PREVALENCE AND CLINICOPATHOLOGIC ASSOCIATION OF LMP1 AND P16 EXPRESSION IN NASOPHARYNGEAL CARCINOMA TISSUE IN HOSPITAL UNIVERSITI SAINS MALAYSIA

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ABSTRAK

Pengenalan : Kajian ini dilaksanakan untuk menentukan ekspresi protein LMP1 dan P16, serta menentukan data patologi klinikal kes kanser nasofarinks di Hospital Universiti Sains Malaysia (HUSM). Kajian ini akan menganalisa dan menentukan hubungkait antara ekspresi protein LMP1 dan P16 dan data patologi klinikal kes kanser nasofarinks.

Kaedah : Ini adalah kajian keratan rentas yang dijalankan di Jabatan Patologi, Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian, Kelantan. Kajian ini melibatkan 70 kes kanser nasofarinks yang didiagnosa di antara tahun 2007 hingga 2017. Daripada jumlah 70 kes hanya 36 kes sahaja sedia untuk digunakan dalam penyelidikan menggunakan protein LMP1 dan P16. Ekspresi LMP1 dilaporkan positif sekiranya lebih dari 10% sel kanser menunjukkan perwarnaan terang pada membran dan sitoplasma. Ekspresi P16 pula dilaporkan positif sekiranya terdapat warna terang pada nukleus dan membran pada lebih dari 30% sel kanser. Prevalens ekpresi protein LMP1 dan P16 dan data patologi klinikal kes kanser nasofarinks di HUSM ditentukan dan hubungkait antara ekspresi protein LMP1 dan P16 dan data patologi klinikal kes kanser nasofarinks dianalisa menggunakan perisian SPSS versi 24.

Keputusan : Daripada jumlah keseluruhan kes kanser nasofarinks yang terlibat dalam kajian ini, majoriti adalah terdiri daripada lelaki 59 (84.3%) dan selebihnya 11 kes (15.7%) adalah wanita. Sebanyak 57 kes (81.4%), adalah dari kumpulan pesakit berumur lebih dari 40 tahun dan selebihnya 13 kes berumur di bawah 40 tahun. Kebanyakan kes kanser nasofarinks adalah pada tahap lV iaitu sebanyak 48 kes (68.6%). Selebihnya adalah pada tahap l, ll, lll dengan peratusan masing masing sebanyak 10.0 %, 14.3 % dan 7.1 %. Sebanyak 67 kes

(95.7%) adalah kes kanser nasofarinks *non keratinizing* dan selebihnya adalah kes kanser nasofarinks *keratinizing* iaitu 3 kes. Daripada 36 kes, sebanyak 16 kes (44.4%) adalah positif LMP1 dan 20 kes (55.6%) adalah negatif. Daripada 16 kes yang positif LMP1, 15 daripadanya adalah lelaki dan hanya seorang sahaja wanita (P>0.05). Keseluruhan kes positif LMP1 adalah dari kumpulan umur lebih 40 tahun. 15 kes adalah terdiri daripada kaum Melayu dan 1 kes daripada kaum Cina (P>0.05). Kesemua kes positif LMP1 adalah pada tahap lV (P<0.05). 15 daripada 34 kes kanser nasofarinks *non keratinizing* adalah positif LMP1. Hanya 1 kes kanser nasofarinks *keratinizing* positif LMP1 (P>0.95). Daripada 36 kes, hanya 1 kes sahaja yang positif P16 (P>0.95). Kes positif P16 ini adalah kes kanser nasofarinks *non keratinizing* pada tahap lV, daripada pesakit lelaki kaum Melayu yang berumur lebih 40 tahun.

Kesimpulan : Kajian ini mendapati kes kanser nasofarinks *non keratinizing* positif untuk LMP1 adalah agak kerap. Terdapat juga kes terpencil kanser nasofarinks *non keratinizing* yang positif untuk P16. Kami melihat terdapat hubungkait antara kes positif LMP1 dengan tahap klinikal. Akan tetapi tiada hubungkait antara ekspresi LMP1 dan P16 dengan data klinikal yang lain. Kajian pada skala lebih besar perlu dijalankan pada masa hadapan.

Kata Kunci: *Kanser nasofarinks, Epidemiologi, Ekspresi protein, LMP1, P16*

ABSTRACT

Introduction : This study is to determine the prevalence of expression of LMP 1 and P16 proteins and the clinicopathology data of the nasopharyngeal carcinoma (NPC) cases in Hospital Universiti Sains Malaysia (HUSM). The association between the expression of LMP1 and P16 proteins in NPC with its clinicopathological parameters was then investigated.

Methodology : This is a cross sectional study that conducted in Department of Pathology, Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian, Kelantan. 70 cases of NPC diagnosed from periods of 2007 to 2017 are included in this study. Out of these 70 cases only 36 cases with its available paraffin embedded tumor were examined for the LMP1 and P16 protein expression. The positivity of LMP1 is based on strong and complete cytoplasm and surface membrane staining in more than 10% of tumor cell. P16 is regarded as positive when there is continuous strong nuclear and cytoplasmic staining pattern in more than 30% of the tumor cells (Block positivity). Prevalence of expression of LMP1 and P16 and clinicopathological data of NPC cases in HUSM is determined and association of the expression of LMP1 and P16 were analyzed using statistical software package SPSS programmed version 24. The strength of associations between variables and LMP1 and P16 is considered statistically significant when p value is same or less than 0.05.

