行政院國家科學委員會補助專題研究計畫成果報告

（計畫名稱）

利用以 DNA 微陣列技術在卵巢癌組織中所發現特定基因蛋白之臨床運用與基礎功能研究

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一、中文摘要

上皮性卵巢癌是婦科癌症中致死率第一位的疾病.這是因為大部分卵巢癌在轉移之前是沒有症狀的,所以三分之二的病例被發現時已經是癌症的後期.目前對上皮性卵巢癌的治療包括減積手術以及以 platinum 為主的化學治療然而,五年存活率仍小於百分之十. 我們利用 DNA 微陣列技術在卵巢癌組織中,發現特定基因蛋白 Edg4/Edg7 會在癌組織中大量表現,因此我們在本年度計畫中進行其臨床與基礎功能研究. 而 Edg4/Edg7 即為 *LPA* (lysophosphatidic acid)的特異性受體.在過去幾年的研究指出 *LPA* 在上皮性卵巢癌的發展中扮演很重要的角色. LPA 是人體內磷脂質的一員,它在人類婦癌中,包括卵巢癌.子宮內膜癌.以及子宮頸癌,被發現在病患血清中濃度有大量表現.在上皮性卵巢癌中大量表現的 LPA 接受體(Edg4/Edg7)和卵巢癌的低存活率有密切的關聯,而且 LPA 似乎可以加強腫瘤細胞的惡性度以及轉移的能力.雖然,尚未有一致的結論,在一些實驗的條件下, LPA 接受體(Edg4/Edg7)的過量表現似乎能導致對化學藥物的抗性.許多的研究顯示,抑制 LPA 接受體(Edg4/Edg7)的表現,能減低 LPA 接受體(Edg4/Edg7)過量表現細胞的惡性度.這些研究強烈顯示, LPA 接受體(Edg4/Edg7)是一個發展抗癌藥物時良好的標的,然而, LPA 接受體(Edg4/Edg7)在腫瘤惡化的過程中扮演何種角色目前仍然未被釐清.在本年度計畫中,我們發現在卵巢癌細胞之 LPA 接受體(Edg4/Edg7)的表現和 IL-6 有關,IL-6 是一個在卵巢癌中表現很強的因子.因此我們嘗試找出 LPA 在卵巢癌中調控 IL-6 表現的角色.我們發現 LPA 主要會透過卵巢癌中大量表現的 LPA 接受體(Edg4/Edg7)經由 Gi/PI3K/Akt 訊息傳遞途徑活化轉譯分子 NF-kB 促進調控 IL-6 的表現,此發現與研究成果已經於 93 年六月投稿於 SCI 級期刊 Carcinogenesis,標題為 **Up-regulation of interleukin-6 in human ovarian cancer cell via a Gi/PI3K-Akt/NF-{kappa}B pathway by lysophosphatidic acid, an ovarian cancer activating factor.並於** 2004 Oct 7 為 Carcinogenesis 接受.

關鍵詞：LPA、卵巢癌、IL-6

二、英文摘要

Bioactive lysophospholipid, lysophosphatidic acid (LPA), is consistently raised in the ascites of patients with ovarian cancer. Interleukin-6 (IL-6) is a pleiotropic cytokine, which is assumed to be involved in ovarian carcinogenesis. However, the regulation of IL-6 in ovarian cancer remains largely unknown. To elucidate the pathogenesis of ovarian cancer, this study investigated how LPA affects IL-6 production in ovarian cancer cells. Experimental results indicated that LPA stimulates IL-6 expression in all ovarian cancer cell lines tested, but not in normal ovarian surface epithelial (NOSE) cells, owing to the lack ōf LPA specific Edg4 and/or Edg7 receptors in NOSE cells. This work demonstrated that LPA transcriptionally activates IL-6 expression, which can be totally blocked by pertussis toxin, indicating that Gi-mediated signaling is critically involved in inducing IL-6 by LPA. Pharmacological and genetic inhibition assays revealed that Gi-mediated PI3K activation phosphorylated downstream Akt and subsequently induced NF-kB activation causes the induction of IL-6 by LPA in SK-OV-3 cells. In sum, dada presented here demonstrate that LPA is an important inducer of IL-6 and LPA regulated IL-6 expression via a Gi/ PI3K-Akt/ NF-kB pathway in ovarian cancer cells, providing molecular therapeutic targets for treating ovarian cancer.

Keywords: LPA、ovarian cancer、IL-6

三、緣由與目的

Epithelial ovarian cancer is characterized by the production of large volumes of ascites and rapid growth of solid intraperitoneal tumors. Malignant ascites from ovarian cancer patients have been found to contain ovarian cancer activating factors that promote tumor growth both in vitro and in vivo [1,2]. Among these, lysophosphatidic acid (LPA) accounts for most of the ability of ascites to activate ovarian cancer cells [3,4]. Various cells, including macrophage, mesothelial cells, activated platelets, endothelial cells, and ovarian cancer cells themselves, contribute to the raised concentrations of LPA in ascites [4-6]. LPA is a naturally occurring phospholipid that exhibits pleiotrophic biological activities via interaction with specific G protein-coupled receptors, LPA1/Edg-2, LPA2/Edg-4, and LPA3/Edg-7 [7]. Several lines of evidence suggest that LPA signaling is involved in the initiation, progression, and metastasis of ovarian cancer. LPA may increase ovarian cancer cell proliferation by directly increasing the level of cyclin D1 [8], and protecting ovarian cancer cells from apoptotic cell death induced by platinum, leading to a poorer prognosis associated for ovarian cancer [9]. LPA also increases production of potent growth factors, such as VEGF and IL-8, which facilitate tumor angiogenesis [10,11]. Furthermore, LPA is likely to promote matrix metalloproteinase activation and up-regulate urokinase plasminogen activator secretion in ovarian cancer cells, resulting in more aggressive behavior by extracellular stromal breakdown [12,13].

