UNIVERSITI PUTRA MALAYSIA

DETERMINATION OF PHOSPHATIDYLINOSITOL-3-KINASE GENE COPY NUMBER AND PROTEIN EXPRESSION IN NASOPHARYNGEAL CARCINOMA BY REAL-TIME PCR AND IMMUNOHISTOCHEMISTRY

MOHAMMAD FIROOZINIA

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By

MOHAMMAD FIROOZINIA

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

June 2013

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DEDICATION

TO

MY BELOVED FAMILY

&

LOVELY WIFE

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FRIENDS

Abstract of the thesis presented to the Senate of University Putra Malaysia in fulfillment of the requirement for the degree Master of Science

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Chairman: Professor. Seow Heng Fong, PhD

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Phosphatidylinositol 3-kinase (PI3K) is a well-known lipid kinase which belongs to a family of enzymes involved in cellular functions such as proliferation, cell growth, motility, and differentiation. The PI3K/AKT pathway is an intracellular signaling pathway, which is important in cancer. The pathway, with *PTEN* gene (tumor suppressor) and *PIK3CA* oncogene, is implicated in the insensitivity of cancer tumors to IGF1 and insulin AKT is recruited to the cell membrane using the PH domain ahead PI3K activation, and phosphatidyl-3,4,5-triphosphate (PIP_3) is created. The PI3K/AKT signaling pathway is involved in many cellular processes associated

with tumorigenesis, together with cell proliferation adhesion and spread. Gene amplification in the *PIK3CA* gene has been reported in many human cancer types such as cancers of colon, breast, brain, liver, stomach and lung.

The purpose of this research is to determine whether there is a significant association of increased *PIK3CA* amplification and PI3K p110α protein expression in nasopharyngeal carcinoma tissues. Real-time quantitative PCR and immunohistochemistry were used to determine the *PIK3CA* gene copy number and to detect protein expression, respectively. The results obtained were analyzed and the ratio of *PIK3CA* to *β-actin* gene copy number was calculated. Positive *PIK3CA* gene amplification was defined as a copy number of ≥4. Also, PI3K p110α protein expression was scored as 0, 1+, 2+ and 3+. The scores of 2+ and 3+ were considered as positive for PI3K p110α protein expression.

Sixty-six nasopharyngeal tumor samples were studied for increased *PIK3CA* gene amplification, and PI3K p110α protein expression was also analyzed in 36 apparently normal adjacent tissues of these 66 tumor samples.

Overall, 26 out of 66(39%) NPC samples showed a significant increase in *PIK3CA* gene copy numbers. 37 out of 66(56%) of tumor sample showed negative staining, and 29 out of 66(44%) showed positive staining for PI3K p110α expression. High frequency of *PIK3CA* gene copy number has been reported in 40% of nasopharyngeal carcinoma in China which is consistent with our results. Correlation of *PIK3CA* amplification showed no significant relationship with age in 4 groups (P= 0.449, P= 0.508, P= 0.625, P= 0.2531), gender (P= 0.230), and race (P= 0.500) in nasopharyngeal carcinoma. But we have identified a significant correlation between *PIK3CA* amplification and histological type (P= 0.001). Correlation of PI3K

p110α protein expression showed no significant relationship with age in 4 groups (P= 0.202, P= 0.154, P= 0.116, P= 0.921), gender (P= 0.300), race (P= 0.785) and histological type (P= 0.824) in nasopharyngeal carcinoma. Correlation between *PIK3CA* gene copy number and PI3K p110α protein expression showed that there is no relationship (p=0.751) between them. There was no significant correlation between *PIK3CA* amplification/expression and age in ovarian clear carcinoma, and there was also no correlation between *PIK3CA* amplification and p110α protein expression in head and neck squamous cell carcinoma has been stated, which is consistent with our finding in nasopharyngeal carcinoma.

