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行政院衛生署九十七年度科技研究計畫

探討肺癌細胞腦部轉移之分子機轉
Novel Signal Pathways involved in Lung Cancer Brain Metastasis

研 究 報 告

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中文摘要

關鍵詞：肺癌, 腦轉移, CXCR4, 訊息傳導路徑, 微環境

這份研究計劃的主要目標是要了解腦部微環境對腦部轉移的肺癌細胞其生物行為的影響。由於我們實驗室先前的研究成果顯示活化的Akt 會誘發CXCR4的表現，並進而促進乳癌轉移；阻斷Akt 的活性可抑制轉移的可能。目前的計劃，將試驗CXCR4在 "肺癌腦轉移" 中是否亦扮演一重要的角色。此項研究計畫提出假設認為當轉移的肺癌細胞穿越血腦屏障(BBB)時，腦部組織的微環境會引起Akt 的活化，進而誘發CXCR4的表現。在此項研究計畫中，我們也將深入研究CXCR4的表現情形是否會促進腦部轉移的肺癌細胞在轉移處的存活能力，並探討CXCR4在腦部轉移的肺癌細胞和其他腦部微環境細胞之間的交互作用。為了達成這樣的目標，我們將重點放在 CXCR4 如何利用新的訊息傳導路徑影響癌化程度、肺癌的腦轉移，並且建立了五個確切的目標(five SPECIFIC AIMS) :

目標一：研究在肺癌細胞穿越血腦屏障(BBB)時CXCR4的生物功能為何。

目標二：對在肺癌細胞腦部轉移的微環境中CXCR4的功能為何以體外試驗模式進行探討。

目標三：利用肺癌細胞腦轉移的動物實驗，證實CXCR4在肺癌腦轉移中的角色，並對CXCR4表現量與人類肺癌病人是否發生腦部轉移潛力的臨床相關性加以研究。

目標四：研究Akt/mTOR&GSK3β訊息傳導路徑於腦轉移肺癌和腦轉移肺癌微環境中的角色。

目標五：證實Akt/mTOR&GSK3 β 訊息傳導路徑於體內腦轉移動物實驗中的重要性，及與人類肺癌病人腦轉移發生的相關性。

根據以上目標，我們經由 Intracardiac 注射 PC14 肺癌細胞株進入 SCID 小鼠中，並在 8~12 週時，取出已經轉移到腦部的 PC14 細胞(PC14BM)繼續進行繼代培養，以期得到高度腦轉移的 PC14 肺癌細胞株。此外我們也比較了 PC14 與 PC14BM 兩株細胞之間 CXCR4 與 VEGF-A 的差異。

英文摘要

Keywords：lung cancer, brain metastasis, CXCR4, signaling pathway, microenvironment

The primary goal of this research project is to understand the influence of brain microenvironment to the biologic behavior of brain metastatic lung cancer cells. Previous work from our lab has shown that activated Akt induces CXCR4 expression and subsequently contributes to the breast cancer lung metastasis, and the blockage of Akt activity inhibits metastatic potentials. The current proposal will test whether CXCR4 also plays a role in the brain metastasis of lung cancer. We now hypothesize that when metastatic lung cancer cells pass through the Blood-Brain Barrier (BBB), the microenvironment of brain will cause the activation of Akt, which in turn induce the expression of CXCR4. We will also ask whether the elevated CXCR4 in the brain metastatic lung cancer cells will promote the interaction between cancer cells with the brain microenvironment and the survival of the cancer cells in the host environment. To reach the goal, we will focus on how CXCR4 may interact with the novel signaling pathways and exert effects on tumor progression and brain metastasis in lung cancer and five SPECIFIC AIMS are proposed.

SPECIFIC AIM 1: To investigate the biological function of CXCR4 in lung cancer cells to cross the Blood-Brain Barrier (BBB) *in vitro*.

SPECIFIC AIM 2: To study the biological function of CXCR4 in microenvironment of brain metastatic lung cancer *in vitro*.

SPECIFIC AIM 3: To validate the role of CXCR4 in lung cancer brain metastasis in *in vivo* brain metastasis animal model and clinical correlation of the expression of CXCR4 with the metastatic potential of human lung cancer.

SPECIFIC AIM 4: To study the role of Akt/mTOR & GSK3 β signaling pathways in brain metastatic lung cancer and microenvironment of brain metastatic lung cancer

SPECIFIC AIM 5: To validate the importance of Akt/mTOR & GSK3 β signaling pathways in brain metastasis animal model and the correlation with the human lung cancer brain metastasis tissue.

According to these specific aims, we have intracardiac injected PC14 lung cancer cell into SCID mice. We collected the PC14 cell which migrated to the brain for primary culture after 8 to 12 weeks to get the highly brain metastasis PC14 lung cell lines (PC14BM). Furthermore, we compared the difference of CXCR4 and VEGF-A expression between PC14 and PC14BM cells.

