UNIVERSITI PUTRA MALAYSIA

IMMUNOHISTOCHEMICAL EXPRESSION OF HEDGEHOG PROTEINS IN DIFFUSE LARGE B-CELL LYMPHOMA

SITI NUR LINA BINTI AZMAN

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By

SITI NUR LINA BINTI AZMAN

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

August 2016

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Specially dedicated to

My parents, brothers, sisters and

Whom I love

For their invaluable love, endless support, understanding, encouragement and patients

"Life is a learning experience, only if you learn"

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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August 2016

Chairman : Maizaton Atmadini Abdullah, PhD
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The Hedgehog (Hh) signalling pathway is a developmental signalling pathway involved in numerous developmental processes, including determination of cell fate, proliferation, patterning, survival and differentiation. In most adult tissues, this pathway remains silent and its aberrant activation has been documented in various types of malignancies including basal cell carcinoma, gastrointestinal, lung and prostate cancers. The aim of this study was to demonstrate the expression of Hh signalling pathway components in 107 DLBCL formalin fixed paraffin embedded (FFPE) tissues from Hospital Kuala Lumpur and determine their association with clinicopathological parameters. We assessed the expression of SHH (ligand), GLI1 (transcriptional effectors of SHH signalling), its receptor Patched (PTCH1) and signal transducer Smoothened (SMO) using immunohistochemistry. SHH was expressed in 42 (42.1%) cases, GLI1 in 61 (57%) cases, PTCH1 in 107 (100%) cases and SMO in 105 (98.1%) cases. Tumours display strong staining intensity for SMO and PTCH1 but low intensity for SHH and GLI1 when compared to normal tonsil tissues. Immunohistochemical staining results showed that SHH, GLI1 and SMO proteins were localised in the cytoplasm of tumour cells, whereas PTCH1 was mainly localised in their nucleus of tumour cells. Survival curves calculated using the Kaplan-Meier method also showed no significant association with sociodemographic distributions (age, gender, race, tumour involvement) and expression between HH signalling pathway proteins. To the best of our knowledge, there is no study investigating the correlation between SHH, PTCH1 and SMO. This study has shown that SHH expression was significantly associated with the expression of PTCH1 (P=0.030) and PTCH1 was significantly associated with SMO (P=0.030) expression. Our findings suggest that high expression of PTCH1 and SMO might be important in the pathogenesis of DLBCL. However, further studies are required to ascertain their roles in DLBCL.

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

EXPRESI PROTIN HEDGEHOG DALAM LIMFOMA SEL B BESAR DIFUS

Oleh

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Laluan Hedgehog (Hh) merupakan laluan signal yang terlibat dalam pelbagai proses pertumbuhan termasuk penentuan nasib sel, pembiakan sel, corak sel, perbezaan dan jangka hayat sel. Dalam kebanyakan tisu-tisu matang, laluan ini tidak aktif, namun pengaktifan laluan ini telah berlaku didalam beberapa jenis tumor termasuk sel basal karsinoma, tumor gastrousus, kanser paru-paru dan kanser prostat. Oleh itu, kajian ini bertujuan untuk mengenal pasti ekspresi protin Hh dalam 107 sampel terendam formalin dan terbenam-parafin daripada DLBCL di Hospital Kuala Lumpur dan hubung kaitnya dengan parameter demografi. Dalam kajian ini, kami menilai ekspresi protein SHH (ligand), GLI1 (faktor transkripsi), reseptor Patched (PTCH1) dan isyarat transduser, Smoothened (SMO) dengan menggunakan teknik immunohistokimia. Ekspresi protin SHH telah menunjukkan ekspresi yang positif dalam 42 (42.1%) kes, GLI1 sebanyak 61 (57%) kes, PTCH1 sebanyak 107 (100%) kes dan SMO sebanyak 105 (98.1%) kes. Tumor DLBCL memaparkan intensiti yang paling kuat bagi protin SMO dan PTCH1 tetapi berintensiti rendah bagi protin SHH dan GLI1 jika dibandingkan dengan tisu tonsil yang normal. Teknik pewarnaan immunohistokimia menunjukkan protin SHH, GLI1 dan SMO kebanyakannya terletak di sitoplasma sel kanser, manakala protin PTCH1 terletak di dalam nukleus sel tumor tersebut. Analisa jangka hayat menggunakan Kaplan Meir menunjukkan tiada sebarang signifikan dengan parameter demografi (umur, jantina, bangsa pesakit, lokasi tumor) dan expresi laluan signal diantara HH protins dalam DLBCL. Sepanjang pengetahuan kami, tiada kajian yang menyiasat hubungkait antara protein SHH, PTCH1 dan SMO. Dalam kajian ini, ekspresi SHH menunjukkan hubungan yang signifikan dengan ekspresi protin PTCH1 (P=0.030), manakala protin PTCH1 telah menunjukkan ekspresi yang signifikan dengan ekspresi protin SMO (P=0.030). Kesimpulan daripada kajian ini kebarangkalian menunjukkan bahawa ekspresi protin PTCH dan SMO yang tinggi adalah penting dalam patogenesis DLBCL. Walaubagaimanapun, kajian selanjutnya harus dilakukan untuk mengenal pasti peranan mereka dalam kanser sel DLBCL.