Amplification of *PIK3CA* was frequent in nasopharyngeal carcinoma. Our finding shows that there is a relationship between *PIK3CA* gene amplification and histological types I (WHO classification, based on light microscopy) in nasopharyngeal carcinoma. In conclusion, our data suggest that *PIK3CA* has an important role in tumors; and gene amplification is a relatively common mechanism in activating PIK3/Akt signaling pathway in nasopharyngeal carcinoma.

The limitation of this study is that the sample for the present study comprised of 74 cases. This sample is only a very small number of the entire population in the country. In particular, the histological type I of nasopharyngeal carcinoma is low in Malaysian patients. Also, during the real-time PCR experiment, the positive control DNA did not show similar Ct values in each experiment, which emerged as one of the limitations of our research. Therefore, research studies with much larger sample size would be required to ensure appropriate generalization of the findings of the study.

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

PENEUTAN BILANGAN SALINAN PHOSPHATIDYLINOSITIDE-3-KINASE GEN DAN ELSPRESI PROTEIN DALAM KARSINOMA NASOFARINKS OLEH REAL-TIME KUANTITATIF PCR DAN IMMUNOHISTOKIMIA

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Phosphatidylinositol 3-kinase (PI3K) adalah kinase lipid yang tergolong dalam keluarga enzim yang melibatkan fungsi dalam sel seperti pemkembangan, pertumbuhan, pergerakan, dan pembezaan sel. PI3K/AKT merupakan laluan isyarat dalaman sel yang memainkan peranan penting dalam kanser. Dalam laluan ini, gen PTEN (penindas tumor) dan onkogen *PIK3CA* terlibat dalam kekurangan sensitif sel kanser terhadap IGF1 dan insulin. AKT dikumpulkan ke membran sel dengan menggunakan domain PH dan ini mengakibatkan pengaktifan PI3K dan penghasilan phosphatidyl-3,4,5-triphosphate (PIP_3). Laluan isyarat PI3K/AKT terlibat dalan

pelbagai proses sel yang boleh dikaitkan dengan perkembangan tumor, berserta dengan perkembangan, perlekatan dan penyebaran sel. Amplifikasi gen *PIK3CA* telah dilaporkan dalam pelbagai jenis kanser manusia seperti kanser usus besar, kanser payudara, kanser otak, kanser hati, kanser perut dan kanser paru-paru.

Tujuan kajian ini adalah untuk menentukan sama ada terdapat hubungan yang ketara diantara peningkatan amplifikasi *PIK3CA* dan ekspresi protein PI3K p110α dalam tisu karsinoma nasofarinks. Teknik real-time PCR kuantitatif dan imunohistokimia telah digunakan untuk menentukan bilangan salinan gen *PIK3CA* dan mengesan ekspresi protein. Penganalisaan keputusan telah dibuat dan nisbah bilangan salinan gen *PIK3CA* kepada β-actin dikirakan. Amplifikasi gen *PIK3CA* yang positif ditakrifkan sebagai bilangan salinan ≥ 4. Selain daripada itu, ekspresi protein PI3K p110α dinilaikan sebagai 0, 1 +, 2 + dan 3+. Skor 2+ dan 3+ dianggap sebagai ekspresi protein PI3K p110α yang positif.

Kami memeriksa bilangan salinan gen *PIK3CA* dan ekspresi protein PI3K p110α dalam 66 sampel tumor nasofarinks. Ekspresi protein PI3K p110α juga dianalisa dalam 36 tisu perdekatan yang kelihatan normal dalam 66 sampel tumor tersebut.

Secara keseluruhan, 26 daripada 66 (39%) sampel NPC menunjukkan peningkatan yang ketara dalam bilangan salinan gen *PIK3CA*. 37 daripada 66 (56%) sampel tumor telah menunjukkan negatif, dan 29 daripada 66 (44%) menunjukkan positif bagi ekspresi PI3K p110α. Bilangan salinan gen *PIK3CA* yang tinggi telah dilaporkan dalam 40% karsinoma nasofarinks di China. Rujukan tersebut adalah konsisten dengan penemuan kita. Korelasi amplifikasi *PIK3CA* menunjukkan bahawa tiada perhubungan yang ketara dengan usia dalam 4 kumpulan (P= 0.449, P= 0.508,

