THE EXPRESSION OF EGFR AND pEGFR IN NASOPHARYNGEAL CARCINOMA PATIENTS IN HUSM

DR. OMAR MOHAMMED ALZALLAL

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF PATHOLOGY
(ANATOMICAL PATHOLOGY)

UNIVERSITI SAINS MALAYSIA

2021

ACKNOWLEDGEMENT

First and foremost, my deepest thank you to Allah, the Most Merciful and Most Gracious for all His Will and shower of Blessings that make it possible for me to finish the dissertation.

My biggest gratitude goes to my supervisor, Dr. Aidy Irman Yajid and to my co-supervisor, Dr. Norzaliana Zawawi for all their guidance and support throughout the entire process of preparing this dissertation.

My appreciation also goes to the lecturers and staffs of Pathology Department, especially En Rosli Jusoh his skillful expertise in materializing this project.

To all my colleagues in the Pathology Department, thank you so much for the continuous moral support and all the ideas that were given throughout for this research.

A special thanks to the lecturer and colleagues, from the Department of Community Health for the statistical analysis courses for which without them this research merely a collection of meaningless numbers and values.

Omar Mohammed Alzallal

PUM 0267/16

September 2020

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
EGFR	Epidermal growth factor receptor
pEGFR	Phosphorylated epidermal growth factor receptor
H&E	Haematoxylin & Eosin
HUSM	Hospital Universiti Sains Malaysia
JAK-STAT	Janus kinases, signal transducer and activator of transcription proteins
IHC	Immunohistochemistry
LIS	Laboratory Information System
M	Distant metastasis
N	Lymph node metastasis
SPSS	Statistical Package for in Social Sciences
T	Primary tumour
TBS	Tris Buffered Saline
K	Keratinised
NK	Non-keratinised
JNK	Jun N-terminal kinase
WHO	World Health Organization
NPC	Nasopharyngeal carcinoma
PCR	Polymers Chain Reaction
SCC	Squamous Cell Carcinoma
HPV	Human Papilloma Virus
CT	Computed Tomography

EBV	Epstein-Barr virus
MRI	Magnetic Resonance Imaging
HLA	Human Leukocyte Antigen
MAPK	Mitogen Activated Protein Kinase
PI3K/AKT	Phosphoinositidyl-3-kinase
FOR	Fossa of Rosenmüller
LMP	Latent Membrane Protein
TGF-B	Transforming Growth Factor-Beta
EBNA-1	Epstein–Barr nuclear antigen 1
BamHI-A	Bacillus amyloliquefaciens
CK	Cytokeratin
EMA	Epithelial Membrane Antigen
ECOG	Eastern Cooperative Oncology Group
HER	Human Epidermal Growth Factor Receptor
ERK	Extracellular signal-Regulated Kinases
COX-2	Cyclooxygenase-2
mAbs	Monoclonal antibodies

ABSTRAK

Pendahuluan: Reseptor faktor pertumbuhan epidermis (EGFR) biasanya meningkat dalam beberapa kanser, di mana pengaktifan EGFR kepada EGFR terfosforilasi (pEGFR) mendorong pertumbuhan sel tumour dan menghalang apoptosis, meningkatkan pencerobohan dan metastasis tumour, serta mendorong berlakunya rintangan terhadap rawatan kemoterapi dan radioterapi. Tambahan lagi, keluarga EGFR berupaya untuk melakukan translokasi (peralihan) dari membran kepada nukleus sel barah melalui jaluran isyarat. Ekspresi berlebihan EGFR dalam tumour primer telah dikaitkan dengan ciri-ciri klinikal yang agresif untuk pelbagai jenis kanser, termasuk kanser pangkal hidung (NPC).

Objektif: Kajian ini menyiasat lokasi, tahap, dan kekuatan ekspresi EGFR dan pEGFR dalam sel kanser pangkal hidung termasuk kemungkinan berlakunya translokasi sel dari membran sel kepada nukleus sel tersebut serta menyelidiki kepentingan klinikal mereka.

Kaedah: Terdapat tiga puluh empat kes kanser pangkal hidung (NPC) telah didiagnosa dan dirawat di Hospital Universiti Sains Malaysia dari tahun 2005 hingga 2018. Ujian imunohistokimia EGFR dan pEGFR telah dilakukan ke atas kes-kes tersebut untuk meneliti lokasi ekspresi dan kemungkinan adanya translokasi sel ke atas EGFR dan pEGFR. Tahap dan kekuatan ekspresi EGFR dan pEGFR ditentukan menggunakan analisis deskriptif. Hubungan antara parameter klinikopatologi dengan ekspresi EGFR dan pEGFR kemudian dianalisis Fisher exact test ujian Chi-square.

Keputusan: Kesemua 34 (100%) kes adalah positif untuk EGFR dan pEGFR berdasarkan ekspresi membran yang jelas pada sel barah NPC, tanpa adanya sebarang perkaitan yang signifikan dengan parameter klinikopatologi. Hanya 23 (67.6%) kes

menunjukkan ekspresi pada sel membran dan nuklear pEGFR yang menandakan berlakunya translokasi sel. Lebih-lebih lagi, 21 dari 23 (91.3%) kes dengan ekspresi pEGFR pada membran dan nuklear tersebut didapati berada pada tahap TNM yang tinggi (tahap IV) (nilai P = 0.217).