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This thesis was submitted to the Senate of University Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

Ab	Antibody
ABC	Activated B-cell
Ag	Antigen
AILD	Angioimmunoblastic
ALCL	Anaplastic large cell lymphoma
AP	Alkaline phosphatase
BCC	Basal cell carcinoma
BcL2	B-cell CLL/Lymphoma 2
BCNS	Basal cell nevus syncdrome
BL	Burkitt lymphoma
DLBCL	Diffuse large B cell lymphoma
DAB	Diaminobenzidine
Dhh	Desert Hedgehog
DPX	Di-N-Butyle Phthalate in Xylene
EBV	Epstein-Barr virus
FDC	Follicular dendritic cells
FFPE	Formalin fixed paraffin embedded
FL	Follicular lymphoma
Fu	Serine/threonine protein kinase Fused
GLI	Glioma-associated oncogene homolog
GCB	Germinal center B-cell
GEP	Gene expression profiling
GPCR	G-protein compound receptor
H_2O_2	Hydrogen peroxide
H&E	Haematoxylin and eosin
HH	Hedgehog
HRP	Horseradish peroxidase
HL	Hodgkin lymphoma
IARC	International Agency for Research on Cancer
IHC	Immunohistochemistry
Ihh	Indian Hedgehog
LSAB	Labelled streptavidin-biotin
LL	Lymphoblastic lymphoma
MOH	Ministry of Health
MZ	Marginal zone
NCS	National Cancer Society Malaysia
NGCB	Non-germinal centre B-like
NHL	Non-Hodgkin lymphoma
NS	Nodular sclerosis subtype
NK	Natural killer lymphocyte
NLPHL	Nodular lymphocyte predominant Hodgkin lymphoma
NMRR	National Medical Research Registration
NOS	Not otherwise specified
OS	Overall survival
PAP	Peroxidase-antiperoxidase
PBS	Phosphate Buffered Saline
PL	Plasmablastic lymphoma
PTCH	Patched
RS	Reed-Sternberg

SD	Standard deviation
SHH	Sonic hedgehog
SLL	Small lymphoblastic lymphoma
SL	Subcutaneous lymphoma
SMO	Smoothened
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Cancer is a very complex disease involving numerous changes in the cell physiology that eventually leads to malignant tumours. It involves a process in which an unregulated division of abnormal cells spreads into nearby tissues or even in the blood. It is often shaped differently from healthy cells; they do not function properly, and they metastasize to other parts of the body. Tumours are clusters of cells that are capable of growing and dividing uncontrollably, and their growth is unregulated. Cancer is not just a disease but rather a group of diseases all of which cause cells in the body to change and grow out of control, invading normal tissues and organs and eventually spreading throughout the body. Cancers are classified according to their tissue of origin like breast cancer, lung cancer, prostate cancer, liver cancer renal cell carcinoma (kidney cancer), oral cancer and brain cancer. It can also be classified into tissue types, for example carcinoma sarcoma, lymphoma or cancers of mixed types which includes mixed mesodermal tumor, carcinosarcoma, adenosquamous carcinoma and teratocarcinoma.