P= 0.625, P= 0.2531), jantina (P= 0.230), dan bangsa (P= 0.500) dalam karsinoma nasofarinks, tetapi kita telah mengenal pasti korelasi yang ketara di antara amplifikasi *PIK3CA* dan jenis histologi (P= 0.001). Korelasi ekspresi protein PI3K p110α menunjukkan bahawa tiada hubungan yang ketara dengan usia dalam 4 kumpulan (P= 0.202, P= 0.154, P= 0.116, P= 0.921), jantina (P= 0.300), bangsa (P= 0.785) dan jenis histologi (P= 0.824) dalam karsinoma nasofarinks. Tiada hubungan yang dapat ditunjukkan di antara bilangan salinan gen *PIK3CA* dan ekspresi protein PI3K p110α (p= 0.751). Rujukan menunjukkan tiada korelasi yang ketara di antara amplifikasi *PIK3CA*/ekspresi dan usia dalam karsinoma ovari dan juga tiada korelasi di antara amplifikasi *PIK3CA* dan ekspresi protein p110α dalam karsinoma sel skuamus di kepala dan leher. Rujukan tersebut adalah konsisten dengan penemuan kita dalam karsinoma nasofarinks.

Amplifikasi *PIK3CA* adalah kerap dalam karsinoma nasofarinks. Penemuan kita menunjukkan bahawa terdapat hubungan di antara amplifikasi gen *PIK3CA* dan jenis histologi I (klasifikasi WHO, berdasarkan mikroskopi cahaya) dalan karsinoma nasofarinks. Kesimpulannya, data kita mencadangkan bahawa *PIK3CA* mempunyai peranan yang penting dalam tumor and amplifikasi gen adalah satu mekanisma yang biasa dalam pengaktifan laluan isyarat PI3K/Akt dalam karsinoma nasofarinks.

Had dalam penyelidikan ini ialah bilangan sampel yang hanya terdiri daripada 74 kes. Bilangan ini adalah sangat kecil berbanding dengan seluruh populasi dalam negara ini. Terutamanya, jenis I histologi dalam karsinoma nasofarinks adalah jarang terdapat dalam pesakit-pesakit Malaysia. Tambahan pula, semasa eksperimen real-time PCR, kontrol positif DNA tidak menunjukkan nilai Ct yang sama dalam setiap eksperimen. Kita menyatakan ini sebagai salah satu had dalam penyelidikan kita. Oleh demikian, penyelidikan yang mempunyai saiz sampel yang lebih besar

diperlukan untuk memastikan penyamarataan yang tepat bagi penemuan dalam penyelidikan.

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

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APPROVAL

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Putra Malaysia or other institutions.

MOHAMMAD FIROOZINIA
Date: 24 June 2013

TABLE OF CONTENTS

LIST OF TABLES

Table **Page**

LIST OF FIGURES

LIST OF ABBREVIATIONS

4EBP	Translational initiation factor 4E-binding protein
AIDS	Acquired immune deficiency syndrome
AJCC	American Joint Committee on Cancer
AKT	v-akt murine thymoma viral oncogene homolog 1
APES	3-aminopropyltrimethoxysilane
BAD	BCL-2 antagonist of cell death
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
BRD7	Bromodomain-containing protein 7
BSA	Bovine serum albumin
C2	Protein-kinase-C homology-2
CEFs	Chicken embryo fibroblasts
CREB	cAMP responsive element binding protein
CT	Computed tomography
CYP2E1	Cytochrome P450 2E1
DAB	3,3'-diaminobenzidine
dNTPs	Deoxy-nucleotide-tri phosphate