Rumusan: Semua kes didapati positif untuk EGFR dan pEGFR berdasarkan ekspresi membran yang jelas pada sel NPC. Semua kes dengan ekspresi EGFR adalah negatif untuk translokasi membran sel ke nuklear, manakala 23 (67.6%) kes ekspresi pEGFR menunjukkan translokasi sel dari membran sel ke nukleus. 21 dari 23 (91.3%) kes yang menunjukkan translokasi nuklear pEGFR didiagnosis sebagai NPC TNM tahap IV, menunjukkan bahawa ekspresi nuklear pEGFR dalam sel NPC membawa prognosis yang buruk.

ABSTRACT

Introduction: Epidermal growth factor receptor (EGFR) is commonly upregulated in several carcinomas, in which EGFR activation into phosphorylated EGFR (pEGFR) promotes tumour cell growth and inhibits apoptosis, enhances tumour invasion and metastasis, and induces chemoresistance and radio-resistance. Furthermore, EGFR family members have the property to translocate (shuttle) from cell membrane of cancer cells into nucleus through signalling pathways. Nonetheless, overexpression of EGFR in primary tumours has been associated with an aggressive clinical course in many cancers including nasopharyngeal carcinoma (NPC).

Objectives: The present study has investigated the cellular localisation, extent, and intensity of EGFR and pEGFR expression on nasopharyngeal cancer cells including the possibility of cellular translocation (shuttle) from the cell membrane into nucleus of cancer cells and investigated their clinical significance.

Methods: Thirty-four cases of nasopharyngeal carcinoma (NPC) were diagnosed and treated in Hospital Universiti Sains Malaysia from year 2005 to 2018. The cases were examined for EGFR and pEGFR immunohistochemistry expression to study the cellular localisation and possible cellular translocation of EGFR and pEGFR. The extent and intensity of EGFR and pEGFR expression were calculated using descriptive analysis. The association between clinicopathological parameters with EGFR and pEGFR expression were then analysed using Fisher's exact test or Chi-square test.

Results: All 34 (100%) cases were positive for EGFR and pEGFR as they showed clear membranous expression on the cancer cells of NPC with no significant association was noted with clinicopathological parameters. Only 23 (67.6%) cases,

showed pEGFR membranous and nuclear localisation in cancer cells which indicated the cellular translocation. Moreover, 21 out of 23 (91.3%) cases that showed membranous and nuclear pEGFR expression were in advanced TNM staging (stage IV) (P value = 0.217).

Conclusion: All cases were positive for EGFR and pEGFR as they showed clear membranous expression on NPC cells. All cases with EGFR expression were negative for cell membrane to nuclear translocation, whereas 23 (67.6%) cases of pEGFR expression showed cellular translocation (shuttle) from cell membrane into nucleus. 21 out of 23 (91.3%) cases that showed pEGFR nuclear translocation were diagnosed as NPC TNM stage IV, indicating that nuclear localisation of pEGFR in NPC cells carry poor prognosis.

CHAPTER 1

INTRODUCTION

Cancer of the head and neck is the sixth most common malignancy worldwide and accounts for approximately 63,000 cases and 13,000 deaths annually in the United States (1). Worldwide, the annual estimation of head and neck cancers are 560,000 cases and 380,000 deaths (2). The most common malignancy of the head and neck is squamous cell carcinoma (SCC) and its variants account for 90%, arising from epithelial mucosal lining of the upper aerodigestive tract including lip and oral cavity, larynx, nasopharynx, tonsils and oropharynx (3).

Nasopharyngeal carcinomas (NPCs) are squamous cell carcinomas arising from the squamous mucosal layer of nasopharynx. The original classification of NPC by World Health Organization (WHO) classification includes: 1) keratinising carcinoma (K), 2) non-keratinising carcinoma (NK) and 3) basaloid SCC (4).

Worldwide, nasopharyngeal carcinoma is relatively uncommon. In 2018, International Agency for Research on Cancer noted that about 129,000 new cases of nasopharyngeal carcinoma, accounting for only 0·7% of all cancers diagnosed in 2018 (5,6). NPC is known of its unbalanced geographic distribution in which 81% of new cases occurred in Asia and 9% in Africa with 67% of the global burden of NPC were seen in China, Indonesia, Vietnam, India and Malaysia (7). Malaysia National Cancer Registry Report (MNCR) classified NPC as the fifth most common cancer for the period of 2012-2016 with higher incidence in male (73.1%) than female (26.9%). Furthermore, MNCR also reported that the incidence of cancer in males increases in

individuals at the ages of 25 years old and above and peaks at the age of 65 years old (8).

Generally, the most frequent risk factors associated with head and neck cancer are smoking, alcohol consumption, human papilloma virus (HPV) infection and Epstein-Barr virus (EBV) infection. HPV infection is commonly associated with oropharyngeal cancers, whereas EBV infection is correlated to nasopharyngeal cancers (9,10).