前言：

Lung cancer is one of the leading causes of malignancy-related mortality worldwide. The virulence of this cancer is mediated in part by the specific and aggressive metastatic pattern of primary neoplastic cells to regional lymph nodes, liver, adrenal glands, contra-lateral lung, brain and the bone marrow. Metastasis is a complex pathophysiological process that is highly organ selective and involves numerous interactions between the cancer cells and the host (1, 2). Although many molecules have been implicated in cancer metastasis, the detailed mechanism of organ-specific tumor metastasis is still not completely understood. The primary goal of this research project is to understand the influence of brain microenvironment to the biologic behavior of metastatic lung cancer cells. Previous work from our lab has shown that activated Akt induces CXCR4 expression (3, 4). The induced CXCR4 expression contributes to the breast cancer lung metastasis, and the blockage of Akt activity inhibits metastatic potentials (5). We now hypothesize that when metastatic lung cancer cells pass through the brain, the microenvironment of brain will cause the activation of Akt, which in turn induce the expression of CXCR4. The induction of CXCR4 will contribute to the brain metastases of lung cancer cells through interaction with its ligand SDF-1α which is selectively expressed in different stage during the CNS development. We will also ask whether the elevated CXCR4 in the brain metastatic lung cancer cells will promote the interaction between cancer cells with the brain microenvironment and the survival of the cancer cells in the host environment. We will explore the signaling pathways that may contribute to the success of cell proliferation and angiogenesis of metastatic lung cancer cells in brain contribute by the brain environment, such as astrocyte and brain endothelial cells.

Specific Aim 1: To investigate the biological function of CXCR4 in lung cancer cells to cross the Blood-Brain Barrier (BBB) *in vitro*.

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Specific Aim 3: To validate the role of CXCR4 in lung cancer brain metastasis in *in vivo* brain metastasis model and clinical correlation of the expression of CXCR4 with the metastatic potential of human lung cancer.

Specific Aim 4: To study the role of Akt/mTOR & GSK3β signaling pathways in brain metastatic lung cancer and microenvironment of brain metastatic lung cancer

Specific Aim 5: To validate the importance of Akt/mTOR & GSK3 β signal pathways in *in vivo* brain metastasis animal model and the correlation with the human lung cancer brain metastasis tissue. In this project, we will identify novel molecular mechanisms involved in lung cancer brain metastasis, both in vitro and in vivo. These findings will be important for the further investigations of novel target therapeutic strategy.

The prognosis of brain metastatic lung cancer

In most cases, central nervous system (CNS) involvement occurs in the late stage of metastatic lung cancer. Usually, patients are already found to have lungs, liver, or bone involvement by the time CNS metastasis is diagnosed. Mean survival from diagnosis of a brain metastasis varies from 2 to 16 months. The mean 1-year survival is estimated only about 20% (12). A prognostic index for brain metastases was formulated by the Radiation Therapy Oncology Group (RTOG), based on the study of 1,200 patients with a variety of solid tumors. Patients with age less than 65-years-old who have controlled primary tumor without extracranial metastases and a better general performance status (Karnofsky performance score greater than 70) usually have better outcomes, with a median survival of 7.1 months. Whereas, patients with an older age and their Karnofsky performance score less than 70 have much more poorer outcomes, with a median survival of only 2.3 months (13). Other favorable prognostic factors include the presence of a solitary brain metastasis, and a longer disease-free interval (13, 14). However, as systemic therapies improve, control of extra-cranial disease may no longer be an important predictive factor for lung cancer patients with CNS metastases. With the control of systemic disease, the incidence of CNS involvement increases, and more of these breast cancer patients are died because of the progressive CNS disease (15).

The prognosis of brain metastatic lung cancer

Treatments for brain metastases of lung cancer include corticosteroids, whole brain radiation therapy, surgical resection, stereotactic radiosurgery and chemotherapy. Corticosteroids are used to relief symptoms by decreasing cerebral edema surrounding brain metastases (16). Whole brain radiation therapy (WBRT) is considered the most common choice of treatment for patients who present with multiple brain metastases. Patients with solitary brain metastasis that are not qualified for either surgical resection or stereotactic radiosurgery are often treated with WBRT. WBRT is able to control neurologic symptoms and therefore improve the quality of life in approximately 75 to

85% of patients. In addition, WBRT is able to prolong the mean survival, compared with corticosteroids alone (16). Surgical resection of brain metastases allows for pathologic diagnosis of the intracranial disease. Besides, it may improve neurologic symptoms and increase quality of life by immediate decompression of tumor mass effect. Surgical resection also improves the overall median survival compared to the supportive care alone. In patients with a single surgically accessible metastasis, good performance status, and stable or absent extracranial disease, there is a survival advantage for surgery, especially with the combination approach of surgery and WBRT, over WBRT alone (16). However, in patients with multiple brain metastases, the role of surgical management is currently considered limited, unless there is an obvious symptomatic lesion (17-19). Stereotactic radiosurgery (SRS) uses either a linear accelerator or multiple cobalt-60 sources to deliver focal radiation to areas smaller than 3.5 cm, which minimizes radiation exposure to the normal surrounding tissues. Because SRS is less invasive than surgical resection, it is given to patients who cannot tolerate surgery or have surgically inaccessible lesions. SRS involvement increases the overall median survival in breast cancer patients with brain metastases. Study showed that the combination approach of SRS and WBRT significantly improved the overall survival of patients with single brain metastasis. However, in spite of the fact that such combination approach could improve the overall performance, it has no survival advantage for patients with multiple brain metastases (16).