Results : From the total of 70 cases of NPC in this study 59 (84.3%) of the cases are male and 11 (15.7%) are female. In 57 cases (81.4%), patients are aged 40 and above and 13 cases aged below 40. Most of the NPC cases are at stage lV (48 cases [68.6%]). Others NPC cases are in stage I, II, and III with percentage of 10.0 %, 14.3 % and 7.1 % respectively. Most of the NPC cases are non keratinizing nasopharyngeal carcinoma (NKNPC), 67 cases (95.7%),

3 cases are keratinizing nasopharyngeal carcinoma (KNPC). Of 36 cases of NPC that proceed with immunohistochemistry staining, 16 (44.4%) cases is LMP1 positive and 20 (55.6%) cases are LMP1 negative. Only 1 (2.8%) out of these 36 cases of NPC is P16 positive. Among 16 cases that are positive for LMP1, 15 of them are male and only 1 case is female (P>0.05). All the positive cases are patients with age group more than 40. 15 cases are Malay and only 1 case is of Chinese ethnicity (P>0.05). All cases that are positive for LMP1 are at the stage lV (P<0.05). 15 out of 34 cases of NKNPC are positive for LMP1. Only1 case of KNPC is positive for LMP1 (P >0.95). Out of our 36 cases of NPC only 1 case is positive for P16 (P>0.95). This 1 case that is positive for P16 is from a male patient, with Malay ethnicity and aged more than 40 years old. The patient is at the stage IV and has NKNPC subtype.

Conclusion : This study found high frequency of LMP1 positive in NKNPC cases. Rare case of P16 positive NKNPC also noted. There is an association of LMP1 with the clinical stage at presentation, however no association is noted between the expression of the LMP1 and P16 with the gender, ethnic, age and histology subtype. Due the relatively limited sample size for the immunohistochemistry study, futher work need to carried out using larger sample size.

Keywords : *Nasopharyngeal carcinoma (NPC), Epidemiology, Protein expression, LMP1, P16*

CHAPTER ONE

RESEARCH INTRODUCTION

1.0 PREAMBLE

This study looks into the prevalence and clinicopathologic association of LMP1 and P16 expression in nasopharyngeal carcinoma (NPC) tissue at Hospital Universiti Sains Malaysia (HUSM). The thesis follows menuscript format and it contains 5 chapters. The first chapter is written to provide the overview of the research to the readers. It pens down the background of the study, research questions, hypothesis, conceptual framework, rationales of the study and research parameter . Through this chapter, it is hope the researcher can prepare readers for the upcoming chapters and subsequently ensure readers understand the contents of this thesis.

1.1 BACKGROUND OF THE STUDIES

Nasopharynx is part of pharynx and it is located adjacent to oropharynx. It is composed of a small tubular passage located behind the nasal cavity and above the soft palate (1,2). The roof is formed by basi-sphenoid and basi-occiput and it posterior wall is formed by the first cervical vertebra. Nasopharynx is separated from the nasal cavity anteriorly by choanae with the eustachian tubes orifices made the both right and left lateral wall of the nasopharynx. Comma-shaped elevation, torus tubarius can be appreciated on the superior and posterior location to this orifices. Pharyngeal recess, fossa of Rosenmuller is just above and behind this torus tubarius. The nasopharynx tapers inferiorly until the level of soft palate, where it later become the oropharynx. Thus nasopharynx is a small area with torus tubarius elevation and the depression of fossa of Rosenmuller. The mucosa of the nasopharynx is covered by respiratory type ciliated pseudostratified columnar epithelium with presence of variable amount of non keratinized stratified squamous epithelium. The presence of crypts due to

invagination of the mucosa can be appreciated. The underlying stroma is composed of lymphoid tissue. Occasional seromucinous glands can also be identified in the subepithelial region (1).

NPC is a squamous carcinoma arising from the mucosa of the nasopharynx. It is the most common type of malignant tumor of the nasopharynx (1). There is other primary malignancy of the nasopharynx but they are uncommon. This includes lymphoma, nasopharyngeal papillary adenocarcinoma, adenoid cystic carcinoma, mucosal melanoma and chordoma. WHO classification in the pathology and genetics of head and neck tumors defined NPC by tumor that arise from the epithelium lining the mucosa of the nasopharynx and display evidence of squamous differentiation which can be seen either with light microscopy or be proven ultrastructurally. NPC classification keep changing and has gone under review for several times. The first 1978 WHO classification identified three histology types of NPC. This three histology types includes keratinizing squamous cell carcinoma also known as WHO type I, non keratinizing squamous cell carcinoma or WHO type II and undifferentiated carcinoma which is WHO type III. In the folowing 1991 WHO classification, the squamous cell carcinoma type in which they refer to the keratinizing squamous cell carcinoma or keratinizing was maintained. The other two type which is non keratinizing carcinoma and undifferentiated carcinoma were put together under one single category of nonkeratinizing nasopharyngeal carcinoma (NKNPC). This non keratinizing carcinoma also futher subdivided into two subtype which is differentiated and undifferentiated subtype. This second WHO classification also remove the use of numerical designation of the WHO type. In the third WHO 2005 classification, the previous 1991 classification was maintain with addition of onother new category which is basaloid squamous cell carcinoma. The current latest WHO classification in WHO 2017 classification follow exactly similar classification as the previous third 2005 WHO classification with no new changes (3). Eventhough the

numerical classification is no longer used, but in our article, there are conditions where the used of the numerical classification will be put in bracket in addition to the current nomenclature. This is particularly true when we refer or cite the previous paper that still using this numerical nomenclature. NPC is rare not only among Caucasian but also throughout most of the world (1). In North America the annual incidence is 0.3-0.7 cases per 100000 population (1). But there is certain well defined geographic area and location where the NPC is frequent. This region includes Southern Asia, Southern China, the Arctic, the middle east and North Africa (3,4). Generally there are three distinct geographic areas that show a significant difference in incidence and prevalence of NPC. The geographic area noted to have among highest frequency includes Guangzhou a city located in southern China (also know as Canton), the incidence is apparently high with occurance of new case up to 18/100 000/year (4–6). Alaska and Greenland also includes as high endemic area with the incidence rate of 8-11/100 000/year. The area of intermediate frequency in which the incidence is noted to have range from 4 to 7/100 000/year is the countries located within Southeastern Asia (5). These regions include Taiwan, Vietnam, Thailand, Malaysia and Philippines. Other regions that also have an intermediate frequency in NPC incidence is Caribbean and Mediterranean including Maghreb and Middle East (5,7). And finally areas of low incidence rate includes North China region, Europe and the United States where the incidence is less than 2/100 000/year (5,7).

