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ESTABLISHMENT OF HYPOXIA-INDUCED INVADOPODIA MODEL AND PHENOTYPE CHARACTERISATION OF MDA-MB- 231 BREAST CANCER CELL LINE

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By

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Breast cancer is one of the most prevalent cancers diagnosed in women. The majority of mortalities attributed to cancer are due to a critical process in which the cancer cells invade and migrate away from the primary site (i.e. the Breast) to another site such as the brain and bones of which is termed metastasis. One of the mechanisms or tools that tumour cells utilize for the invasion of cancer cells is through the degradation of the extracellular matrix (ECM) by specific actin-foot protrusions characterized as invadopodia. In solid tumours, the invasiveness of cancer cells markedly increases due to hypoxia (low oxygen) that exists in 80% of cancer patients. Previous reports have investigated several mechanisms of invadopodia formation, such as invadopodia components, signalling pathways, and microenvironments. However, limited information is available regarding the utilization of carbon and nitrogen sources in hypoxia-induced MDA-MB-231 cells that may be required for invadopodia formation. Therefore, to achieve the primary objective of this study, a number of methods were employed. First, an invadopodia assay was used to determine the effect of the passage number on invadopodia formation. This was also used to determine the effect of the hypoxia condition on the invadopodia formation treated with 0.5 mM DMOG and incubated in a hypoxia chamber. The second method was the western blot technique. This method was used to investigate the expression of the fundamental proteins of hypoxia, including the hypoxia-inducible factor 1α (HIF-1α) and the vascular endothelial growth factor (VEGF).

In addition, to detect the expression of the essential proteins involved in invadopodia formation, including matrix metalloproteinase-2 (MMP-2), the Rho guanine nucleotide exchange factor 7 (β-PIX) under hypoxia was also used. The third method, Phenotype microarray for Mammalian cells (PMM), was used to ascertain the essential chemical substrates as a nutrient for MDA-MB-231 cells in forming invadopodia

under hypoxia. This powerful tool provides a platform to extensively analyse living cellular phenotypes in response to microenvironment changes or chemical treatments through 96 well plates preloaded with carbon-energy and nitrogen substrates. PMM technology is a new colorimetric assay which is used to measure the redox energy generated when cells oxidise chemical substrates.

The results of this study showed that invadopodia formation was significantly affected by the cell line passage number making the cells unable to accomplish gelatin degradation in passage numbers 15 to 35. Therefore, for the following experiments, a low passage number was considered in order to achieve accurate results. The results also revealed that the hypoxia condition using 1% O_2 in the hypoxia chamber and 0.5 mM DMOG treatment led to an increase in the number of cells forming invadopodia and induced gelatin degradation. At the molecular level, the western blot analysis proved that HIF-1α in normoxia was degraded while under hypoxia, it dramatically increased.

Furthermore, VEGF, MMP-2 and β-PIX levels significantly increased in hypoxia compared to normoxia. Here, the proteins played an essential function in invadopodia formation. Finally, the PMM results elucidated that 11 chemical substrates were strongly nourished by MDA-MB-231 cells as a single source for survival under hypoxia condition, such as Dextrin. These substrates could potentially be required for invadopodia formation under a hypoxic condition.

Accordingly**,** these substrates could prove to be potential energy sources for cancer invasion. The findings of this study also propose utilizing certain types of carbon sources such as dextrin as drug carriers in order to enhance the effectiveness of chemotherapy against the hypoxia region and invadopodia formation. As with many research studies, there are certain limitations. However, the author of this study believe that the findings will offer a useful starting point in the cancer invasion field of study, particularly invadopodia formation. Future work could be undertaken to confirm the findings of this study by using a phenotype microarray on other types of highly invasive cells and to confirm the effect of each significant chemical substrate on invadopodia formation.