Generally, chemotherapy has not yet been considered a useful strategy in the management of brain metastases because the tight junction of blood brain barrier precludes the entry of most chemotherapeutic agents into the CNS. However, some drugs did show the promise in combination with radiation therapy (16). For example, Efaproxiral, which can increase tumor oxidation and therefore increase the radiation sensitivity, demonstrated an improvement of median survival in cancer patients with brain metastases when used in combination with WBRT compared to those using WBRT alone (16, 20). Several new techniques to deliver chemotherapeutic agents are now being studied. For example, an attempt to place BCNU (carmustine) in the resection cavity at the time of surgery is under investigation. BCNU is an impregnated polymer wafer that can protect hydroxylasing and allow slow releasing of chemotherapeutic agents. This technique has been shown successfully in brain primary tumors and is now being studied for metastasis cancers (16, 21). Another newly developed technique is direct intracerebral microinfusion (convection-enhanced

delivery). This approach has been tested in the animal study and showed the effectiveness (22). However, human trials have not yet been conducted. A better understanding of the molecular events that lead to brain metastasis and of the complex interactions between metastatic cells and host factors is essential for the design of more effective cancer therapies.

The microenvironment of brain metastatic lung cancer--Blood brain barrier (BBB)

There is a growing body of evidence that the numerous interactions between the cancer cells and the host microenvironment play important roles in the progression and metastasis of cancers. The interactions of cancer cells with the primary microenvironment, including disruption of basement membrane and extracellular matrix, facilitate the metastatic cancer cells to escape from the primary tumor. While the interactions of cancer cells with a tissue microenvironment that is distant from the primary organ, for example the formation of new vascular networks and evasion of the host immune system, enable the colonizing in the distant site (43, 44). Vascular endothelial growth factor (VEGF), a heparin-binding glycoprotein which is considered to be the most selective mitogen for endothelial cells and also a vascular permeability factor, is expressed about four-fold higher in the primary breast cancer patients with brain metastasis compared to those without brain metastasis (45). In vitro study indicated that VEGF might contribute to breast cancer brain metastasis by enhancing the transendothelial migration of tumor cells through the down-regulation of endothelial integrity and increasing the adhesion of tumor cells onto the human brain microvascular endothelial cell (HBMEC) monolayer (46). Targeting endothelial cells with a VEGF receptor specific tyrosine kinase inhibitor reduced angiogenesis and restricted the growth of the brain metastases (47). These studies indicate that angiogenesis, especially the function of VEGF, is involved in promotion of cancer brain metastasis. Blood brain barrier (BBB) consists of astrocytes, pericytes, capillary endothelial cells and basement membrane. Astrocytes that form the blood brain barrier have tight junctions between each other, which can enclose the capillaries on all sides. Distinctive from endothelial cells of other organs, endothelial cells within the blood brain barrier is nearly leak-proof. They join together by connective elements or continuous tight junctions and are equipped with a selective substance permeability which allows only particles with a diameter of less than 20 nm to cross over. The structure of blood brain barrier is so constructed in order to build an effective shield against higher molecular substances and

organisms which may be harmful to brain function and let only necessary small substances present in the blood to immediately pass through (23). In cases of tumors burden, the tight junctions between the endothelial cells become stretched out and result in the increased vascular permeability, which allows circulating tumor cells to move out of the vessel and go into the brain. Studies showed that cancer cells that express high level of chemokine receptor CXCR4 can increase the permeability of brain endothelial cell and facilitate the invasion of these cancer cells into the brain (24). However, even with the increased permeability of brain endothelial cells in the present of tumor burden, blood brain barrier is still a formidable diffusion barrier. As a result, most systemic chemotherapeutic agents are too large to cross the blood brain barrier and result in a poor drug delivery. Therefore, new therapeutic approaches for metastatic brain tumors could be either to increase the permeability of blood brain barrier or to develop small molecular weight drugs. Also, an improved understanding of the interactions between tumor and epithelial cells could assist in the development of prevention strategies which aims to block the invasion of tumor cells into the brain. Human brain microvascular endothelial cell (HBMEC) is now widely used in vitro as a model system to mimic the in vivo human blood brain barrier. There are several characteristics of HBMEC indicating that it does maintain the signature properties of human brain endothelial cell that forms the blood brain barrier, including the formation of tubular-like networks on matrigel, the ability to uptake acetylated low-density lipoprotein (AcLDL) and to produce von Willebrand factor and γ -glutamyl transpeptidase endothelial-specific markers (24, 25). In about 4 to 5 days after plating, HBMEC can form the tight junction between each other, which can be detected by increased measurements of trans-endothelial electrical resistance (TEER) (26). HBMEC can be used in the transendothelial migration assay to study the invasion ability of tumor cells. In this assay, HBMEC is cultured in fibronectin-coated Boyden chambers for 5 days allowing the formation of tight junctions before the migration measurements. HBMEC can also be used to measure the adhesion ability of tumor cells (24). Although lacking the contribution of astrocyte in this system, it is so far one of the best methods to mimic blood brain barrier in vitro.

The role of CXCR4/SDF-1α pathway in cancer metastasis

Metastasis is a complex pathophysiological process that is highly organ selective.