Besides having a very distinctive geographic distribution with different region has their own incidence and prevalence, NPC also has difference preponderence and prevalence in certain ethnic group which this highlighted genetic factor as one of the important contributor in the development of NPC. NPC is noted more common among certain ethnic groups including the Inuit, Northern Africans and Chinese from Southeastern Asia (4). Even within similar race live within same environment, there is difference in incidence of NPC,

this can be seen in different subethnic group. In study conducted by R. W. Amstrong, they found that within Chinese subethnic group in Selangor, they noted that there is higher rate in Cantonese, moderate rate in Khek and subethnic Hokkien and Teochiu show the lowest rate of NPC (8).

NPC has a multifactorial etiology and currently many available data propose a complex relationship between genetic and dietary factors. Besides the significant different in incidence of NPC based of geographic area and also its high rates of incidence in certain ethnic group, NPC also closely related with the certain environmental exposure includes smoking and alcohol consumption.

Studies noted that there is increase incidence of NPC with tobacco smoking and alcohol consumption. It is well known that tobacco smoking has been implicated with the development of many carcinoma particularly lung carcinoma (9). Other carcinoma including hepatocellular carcinoma and urothelial carcinoma also was noted to have association with the tobacco smoking habit (10,11). A review article by Khani et al in 2018 presented the unique relationship between the habit of tobacco smoking with the risk factor for the development of certain type of cancer and the protective role that may be associated with the smoking habit for the other cancer type (12). They found out that tobacco smoking increase the risk of development of upper erodigestive tract, lung, esophagus, stomach, pancreas, kidney, bladder, prostate, and colorectal cancer (12). A population base case control Study by Vaughan et al in a low risk population concluded the association of tobacco smoking with risk for developing keratinizing nasopharyngeal carcinoma (KNPC) (13). They found that tobacco smoking and alcohol consumption are one of the factor noted to have association and can contributes to the development of the KNPC but no association with the other type of NPC appreciated. Paper published by Vaughan et al found that there is no relation of smoking and alcohol consumption with the development of non keratinizing nasopharyngeal

carcinoma (NKNPC) but they agree that both are significant risk factor for the development of KNPC (13). High consumption of fermented and salted food with high content of nitrosamine is recognized as contributing factor to the development of NKNPC (5,7,14).

Another factor that has been implicated to be involved in the development of NPC is infection by Ebstein-Barr virus (EBV). EBV is associated to the development of cancer arising from the lymphoid and epithelial cell. It has been classified as a type I carcinogen by the World Health Organization (WHO). It is linked and implicated in the pathogenesis of several human malignancies including Burkitt's and Hodgkin's lymphomas, EBV positive gastric carcinoma and NPC (15). Many evidence show oncogenic role of EBV in the genesis of NPC, this include serology evidence particularly high IgA titre in patient, presence of EBV DNA and RNA in all tumor cell and expression of LMP1 and EBNA1 (EBV nuclear antigen-1) (16). EBV infection in NPC is classified as type II latent infection in which only EBV nuclear antigen-1(EBNA1), latent membrane protein-1(LMP1), LMP2, and EBV early RNA (EBER) expressions can be detected (17). Once cells have become infected, EBV latent genes provide growth and survival benefits, resulting in the development of NPC. Latent infection causes cells to enter cell cycle and maintain continuous proliferation and prevent apoptosis (18).

Among the molecules of EBV latency, LMP1 is the main oncogene of EBV. It has the ability to recruit an array of cellular genes and to inhibit apoptosis by elevating Bcl2 levels (19). The LMP1 molecule includes 6 transmembrane domains and carboxy terminus containing 2 signaling domains called C terminal activating regions 1 and 2 (CTAR 1 and 2). The transmembrane domains allow LMP1 to associate with the host membrane, whereas CTAR region directly activated a number of signalling pathway. LMP1 has a role as antiapoptotic and LMP1 positive cell has greater mobility leading to higher metastatic

potential and faster disease progression. LMP1 is also involved in suppressing immunogenic response against NPC (15,17) .

Among the subtype of NPC, NKNPC is noted to be associated with the presence of EBV infection (1). Old et al in his study using precipitation antibody in human serum to an antigen present in cultured Burkitt's lymphoma cells is among the first to discover the association of NPC with EBV (20). In this study, they found that most patient with NKNPC has positive EBV serology (1). Study by Adam et al prove the presence of EBV in all of the cases of NKNPC (21). These consistent finding indicates the possible role of EBV in the pathogenesis of NPC.

Among the diagnostic tools used to detect the present of EBV is by detection of IgA antibody against EBV viral capsid antigen and IgG/IgA against early antigen (1). EBV in NPC tissue can be demonstrated by detection of EBER using in situ hybridization and by immunohistochemistry staining method. PCR also can be use to detect the presence of EBV, but as the bystander EBV positive lymphocytes can give rise to false positive result making it not specific. Saleh Aidahri et al in the study on a 61 Saudi patient using in situ hybridization technique to detect latent infection of EBV found that 98.4% cases of NPC is positive compared with only 6.6% positivity in normal mucosa (22). In the small number of these positive cases in the normal mucosa, they found that there is actually presence of associated adjacent tumor tissue. By this, they conclude that positive finding of EBV in adjacent normal mucosa can be used as indication of early or recurrent disease. Adam et al in his study using normal nasopharyngeal epithelium as a control able to demonstrated the presence of EBV in the NPC tissue but not in the normal control tissue (21). The absent of EBV in this normal control nasopharyngeal epithelium with no adjacent tumor tissue is consistent with the previous study.