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

PEMBENTUKAN MODEL INVADOPODIA CETUSAN HIPOKSIA DAN PENCIRIAN FENOTIP TITISAN SEL KANSER BUAH DADA MDA-MB-231

Oleh

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Kanser buah dada merupakan salah satu kanser yang paling tersebar luas dalam kalangan wanita seluruh dunia. Kebanyakan mortaliti kanser disebabkan oleh proses kritikal di mana sel kanser menyerang dan berhijrah jauh daripada tapak utama (Buah dada) ke tapak lain seperti otak dan tulang, iaitu proses ini dikenali sebagai metastasis. Salah satu alat yang digunakan sel tumor untuk serangan sel kanser ialah degradasi matrik ekstraselular (ECM) oleh bonjolan tapak aktin yang dicirikan sebagai invadopodia. Dalam tumor tumpat, serangan sel kanser meningkat secara berbeza disebabkan hipoksia (oksigen rendah) yang wujud dalam 80% pesakit kanser. Laporan terdahulu telah mengkaji beberapa mekanisme pembentukan invadopodia seperti komposisi invadopodia, laluan berisyarat, dan mikropersekitaran. Walau bagaimanapun, pembentukan terhad yang sedia ada berkaitan utilisasi sumber karbon dan nitrogen dalam sel MDA-MB-231 cetusan hipoksia mungkin diperlukan bagi pembentukan invadopodia. Oleh sebab itu, bagi mencapai ojektif utama kajian , kaedah berikut telah dijalankan , kaedah pertama , asai invadopodia, digunakan untuk menentukan kesan bilangan saluran ke atas pembentukan invadopodia dan menentukan kesan keadaan hipoksia yang dirawat dengan 0.5 mM DMOG dan dieramkan dalam ruang hipoksia. Kaedah kedua, teknik blot barat, digunakan untuk menyelidiki ekspresi protein asas hipoksia termasuk faktor cetusan hipoksia 1α (HIF-1α) dan faktor pertumbuhan endotelial vaskular (VEGF). Tambahan lagi, bagi mengesan ekspresi protein penting yang terlibat dalam pembentukan invadopodia termasuk matrik metaloproteinase-2 (MMP-2) dan faktor pertukaran Rho guanina nekleade 7 (β-PIX) di bawah hipoksia juga. Kaedah ketiga, mikroasai Fenotip bagi sel mamalia (PMM) digunakan untuk menentukan substrat kimia penting sebagai nutrien bagi sel MDA-MB-231 bagi pembentukan invadopodia di bawah hipoksia. Alat paling kuat ini menyediakan landasan bagi penganalisisan fenotip selular hidup yang komprehensif sebagai respon kepada perubahan mikropersekitaran atau rawatan kimia melalui piring berlubang 96 yang diprabeban dengan tenaga karbon dan substrat nitrogen.

Tambahan lagi, teknologi PMM merupakan sebuah asai kolometrik baharu yang mengukur tenaga redoks yang terhasil ketika sel mengoksida substrat kimia. Dapatan kajian menunjukkan bahawa pembentukan invadopodia sangat dipengaruhi oleh bilangan saluran titisan sel yang menyebabkan sel tidak berupaya untuk melaksanakan degradasi gelatin dalam saluran nombor 15 hingga 35. Oleh sebab itu, bagi eksperimen berikutnya, bilangan saluran yang rendah telah diambil kira bagi mendapatkan keputusan yang tepat. Dapatan juga menunjukkan bahawa keadaan hipoksi menggunakan 1% O_2 dalam ruang hipoksia dan 0.5mM rawatan DMOG mengakibatkan peningkatan bilangan sel pembentukan invadopodia dan mencetuskan degradasi gelatin. Pada tahap molekular, analisis blot barat mengesahkan bahawa HIF-1α dalam normosia telah dinyah gred manakala di bawah hipoksia meningkat secara drastik. Tambahan lagi, tahap VEGF, MMP-2 dan β-PIX secara signifikan meningkat dalam hipoksia berbanding dengan normosia. Protein tersebut memainkan peranan yang penting dalam pembentukan invadopodia. Akhirnya, dapatan PMM menjelaskan bahawa 11 substrat amat diperkaya oleh sel MDA-MB-231 sebagai sumber tunggal bagi kelangsungan di bawah keadaan hipoksia seperti Dekstrin.

Substrat tersebut mungkin berpotensi diperlukan bagi pembentukan invadopodia di bawah keadaan hipoksia. Kesimpulannya, substrat tersebut mungkin merupakan tenaga yang berpotensi untuk serangan kanser. Dapatan juga mengesyorkan peluang untuk menggunakan beberapa jenis sumber karbon seperti dekstrin sebagai pembawa bagi menggalakkan keberkesanan kimoterapi terhadap bahagian hipoksia dan pembentukan invadopodia . Kajian ini jelasnya mempunyai beberapa limitasi. Di samping mempercayai bahawa dapatan ini mungkin merupakan titik permulaan dalam bahan serangan kanser terutama pembentukan invadopodia. Penyelidikan masa hadapan seterusnya harus membangun dan mengesahkan dapatan awal ini dengan menggunakan mikroasai fenotip ke atas jenis sel yang amat invasif lain dan mengesahkan kesan setiap substrat kimia yang signifikan ke atas pembentukan invadopodia.