Chemokines and their receptors regulate leukocyte migration to inflammation sites and play an important role in the regulation of hematopoiesis, homing of hematopoietic stem cell in bone marrow and T and B lymphocytes in lymphoid tissue, and in the trafficking of dendritic cells. Recently, it was suggested that chemokines and their respective receptors are involved in the development of targeted metastases of primary tumors. Recently, it was suggested that chemokine stromal cell derived factor-1α (SDF-1α, also known as CXC chemokine ligand 12, a kind of α-chemokine), and its specific receptor CXCR4 (a G protein-coupled seven-transmembrane receptor) are involved in regulating migration and metastasis of a variety of cancers, including breast and lung cancers (27-30). Chemokines such as SDF-1 α are released in high amounts by certain organs, such as lung, bone, and liver. Malignant cancer cells, which express the chemokine receptor CXCR4, invade the extracellular matrix and circulate in the blood and lymphatic vessels. The attraction between SDF-1 α and CXCR4 causes cancer cells to leave the circulation and migrate into organs with large amounts of chemokines, where cancer cells proliferate, induce angiogenesis, and form metastatic tumors (27). In the brain, SDF-1 α is selectively expressed both in the developing and mature CNS. In addition, the expression of CXCR4 is consistently higher in primary lung tumor cells than in normal lung epithelial cells. In vitro study showed that SDF-1 α could induce blood vessel instability, through an increased vascular permeability, resulted in the penetration of breast tumor cells through the human brain microvascular endothelial cells. Blockade of the CXCR4/SDF-1 α pathway with anti-CXCR4 antibody decreased transendothelial cancer migration as well as vascular permeability (24). In an in vitro model, it has further been shown that SDF-1α/CXCR4 may be involved in the penetration of blood brain barrier for breast cancer cells suggesting their possible roles in brain metastasis of breast cancer (30). The current proposal will test whether CXCR4 also plays a role in the brain metastasis of lung cancer.

Signaling pathways involve in cancer metastasis

The increased motility and invasiveness of cancer cells is the first phase of metastasis are reminiscent of epithelial-mesenchymal transition (EMT) during embryonic development. The plasticity generated by EMT is associated with the functional loss of E-cadherin, a cell-cell adhesion molecule that participates in a calcium-dependent interaction to form the epithelial adherent junction (31-33). The second phase of

metastasis (metastatic colonization) requires the reverse EMT process that is used by cancer cells to make up micrometastasis to establish a secondary carcinoma. It therefore makes sense that the initial invasion stage probably requires a rapid and effective repression of E-cadherin while the regrowth metastasis of secondary tumor requires the reexpression and maintenance of E-cadherin in the secondary carcinoma. The Glucogen synthase kinase 3 β (GSK3 β) is the upstream signal pathway involve in the regulation of E-cadherin expression (31-32). The phosphorylation of GSK3 β at Ser-9 leads to the inactivation of this protein and down expression of E-cadherin, regain of GSK3 β activity can induce the E-cadherin expression. We hypothesis that the dynamic change of GSK3 β activity may play an important role of the lung cancer tumor cell through systemic circulation to the brain and then penetrate the vessel endothelial cell to invasive brain parenchyma. Constitutive activation of cell survival signaling is a general mechanism underlying tumor development and resistance to therapy and constitutes a major clinical problem in cancer (34-36). One of the key survivals signaling mechanism is the Akt pathway that can be activated by oncogene. Hyperactivation of Akt is associated with resistence to apotosis, increased cell growth, cell proliferation and cell energy metabolism (37-42). Akt can potentially phosphorylate many proteins in mammalian cells. However, it remains to be determined which downstream effectors of Akt are most critical for the genesis of cancer. Several lines of evidence point to the two most evolutionarily conserved downstream effectors, the fork-head family of transcription factor, FOXO, and the mammalian target of rapamycin, mTOR. FOXO transcription factors, which inhibit mammalian cell proliferation, are directly phosphorylated and inactivation by Akt, whereas mTOR, which is associated with increased cell proliferation, is indirectly activated by Akt. One mechanism that Akt can activate mTOR is through direct phosphorylation of tuberous sclerosis complex 2 (TSC2). Tuberous sclerosis complex1 (TSC1) and TSC2 form a heterodimer with GTPase activity that inhibits the activity of Rheb, a small GTPase required for the mTOR activation. Upon activation, mTOR, which forms a rapamycin-sensitive complex with Raptor, increase mRNA translation via activation of S6 kinase and inhibition of eIF4E binding protein. Our preliminary results showed that brain metastasis human lung cancer cells have a higher Akt activity than the low metastatic parental lung cancer cells. In addition, the Akt activity of parental lung cancer cells was significantly increased after the cells were co-culture with murine brain endothelial cells or murine astrocytes

for 24 hours and the GSK 3 β activity is down regulated after 48 hours co-culture with brain endothelial cell. To identify the signaling pathways in lung cancer brain metastasis become an important issue. In this project, we will identify novel molecular mechanisms involved in lung cancer brain metastasis, both in vitro and in vivo. These findings will be important for the further investigations of novel target therapeutic strategy.

材料與方法

Specific Aim 1: To investigate the biological function of CXCR4 in lung cancer cells to cross the Blood-Brain Barrier (BBB) *in vitro*.