Salted fish contains nitrosamine compound which is apart from being carcinogenic, also act as EBV reactivating substance (3). Assayed of the immediate EBV early protein and quantitative mRNA analysis performed on the cells line derived from NPC patient noted a significant increase of the level in a cell line treated by nitrosamine compound N-Methyl-N-Nitro-N-Nitrosoguanidine (23).

Oncogenic high risk Human Papillomavirus (HPV) also been implicated in the pathogenesis of NPC. Infection with HPV has long known as the cause of cervical cancer. Evidence show that HPV is also a causative agent for some head and neck squamous cell carcinomas (HNSCC) particularly oropharyngeal carcinoma (15). Due to the close location and also the similarities of the mucosal lining and underlying lymphoid rich tissues, there is also possibilities of association of the HPV with the NPC. HPV is a virus with a double stranded DNA. It is a non enveloped virus under the family Papilloviridae. HPV encode four conserved proteins which is replication factor E1 and E2, and capsid protein L1 and L2. There is four accessory protein protein including E4, E5, E6 and E7 (24).

Immunoexpression of P16 is widely use as a surrogate marker for detection of HPV in carcinoma. P16 is tumor suppressor protein encoded by CDKN2A gene. In normal cellular state, it prevent progression of cell cycle into synthesis phase by inhibiting cyclin D dependent protein kinases (CDK) from phosphorylating the retinoblastoma protein (pRb). This prevent conformational changes and release of the E2F transcriptor factor. This result in inhibition of the cell cycle to enter the synthesis phase and prevent gene replication and cell division(6). In normal circumstance, expression of P16 is not detected by immunohistochemistry method. In cell infected by high-risk subtype of HPV, there is inactivation of the pRb by binding of the E7 gene product. This lead to functional inactivation of the pRb gene. This result in overexpression of P16, loss of tumor suppressor function and release of the E2F transcriptional factor (24,25). Therefore, this study is using

immunohistochemistry of P16 as surrogate marker to detect the presence of HPV oncogenic virus in NPC cases.

Over the last three decades association of high risk HPV infection in a oropharyngeal carcinoma is well established (3). As many as 71% of head and neck tumor are positive for HPV in study done by Singhi et al. Using both in situ hybridization technique (ISH) and immunohistochemistry (IHC) method they found that 82% out of the positive HPV case are from oropharyngeal carcinoma (25). A study done by Dogan et al using 67 cases in a low incidence population test for present of HPV virus by using in situ hybridation method (ISH) and P16 IHC found that among NPC patient who are negative for EBV ISH, 24 % is positive in HPV detection (27). Among subtype of NPC, HPV is usually associated with the NKNPC subtype. But there is one study in Greek patient using PCR technique to detect DNA of HPV in 63 cases, they found that 44% of KNPC cases are positive for HPV (28). No co-infection was noted in the study showing that both are independent risk factors for the development of NPC. However, there is another study that shows co-infection of EBV and HPV in NPC. A study done by Rassekh et al stated that 52.9% of cases demonstrated co-infection with EBV and HPV in which all of the HPV positive cases are EBV positive (29). They also found that HPV positive NPC is associated with NKNPC (29). None of the KNPC cases in the study are positive for HPV. HPV was found to be associated with NPC and bear a favorable prognosis (27). In the study done by Dogan et al, the overall survival of HPV positive NPC is statistically better than the HPV negative NPC (20). Other possible contributing factors for the development of NPC includes occupational exposure to dust, wood dust, chemical fumes, and formaldehyde (3).

The symptom of NPC is usually non specific, and the most common presenting symptom is painless enlargement of the upper cervical lymph node. This is non specific symptom and usually happened due to advanced disease because the carcinoma has already

metastasized to the lymph node. Specific symptom of the NPC is due to presence of nasopharyngeal mass. This includes nasal symptoms, which are epistaxis, blood stained post nasal drips and obstruction. The presence of mass can also cause Eustachian tubes dysfunction manifested as hearing impairment, serous otitis media and tinnitus. Skull base involvement due to locally advanced disease may be associated with fifth and sixth cranial nerve palsy and patient may present with headache, diplopia, and facial pain (3,30). Ten percent of NPC patient are asymptomatic (4). In endemic area, as much as 12% of patients may come with dermatomyositis as a presentation of the underlying NPC. As the symptom is vague and many are non specific, this sometimes can delay the exact management. Therefore, Malaysian Clinical Practice Guideline in management of NPC recommend that patient who present with any symptom including painless neck lump, blood stained nasal discharge or saliva, unilateral ear block or hearing loss, unilateral headache, facial numbness or diplopia to be referred to otorhinolaryngologist as soon as possible to rule out NPC (31).

NPC are considered highly malignant due to its early local infiltration, early lymphatic spread, and disproportionately high incidence of hematogenous dissemination (1). Thus, the presenting stage is the most important prognostic factor and patient without distant metastasis showed 5 year disease specific survival (DSS) of 81% and overall survival of 75% with radiation therapy. Treatment of NPC remains a challenge because of the non specific symptom and late presentation, high relapse and difficulty in thorough nasopharyngeal examination. For stage 1 NPC in which the tumor is limited to nasopahrynx or only extend to the oropharynx or nasal cavity, radiotherapy alone is the main treatment. For stage ll, lll, lVA and lVB patient, concurrent chemoradiotherapy is usually offered (32). Clinical practice guideline for NPC also recommend for patient with persistent disease or recurrent NPC, the management option include nasopharyngectomy or irradiation for patient with local disease.