Routine evaluation should include clinical history and physical examination, haematology and biochemistry work up. Other studies should be included are chest radiograph, computed tomography (CT), magnetic resonance imaging (MRI) of the nasopharynx, skull base, and neck and nasopharyngoscopy. While, a definitive diagnosis is made by endoscope-guided biopsy of the primary tumour. Confirmatory tests such as polymers chain reaction (PCR) is also important in case of Epstein-Barr virus (EBV) or Human Papillomavirus (HPV) related aetiology as their presence has a prognostic significance (11–13).

The prognosis and predictive factors related to NPC stated by World Health Organization (WHO, 2017) include NPC staging at presentation, histopathologic type (keratinised vs non-keratinised), age, gender, CNS symptoms at presentation and HPV or EBV related NPC all of which have their influence in the outcome (14).

NPC has become one of the major health issues within the Malaysian population. The cancer has been recognised as one of the top five malignancies in Malaysia and has affected many Malaysians including one of the most prominent figure in Malaysia, Dato' Lee Chong Wei (8,15).

Recently, there is a crucial role of EGFR in the development and progression of NPC, in addition, EGFR has been proposed as a new target for NPC therapy because of its anti-neoplastic effect (16,17). However, data regarding the role of pEGFR in NPC is scarce, prompting us to conduct this study.

Several studies have shown that EGFR family members can be translocated (shuttled) from the cell membrane to the nucleus of the cancer cells either in full-length or fragmented. Furthermore, the increased nuclear localisation of EGFR is associated with poor prognosis (18, 19).

The present study was carried out to investigate the expression of EGFR and pEGFR by immunohistochemistry in NPC and to correlate the results with clinicopathological variables associated with NPC cases. The study will provide additional knowledge into our understanding regarding the role of EGFR and pEGFR in NPC.

The study has focused on cellular localisation, extent and intensity of the EGFR and pEGFR expression on cancer cells of NPC cases with its clinical significance. Also, the study has investigated the possibility of cellular translocation (shuttle) from the cell membrane into the nucleus of nasopharyngeal cancer cells and its clinical significance.

The method adapted in this study was immunohistochemistry stains to detect the expression of EGFR and pEGFR antibodies to cancer cells of NPC cases. Immunohistochemistry analysis was performed on samples obtained from 34 patients diagnosed with NPC at HUSM. The association of clinicopathologic data of NPC patients with EGFR and pEGFR expression was analysed by using SPSS.

CHAPTER 2

LITERATURE REVIEW

2.1 Background of respiratory system

Respiratory system comprises of two main tracts, the upper and lower respiratory tracts. The upper respiratory tract is composed of a system of interconnected cavities involved in filtering, humidifying and warming inspired air. These cavities include the nasal cavity, paranasal sinuses and nasopharynx, which are involved in sense of smell, resonance chambers of speech and integral in the regulation of air pressure within the middle ear with external environment via auditory (Eustachian) tubes to middle ear cavities. While the lower respiratory tract begins with larynx and continues with trachea and then divided into small and smaller airways till reach the alveoli. Histologically, the upper respiratory tract is lined by stratified squamous epithelium or pseudostratified columnar epithelium with numerous goblet cells (20,21).

From a pathologic standpoint, the lateral wall of the nasopharynx is one of the most important areas. The lateral wall contains the site of the opening of the Eustachian tube, which forms a triangular prominence, the torus tubarius. Fossa of Rosenmüller is a depression located posteriorly to the torus tubarius includes Morgagni sinus, nasopharyngeal fossa and pharyngeal recess. The fossa is formed by a herniation of the nasopharyngeal mucosa through a deficiency between the skull base and the most superior fibres of the superior constrictor muscle. An extensive network of lymphatics drains the nasopharynx from areas such as right and left retropharyngeal lymph nodes (nodes of Rouvière), cervical chain and the spinal accessory nodes (22–24).

2.2 Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is a squamous cell carcinoma arising from the mucosal epithelium of the nasopharynx. Although NPC is one of the head and neck carcinomas but differs from others in epidemiology, clinical history, histology and the treatment response (25). The World Health organization (WHO) classifies NPC as 1) keratinising carcinoma, 2) non- keratinising carcinoma and 3) basaloid SCC (14).

The incidence of NPC is noticeably different per population and global distribution. NPC is rare in United States and Western Europe. By contrast, NPC is endemic in Southern China (e.g., Hong Kong) and South-East Asia. The highest number of NPC cases was seen in China (33,198 cases), Indonesia (13,084 cases), Vietnam (4,931cases), India (3,947 cases) and Malaysia (2,030 cases) (26).

In high-risk populations, NPC incidences rise after the age of 30 years, peaks at 40-60 years with two to three fold higher in males compared to females but rare cases are seen in the paediatric population (27,28).

The causative carcinogens of NPC have not yet been definitively identified (14). However, the major aetiological factors for endemic NPC are genetic susceptibility, early-age exposure to chemical carcinogens, and Epstein-Barr virus (EBV) infection. Moreover, the environmental factors, such as smoking, alcohol intake and the high consumption of salted and fermented foods with high nitrosamine content has been implicated in non-keratinising NPC as contributory factors (27). It is also worth to mention that the risk of developing NPC is linked to genes coding for certain tissue antigens (i.e., HLA genes). In Chinese populations, HLA-A*02 alleles and HLA-8*46 alleles are associated with a high risk of NPC development (29).