There is no one single cause for cancer. According to Foster and Sarelius, 2015, the medical reviewer of University of Rochester Medical Center, believe that there are many factors that may conrtribute to cancer. The factors involved may be genetic, environmental, or biological characteristics of the individual for example age, gender and ethnicity. Diagnosis, treatment and prognosis for childhood cancers are different from adult cancers because childhood cancer is more responsive to therapy, and a child can tolerate more aggressive therapy (Foster & Sarelius, 2015). The overall five-years survival rate for common childhood cancers are acute lymphocytic leukemia (89%), acute myelogenous leukemia (65%), brain and other CNS (72%), wilms tumours (92%), hodgkin lymphoma (98%) and non-hodgkin lymphoma (89%). The overall five-years survival rate for common adult cancers are prostate (99%), melanoma of the skin (92%), breast (89%), urinary bladder (77%) colorectum (65) and lung and bronchus (17%) (American Cancer Society, 2015).

Cancer is one of the leading causes of death worldwide and the pattern continues to increase with time. These causes might have been due to avoidable risk factors that include tobacco exposure, smoking, poor diet, inadequate exercise and being overweight. Worldwide, one in eight deaths is due to cancer (American Cancer Society, 2008). When countries are grouped according to economic development, cancer is the leading cause of death in developing countries and the second-leading cause of deaths in emerging countries. According to the International Agency for Research on Cancer (IARC) Globocan of the World Health Organisation (WHO), there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012 worldwide (Jacques Ferlay, Isabelle Soerjomataram, Rajesh Dikshit, 2012).

In Malaysia, the incidence of cancer has become the fourth leading cause of death and is expected to increase by year with an estimated of 30,000 cases annually. Cancer is one of the top ten causes of hospitalisation and the fifth cause of death in both Ministry of Health and private hospitals (Ministry of Health Malaysia, 2013). A newspaper

online article 'Rise in cancer deaths in Malaysia' 2014, stated that the incidence of cancer in Malaysia has increased from 32, 000 new cases in 2008 to about 37,400 cases in 2012. This number, however, is expected to rise in the years to come if no actions such as living a healthy lifestyle, avoiding exposure to known cancer-causing substances and taking medicines or vaccines are taken (The Star Online, 2014). According to the National Cancer Registry of Malaysia, the most common cancer among Malaysian population is breast cancer, with one in 19 Malaysian developing breast cancer, one in 33 developing colorectal cancer, and one in 40 developing lung cancer followed by nasopharyngeal cancer, cervical cancer, lymphomas, leukaemia, ovarian cancer, stomach cancer and also liver cancer. Within different ethnic groups, cancer seems to be predominant among Chinese compared to Malays and Indians (Zainal & Nor Saleha, 2011). The National Cancer Society Malaysia (NCSM) estimated that in 2015, 90,000 to 100,000 Malaysians are living with cancer. Less than 10% of cancers occurred among children while over 50% in men and 35% in women aged 50 and above. However, it is estimated that a ratio of one in four Malaysian (1:4) will develop cancer at the age of 75 years old. It is also expected that by 2030, the global burden of cancer will increase to 21.4 million new cancer cases and 13.2 million deaths from cancer annually.