For patient presenting with regional disease the management option include neck dissection, reirradiation and chemotherapy (32).

1.2 RESEARCH QUESTIONS

The researcher has designed 3 research questions, as follows;

a) What is the prevalence of expression of LMP1 and P16 immunohistochemistry in NPC cases in HUSM?

b) What is the clinicopathological data of NPC in HUSM Kelantan?

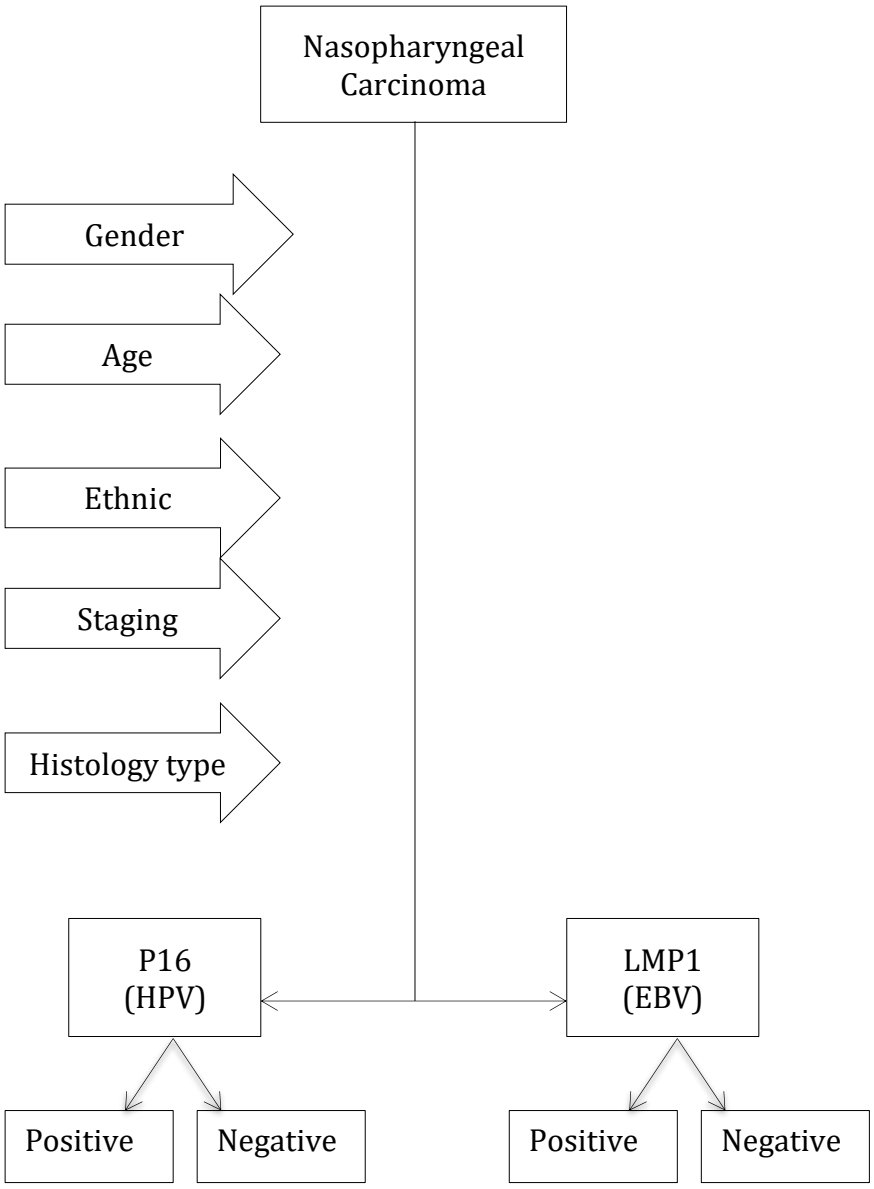
c) What is the association between expression of LMP1 and P16 immunohistochemistry with clinicopathology data?

1.3 RESEARCH HYPHOTHESES

The hypothesis of this study is as follows :

Expression of LMP1 and P16 is associated with its clinicopathology parameters.

1.4 CONCEPTUAL FRAMEWORK



1.5 RATIONALE OF THE STUDY

NPC is common in Southern Asia and it is the fifth of among most common tumor in Malaysia and is the third most common tumor in male age 25 to 59 (6,33). As such, it is important to determine the prevalence and establish epidemiological data of the nasopharyngeal carcinoma in the HUSM.

Many studies implicated the important roles of EBV in the pathogenesis of NPC (4,6,34). Data suggested that EBV is strongly associated with NKNPC. This study is

interested to determine the prevalence of LMP1 positivity and its association with histology subtype of NPC and also clinicopathology data of NPC cases at HUSM.

HPV has emerged as the major etiologic event in the squamous cell carcinoma of the oropharynx (35). Due to close relationship of the location with the nasopharynx, we are interested to know the prevalence of the HPV infection with the NPC by using the surrogate P16 immunohistochemistry marker.

Overexpression of P16 in EBV positive NPC associated with improved survival and longer time to locoregional failure (36). Other studies also show that overexpression of P16 is associated with good prognosis and correlates with good response to treatment, good behavior and good outcome (27). Therefore, we are interested to know the prevalence of NPC that are positive for LMP1 and P16 IHC marker and establish the data.

The purpose of this study is to highlight the prevalence of expression of both LMP1 and P16 in NPC cases in HUSM. We also would to see the association of both marker with patient's clinicopathological data. We hope that the findings can provides further insight into the probability of future use of LMP1 and P16 as novel marker, to provide more detailed and comprehensive guidance in determining the prognosis and predicting treatment outcome for patient diagnosed with NPC. Since this study is contectualized to Malaysian population, the study will add values and knowledge to the topic discussed, and benefit those who are keen in this research field.