EBV has been proposed as a primary etiologic agent in the pathogenesis of this cancer due to the discovery that NPC cells express specific subgroup of EBV-latent proteins, including EBNA-1 and two integral membrane proteins, LMP-1 and LMP-2, along with the BamHI-A fragment of the EBV genome (30). While, the role of human papillomavirus (HPV) as an etiologic agent for nasopharyngeal carcinoma is less well defined than that of EBV, and its relative frequency may differ substantially in endemic and non-endemic regions (31). HPV-related NPCs most frequently show non-keratinising histology. The prognosis for patients with HPV associated nasopharyngeal carcinoma is significantly better on a stage-by-stage basis than the prognosis for patients whose disease is associated with EBV (32,33).

The most common site of origin is fossa of Rosenmüller whereas, the next most common site is the superior posterior wall of the nasopharynx (14). Since this is a clinically occult site, patients may remain asymptomatic for a prolonged period of time. Majority of NPC patients present with local and/or regional advanced disease mostly due to prolonged asymptomatic phase but in certain cases, because of missed diagnosis. However, the presenting symptoms are related to the presence of a mass in the nasopharynx with common complains such as obstruction, epistaxis or blood-stained postnasal drip and painless neck mass due to lymph node metastasis. Symptoms associated with cranial nerve involvement such as headache, diplopia, facial numbness or paraesthesia are seen. Aural symptoms like tinnitus, discharge, earache, deafness or serous otitis media can occur secondary to Eustachian tube dysfunction (34). Paraneoplastic syndromes such as hypertrophic osteoarthropathy syndrome (Pierre Marie syndrome), leukemoid reaction, and fever of unknown origin also can be seen (35).

Diagnostic work-up should include clinical history and physical examination, haematology and biochemistry work ups (such as complete blood count, liver, and kidney function tests, blood glucose levels), imaging studies such as chest radiography, computed tomography (CT), magnetic resonance imaging (MRI) of the nasopharynx, skull base, neck and nasopharyngoscopy. However, a definitive diagnostic test is made by endoscope-guided biopsy of the primary tumour. It is worth mentioning that MRI has 100% sensitivity for cancer detection and therefore, frequently used to complement endoscopy and endoscopic biopsy. Molecular tests such as polymers chain reaction (PCR) is also important in case of Epstein-Barr virus (EBV) or Human Papillomavirus (HPV) related aetiology as their presence has a prognostic significance (11–13,36,37).

Aspiration cytology of metastatic NPC can be used in diagnosis of NPC and the diagnosis can be readily confirmed by immunostaining for cytokeratin and in situ hybridization for EBER (38).

Screening for NPC is challenging, because there is no clear pre-malignant phase, as seen in other malignancies such as adenoma-carcinoma sequence in colorectal cancer.

The screening is advocated in populations where the NPC is endemic. Screening family members of NPC patient is important because first-degree relatives of NPC patients have a higher risk (4-to-10 folds) of developing NPC in compared to patients with no family history of NPC. Moreover, the benefit of screening is aiding the early detection of NPC in its early stage and that has influence to reduce the morbidity caused by NPC treatment. In the early stage disease, NPC is treated with radiation therapy alone, compared to advanced disease, which is treated with combination of

chemotherapy and radiation therapy which leads to increase the morbidity caused by treatment related complications (39,40).

Moreover, screening is useful in detection of plasma EBV DNA in high-risk population for NPC. The plasma EBV DNA detection for nasopharyngeal carcinoma screening has 97·1% sensitivity and 98·6% specificity (41).

On the other hand, a study was done by Yang *et al*, (42), showed that no available data from randomised controlled trial or controlled clinical trials were allowed to determine the efficacy of screening for nasopharyngeal cancer, or the cost-effectiveness and cost-benefit of a screening strategy. Furthermore, in 2011, a study was done by Health Technology Assessment Section (MaHTAS), Ministry of Health Malaysia, concluded that there was no evidence on the effectiveness of NPC screening in terms of reduction in mortality rate or increase in quality adjusted life years (43).

2.3 Histopathological features of NPC

Macroscopically, the tumour can range from barely noticeable lesion to a discrete raised nodule with or without surface ulceration, infiltrative fungating mass or as a smooth bulge in the mucosa (14).

While microscopically the World Health Organization (WHO) classifies nasopharyngeal carcinoma into three histopathologic types. Keratinising squamous cell carcinomas are a group of invasive carcinomas showing obvious squamous differentiation in the form of intercellular bridges and/or various degrees of keratinisation, accompanied by a desmoplastic stroma (Figure 2.1 A). Keratinising-NPC can arise de novo or (more rarely) secondary to radiotherapy. On the other hand, non-keratinising carcinoma exhibits a variety of architectural patterns, ranging from solid sheets to irregular islands, trabeculae, and dis-cohesive sheets of malignant cells intermingled with variable lymphocytes and plasma cells (Figure 2.1 B). Undifferentiated and differentiated are sub classification of non-keratinising NPC. However, the subtypes have no clinical or prognostic value. Lastly, basaloid squamous cell is a type of tumour that is morphologically identical to analogous tumours more commonly occurring in other head and neck sites and has infrequently been reported to occur as a primary tumour of the nasopharynx. The routinely used immunohistochemistry stains (IHC) in NPC diagnosis are p63 and Pancytokeratin in which both show strong expression in tumour cells. Other IHC stains, low-molecular-weight cytokeratins and EMA staining show patchy positivity, while, CK7 and CK20 are found to be negative in tumour cells (14).