In 2010, the World Health Organization (WHO) recorded that lymphoma was the 21st top killer disease with 0.97% of the total death (World Health Organization, 2010). Almost one thousand patients are diagnosed every day with one million people worldwide living with lymphoma. According to the National Cancer Registry, 2007, lymphoma is the 6th commonest cancer among Malaysians. It is the sixth most common cancer in males and the eighth most common cancer in females (Zainal & Nor Saleha, 2011). There are two main subgroups of lymphoma, which are non-Hodgkin lymphoma (NHL) and Hodgkin's lymphoma (HL). HL is divided into four subtypes, and NHL is divided into more than 61 subtypes. The data collected at the GLOBOCAN, 2012, estimated that the incidence of HL patients is about 0.5% per 1,000,000 populations while for NHL is about 2.9% per 1,000,000 populations. In 2015, approximately about 9,050 new cases of HL and 4% case of NHL were expected to occur. There are approximately 1,150 deaths and 19,790 deaths from HL and NHL respectively (American Cancer Society, 2015). The most common subtype of NHL is diffuse large B-cell lymphoma (DLBCL) and are known to be the aggressive subtype. It develops when the body makes abnormal B-lymphocytes which is the lymphoma cells. B-cell lymphocytes are white blood cells that fight infection. Sometimes lymphoma cells begin in other parts of the body known as extranodal disease. The term diffuse refers to the fact that the cancer cells are spread around and not concentrated in one particular part of the node or in clusters within a part of the node. In other words the cancer cells don't clump together very well.

Although tumours differ based on their tissues of origin, all cancers share the fundamental features of propagated mutation to daughter cells. These propagated mutations generally lead to deregulated signalling pathways that gives a variety of clinical consequences and indications for new therapeutic involvement (Rajeev et al., 2011). Cancer cells aberrantly reactivate oncogenic developmental pathways such as Notch, Wnt and Hedgehog to provide growth and survival advantage to tumour cells. The Hedgehog pathway (Hh) protein family is a group of secreted signalling molecules for mammalian development and homeostasis of renewal adult tissues (McMahon, Ingham, & Tabin, 2003). It is normally activated during embryonic development

(Ingham, 2001) and promotes vascularization (Nagase, Nagase, & Machida, 2008). The basic components of Hh ligands refer to the three family members which include Sonic (Shh), Indian (Ihh) and Desert (Dhh) Hedgehog.

In adults, HH ligands are thought to be important for the renewal of hematopoietic stem cells (Varjosalo & Taipale, 2008). Ligands of the pathway are expressed in the hematopoietic niche and have been shown to be important for survival signals of leukemia, lymphoma and myeloma cancer stem cells by bone marrow imaging and transgenic Eμ-Myc mice model, whereas inhibiting the pathway suppresses disease progression (Dierks et al., 2007, 2008; Zhao et al., 2009). The ligands Ihh and Shh promote survival of B-cell malignancies such as lymphoma (Lowrey et al., 2002; Stewart et al., 2002). Unlike solid tumours which produce HH ligand, Ihh and Shh are produced by surrounding non-malignant cells in the lymph nodes, bone marrow and spleen to permit the growth of lymphoma cells.

1.2 Problem Statement

Despite treatment with conventional anthracyclin-based chemotherapy, approximately half of all DLBCL patients eventually die of this disease. Inappropriate activation of the HH signalling pathway has been found to occur in haematological neoplasms. This study is conducted to study the expression of HH signalling proteins expression (SHH, GLI1, PTCH1 and SMO) in DLBCL. This is important to unlock some possible diagnostic and treatment strategies in DLBCL.

1.3 Significance of Study

The study will provide an overview on the expression of HH pathway proteins in DLBCL. Hence further in depth research can be performed to evaluate their functions in tumour progression and the possibilities of targeted therapeutic interventions.