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

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This is to confirm that:

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LIST OF ABBREVIATIONS

mM	Milli Molar
µL	Micro liter
ATCC	American type culture collection
CO_2	Carbon dioxide
DMEM	Dulbecco‟s Modified Eagles Medium
DMOG	Dimethyloxaloylglycine
DMSO	Dimethyl sulfoxide
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
HIF	Hypoxia Inducible Factor
DNA	Deoxyribonucleic acid
HRP	Horseradish Peroxidase
KDa	Kilodalton
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteases
MT1-MMP	Membrane type-1 matrix metalloproteases
PBS	Phosphate Buffered Saline
PHD	Prolyl Hydroxylase Domain Protein
VHL	Von Hippel-Lindau protein
RPMI	Roswell Park Memorial Institute medium
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
VEGF	Vascular endothelial growth factor

TNBC	Triple negative breast cancer
MMP-2	Matrix metalloproteinase-2
PMM	Phenotype microarray for mammalian cells
HER -2	Human epidermal growth factor receptor 2
ER	Estrogen receptors
PR	Progesterone receptors
2D	2 Dimension
3D	3 Dimension
EMT	Epithelial-mesenchymal transition
N_2	Nitrogen
ATP	Adenosine triphosphate
NADH	Nicotinamide adenine dinucleotide
ddH_2O	Double distilled water
NaHCo3	Sodium bicarbonate
BSC	Biosafety cabinet
CGM	Complete growth media
RIPA buffer	Radioimmunoprecipitation assay buffer
GFP	Green fluorescence protein

CHAPTER 1

INTRODUCTION

Breast cancer is recognised globally as one of the most commonly occurring cancers found in women and is the second most common cancer overall. In 2018, there were over two million new cases of breast cancer reported (WHO, 2018) and is sadly the leading cause of mortality found among women in Malaysia. The percentage of breast cancer patients compared to other cancers is 31% and is commonly found in women with only 1% of males diagnosed with this form of cancer. Close to 3,500 breast cancer cases are detected each, and about 1 in 20 women are at risk of contracting breast cancer (Yip *et al.,* 2006).

Numerous studies have revealed that the risk of contracting breast cancer is due to a combination of many factors, such as environmental changes (Kamińska *et al.,* 2015). The most aggressive form of breast cancer is triple-negative breast cancer (TNBC), which contributes to between 15 and 20% of all breast cancer cases. The leading cause of mortality among breast cancer patients is due to the spread of cancer cells to distant organs through a process called metastasis. Metastatic breast cancer is classified as stage 4 of breast cancer where cancer has spread to other parts of the body which typically includes the lungs, liver, bones or brain (Hassan *et al.,* 2017).

The spread of cancer normally occurs through one or more stages, for example; (1) the cancer cells invade nearby healthy cells having the ability to replicate more cancer cells, and (2) the cancer cells penetrate the circulatory or lymph system where they then travel through the walls of nearby lymph vessels or blood vessels. This is followed by the migration of these cells through blood circulation. The lymph system and the bloodstream also carry cancer cells to other parts of the body (Xie *et al.,* 2017). Cancer cells will refrain from moving any further once they are embedded in the capillaries at distant locations and divide and migrate into the surrounding tissues. Consequently, new small tumours grow in which cancer cells form small tumours at the new location called micrometastasis (Paz *et al.,* 2014). Statistical data has shown that 90% of cancer mortalities result from metastasis (Lujambio *et al.,* 2008). Breast cancer is potentially curable under current treatment unless the cancer cells are not metastasis to distant organs (Xie *et al.,* 2017).

One of the pre-requisites of cancer metastasis is the ability of cancer cells to break tissue barriers and to invade surrounding tissues. This step is believed to occur through the assistance of actin-rich protrusions called invadopodia (Paz *et al.,* 2014). These finger-like protrusions are present only in highly invasive cancer cells, including MDA-MB-231 cells. Previous studies suggested that the primary role of invadopodia is to degrade the ECM, which contributes to the process of metastasis, such as intravasation and extravasation (Lee *et al.,* 2014).