BBB, with its intact structure which is composed of a special kind of astrocyte and endothelial cells with tight junctions, precludes the entry of any substances which may harm brain functions. Therefore, cancer cell must first cross the Blood-Brain Barrier (BBB) to form the secondary lesion. To better understand the biological function of CXCR4 in the lung cancer cells to cross the BBB, *in vitro* transendothelial adhesion and invasion assays will be performed. Human brain microvascular endothelial cells (HBMEC) constitute the major component of the BBB and are critical in maintaining its structural and functional integrity. Therefore, HBMEC will be used directly as a model system for the *in vivo* human BBB. In this model, HBMEC forms tubular-like networks on matrigel and has the ability to uptake acetylated low-density lipoprotein (AcLDL), indicating that these cells maintains the signature properties of human BBB. These cells will also produce von Willebrand factor and γ-glutamyl transpeptidase endothelial-specific markers. We will examine and compare the invasive activity within a set of cell lines, PC14 *vs* PC14 BM (the brain metastatic clone of the PC14, in which the expression of CXCR4 is 1.58 fold higher than PC14), by *in vitro* transendothelial invasion assay. This experiment will tell us if brain metastatic lung cancer cell would have advantage to invade the BBB. If yes, we will further justify if the increased transendothelial invasive activity is through the upregulation of CXCR4. We plan to add CXCR4 blocking antibody in dose-increase manner to the transendothelial invasion assay. We will also use siRNA strategy to knock down the expression of CXCR4 in PC14 BM cell for the transendothelial invasion assay described above. If CXCR4 plays a role in lung cancer cell invasion through BBB, we will expect to see that the increased invasive activity in brain metastatic cell line (PC14 BM) will be inhibited by CXCR4-blocking antibody and CXCR4 siRNA. Furthermore, to determine if the SDF-1 expressed in the BBB acts as a chemoattractant which contribute to the metastatic lung cancer cells to cross the BBB through interacting with upregulated CXCR4 in these cancer cells, the normal *in vitro* invasion activity will be used as control. The same strategy described above will be used for the *in vitro* transendothelial adhesion assay.

Specific Aim 2: To study the biological function of CXCR4 in microenvironment of brain metastatic lung cancer *in vitro*.

Once the cancer cell passes through the BBB, it will still undergo processes like growth, survival from apoptosis and secondary angiogenesis to be able to form the metastatic tumor. The outcome of the metastatic process depends on multiple and complex interactions of metastatic cells with the organ microenvironment. To better understand the role of CXCR4 in interaction of the lung cancer cells to brain microenvironment, *in vitro* cancer cell-astrocyte adhesion assay and *in vitro* cancer cell-endothelial cell adhesion assay will be performed. We will examine and compare the adhesion activity to brain astrocyte and endothelial cell within PC14 and PC14 BM cell lines. This experiment will tell us if brain metastatic lung cancer cell would have advantage to adhere to the brain microenvironment.

If yes, we will further investigate if the increased adhesion is through upregulated CXCR4. We plan to add CXCR4 blocking antibody in dose-increase manner and use SiRNA strategy to knock down the expression of CXCR4 in PC14 BM cell to the adhesion assays described above. If CXCR4 plays a role in lung cancer cell adhering to the brain microenvironment, we will expect to see that the increased adhesion activity in brain metastatic cell line (PC14 BM) will be inhibited by CXCR4-blocking antibody and CXCR4 siRNA. During the metastaisis process, tumor cells must gain the ability to survive under adverse

conditions. Thus, we will next investigate whether the CXCR4 induced adhesion of lung cancer cells to the microenvironment would protect lung cancer cells from etoposide-induced apoptosis. PC14 and PC14 BM cells will be co-cultured with brain astrocyte or endothelial cell and then treat with etoposide. The viability of the cancer cell will be determined by FACS analysis. If the microenvironment of the brain would provide the protective effect on the brain metastatic lung cancer cells, we would expect to see less apoptotic cells in the PC14 BM-astrocyte and PC14 BM-endothelial co-culture system. Then CXCXR4 blocking antibody and CXCR4 siRNA will be used to the above assay to determine whether the protective effect of the brain microenvironment to the metastatic lung cancer cells is mediated by CXCR4.

Specific Aim 3: To validate the role of CXCR4 in lung cancer brain metastasis in *in vivo* brain metastasis model and clinical correlation of the expression of CXCR4 with the metastatic potential of human lung cancer.

PC14 BM and PC14 BM/siCXCR4 and parental PC14 will be injected into the nude mice through intra-internal carotid artery to develop the *in vivo* brain metastasis model. This approach will permit us to measure brain metastasis through circulation system.

We expect that PC14 BM which has the higher CXCR4 expression should enhance tumor growth in brain while the brain metastases will be reduced in PC14 BM/siCXCR4 transfectant cells in which CXCR4 expression is reduced. Specimens of lung cancer with or without brain metastases will be obtained from China Medical University Hospital, National Taiwan University Hosipital, and M. D. Anderson Cancer Center in Houston, Texas USA. CXCR4 expression pattern will be determined by immunohistochemisty in each sample. Cox multivariate regression analysis will be used to examine the significance of CXCR4 in predicting the outcome of brain metastases. The survival curve will be calculated by the method of the Kaplan and Meier. All tests will be two-sided and the level of significance will be set at 0.05.

Specific Aim 4: To study the role of Akt/mTOR & GSK3 β signaling pathways involved in the lung cancer cells brain metastasis and the interaction of cancer cell with the brain endothelial cell & brain astrocyte cell.

Three different lung cancer cell lines: parental lung cancer cell (PC14), lung cancer cell with malignant pleural effusion (PC14PE6), PC14 lung cancer cell with brain metastasis (PC14 BM) are carefully studied with western blot methods to detect the signal protein expression in these cells. The preliminary results revealed that the phosphorylation Akt and phosphorylation GSK3 β are over expression in PC14 BM cell which has the highly metastasis activity than PC14 cell. The other upstream and downstream signal pathway (such as growth factor receptor, m-TOR, S6 kinase, Erk,

E-cadherin) of Akt and GSK3 β will be further examined. The brain microvascular endothelial cell (Brain EC cell) constitutes the major component of the blood brain barrier (BBB) and is critical in maintaining its structural and functional integrity.

