-Box 2
TBST-20	TBS plus tween 20 solutions
TCC	Transitional Cell Carcinoma
TKI	Tyrosine Kinase Inhibitor
TURBT	Transuretheral Resection of Bladder Tumour
TABSHO	Total Abdominal Hysterectomy Bilateral Salpingo Oophorectomy

VEGRFRs	Vascular Endothelial Growth Factor Receptors
WHO	World Health Organization
ZFHX3	Zinc Finger Homeobox-3
%	percent
°C	degree celcius
µl	microliter

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Cancer is the main cause of death in economically developed countries. In 2008, about 12.7 million cancer cases and 7.6 million cancer deaths were reported worldwide (Jemal *et al*., 2008). Based on the Malaysia Cancer Statistic 2006, a total of 21733 of people in peninsular Malaysia were diagnosed with cancer and registered with the National Cancer Registry. The total number of patients comprised of 9974 males and 11799 females. Among the cancer cases reported, bladder cancer was the tenth leading cause of death worldwide. However, the incidence of this cancer varies worldwide and it was highly reported in North America and Europe. In Peninsular Malaysia bladder cancer is the sixth most common cancer in males but is less common in females (Omar *et al*., 2006). Bladder cancer is the second most frequent cancer that occurs in the urogenital system after prostate cancer. Early diagnosis and persistent surveillance can reduce the risk of death among patients because of its high recurrent rate and risk of metastasis if there is a delay in the diagnosis and treatment.

There are various histological types of bladder cancer in humans. Examples of histopathological subtypes are transitional cell carcinoma (TCC), squamous cell carcinoma and adenocarcinoma. The most common type of bladder cancer diagnosed is TCC which accounts for more than 90% of all bladder cancer cases (Braud *et al*., 2002). Transitional cell carcinoma is currently known as urothelial carcinoma. Urothelial carcinoma of the bladder starts in the cells that line the bladder spreads to bladder wall and will later involve to the neighbouring organs or other body parts if it is not treated at an early stage. Smoking, chemical exposure, age, sex and chronic bladder inflammation are risk factors that contribute to the development of bladder cancer. However, the pathogenesis of bladder cancer is still largely unknown. Urothelial carcinoma is treated based on the tumour stage and grade. Besides the treatment protocols, the incidence of therapeutic resistance and failure is often reported.

The exact genetic events that lead to urothelial transformation involve the activation or gain of oncogenes, inactivation or loss of tumour suppressor genes and alterations in apoptotic gene modulators (Sandberg *et al*., 1994). A study showed that there are a lot of genetic abnormalities but the specific genes involved are still unidentified. This is because there are a lot of genetic mutations that have been detected in urothelial carcinoma (Cantile *et al*., 2003). One of the gene families which was found to be involved in the development of urothelial carcinoma is homeobox gene. Homeobox gene is a family of regulatory genes containing a common 183-nucleotide sequence (homeobox) and coding for specific nuclear proteins (homeoproteins) which act as transcription factors (Cillo *et al*., 1999). Homeobox genes are involved in the development of adult organ tissue and are also important in the development of the central nervous system, axial skeleton, limb and

urogenital tract (Goodman *et al*., 2001). The oncogenic potential of homeobox genes has clearly been implicated in leukaemia (Calvo *et al*., 2000). Homeobox genes are involved in cell proliferation, differentiation, apoptosis and metastasis by regulating other genes. For example, HOXA9 was found to suppress BRCA1which is involved in the development of breast cancer (Gilbert *et al*., 2010). As a result, the deregulation of homeobox genes was shown to be associated with cancer development and malignant progression such as invasion and metastasis (Takahashi *et al*., 2003).