1.4 Research Objective

1.4.1 General Objective

To evaluate the expression of Hh signalling pathway proteins in DLBCL

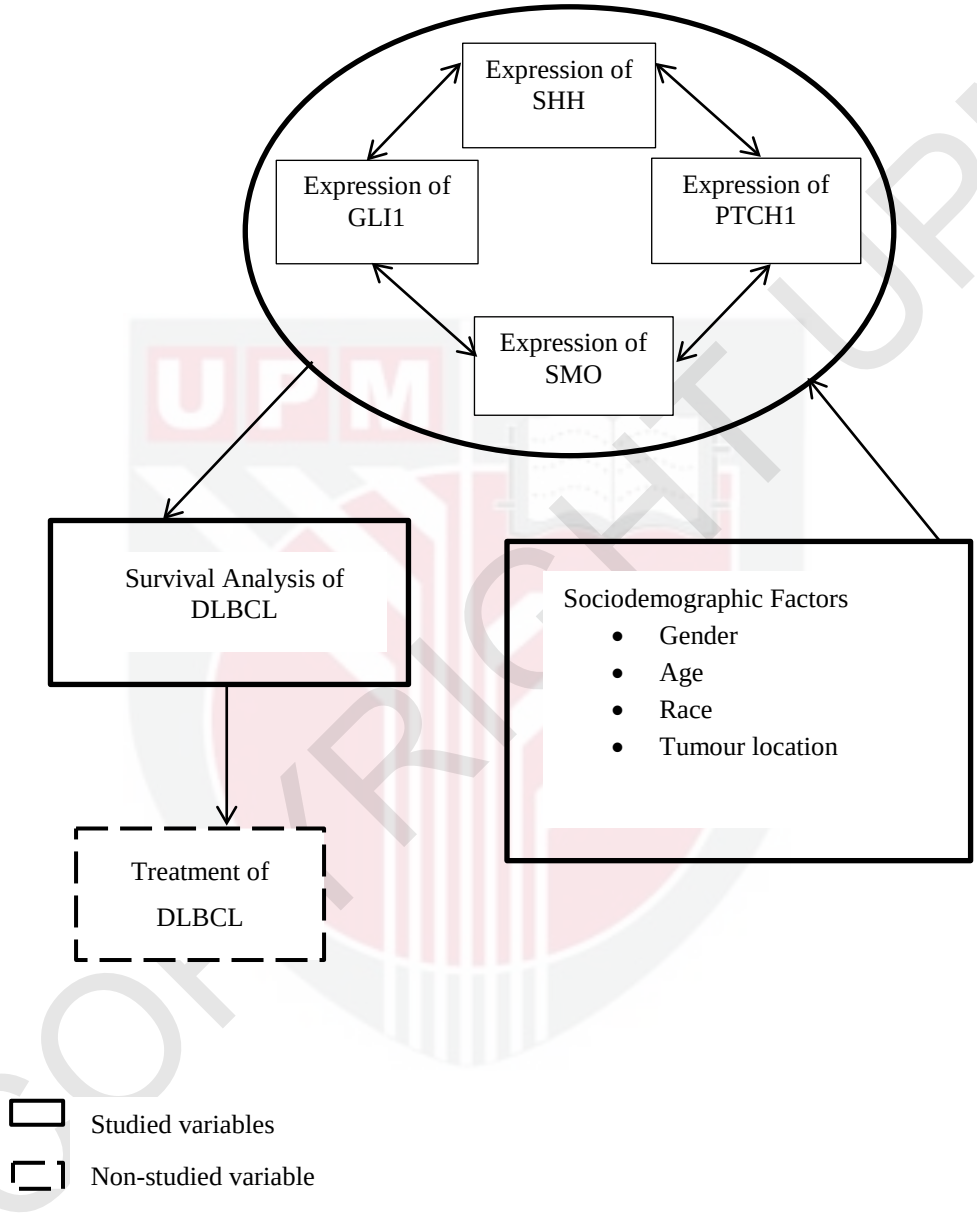
1.4.2 Specific Objectives

1. To determine the sociodemographic distribution of patients with DLBCL.
2. To analyze the immunohistochemical expression of four Hedgehog pathway proteins (SHH, GLI, PTCH1 and SMO) in DLBCL.
3. To determine the correlation of immunohistochemical expression between Hedgehog pathway proteins and socidemographic factors in DLBCL.
4. To analyze the association between Hedgehog pathway proteins and sociodemographic factors with overall survival in DLBCL.

1.5 Hypothesis

1. There is high expression of Hedgehog pathway proteins in DLBCL.
2. There is significant association between the expression of four different HH pathway proteins (SHH, GLI1, PTCH1 and SMO) in DLBCL.
3. There is significant association between the expression of Hegehog pathway proteins with sociodemographic factors and overall survival in DLBCL.

1.6 Conceptual Framework



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