The brain astrocyte cell is the interstitial cell of the brain parenchyma and may play an important role in regulating cancer cell metastasis to brain microenvironment. Cancer cells invasion to brain involve sequential steps to interact with brain EC cell and brain astrocyte. We use the parental lung cancer cell (PC14) to co-culture with the brain endothelial cell & brain astocyte 6 hours, 12 hours, 24 hours, 48 hours than detect the time sequence expression of protein and try to determine the signal pathway interaction between the cancer cell and brain endothelial cell & astrocyte. As the preliminary result reveal that the Akt expression is highly expressed when co-cultured with the brain EC cell and astrocyte and the GSK 3 β activity has the dynamic change during the time sequence interaction. The E-cadherin expression related to the metastastic and invasion

activity of lung cancer cell will also be examined in this project. Cancer cell brain invasion involves a sequential series of critical steps, including adhesion to endothelial cells and migration toward the brain microenvironment than cancer cell survival, proliferation, and angiogenesis process. To better understand the biological function of the interaction of brain endothelial cell, astrocyte cell and lung cancer cell, we will examine and compare the invasive activity within two cell line, p-Akt expression lung cancer cell lines vs Akt siRNA stable transfection lung cancer cell line, by in vitro transendothelial invasion assay. This
experiment will tell us if Akt activating lung cancer cell would have the advantage to invade the blood brain barrier.

When lung cancer cell is co-cultured with brain endothelial or astrocyte in the transwell dish, we will use the commercial available PI3K inhibitor LY294003 (or other related compounds) to block the activity of Akt then study the migration and invasion activity of cancer cell. This experiment may help us to further prove the importance of PI3K/Akt pathway in cancer cell brain metastasis mechanism. Furthermore, to determine if the astrocyte could secrete some cytokine and attract the cancer cell to cross blood brain barrier than migrate into the brain environment, the cytokine assay will perform in vitro transwell lung cancer cell co-cultured with astrocyte system.

Specific Aim 5: To validate the importance of Akt signal pathway in vivo brain metastasis animal model and positive correlation with the human lung cancer brain metastasis tissue.

Parental lung cancer cell line will be injected into the nude mice through internal carotid artery to develop the in vivo brain metastasis mode. Brain metastasis lesion will use the histological stain to evaluate the protein expression in brain metastasis and parenchyma microenvironment. Use the stable transfection Akt siRNA parental lung cancer cell and these stable transfected cells will be inject into internal carotid artery of the nude mice and measure the numbers of the brain metastasis lesions, we expect the cancer cell brain metastasis ability will significant decrease in the Akt siRNA transfect ion cancer cells. Use the histochemical stain method to compare the protein expression of the human primary lung cancer and brain metastasis samples, there will be the high incidence of Akt expression in brain metastasis lesion. The incidence of brain metastases of lung cancer is increasing as the improvement of systemic disease is achieved. Because of the progressive neurological disability of brain metastases and the lack of effective

treatment due to the unique structure of blood brain barrier, brain metastases of lung cancer is becoming more important and urgent as it affects both the survival and quality of life of the patients. However, so far little is known about the mechanisms of lung cancer metastasis to the brain, as well as the interaction of the metastatic cancer cells with the surrounding microenvironment. An improved understanding of these mechanisms will help us to prevent the metastases of cancer cells to the brain, or to develop better therapeutic strategies for the brain metastases, therefore improve the survival and quality of life of lung cancer patients. The current proposal using the *in vitro* co-culture system has unraveled interesting phenomenon, suggesting that CXCR4, Akt, GSK3 β and E-cadherin may be involved in the tumor microenvironment of brain metastasis of lung cancer. Dissection of the signaling networks and it' s relationship with BBB and tumor microenvironment of brain metastasis of lung cancer will certainly help us understand better the deadly disease. Success of this current proposal may also help for identify possible clinical intervention for brain metastasis of lung cancer, which currently has no cure.

結果

In addition to the published work described above, we have also obtained significant progress to serve as preliminary results for the current grant application. In the following section, we will present the data directly relevant to the current proposal. It was suggested that SDF-1α/CXCR4 axis is involved in regulating migration and metastasis of a variety of cancers. The attraction between SDF-1α and CXCR4 causes cancer cells to leave the circulation and migrate into organs with large amounts of chemokines, where the cancer cells proliferate, induce angiogenesis, and form metastatic tumors. Figure 1 indeed validates that expression of CXCR4 and VEGF may contribute to lung cancer metastasis to brain tissue. Therefore, it is important and timely to further characterizes in detail for the proposed role of CXCR4 in lung cancer metastasis to brain and will be further pursued in the SPECIFIC AIMs 1-3.