In a solid tumour, the aggressiveness and angiogenesis of cancer cells increase due to hypoxia (low oxygen) that exists in the majority of malignant tumours, which are known to resist chemotherapy and radiotherapy (Muz *et al.,* 2015). The generation of a hypoxic condition and activation of its main effector, hypoxia-inducible factor-1 (HIF-1), are common features found in metastatic cancers. (Hockel and Vaupel, 2001). Hypoxia enables several events to occur in the tumour microenvironment leading to the expansion of an aggressive phenotype from the heterogeneous tumour cells, promoting lethality in cancer patients (Hockel and Vaupel, 2001).

Previous studies have investigated the molecular components and signalling of invadopodia. Most of the components and signalling are elevated due to the expression of the hypoxia-inducible factor-1α (HIF-1α), which is considered as a master transcriptional regulator of the adaptive response to hypoxia. HIF-1α activates several proteins, including the vascular endothelial growth factor (VEGF) (Parekh and Weaver, 2016; Artym *et al*., 2006). Studies have also identified a few molecular players that have essential roles in the matrix-degrading ability of invadopodia such as MMPs, N-WASp, Cortactin, β-PIX, Arp2/3, LPA1 receptor and EGFR cooperation (Harper, *et al.,* 2018; Kumar *et al*., 2018; Munoz-Najar *et al.,* 2006; Hashim *et al.,* 2013).

However, one area that has yet to be investigated is the utilization of carbon sources in which MDA-MB-231 cells are consumed as an energy provider before the formation of invadopodia. Therefore, to extend the study on hypoxia-induced invadopodia formation in MDA-MB-231 cells, a phenotype microarray is used. As a new technology, it is currently used in determining the phenotypic changes in living cells through the utilization of chemical substrates; termed as a phenotype microarray for mammalian cells (PMM). PMM can be described as analogous two-dimensional array technology that provides a specific 96-well plate system pre-loaded with carbon energy and nitrogen substrates in each well. Since cancer cells require sufficient carbon energy and nitrogen sources in order to grow and metastasise, the investigation of the utilisation of carbon in cancer cells may add further insight into cancer research (Yin *et al.,* 2012). PMM has four different plates, each containing essential carbon energy and nitrogen sources, beginning from PM-M1 to PM-M4 (Bochner *et al.,* 2011).The research of this study was undertaken based on the existing problem associated with elusive metastasis treatment especially in tumour hypoxia, where the hypoxic region is resistant to chemotherapy and radiotherapy treatment (Sowa *et al.,* 2017). Notably, while the survival rate of patients diagnosed with breast cancer metastasis remains low (Yates *et al.,* 2017), this could possibly be due to the lack of understanding concerning the metabolism changes within the metastatic cancer cells. Especially in controlling the invasion and migration of aggressive cancer cells to distant sites within the body (Wang *et al.,* 2017).

The general objective of this study is:

- To develop a hypoxia-induced invadopodia model and phenotype characterization of the MDA-MB-231 breast cancer cell line.

Specifically, in achieving the primary objective of this study, a number of supporting objectives are presented:

- To investigate the effect of the passage number on invadopodia formation.
- To identify the invadopodia formation in normoxia and hypoxia.
- To identify the expression level of HIF-1α, VEGF, β-PIX and MMP-2 expression in a hypoxic condition.
- To characterize the utilization level of carbon and nitrogen sources in hypoxia-induced MDA-MB-231 cells that form invadopodia.

Accordingly, it is hypothesised that invadopodia formation absent in cells with high passage number and hypoxia conditions will increase the number of cells to form invadopodia and increase gelatin degradation. It is also anticipated that the expression levels of the following proteins HIF-1α, VEGF, β-PIX, and MMP-2 will be enhanced in hypoxia. Moreover, some types of chemical substrates in PMM plates that include carbon sources will increase in hypoxia as a single source for the survival of MDA-MB-231 cells.

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BIODATA OF STUDENT

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LIST OF PUBLICATIONS

Hamad, H. A., Kqueen, C. Y., & Hashim, N. F. M. (2018). Invadopodia Formation is a Critical Step in Cancer Cell Invasion: The Effect of Passage Number on Invadopodia Formation in MDA-MB-231 Breast Cancer Cell Line. *Life Sciences, Medicine and Biomedicine*, *2*(3).

Phytochemical Ability in Inhibition of Invadopodia Formation and Hypoxia-Inducible Factor 1 in Metastasis of Hypoxic Breast Cancer Cells. Review paper, accepted in Malaysian Journal For Medicine and Health Sciences. Scopus indexed journal.

Identification of Potential Chemical Substrates as Fuel for Invadopodia Formation in Hypoxia-Induced MDA-MB-231 Breast Cancer Cell Line. Major revision received from cells Journal (Q1, IF: 5.656).

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