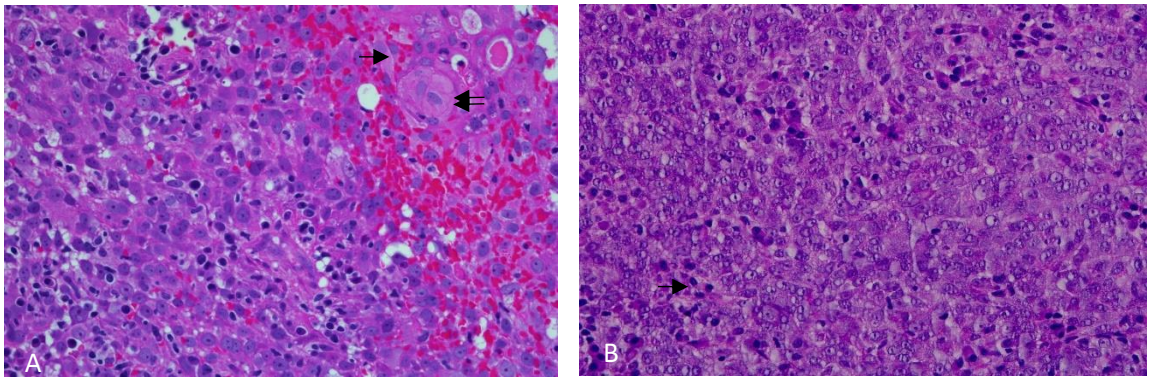

Figure 2.1: Nasopharyngeal carcinoma (NPC) tissues were stained with haematoxylin and eosin stain (H&E) and viewed under light microscope at x400 magnification (A) Keratinised NPC shows presence of intercellular bridges (→) and keratinisation (⇒), (B) Non-keratinised NPC shows sheets of tumour cells with presence of variable lymphoplasma cells (→) intermingled with tumour cells . (Source: Archives of HUSM, Pathology Department).

2.4 TNM Staging

NPC has a highly malignant behaviour for tumour spread as locoregional spread or distant metastasis. 5% to 11% of patients have distant metastases at the time of presentation (44). This tumour type was also shown to have propensity for early lymphatic spread and extensive locoregional infiltration with erosion of skull base and paranasal sinuses, intracranial spread and infiltration of cranial nerves. NPC can thus metastasise to distant organs such as bone, lung, liver or distant nodes (45,46).

The staging system for NPC is TNM classification (TNM: tumour/node/metastasis) by The American Joint Committee on Cancer (AJCC) this system is customised for the tumour behaviour and therapeutic needs for NPC. The staging system is illustrated in (Table 2.1) and (Table 2.2) (47).

Table 2.1: NPC TNM Classification by AJCC (47)

Primary Tumour (T)	Regional Lymph Node (N)	Distant Metastasis (M)
TX: Primary tumour cannot be assessed	**NX**: Regional lymph nodes cannot be assessed	**M0**: No distant metastasis
T0: No tumour identified, but EBV-positive cervical node(s) involvement	**N0**: No regional lymph node metastasis	**M1**: Distant metastasis
Tis: Tumour in situ	**N1**: Unilateral metastasis in cervical lymph node(s) and/ or unilateral or bilateral metastasis in retropharyngeal lymph node(s), 6 cm or smaller in greatest dimension, above the caudal border of cricoid cartilage	
T1: Tumour confined to nasopharynx, or extension to oropharynx and/or nasal cavity without parapharyngeal involvement	**N2**: Bilateral metastasis in cervical lymph node(s), 6 cm or smaller in greatest dimension, above the caudal border of cricoid cartilage	
T2: Tumour with extension to parapharyngeal space, and/or adjacent soft tissue involvement (medial pterygoid, lateral pterygoid, prevertebral muscles)	**N3**: Unilateral or bilateral metastasis in cervical lymph node(s), larger than 6 cm in greatest dimension, and/ or extension below the caudal border of cricoid cartilage	
T3: Tumour with infiltration of bony structures at skull base, cervical vertebra, pterygoid structures, and/or paranasal sinuses		
T4: Tumour with intracranial extension, involvement of cranial nerves, hypopharynx, orbit, parotid gland, and/ or extensive soft tissue infiltration beyond the lateral surface of the lateral pterygoid muscle		

Table 2.2: Stage group (WHO Classification of Head and Neck Tumours, 2016) (47)