E1047 Ethylenediaminetetraacetic Acid Tetrasodium Salt

EBCT *Electron-beam computed tomography*

EBV Epstein-Barr virus

EGF Epidermal growth factor

ER Estrogen receptor

FOXO Forkhead box protein

GSK3β Glycogen synthase kinase 3 beta

GSTM1 Glutathionine S-transferase M1

HLA Human leukocyte antigen

hOGG1 Human homolog of 8-oxoguanine DNA glycosylase

IHC Immunohistochemistry

IKK IκB kinase

IκB Inhibitor of nuclear factor kappa B kinase

LOH Loss of heterozygosity

LKB1 Upstream Kinase in the AMP-Activated Protein Kinase Cascade

MAPK Mitogen-acivated protein kinase

MRI Magnetic resonance imaging

NFκB Nuclear factor kappa B

NIH 3T3 Murine fibroblast

NPC	Nasopharyngeal carcinoma
P110a	p110 isoform of PI 3-kinase
P110β	p110 isoform of PI 3-kinase
P110δ	p110 isoform of PI 3-kinase
$P21^{cip1}$	Cyclin-dependent kinase inhibitor 1A
$P27^{kip1}$	Cyclin-dependent kinase inhibitor 1B
p53	Tumor suppressor protein p53
p85	Phosphinositide-3-kinase, regulatory subunit 1 (p85 alpha)
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase 1
PET	Positron emission tomography
PI3K	Phosphoinositide-3-kinase
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide gene
PIP2	Phosphatidylinositol 4,5 bisphosphate
PIP3	Phosphatidylinositol 3,4,5 triphosphate
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RASSF1A	Ras association (RalGDS/AF-6) domain family 1
RBD	*RAS*-binding domain

REL-A	v-rel reticuloendotheliosis viral oncogene homolog A
RHEB	Ras homologue enriched in brain
RTK	Receptor tyrosine kinase
S6K	p70 S6 kinase
STK11	Serine/threonine kinase 11
TBS-T	Tris-buffered saline with Tween-20
TKR	Tyrosine kinase receptor
TNM	Cancer staging system
TOR	Target of rapamycin
TSC	Tuberous sclerosis complex
WCR	World cancer report
WHO	World Health Organization
XRCC1	X-ray repair cross-complementing group 1

CHAPTER 1

INTRODUCTION

It is widely known that the uncontrolled growth of abnormal cells occurs in malignant cells. Cancer begins when cells start growing out of control. The majority of the human malignancies is caused by somatic alterations within the cancer genome, mainly due to the action of oncogenes and inactivation of tumor gene suppressors. Genetic alterations within the genome sequence, for example, germline mutations, point mutations, gene amplification, over-expression, and retroviral transduction lead to deregulation of cancer-related genes (Moscow and Cowan 2011). Amongst the mechanisms described, somatic alteration appears to be a main causal factor in nearly all of the human malignancies (Weir *et al*., 2004). A large amount of the oncogenes is mutated kinase genes. Both (lipid and the protein) kinases are altered genes in cancer cells. Therefore, the proteins belonging to the kinase group have been chosen by cancer gene hunters. Kinase inhibitors are the biggest class of new cancer drugs. In fact, kinases and their direct regulators are the most frequently mutated oncogenes and tumor suppressors. Kinase inhibitors have been used in treating cancers that have high mortality rates, such as lung, breast, and colorectal, pancreatic, prostate and nasopharyngeal cancer (Zhang *et al*., 2009; Edward *et al*., 2003). The most well-known of kinases example include the oncogenic kinase *PIK3CA*, which activates PI3K. The kinase activity of

phosphatidylinositol 3-kinase (PI3K) was first described in viral oncoprotein and with activated tyrosine-protein kinases.

PIK3CA is a 34kb gene found on human chromosome 3q26.3. It contains 20 exons, which codes for 1068 amino-acids corresponding to a 124 kDa size protein (Karakas *et al*., 2006). Amplification of genes, deletions of somatic missense mutations of the *PIK3CA* gene have been found in many human cancer types such as colon, breast, brain, liver, stomach and lung cancers (Shayesteh *et al*., 1999; Campbell *et al*., 2004; Phillip *et al*., 2006; Kozaki *et al*., 2006).