1.6 PARAMETERS OF THE STUDY

In this study, the major parameter or limitation that must be noted is in the number of cases available for this research. Due to difficulties in getting financial aid for this study, the findings can only be generalized to the hospital or target participants population only.

1.7 CHAPTER SUMMARY

Through this chapter, the researcher has explained and justified why this study merits our attention and why there is a need to conduct this study through its background of the study, research questions, hypothesis and rationale of the study. In the next chapter, the researcher will discuss further on matters related to the objective of the study.

CHAPTER TWO

OBJECTIVES

2.1 RESEARCH OBJECTIVES

General objectives are as follows:

- To determine the expression of LMP 1 and P16 immunohistochemistry in the NPC cases in HUSM.

The specific objectives are as follows;

- To determine the association between the expression of LMP1 immunohistochemistry in NPC with its clinicopathological parameters.
- To determine the association between the expression of P16 immunohistochemistry in NPC with its clinicopathological parameters.

All of the above objectives are reported in the proposed manuscript for publication.

CHAPTER THREE

MANUSCRIPT

TITLE PAGE: PREVALENCE OF LMP1 AND P16 EXPRESSION AND ITS CLINICOPATHOLOGIC ASSOCIATION IN NASOPHARYNGEAL CARCINOMA CASES IN HOSPITAL UNIVERSITI SAINS MALAYSIA

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3.1 ABSTRACT

Introduction : This study is to determine the prevalence of expression of LMP1 and P16 proteins and the clinicopathology data of the nasopharyngeal carcinoma (NPC) cases in Hopital Universiti Sains Malaysia (HUSM). The association between the expression of LMP1 and P16 proteins in NPC with its clinicopathological parameters was then investigated.

Methodology : This is a cross sectional study that conducted in Department of Pathology, HUSM, Kubang Kerian, Kelantan. Seventy cases of NPC diagnosed from periods of 2007 to 2017 are included in this study. Out of these 70 cases only 36 cases are examined for the LMP1 and P16 protein expression. The positivity of LMP1 is based on strong and complete cytoplasm and surface membrane staining in more than 10% of tumor cell. P16 is regarded as positive when there is continuous strong nuclear and cytoplasmic staining pattern in more than 30% of the tumor cells (Block positivity). Prevalence of expression of LMP1 and P16 and clinicopathological data of NPC cases in HUSM is determined and association of the expression of LMP1 and P16 were analyzed using statistical software package SPSS programmed version 24. The strength of associations between variables and LMP1 and P16 is considered statistically significant when P value is same or less than 0.05.

Results : From the total of 70 cases of NPC in this study 59 (84.3%) of the cases are male and 11 (15.7%) are female. In 57 cases (81.4%), patients are aged 40 and above and 13 cases aged below 40. Most of the NPC cases are at stage lV (48 cases [68.6%]). Others NPC cases are in stage I, II, and III with percentage of 10.0 %, 14.3 % and 7.1 % respectively. Most of the NPC cases are non keratinizing nasopharyngeal carcinoma (NKNPC), 67 cases (95.7%) and 3 cases are keratinizing nasopharyngeal carcinoma (KNPC). Of 36 cases of NPC that proceed with immunohistochemistry staining, 16 (44.4%) cases are LMP1 positive and 20

(55.6%) cases are LMP 1 negative. Only 1 (2.8%) out of these 36 cases of NPC is P16 positive. Among 16 cases that are positive for LMP1, 15 of them are male and only 1 case is female (P>0.05). All the positive cases are patients with age group more than 40. 15 cases are Malay and only 1 case is of Chinese ethnicity (P>0.05). All cases that are positive for LMP1 are at the stage lV (P<0.05). 15 out of 34 cases of NKNPC are positive for LMP1. Only1 case of KNPC is positive for LMP1 (P>0.95). Out of our 36 cases of NPC only 1 case is positive for P16 (P>0.95). This 1 case that is positive for P16 is from a male patient, with Malay ethnicity and aged more than 40 years old. The patient is at the stage Ⅳ and has NKNPC subtype.

Conclusion : This study found high frequency of LMP1 positive in NKNPC cases. Rare case of P16 positive NKNPC also noted. There is an association of LMP1 with the clinical stage at presentation, however no association is noted between the expression of the LMP1 and P16 with the gender, ethnic, age and histology subtype. Due the relatively limited sample size for the immunohistochemistry study, futher work need to carried out using larger sample size.

Keywords: *Nasopharyngeal carcinoma (NPC), Epidemiology, Protein expression, LMP1, P16*

3.2 INTRODUCTION

Nasopharynx is part of pharynx and it is located adjacent to oropharynx. It is composed of a small tubular passage located behind the nasal cavity and above the soft palate (1,2). The roof is formed by basi-sphenoid and basi-occiput and it posterior wall is formed by the first cervical vertebra. Nasopharynx is separated from the nasal cavity anteriorly by choanae with the eustachian tubes orifices made the both right and left lateral wall of the nasopharynx. Comma-shaped elevation, torus tubarius can be appreciated on the superior and posterior location to this orifices. Pharyngeal recess, fossa of Rosenmuller is just above and behind this torus tubarius. The nasopharynx tapers inferiorly until the level of soft palate, where it later become the oropharynx. Thus nasopharynx is a small area with torus tubarius elevation and the depression of fossa of Rosenmuller. The mucosa of the nasopharynx is covered by respiratory type ciliated pseudostratified columnar epithelium with presence of variable amount of non keratinized stratified squamous epithelium. The presence of crypts due to invagination of the mucosa can be appreciated. The underlying stroma is composed of lymphoid tissue. Occasional seromucinous glands can also be identified in the subepithelial region (1).