NANOG is one of the homeobox genes that act as a transcription factor and is involved in maintaining pluripotency and self-renewal of embryonic stem cells. Recent studies have reported that NANOG is also involved in self-renewal and the tumourigenicity of cancer stem cells in a variety of human cancers (Boyer *et al*., 2005; Wang *et al*., 2006; Hu *et al*., 2010 and Wen *et al*., 2010). The upregulation of NANOG was found to be associated with tumour metastasis and poor prognosis in various human malignancies including prostate cancer, lung adenocarcinoma, gliomas, rectal cancer, gastric cancer and oral squamous cell carcinoma (Luo *et al*., 2013). In bladder cancer cases, it was proven to show that increased expression of NANOG was associated with the increase in pathological grade (Zhang Y *et al., 2012)*. However, the relationship of NANOG expression with other clinicopathological parameters and the pathways involved in the increase of its expression is still unknown.

Thus far, 353 genes have undergone further analyses in the pursuit to identify the transcriptionally active and inactive genes that are co-regulated with NANOG expression. Transcriptionally inactive genes co-regulated by NANOG are the genes that specify transcription factors which are important for differentiation into extra-embryonic, endodermal, mesodermal and ectodermal lineages. NANOG also found to be potential therapeutic target for cancers. Among the 353 genes, LHX5 and ISL1 are homeobox genes that are involved in lineage differentiation. LHX5 was believed to be involved in ectoderm differentiation, while ISL1 takes part in both ectoderm and endoderm differentiation.

Studies done on ISL1 and LHX5 also showed that they played important role in a few types of cancer. For example, ISL1 was found to play an important role in development of pancreatic endocrine tumours, diffuse large B-cell lymphoma (DLBCL) and gastric cancer. LHX5 was found to have a prognostic role in breast cancer. Thus understanding on how these genes are co-expressed in bladder cancer will aid in delineating the role of these genes in bladder cancer development. Besides, the study will help in the current management and treatment as bladder cancer has high tendency rate to recur from low grade to high grade of tumour.

Therefore, a study to delineate the expression of homeobox genes (NANOG, ISL1 and LHX5) in urothelial carcinoma cells across its different stages and grades is necessary in order to identify the relationship between the expression of ISL1 and LHX5 with NANOG. The relationship of these genes expression will also be correlated with clinicopathological parameters to assess the utility of the protein

expression of these genes as potential biomarkers for prognostication. The identification of cancer cell populations that express or suppress these expressions of these genes will help in the discovery of new and effective therapies for bladder cancer in the future.

1.2 Problem statement

The diagnosis of urothelial carcinoma of the bladder is generally achieved through cystoscopy and biopsy. Urothelial carcinoma has a very high frequency of recurrence and therefore requires follow-up cystoscopy as well as urine cytology for periodic surveillance to identify early recurrence. Failure to treat this cancer at an early stage will lead to the development of advance stage disease which may further complicate the management of the patient. Radical cystectomy is the current treatment for urothelial carcinoma. As this cancer needs long-term follow-up and surveillance procedures to monitor for tumour recurrence, many of the patients tend to default their follow up or suffer the morbidities associated with the procedure. This study was conducted to delineate the association of homeobox protein (NANOG, IS1 and LHX5) expression with the progression of urothelial carcinoma in the hope that new biomarkers that can predict bladder cancer progression can be developed. These biomarkers may help urologists make clinical decisions that will reduce the need for periodic cystoscopy surveillance and improve patient outcomes by identifying potentially aggressive disease early.

1.3 Objective

1.3.1 General objective

To characterise the protein expression of NANOG, ISL1 and LHX5 in urothelial carcinoma of the bladder.

1.3.2 Specific objectives

1. To determine the protein expression and localization of NANOG, ISL1 and LHX5 in urothelial carcinoma of the bladder.
2. To determine the pattern of protein expression of NANOG, ISL1 and LHX5 across different stages and grades of urothelial carcinoma of the bladder.
3. To determine the association between the protein expression of NANOG, ISL1 and LHX5.
4. To determine the association between the protein expression of NANOG, ISL1 and LHX5 and patient demographic factors as well as clinicopathological parameter

