UNIVERSITI PUTRA MALAYSIA

EFFECTS OF SMALL MOLECULE INHIBITORS TARGETING PI3K, EGFR, IGF-1R, MTOR, SMAD3 AND MEK IN TUBE FORMATION AND 3-D SPHEROID ASSAY

NG CHIN TAT

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By

NG CHIN TAT

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

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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November 2014

Chairman: Professor Seow Heng Fong, PhD

Faculty: Medicine and Health Sciences

Angiogenesis is the process where new blood capillaries are formed from the preexisting blood vessels. The MEK/ERK and PI3K/Akt signaling pathways are involved in the processes that drive cancer progression such as cell motility, metastasis and angiogenesis. The cellular events in angiogenesis which include endothelial cell migration, invasion and differentiation contribute significant role to cancer metastasis. Inhibition of angiogenesis via disruption of signaling pathways appears to be a rational therapeutic strategy. Hence, there is need to investigate the role of PI3K-AKT and MEK-ERK signaling in microvascular endothelial cells. In this study, we investigated the role of PI3K/AKT and MEK/ERK pathway in two microvascular endothelial cell lines, namely, HMEC-1 (SV40-immortalized) and TIME (telomerase-immortalized). The specific objectives of the study were (i) to investigate the effect of blocking PI3K/AKT and MEK/ERK pathways on tube formation by HMEC-1 and TIME by using small molecules inhibitors targeting phosphoinositide 3-kinase (PI3K), epidermal growth factor receptor (EGFR), insulin-like growth factor I receptor (IGF-1R), mammalian target of rapamycin (mTOR), mothers against decapentaplegic homolog 3 (SMAD3), and mitogen-activated protein kinase kinase (MEK), (ii) to investigate the effect of blocking PI3K/AKT and MEK/ERK pathways on cell invasion by HMEC-1 and TIME in a 3-D spheroid invasion model by using small molecule inhibitors targeting PI3K, IGF-1R, EGFR, mTOR, Smad3, and MEK. and (iii) to investigate the effect of small molecule inhibitors targeting PI3K, EGFR, IGF-1R, mTOR, Smad3, and MEK on phosphorylation status of AKT and ERK by HMEC-1 and TIME. *In vitro* angiogenesis was examined using tube formation whereas the invasion properties were assessed using three-dimensional (3D) spheroid *in vitro* invasion assays. PD0325901 and NVP-AEW541 were able to inhibit tube formation by TIME cells in a dose-dependent manner but had no effect on HMEC-1. Western Blot showed MEK inhibitor PD0325901 and IGF-1R inhibitor NVP-AEW541 suppressed phosphorylation of ERK and AKT, respectively, in HMEC-1 and TIME cells. NVP-BKM 120 inhibited tube formation and suppressed phosphorylation of AKT in both cell lines in a dose-dependent manner. TIME spheroids

treated with inhibitors (NVP-AEW541, NVP-BKM120 and PD0325901) showed a significant reduction in invasion and a similar trend were observed in suppression of tube formation. However, treatment with PD0325901 showed inhibition in HMEC-1 spheroids invasion whereas there is no suppression in tube formation. HMEC-1 spheroids treated with inhibitors (NVP-BKM120, NVP-BKM120 combination with PD0325901) showed a significant reduction in invasion and a similar trend were observed in suppression of tube formation. This result suggested the different results obtained in response to inhibitors might be due to the different models chosen. In conclusion, our results indicated that tube formation of TIME cell was inhibited when MEK-ERK pathway and/or PI3K-AKT pathway was blocked. In contrast, the angiogenic activity of HMEC-1 cell was inhibited via blockade of PI3K-AKT pathway but not MEK-ERK pathway.

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

KESAN-KESAN PERENCAT MOLEKUL KECIL MENSASARKAN PI3K, EGFR, IGF-1R, MTOR, SMAD3, DAN MEK PADA FORMASI TUBE DAN 3-D SFEROID ASSAL