The plasticity generated by EMT is associated with the functional loss of E-cadherin, a cell-cell adhesion molecule that participates in a calcium-dependent interaction to form the epithelial adherent junction. The second phase of metastasis (metastatic colonization) requires the reverse EMT process that is used by cancer cells to make up micrometastasis to establish a secondary carcinoma. It therefore makes sense that the initial invasion stage probably requires a rapid and effective repression of E-cadherin while the regrowth metastasis of secondary tumor requires the reexpression and maintenance of E-cadherin in the secondary carcinoma. The PC14 BM cell is established by injection of PC14 cell into mice internal carotid artery and select the parenchymal brain lesion for primary culture than re-injected the primary culture tumor cell into the mice internal carotid artery and repeat select the highly brain metastasis cell lines (PC14 BM). The prelimary data revealed that the PC14 BM could induce (80-100%) brain metastasis lesion by injecting the PC14 BM cell into mice internal carotid artery so we can compare the highly brain metastasis potential PC14 BM with it's parental cell PC14. We found that epithelial markers, such as E-cadherin and α-catenin, were dramatically decreased in brain metastatic lung cancer cells (PC14 BM) (Figure 2). Furthermore, the expressive level of mesenchymal markers, such as vimentin and N-cadherin, were significantly increased in PC14 BM cells (Figure 2). The Glucogen synthase kinase 3 β (GSK3 β) is the upstream signal pathway involve in the regulation of E-cadherin expression. The phosphorylation of GSK3 β at Ser-9 leads to the inactivation of this protein and down expression of E-cadherin, regain of GSK3 β

activity can induce the E-cadherin expression. Hyperactivation of Akt is associated with resistence to apotosis, increased cell growth, cell proliferation and cell energy metabolism. To define the expression level of GSK3 β and Akt in lung cancer with or without brain metastasis, PC14 and PC14 BM cells were co-culture with or without astrocytes or brain vascular endothelial cells and analyzed the activation status of GSK3 β and Akt by western blot assay (Figure 3 and Figure 4). We also defined the expression of epithelial-mesenchymal transition markers in lung cancer cells co-cultured with astrocytes or brain vascular endothelial cells. We found that lung cancer cells co-cultured with astrocytes or brain endothelial cells (Brain EC) dynamically altered the EMT process. Further investigation of mechanism will be continued in the SPECIFIC AIMs 4-5.

討論

Success of this project will help us to establish signaling networks to understand better the molecular mechanisms that may contribute to the cell proliferation and angiogenesis of metastatic lung cancer cells in brain contribute by the brain environment, such as astrocyte and brain endothelial cells. Identify novel molecular mechanisms involved in lung cancer brain metastasis, both *in vitro* and *in vivo*, will be important for the further investigations of novel target therapeutic strategy. We will also get anticipated research results as following:

1. The biological functions of CXCR4 in lung cancer cell to cross Blood-Brain Barrier and the effects on lung cancer brain metastasis will be identified.
2. The role of CXCR4 in the microenvironment of brain metastatic lung cancer cells will be investigated by study the interaction between brain metastatic lung cancer cells and brain environment, such as astrocyte and brain endothelial cells.
3. The *in vivo* biological role of CXCR4 in lung cancer brain metastasis will be identified by using animal model.
4. The clinical significance and correlation of the expression of CXCR4 in brain metastatic lung cancer patients will be clarified.
5. The signaling pathways involved in brain metastasis and EMT transition process of lung cancer cells will be identified, such as Akt/mTOR & GSK3 β signaling pathways.
6. The *in vivo* role of signaling pathways, such as Akt/mTOR & GSK3 β signaling pathways, in lung cancer brain metastasis will be identified by using animal model.
7. The clinical significance and correlation of identified signaling pathways in brain metastatic lung cancer patients will be clarified.

結論與建議(此係研究上的)

目前計畫執行期間僅六個月，根據先前的實驗資料，我們將進一步確認目前從動物體內取出之肺癌細胞株在腦轉移能力的差異，並以動物模式做更深入的探討。此外正在建立 siCXCR4 的肺癌細胞株，為後續的實驗作準備。

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圖、表

Figure 1

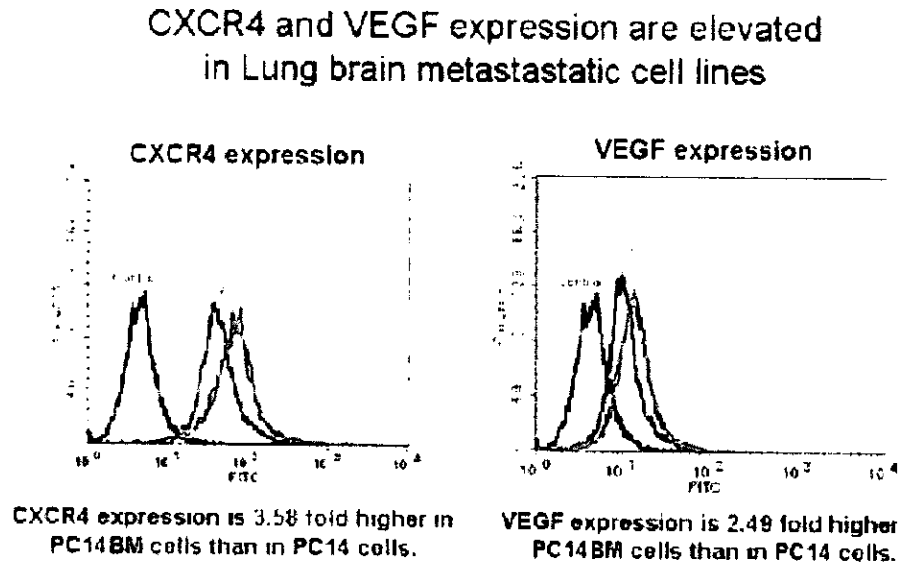


Figure 1, CXCR4 and VEGF-A are elevated in brain metastatic lung PC14 cancer cells (PC14 BM). FACScan analysis of expression of CXCR4 and VEGF protein in PC14 and PC14 BM lung adenocarcinoma cells.