AKT (a serine/threonine kinase) is known as a protein kinase that has an important role in cellular processes like apoptosis, transcription and cell proliferation. AKT is recruited to the cell membranes by direct interaction of p85 subunit with the activated receptors, and phosphatidyl-3,4,5-triphosphate (PIP3) is formed. The phosphoinositide-3-kinase (PI3K) -AKT signaling pathway controls primary cellular progression involved in tumorigenesis, together with cell proliferation, adhesion, survival, motility and spread. PI3K increases intracellular pools of phosphatidyl inositol-3,4,5-tri-phosphate (PIP_3) via phosphorylating the phosphatidylinositol 4,5 biophosphate (PIP_2).

The signaling pathway of PI3K/AKT is activated in many carcinomas, due to oncogenic alteration. Activation is possibly due to activating mutations of *PIK3CA* genes or inactivating mutations in the tumor suppressor genes. Genetic alteration is generally found in the genes encoding biomolecules of the signaling pathway of PI3K/AKT. It plays an imperative role during the pathogenesis and tumorigenesis of a large spectrum of human cancers. Phosphatidylinositol-3-kinases are significant mediators of cellular

enlargement, proliferation, adhesion, survival, and motility (Cantley *et al*., 2002). Several *PIK3CA* gene mutations have been found to activate the enzymatic action of *PIK3CA*, leading to activation of Akt-signaling, increased growth factor-independent growth, invasion and metastasis of cancer cells (Samuels *et al*., 2004; Kang *et al*., 2005; Samuels and Ericson 2006). Two *PIK3CA* mutational 'hotspots' were found in exons 9 and 20 and activating mutations in these regions have been seen in various human cancers (Samuels *et al*., 2004; Hayes *et al*., 2006; Karakas *et al*., 2006; Kozaki *et al*., 2006; Philips *et al*., 2006; Qiu *et al*., 2006; Schonleben *et al*., 2006).

Amplification of the *PIK3CA* gene was found in several cancers, such as ovarian carcinoma (Shayesteh *et al*., 1999), cervical tumor (Ma *et al*., 2000), brain cancers (Hui et al., 2001), small cell /non-small cell lung cancer (Massion *et al*., 2002), squamous cell cancer (Woenckaus *et al*., 2002), gastric malignancy (Byun *et al*., 2003), esophageal adenocarcinoma (Miller *et al*., 2003), thyroid carcinoma (Wu *et al*., 2005), oral squamous cancers cell (Qiu *et al*., 2006), and oral squamous cell cancers (Patel *et al*., 2007). Also, genetic amplification, movement, activation of *PIK3CA* and Akt phosphorylation have been reported in human tumors (Shayesteh *et al*., 1999; Ma *et al*., 2000; Massion *et al*., 2002; Byun *et al*., 2003; Samuels *et al*., 2004; Wu *et al*., 2005; Kozaki *et al*., 2006).

PI3K p110α expression has been correlated with cell growth, proliferation, and resistance to apoptosis (Kobayashi *et al*., 2006; Bondar *et al*., 2002; Shinohara *et al*., 2007; Bedogni *et al*., 2004; Leger *et al*., 2007; Longo *et al*., 2008). Correlation between *PIK3CA* amplification/expression and age in ovarian clear cell carcinoma has also been evaluated (Abe *et al*., 2013). The relationship of p53 protein expression and MDM2

gene expression (Luo *et al*., 2000) , expression and relationship between p53 oncoproteins and proliferating cell nuclear antigen (Cheng *et al*., 2005), and multidrug-resistant genes expression (Tao *et al*., 2005) has been investigated in nasopharyngeal carcinoma.

Our hypothesis states that there is a significant increase in *PIK3CA* gene copy number and PI3K p110α protein expression in nasopharyngeal carcinoma.

The objectives of the present research are:

1) To determine frequency of *PIK3CA* gene amplification and PI3K p110α protein expression in nasopharyngeal carcinoma.
2) To determine the relationship between *PIK3CA* gene amplification and PI3K p110α protein expression
3) To determine the relationship of *PIK3CA* gene amplification with patient demographics and histological type
4) To determine the relationship of PI3K p110α expression with patient demographics and histological type

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