Stage	T	N	M
Stage 0	Tis	N0	M0
Stage l	T1	N0	M0
Stage ll	T1	N1	M0
	T2	N0-1	M0
Stage lll	T1-2	N2	M0
	T3	N0-2	M0
Stage IV A	T4	N0-2	M0
Stage IV B	Any T	N3	M0
Stage IV C	Any T	Any N	M1

Multiple clinical and biological characteristics can help in determining prognosis. The staging system is necessary for indicating prognosis, treatment strategy, and evaluating treatment outcome. The most powerful prognostic factor of NPC is cancer stage at presentation. A study using the 2002 TNM staging system found that the 5-year disease-specific survival rate for stage I disease was 98%; for stage IIA- B, 95%; for stage lll 86%; and for stage IVA-B, 73% (14, 48). The histopathologic type (keratinising vs non-keratinising) also plays an important role in prognosis. Keratinised NPC has a worse prognosis than non-keratinising NPC as keratinising NPC shows a greater propensity for locally advanced tumour growth as it has less responsive to radiation therapy. Moreover, keratinised NPC has lower overall survival with a greater than 2-fold higher hazard of death compared to NK-NPC (49,50). Factors such as patient age > 40 years old, male sex, cranial nerve palsy, and ear symptoms at presentation and increasing in tumour volume all are unfavourable

prognostic factors in NPC (51–53). Moreover, the Eastern Cooperative Oncology Group (ECOG), Zubrod, or Karnofsky performance scale (Table-2.3) can be used along with standard staging information in case of co-morbidity to help in predicting survival (47).

Table 2.3: Zubrod/ECOG Performance Scale (47).

Scale	Level of activity
0	Fully active, able to carry out all predisease activities without restriction (Karnofsky 90–100)
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature. For example, light housework, office work. (Karnofsky 70–80)
2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50 % of waking hours. (Karnofsky 50–60)
3	Capable of only limited self-care, confined to bed or chair 50 % or more of waking hours (Karnofsky 30–40)
4	Completely disabled. Cannot carry out self-care. Totally confined to bed. (Karnofsky 10–20)
5	Death (Karnofsky 0)

2.5 Role of EGFR and pEGFR in NPC

Epidermal growth factor receptor (EGFR) (also known as ErbB1/HER1) is a receptor tyrosine kinase that is commonly upregulated in cancers such as in non-small-cell lung cancer and in head and neck carcinomas. EGFR is a proto-oncogene and prototype of the EGFR family that has been shown to play a key role in the development and growth of tumour cells by promoting cell proliferation and opposing apoptosis. The EGFR family comprises four distinct receptors: EGFR/ErbB-1, HER2/ErbB-2, HER3/ErbB-3 and HER4/ErbB-4 (54,55).

Nearly all cell types possess ErbB family members except the haematopoietic cells. In normal cells, the expression of EGFR is estimated to be from 40,000–100,000 receptors per cell, whereas overexpression of more than 10^6 receptors per cell is observed in cancer cells (56). The EGFR protein from N-terminal to C-terminal consists of three domains (i) an extracellular ligand binding and dimerization arm (exons 1–16), (ii) a hydrophobic transmembrane domain (exon 17), and (iii) the intracellular tyrosine kinase and C-terminal tail domains (exons 18–28) (57).

The initiation of signal transduction occurs when EGFR is activated by its ligands, causing a sequence of events that starts with receptor dimerization, followed by transphosphorylation of the C-terminal tail and ends with propagation of the signal through different complex signaling pathways to stimulate new genes expression. (56).

The signalling pathways of EGFR signals are many including the ERK MAPK, PI3K-AKT, SRC, PLC- 1-PKC, JNK, and JAK-STAT pathways. Inter-linking of the pathways leads to activation of EGFR in which stimulates an entire signaling network leading to outcomes such as, cell proliferation, growth differentiation, migration, and

inhibition of apoptosis. Activation of EGFR (phosphorylated EGFR (pEGFR)) is stimulated by several different signal transduction pathways such as Rat sarcoma (Ras)/Mitogen Activated Protein Kinase (MAPK), Phosphatidylinositol-3 Kinase (PI3K)/Act pathway, Phospholipase-Cγ (PLCγ)/PLC protein kinase C pathway, Signal Transducer and Activators of Transcription Pathway (STAT) and Sarcoma (Src) Kinase Pathway in which Src is a proto-oncogene that encoding a non-receptor tyrosine kinase. EGFR phosphorylation (pEGFR) is also formed by the adaptor proteins such as growth Factor Receptor bound protein 2 (Grb2) in which Grb2 act as key molecule in intracellular signal transduction, linking activated cell surface receptors to downstream targets by binding to specific phosphotyrosine-containing and proline-rich sequence motifs and other adaptor proteins like, Son of Sevenless (Sos) which is a set of genes encoding guanine nucleotide exchange factor (GEF) plays a critical role in signal transduction by activating Ras. pEGFR binds directly, or through association with the adaptor molecule Shc, to specific docking sites on the receptor (56,58–63). Formation of pEGFR-Shc complex is important as the presence of Shc family assists the transduction of extracellular signals into intracellular that allows for signal transduction to occur (64)

Intracellular communication between EGFR and Src, in addition to elevated levels of Src and EGFR are often linked to the development of variety of cancer cells (65). EGFR mutations have also been found in several human tumour types in which they occur at mutational “hotspots” in the extracellular region, the kinase domain, and the C-terminal tail (54).