Oleh

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Angiogenesis merupakan satu proses di mana pembetukan kapilari darah baru daripada saluran darah yang wujud. Lintasan isyarat MER/ERK dan PI3K/AKT yang terlibat dalam proses yang mendorong progresi kanser seperti motiliti sel, metastasis, dan angiogenesis. Peristiwa-peristiwa sel-sel dalam angiogenesis termasuk migrasi, invasi dan pembezaan sel endothelial menyumbangkan perkembangan kepada metastasis kanser. Perencatan angiogenesis melalui gangguan laluan isyarat akan menjadi satu strategi terapeutik yang rasional. Oleh itu, pensiasatan peranan pada lintasan isyarat PI3K-AKT dan MEK-ERK dalam sel endothelial mikrovesikel adalah diperlukan. Objektif-objektif khusus kajian ini adalah (i) untuk mengkaji kesan menyekat laluan PI3K / AKT dan laluan MEK / ERK pada formasi tuib oleh HMEC-1 dan TIME dengan menggunakan perencat-perencat molekul kecil mensasarkan óphosphoinositide 3-kinaseô (PI3K), óepidermal growth factor receptorô (EGFR), óinsulin-like growth factor I receptorô (IGF-1R), ómammalian target of rapamycinô (mTOR), ómothers against decapentaplegic homolog 3ô (SMAD3), dan ómitogen-activated protein kinase kinaseô (MEK). (ii) untuk mengkaji kesan menyekat laluan PI3K / AKT dan laluan MEK / ERK pada invasi sel oleh HMEC-1 dan TIME dalam tiga dimensi (3D) sferoid assal dengan menggunakan perencat-perencat molekul kecil mensasarkan PI3K, IGF-1R, EGFR, mTOR, SMAD3, dan MEK dan (iii) untuk mengkaji kesan perencat-perencat molekul kecil mensasarkan PI3K, EGFR, IGF-1R, mTOR, SMAD3, and MEK trehadap status pemfosforilan AKT dan ERK pada HMEC-1 dan TIME. Angiogenesis *in vitro* diperiksa dengan menggunakan formasi tiub manakala sifat invasi dikaji dengan menggunakan tiga dimensi (3D) sferoid assal invasi *in-vitro*. PD0325901 dan NVP-AEW541 boleh merencatkan formasi tiub oleh sel-sel TIME dengan cara yang bergantung kepada dos tetapi tidak mempunyai kesan terhadap HMEC-1. Pemendapan Western menunjukkan PD0325901 perencat kepada MEK dan NVP-AEW541 perencat kepada IGF-1R menghalang pemfosforilan ERK dan AKT dalam sel-sel HMEC-1 dan TIME masing-masing. Perencat NVP-BKM120 menghalang pembentukan tiub dan pemfosforilan bagi AKT dalam kedua-dua jenis titisan sel dengan cara yang

bergantung kepada dos. Rawatan sferoid TIME dengan perencat-perencat (NVP-AEW541, NVP-BKM120 and PD0325901) menunjukkan pengurangan yang ketara dalam invasi dan trend yang sama diperhatikan dalam halangan bagi formasi tiub. Walau bagaimanapun, rawatan dengan PD0325901 menunjukkan perencatan dalam invasi terhadap sferoid HMEC-1 manakala tidak ada perencatan dalam tiub formasi. Rawatan sferoid TIME dengan perencat-perencat (NVP-BKM120, NVP-BKM120 combinasi dengan PD0325901) menunjukkan pengurangan yang ketara dalam invasi dan trend yang sama diperhatikan dalam halangan bagi formasi tiub. Keputusan ini mencadangkan keputusan yang berbeza diperolehi dalam gerak balas terhadap perencat-perencat yang mungkin disebabkan oleh model kajian yang berlainan dipilih. Kesimpilannya, keputusan kami menunjukkan bahawa angiogenesis bagi sel-sel TIME telah direncatkan apabila laluan MER-ERK dan/atau laluan PI3K-AKT telah disekatkan. Sebaliknya, aktiviti angiogenik bagi sel HMEC-1 telah direncatkan melalui sekatan laluan PI3K-AKT tetapi bukan laluan MEK-ERK.

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

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LIST OF ABBREVIATIONS

%	Percent
µ	Micro
AKT	v-akt murine thymoma viral oncogene homolog
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CR	Conserved region
DAPI	4û,6-diamidine-2û-phenylindole
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitrix oxide synthase
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
GDP	Guanosine diphosphate
Grb2	Growth factor receptor-bound protein 2
GPCR	G-protein-coupled receptor
GSK	Glycogen synthase kinase
GTP	Guanosine triphosphate
HER2	Human epidermal growth factor receptor 2
HIF-1Ŭ	Hypoxia-inducible factor-1 alpha
H-Ras	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
IC_{50}	Half maximal inhibitory concentration
IGF-1	Insulin-like growth factor 1
IRS1/2	Insulin receptor substrate 1 or 2
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MEK1/2	MAPK/ERK kinase 1 or 2
mTOR	Mammalian target of rapamycin
mTORC2	Rapamycin-insensitive mTOR complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NF-ŇB	Nuclear factor-ŇB
NIH	National Institutes of Health
PBS	Phosphate buffered saline
PDGFR	Platelet-derived growth factor receptor
PDK1	3-Phosphoinositide-dependent kinase-1
PH domain	Pleckstrin homology domain
PI	Propidium iodide
$PI(3,4)P_2$	Phosphatidylinositol 3,4-bisphosphate
$PI(3,4,5)P_3$	Phosphatidylinositol 3,4,5-trisphosphate
$PI(4,5)P_2$	Phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphatidylinositol 3-kinase