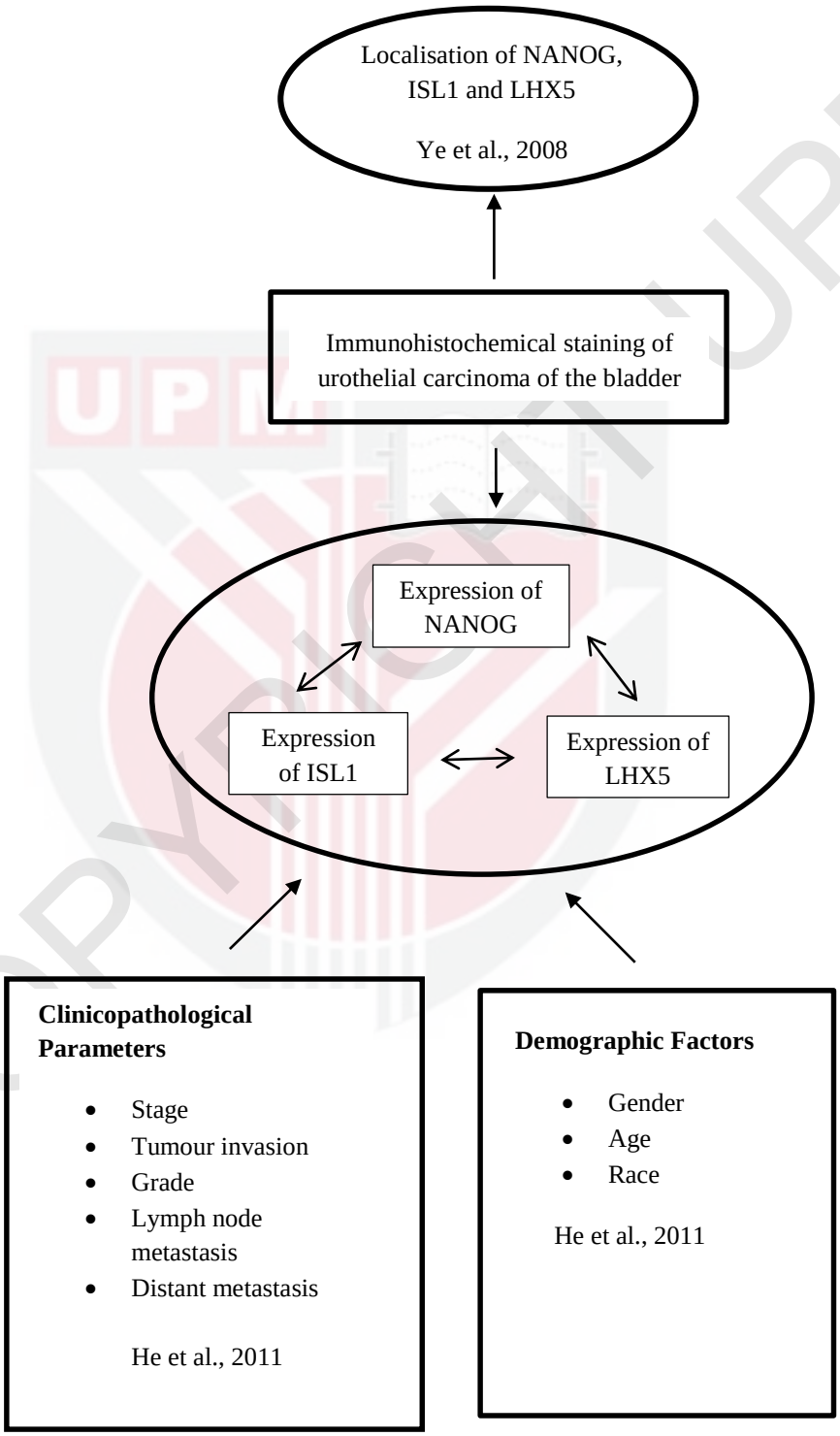
1.4 Research Hypothesis

1.4.1 There is a significant difference in the expression pattern of NANOG, ISL1 and LHX5 in different stages and grades of urothelial carcinoma of the bladder.

1.4.2 There is a significant association between the expression of ISL1, LHX5 and NANOG.

1.4.3 There is a significant association between the expression of NANOG, ISL1 and LHX5 and patient demographic factors as well as clinicopathological parameters.

1.5 Conceptual Framework



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