EGFR mutations can occur either due to deletions of EGFR mRNA or no deletion mutants. Deletions of EGFR mRNA are found to involve both regions that encodes the extracellular domain of EGFR and in the intracellular region of the EGFR in which

caused by genomic rearrangements, resulting in alternative splicing of the mRNA. There are three different deletions of the extracellular domain of EGFR (EGFRvI, II and III). EGFRvI is a complete deletion of the extracellular domain, previously identified in a single tumour cell line derived from a xenograft originated from human glioma. EGFRvII is deletion of 83 amino acids in domain IV of the extracellular domain represent only 7% of the total polypeptide mass of EGFR. EGFRvIII is an intragene rearrangements that result in overexpression of transcripts lacking exons 2-7 and, in some cases EGFRvIII occur due to alternative splicing of the mRNA. These deletions have been observed in different types of neoplasia such as glioblastoma, non-small-cell lung carcinomas, breast cancer, paediatric gliomas, medulloblastomas, and ovarian carcinomas. On the other hand, no deletion mutants have been found to occur in the transmembrane or the tyrosine kinase domains. The elimination of the transmembrane domain results in the inability of the receptor to anchors itself to the membrane, which may remove the interaction with the cell membrane related substrates for the tyrosine kinase. The loss of tyrosine kinase domain could abolish the function of EGFR completely, that will stop the ligand induced signal transduction, even if growth factors were available (66).

EGFR is commonly overexpressed in NPC and is related to its pathogenesis. Moreover, overexpression of EGFR in primary tumours was associated with tumour metastasis, recurrence, and poor survival in patients with NPC (16). About 85% of the Chinese patients with NPC have moderate to strong expression of EGFR (67).

The role and mechanisms of EGFR in the NPC are not fully understood however, activation of EGFR pathways promotes tumour cell growth, invasion and metastasis, prevents apoptosis and induces chemoresistance and radioresistance (17).

Anti-EGFR represents a promising new therapeutic target in cancer. EGFR is highly expressed in most human epithelial carcinomas and has been correlated with a more aggressive phenotype, greater resistance to treatment, and poor prognosis (68). Anti-EGFR monoclonal antibodies (anti-EGFR mAbs) bind to the extra-cellular domain of EGFR and prevent activation of downstream signalling pathways, and therefore exert anti-neoplastic effects (17).

Nimotuzumab (NTZ) and cetuximab (CTX) are the main anti-EGFR monoclonal antibody (mAbs) that have been commonly used for NPC treatment. Many studies have shown that CTX or NTZ augments the efficacy of chemoradiotherapy for locoregional advanced NPC. On the other hand, Platinum-based chemotherapy is the standard first-line treatment for recurrent or metastatic nasopharyngeal carcinoma (69).

CHAPTER 3

AIMS AND OBJECTIVES

3.1 General Objectives

To study the expression of EGFR and pEGFR in nasopharyngeal carcinoma cases reported at the Hospital Universiti Sains Malaysia.

3.2 Specific Objectives

1. To determine the extent and intensity of EGFR expression.

2. To determine the extent and intensity of pEGFR expression.

3. To determine the association between the clinicopathological characteristics of NPC with the EGFR and pEGFR expression.

CHAPTER 4

RESEARCH METHODOLOGY

4.1 Study design

A retrospective cross-sectional study was conducted in Hospital Universiti Sains Malaysia (HUSM) from the year 2005 till 2018. This study was approved by the Human Ethical Committee of the School of Medical Sciences, Universiti Sains Malaysia. This study was approved by the Human Research Ethics Committee, Universiti Sains Malaysia (**USM/JEPeM/19040245**).

4.2 Study Population and Sample Size Determination

This study consists of 34 patients diagnosed and treated at HUSM for nasopharyngeal carcinoma during the period of 13 years from 2005 to 2018. These patients were selected from 67 cases from which 33 cases were excluded.

4.2.1 Study criteria

Inclusion criteria: All histopathologically confirmed nasopharyngeal carcinoma patients in HUSM from 2005 to 2018.

Exclusion criteria: (a) Missing tissue paraffin blocks, (b) Tissue exhaustion either due to inadequate or unsuitable material, (c) Incomplete patient data

4.2.2 Sample size

The sample size was calculated based on objectives. For first and second objectives, no sample size need to be calculated because it only involved descriptive statistics. The sample size calculation for third objective was calculated following Post-Hoc

power for two-sample proportion using PS Power and Sample Size Calculations software version 3.1.2. The sample size estimation was 80 cases following rule of thumb for 8 variables (Geoffrey R. Norman, David L. Streiner) (70).

In view of 34 sample size of the study, we have calculated the Post-Hoc power for two-sample proportion using PS Power and Sample Size Calculations software. The publication used as a reference for the sample size calculation was T C Putti *et al*, (71). The data input as follow:

α= 0.05 n= 34 P_0= 0.7 P_1= 0.96 m= 1

Post-Hoc power= 83%

α: The Type I error probability for a two-sided test.

n: The number of case patients.