PIK3CA	Phosphatidylinositol 3-kinase catalytic subunit
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PKB	Protein kinase B
PTC	Papillary thyroid carcinoma
PTEN	Phosphatase and tensin homolog
Raf	v-raf-1 murine leukemia viral oncogene homolog
RPMI 1640	Roswell Park Memorial Institute 1640
RTKs	Receptor tyrosine kinases
SCLC	Small cell lung carcinoma
SD	Standard deviation
SDS	Sodium dodecyl sulfate
Ser	Serine
SH2	Src homology 2
SHIP	SH2 domain-containing inositol phosphatase
Src	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
TGF-ϐ	Transforming growth factor beta
Thr380	Threonine380
TSC1/2	Tuberous sclerosis complex 1/2
Tyr	Tyrosine
VEGFR	Vascular endothelial growth factor receptor

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Angiogenesis is a process in which new bloods vessels are formed (Mikirova, Casciari, & Riordan, 2010) and is characterized by a number of cellular events including endothelial cell migration, invasion and differentiation into capillaries. Oxygen and nutrients are delivered via blood vessels to every part of the body, and also nourish malignant tumors (Carmeliet & Jain, 2011). Tumor angiogenesis is a process based upon a sequence of interplay between endothelial cells and tumor cells. It plays an important role for local tumor progression and development of distant metastasis (Khodarev, 2003).

Active form of PI3K is an oncogene, and amplifications and mutations of PI3K are commonly found in many kinds of human cancers (Okumura *et al*., 2012). In endothelial cells, the PI3K/AKT pathway is also involved in angiogenesis through its regulation of nitric oxide (NO) signaling (Karar & Maity, 2011) and endothelial nitric oxide synthase (eNOS) which are important in VEGF-induced angiogenesis and vascular permeability (Fukumura *et al*., 2001). RAS/RAF/MEK/ERK pathway is deregulated in approximately one third of all human cancers (Neuzillet *et al*., 2014) and is involved in the promotion of tumor growth, invasion, angiogenesis, and metastasis (Emuss & Marais, 2008).

Recently, many studies have revealed the importance of PI3K/AKT and MEK/ERK signaling in angiogenesis. However, the role of these two signaling pathways in microvascular endothelial cells is less-well studied. In our study, two microvascular endothelial cell, namely human dermal microvascular endothelial cell (HMEC-1) and telomerase-immortalized microvascular cell (TIME) were selected. HMEC-1 is immortalized via transfection with a plasmid containing SV40A gene and has been used for a wide range of application in endothelial cell research and drug development. To represent the population of microvascular endothelial cell, TIME is also included in this study. These cell lines are immortalized and provide a convenient model for *in vitro* studies. The human endothelial cell line, HUVEC, is not immortalized and dies off after a number of passages.

In our study, we aim to investigate the role of PI3K/AKT and MEK/ERK pathway in angiogenesis by using tube formation assay and three dimensional (3D) spheroid invasion assay with TIME and HMEC-1. Our hypothesis is that, blockade of PI3K/AKT and MEK/ERK signaling pathway with small molecule inhibitors are able to suppress tube formation, phosphorylation of AKT and ERK, and inhibit cell invasion in a collagen-embedded three dimensional (3D) model.

Tube formation assay is chosen for this study due to its simplicity, rapidness, quantitative, reliability and more comprehensive than other *in vitro* assays. 3D spheroid invasion assay closely mimics invasion *in vivo* due to invasion occurring from cell clusters with well-established cell–cell interactions rather than from single cells. Therefore, it is a good model to study HMEC-1 and TIME cell invasion.

1.2 Objectives of Study

The objectives of the study were:

1. To investigate the effect of blocking PI3K/AKT and MEK/ERK pathways on tube formation by HMEC-1 and TIME by using small molecules inhibitors targeting phosphoinositide 3-kinase (PI3K), epidermal growth factor receptor (EGFR), insulin-like growth factor I receptor (IGF-1R), mammalian target of rapamycin (mTOR), mothers against decapentaplegic homolog 3 (SMAD3), and mitogen-activated protein kinase kinase (MEK).

2. To investigate the effect of blocking PI3K/AKT and MEK/ERK pathways on cell invasion by HMEC-1 and TIME in a 3-D spheroid invasion model by using small molecules inhibitors targeting PI3K, IGF-1R, EGFR, mTOR, Smad3, and MEK.

3. To investigate the effect of small molecules inhibitors targeting PI3K, EGFR, IGF-1R, mTOR, Smad3, and MEK on phosphorylation status of AKT and ERK by HMEC-1 and TIME.

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