Figure 2

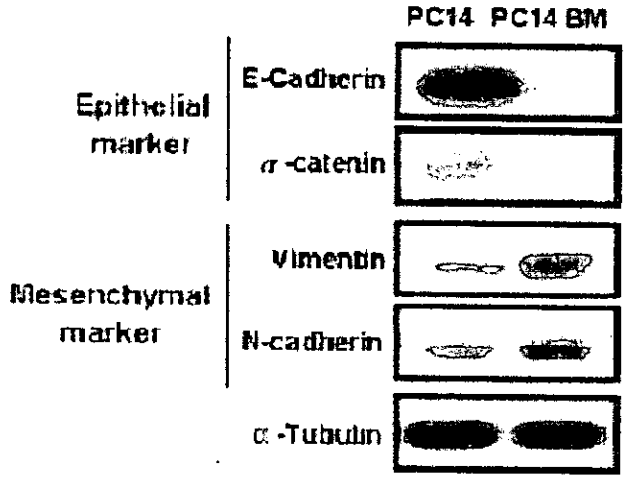


Figure 2, PC14 brain metastatic cells have higher EMT ability than PC 14 cells. The epithelial-mesenchymal transition activity of PC14 and PC14 BM cells were analyzed by western blot.

Figure 3

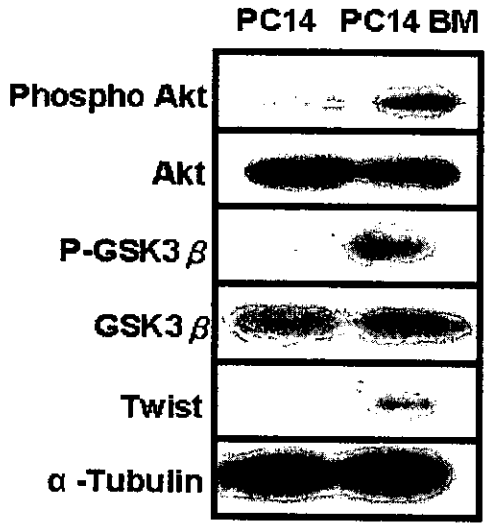
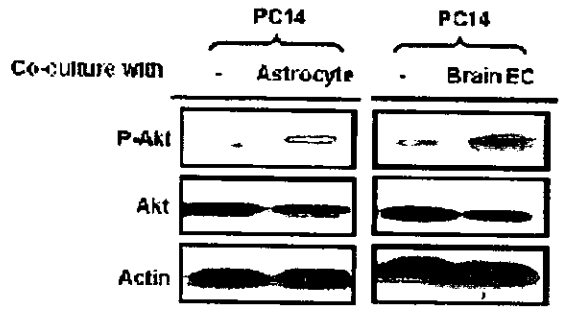


Figure 3, The expression of Akt and GSK3 β activity in PC14 and PC14 brain metastatic cells (PC14 BM) . Akt and GSK3 β activation were analyzed by western blot assay by specific anti-phospho-Akt and anti-phospho- GSK3 β antibodies in PC14 and PC14 BM cells.

Figure 4

(A)



(B)

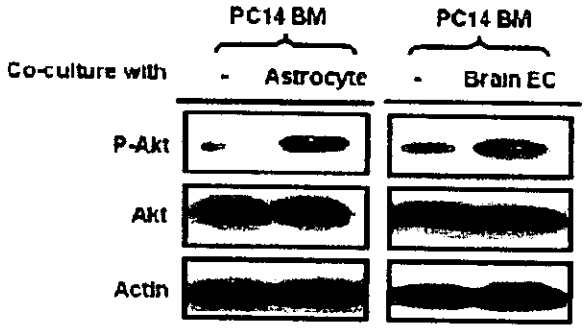


Figure 4, Co-culture with astrocytes or brain endothelial cells (Brain EC) increases Akt activation in lung cancer cells. Akt activation was analyzed by western blot assay by specific anti-phospho-Akt antibody in PC14 (A) and PC14 BM cells (B) co-culture with or without astrocytes (for 48 hours) or brain vascular endothelial cells (for 48 hours).

Figure 5

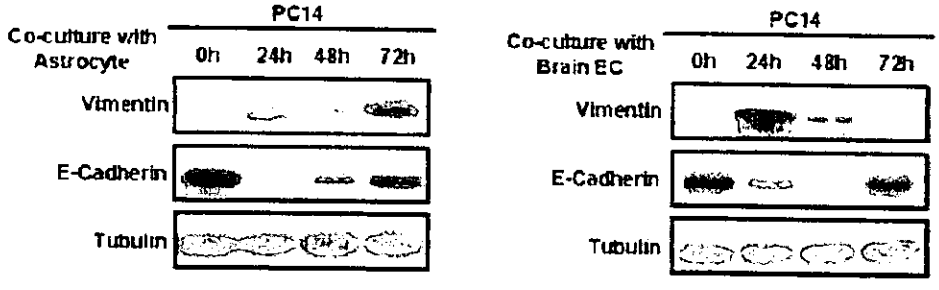


Figure 5, Co-culture with astrocytes or brain endothelial cells (Brain EC) dynamically altered the EMT process in lung cancer cells. PC14 cells were co-culture with astrocytes (left panel) or brain vascular endothelial cells (right panel) for indicated time. The expression of vimentin (mesenchymal marker) and E-cadherin (epithelial marker) were analyzed by western blot assay.