P_0: The probability of exposure in controls.

P_1: The probability of exposure in cases

m: The ratio of control to experimental subjects.

Note: The targeted sample size in this study (80 cases) cannot be determined, because the total registered cases with confirmed diagnosis of NPC was only 67 cases from 2005 to 2018. The searching methods of NPC cases were done via computerized record collection system (LIS and PATHORS) from the pathology department in HUSM and also was done manually through reading the final pathology reports from 2005 to 2018 in the record room at pathology department in HUSM. 33 out 67 cases were excluded because of unavailable tissue paraffin blocks or inadequate or unsuitable material.

4.3 Clinicopathological Review

All the histological sections were retrieved from the archives and reviewed. The sections of the primary tumours were examined. For each case, the histological classification was done by using WHO classification for keratinised, non-keratinised and basaloid subtypes. Other clinicopathological data including age, gender, race, presentation, co-morbidity, and tumour staging were taken from the histopathological reports and clinical records.

4.4 Research tools

1. Anti-EGFR antibody

The primary antibody is a rabbit monoclonal, [EP38Y] ab52894 (abcam).

2. Anti-EGFR (phospho Y1068) antibody

The primary antibody is a rabbit monoclonal, [EP774Y] ab40815 (abcam).

4.5 Methods of data collection

The details of patients including demographic data and histopathological examination reports were retrieved from the registry of Pathology Department, School of Medical Sciences, Hospital USM, Kubang Kerian, Kelantan. All the cases that fulfilled the inclusion criteria were selected. The corresponding original histologic slides with a final histopathologic diagnosis of nasopharyngeal carcinoma were reviewed to confirm the diagnosis and to select the paraffin blocks. Some of the cases were excluded from the study according to the exclusion criteria. Selection was carried from the year 2005 to the year 2018. Care was taken to ensure patients' confidentiality in this study.

4.5.1 Steps of sample and data collection

- The availability of specimens is searched via computerized record collection system (LIS and PATHORS) from the pathology department in HUSM.
- The entire nasopharyngeal carcinoma specimens' records that had been obtained from the first step are transferred into Microsoft excel programs and sorted by their date.
- All histopathologically confirmed nasopharyngeal carcinoma cases are selected manually.
- The histological slides for confirmation, paraffin blocks for re-staining for EGFR and pEGFR immunostains and the formal pathological report are obtained according to the protocol of the respective center.
- After the slides are obtained, re-confirmation of the diagnosis, and screening for the suitability of the specimen will followed. If the slides have poor staining (due to aging), re-sectioning and re-staining for haematoxylin and eosin will be done.
- If the slides are suitable for this research, the paraffin blocks for the respective slides are send for sectioning and staining for the EGFR and pEGFR immunostains.
- Once the EGFR and pEGFR immunostains are ready, examination of the immunostained slides will be started following the adapted evaluation scale from the reference publication to evaluate the extent and intensity of EGFR and pEGFR expression.
- The patients' records are obtained from the record unit by following the protocol of the respective center. These records are used to obtained data for the socio-demographic, patient presentation, co-morbid disease, smoking status, laboratory, tumour metastasis and TNM staging.
- Both the data collected from the histological slides and patients' records are combined for further statistical analysis.

4.6 Immunohistochemical staining procedure of EGFR and pEGFR

Two immunohistochemistry (IHC) stains were used; 1) rabbit monoclonal anti-EGFR antibody [EP38Y], 2) rabbit monoclonal anti-EGFR (phosphor Y1068) antibody [EP774Y]. These IHC tests were performed using a semi-automated method, according to the standard laboratory protocol and manufacturer guidelines.

Tissue sections of 3-4µm thickness were cut and transferred to poly-L-lysine precoated slides. This was followed by deparaffinization and rehydration process. Antigen retrieval process was carried out by using heat-induced epitope retrieval method via Dako (PT Link). The slides were processed in Dako (PT Link) at 95°C for 15 minutes. Peroxidase blocking agent was applied and incubated for 5 minutes. The slides were then incubated with the primary antibody (anti-EGFR; 1:200, anti-pEGFR; 1:200) for 30 minutes at room temperature using Squenza Immunostainer (Shandon Sequenza). Subsequently, after washing with Tris-buffered solution (TBS), the secondary antibody (labelled K8024, EnVision FLEX, HRP) was applied and incubated for 20 minutes at room temperature. The slides were then incubated with 3,3'-diaminobenzidine (DAB) solution for 5 minutes. Finally, counterstained with Harris Haematoxylin for 5 seconds, followed by dehydration process and placing cover slip using Cytoseal XYL mounting medium. For the control tissue, cervical squamous cell carcinoma was used for anti-EGFR antibody and anti-pEGFR antibodies.

4.7 Immunohistochemical scoring of EGFR and pEGFR

The expression of EGFR and pEGFR at cell membrane (membranous) of nasopharyngeal cancer cells is considered as positive expression (72-74). Upon reviewing the literatures, it has been clearly elucidated that EGFR family members have the property to translocate (shuttle) from cell membrane of cancer cells into