NPC is a squamous carcinoma arising from the mucosa of the nasopharynx. It is the most common type of malignant tumor of the nasopharynx (1). There is other primary malignancy of the nasopharynx but they are uncommon. This includes lymphoma, nasopharyngeal papillary adenocarcinoma, adenoid cystic carcinoma, mucosal melanoma and chordoma. WHO classification in the pathology and genetics of head and neck tumors defined NPC by tumor that arise from the epithelium lining the mucosa of the nasopharynx and display evidence of squamous differentiation which can be seen either with light microscopy or be proven ultrastructurally. NPC classification keep changing and has gone under review for several times. The first 1978 WHO classification identified three histology

types of NPC. This three histology types includes keratinizing squamous cell carcinoma also known as WHO type I, non keratinizing squamous cell carcinoma or WHO type II and undifferentiated carcinoma which is WHO type III. In the folowing 1991 WHO classification, the squamous cell carcinoma type in which they refer to the keratinizing squamous cell carcinoma or keratinizing was maintained. The other two type which is non keratinizing carcinoma and undifferentiated carcinoma were put together under one single category of nonkeratinizing nasopharyngeal carcinoma (NKNPC). This non keratinizing carcinoma also futher subdivided into two subtype which is differentiated and undifferentiated subtype. This second WHO classification also remove the use of numerical designation of the WHO type. In the third WHO 2005 classification, the previous 1991 classification was maintain with addition of onother new category which is basaloid squamous cell carcinoma. The current latest WHO classification in WHO 2017 classification follow exactly similar classification as the previous third 2005 WHO classification with no new changes (3). Eventhough the numerical classification is no longer used, but in our article, there are conditions where the used of the numerical classification will be put in bracket in addition to the current nomenclature. This is particularly true when we refer or cite the previous paper that still using this numerical nomenclature. NPC is rare not only among caucasian but also throughout most of the world (1). In North America the annual incidence is 0.3-0.7 cases per 100000 population (1). But there is certain well defined geographic area and location where the NPC is frequent. This region includes Southern Asia, Southern China, the Arctic, the middle east and North Africa (3,4). Generally there are three distinct geographic areas that show a significant difference in incidence and prevalence of NPC. The geographic area noted to have among highest frequency includes Guangzhou a city located in southern China (also know as Canton), the incidence is apparently high with occurance of new case up to 18/100 000/year (4–6). Alaska and Greenland also includes as high endemic area with the incidence rate of 8-

11/100 000/year. The area of intermediate frequency in which the incidence is noted to have range from 4 to 7/100 000/year is the countries located within Southeastern Asia (5). These regions includes Taiwan, Vietnam, Thailand, Malaysia and Philippines. Other region that also have an intermediate frequency in NPC incidence is Caribbean and Mediterranean including Maghreb and Middle East (5,7). And finally areas of low incidence rate includes North China region, Europe and the United States where the incidence is less than 2/100 000/year (5,7).

Besides having a very distinctive geographic distribution with different region has their own incidence and prevalence, NPC also has difference preponderence and prevalence in certain ethnic group which this highlighted genetic factor as one of the important contributor in the development of NPC. NPC is noted more common among certain ethnic groups including the Inuit, Northern Africans and Chinese from Southeastern Asia (4). Even within similar race live within same environment, there is difference in incidence of NPC, this can be seen in different subethnic group. In study conducted by R. W. Amstrong, they found that within Chinese subethnic group in Selangor, they noted that there is higher rate in Cantonese, moderate rate in Khek and subethnic Hokkien and Teochiu show the lowest rate of NPC (8).

NPC has a multifactorial etiology and currently many available data propose a complex relationship between genetic and dietary factors. Besides the significant different in incidence of NPC based of geographic area and also its high rates of incidence in certain ethnic group, NPC also closely related with the certain environmental exposure includes smoking and alcohol consumption.

Studies noted that there is increase incidence of NPC with tobacco smoking and alcohol consumption. It is well known that tobacco smoking has been implicated with the development of many carcinoma particularly lung carcinoma (9). Other carcinoma including

hepatocellular carcinoma and urothelial carcinoma also was noted to have association with the tobacco smoking habit (10,11). A review article by Khani et al in 2018 presented the unique relationship between the habit of tobacco smoking with the risk factor for the development of certain type of cancer and the protective role that may be associated with the smoking habit for the other cancer type (12). They found out that tobacco smoking increase the risk of development of upper erodigestive tract, lung, esophagus, stomach, pancreas, kidney, bladder, prostate, and colorectal cancer (12). A population base case control Study by Vaughan et al in a low risk population concluded the association of tobacco smoking with risk for developing keratinizing nasopharyngeal carcinoma (KNPC) (13). They found that tobacco smoking and alcohol consumption are one of the factor noted to have association and can contributes to the development of the KNPC but no association with the other type of NPC appreciated. Paper published by Vaughan et al found that there is no relation of smoking and alcohol consumption with the development of non keratinizing nasopharyngeal carcinoma (NKNPC) but they agree that both are significant risk factor for the development of KNPC (13). High consumption of fermented and salted food with high content of nitrosamine is recognized as contributing factor to the development of NKNPC (5,7,14).

Another factor that has been implicated to be involved in the development of NPC is infection by Ebstein-Barr virus (EBV). EBV is associated to the development of cancer arising from the lymphoid and epithelial cell. It has been classified as a type I carcinogen by the World Health Organization (WHO). It is linked and implicated in the pathogenesis of several human malignancies including Burkitt's and Hodgkin's lymphomas, EBV positive gastric carcinoma and NPC (15). Many evidence show oncogenic role of EBV in the genesis of NPC, this include serology evidence particularly high IgA titre in patient, presence of EBV DNA and RNA in all tumor cell and expression of LMP1 and EBNA1 (EBV nuclear antigen-1) (16). EBV infection in NPC is classified as type II latent infection in which only EBV

nuclear antigen-1(EBNA1), latent membrane protein-1(LMP1), LMP2, and EBV early RNA (EBER) expressions can be detected (17). Once cells have become infected, EBV latent genes provide growth and survival benefits, resulting in the development of NPC. Latent infection causes cells to enter cell cycle and maintain continuous proliferation and prevent apoptosis (18).

Among the molecules of EBV latency, LMP1 is the main oncogene of EBV. It has the ability to recruit an array of cellular genes and to inhibit apoptosis by elevating Bcl2 levels (19). The LMP1 molecule includes 6 transmembrane domains and carboxy terminus containing 2 signaling domains called C terminal activating regions 1 and 2 (CTAR 1 and 2). The transmembrane domains allow LMP1 to associate with the host membrane, whereas CTAR region directly activated a number of signalling pathway. LMP1 has a role as antiapoptotic and LMP1 positive cell has greater mobility leading to higher metastatic potential and faster disease progression. LMP1 is also involved in supressing immunogenic response against NPC (15,17) .

Among the subtype of NPC, NKNPC is noted to be associated with the presence of EBV infection (1). Old et al in his study using precipitation antibody in human serum to an antigen present in cultured Burkitt's lymphoma cells is among the first to discover the association of NPC with EBV (20). In this study, they found that most patient with NKNPC has positive EBV serology (1). Study by Adam et al prove the presence of EBV in all of the cases of NKNPC (21). These consistent finding indicates the possible role of EBV in the pathogenesis of NPC.

Among the diagnostic tools used to detect the present of EBV is by detection of IgA antibody against EBV viral capsid antigen and IgG/IgA against early antigen (1). EBV in NPC tissue can be demonstrated by detection of EBER using in situ hybridization and by immunohistochemistry staining method. PCR also can be use to detect the presence of EBV,

but as the bystander EBV positive lymphocytes can give rise to false positive result making it not specific. Saleh Aidahri et al in the study on a 61 Saudi patient using in situ hybridization technique to detect latent infection of EBV found that 98.4% cases of NPC is positive compared with only 6.6% positivity in normal mucosa (22). In the small number of these positive cases in the normal mucosa, they found that there is actually presence of associated adjacent tumor tissue. By this, they conclude that positive finding of EBV in adjacent normal mucosa can be used as indication of early or recurrent disease. Adam et al in his study using normal nasopharyngeal epithelium as a control able to demonstrated the presence of EBV in the NPC tissue but not in the normal control tissue (21). The absent of EBV in this normal control nasopharyngeal epithelium with no adjacent tumor tissue is consistent with the previous study.

Salted fish contains nitrosamine compound which is apart from being carcinogenic, also act as EBV reactivating substance (3). Assayed of the immediate EBV early protein and quantitative mRNA analysis performed on the cells line derived from NPC patient noted a significant increase of the level in a cell line treated by nitrosamine compound N-Methyl-N-Nitro-N-Nitrosoguanidine (23).

Oncogenic high risk Human Papillomavirus (HPV) also been implicated in the pathogenesis of NPC. Infection with HPV has long known as the cause of cervical cancer. Evidence show that HPV is also a causative agent for some head and neck squamous cell carcinomas (HNSCC) particularly oropharyngeal carcinoma (15). Due to the close location and also the similarities of the mucosal lining and underlying lymphoid rich tissues, there is also possibilities of association of the HPV with the NPC. HPV is a virus with a double stranded DNA. It is a non enveloped virus under the family Papilloviridae. HPV encode four conserved proteins which are replication factor E1 and E2, and capsid protein L1 and L2. There are four accessory protein including E4, E5, E6 and E7 (24).

Immunoexpression of P16 is widely used as a surrogate marker for detection of HPV in carcinoma. P16 is tumor suppressor protein encoded by CDKN2A gene. In normal cellular state, it prevent progression of cell cycle into synthesis phase by inhibiting cyclin D dependant protein kinases (CDK) from phosphorylating the retinoblastoma protein (pRb). This prevent conformational changes and release of the E2F transcriptor factor. This result in inhibition of the cell cycle to enter the synthesis phase and prevent gene replication and cell division (6). In normal circumstance, expression of P16 is not detected by immunohistochemistry method. In cell infected by high risk subtype of HPV, there is inactivation of the pRb by binding of the E7 gene product. This lead to functional inactivation of the pRb gene. This result in overexpression of P16, loss of tumor suppressor function and release of the E2F transcriptional factor (24,25). Therefore, this study is using immunohistochemistry of P16 as surrogate marker to detect the presence of HPV oncogenic virus in NPC cases.

Over the last three decades association of high risk HPV infection in a oropharyngeal carcinoma is well established (3). As many as 71% of head and neck tumor are positive for HPV in study done by Singhi et al. Using both in situ hybridization technique (ISH) and immunohistochemistry (IHC) method they found that 82% out of the positive HPV case are from oropharyngeal carcinoma (26). A study done by Dogan et al using 67 cases in a low incidence population test for present of HPV virus by using in situ hybridation method (ISH) and P16 IHC found that among NPC patient who are negative for EBV ISH, 24 % is positive in HPV detection (27). Among subtype of NPC, HPV is usually associated with the NKNPC subtype. But there is one study in Greek patient using PCR technique to detect DNA of HPV in 63 cases, they found that 44% of KNPC cases are positive for HPV (28). No co-infection was noted in the study showing that both are independent risk factors for the development of NPC. However, there is another study that shows co-infection of EBV and HPV in NPC. A