Interleukin-6 (IL-6) is a secreted, multifunctional glycoprotein. IL-6 acts through a hexametric cytokine receptor complex composed of an IL-6-specific receptor alpha chain (gp80) and a signal transducer gp130, and then triggers signaling cascades through the Jak/STAT, Ras/MAPK, and PI3K/Akt pathway [14]. Various arguments have indicated that IL-6 is important in the ovarian carcinogenesis. Moreover, in vitro experiments have shown that epithelial ovarian cancer cells constitutively produce IL-6, thus modulating host immune response to ovarian cancer [15,16]. Increased IL-6R alpha expression and constitutive STAT3 activation also have been found to associate with ovarian cancer proliferation [47]. In ovarian cancer patients, IL-6 level in serum and ascites was markedly elevated and appears to be a significant prognostic factor [17,18]. Additionally, elevated concentrations of IL-6 correlated with a poor initial response to paclitaxel chemotherapy, and ovarian cancer cells expressing IL-6 may prevent cisplatin-induced apoptosis [19,20]. Furthermore, IL-6 displayed increased chemotactic and/or chemokinetic activity and overall invasiveness for ovarian cancer cells [21]. Despite IL-6 being critically involved in ovarian carcinogenesis, the mechanism for the regulation of IL-6 in ovarian cancer remains largely mysterious.

Recently, LPA has been found to induce IL-6 expression in ovarian cancer cells [22], and a new LPA3 receptor-selective agonist, metabolically stabilized LPA analogue (2S)-OMPT was shown to substantially induce IL-6 [23]. This study demonstrated that LPA enhanced IL-6 expression in ovarian cancer cells but not in normal ovarian surface epithelial cells. The selective induction of IL-6 by LPA was attributed to the expression of Edg4/Edg7 receptors in ovarian cancer cells. Furthermore, Gi-mediated PI3K-Akt/NF-kB activation is the major signaling pathway responsible for IL-6 induction by LPA. The experimental results presented here clarify the role of LPA in the oncogenesis of ovarian cancer.

四、材料與方法

*Cell culture:*The ovarian cancer cell lines OVCAR-3, Caov-3, and SK-OV-3 were obtained from the American Type Culture Collection (Manassas, VA, USA). OVCAR-3 was cultured in RPMI-1640 medium with 20% fetal bovine serum (Life Technologies Ltd, Paisley, UK) and 10ug/ml insulin (Sigma, St Louis, MO, USA). Moreover, SK-OV-3 and Caov-3 were cultured in Dulbecco's modified Eagle medium with 10% FCS. Normal ovarian surface epithelial (NOSE) cells were harvested as described previously [24]. Primary NOSE cells were cultured in a 1:1 mixture of M199/MCDB105 medium (Sigma, St Louis, MO, USA) supplemented with 15% fetal bovine serum (Life Technologies Ltd, Paisley, UK) and penicillin-streptomycin (100 IU/ml -100 μg/ml; Life Technologies Ltd). Moreover, human

umbilical veins endothelial cells (HUVECs) were cultured in M199 medium supplemented with 20% fetal bovine serum, endothelial cell growth supplement (Intracel, Rockville, MD, USA), heparin, L-glutamine, penicillin, and streptomycin, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. To prevent the various individual specimens from causing genetic variation in the sample, HUVECs from five or more different donors were pooled together. The following experiments were performed using HUVECs at no more than five passages.

Antibodies and reagents

1-oleoyl-LPA, pertussis toxin (PTX), LY294002, Wortamanin, PD98059 and PDTC were purchased from Sigma (St. Louis, MO, USA). LPA was dissolved in (vehicle) PBS containing 1% fatty acid-free bovine serum albumin (Sigma, St. Louis, MO, USA). Recombinant human IL-6 and human IL-6 neutralizing antibody were obtained from R&D systems (Minneapolis, MN, USA). Antibody to human phospho-Erk, Erk, phospho-p38, p38 and p65 were from Santa Cruz Biotechnology. Antibodies to Akt and phospho-Akt were purchased from Upstate Biotechnology. Finally, dominant-negative mutant of Akt vector was kindly provided by Dr RH Chen, of the Institute of Molecular Medicine, National Taiwan University, Taipei, Taiwan.

*Enzyme Immunoassay:*The IL-6 levels of the cell culture supernatant were determined using enzyme immunoassay (EIA) with commercially available kits (R&D Systems). Each measurement was performed in triplicate, and the average value then was recorded as pg/ml.

*Plasmid construction:*The hIL-6 promoter in a luciferase activity reporter system was constructed and modified as described elsewhere [25]. Briefly, the 1.2-kb hIL-6 promoter region was amplified from SK-OV-3 total cellular DNA using primers F (5'-GGAAGATCTCTCCTGCAAGAGACACCATCCTGA-3') and R (5'-CGGAAGCTTAGGGCAGAATGAGCCTCAGAGACAT3-3'); the underlined nucleotides represent the BglII and HindIII sites, respectively. Moreover, the PCR fragment was cloned into pGL2-basic vector (Clontech, Palo Alto, CA) to produce phIL6-1.2K.

*Reporter assays:*Transfections of phIL6-1.2K into SK-OV-3 cells were performed in six-well plates using the Transfast Transfection Reagent (Promega) method. At 24 h after transfection, cells were serum starved for 24 h, and then pre-treated with various reagents. To control the transfection efficiency, cells were cotransfected with pSV-ß-galactosidase, and data normalizations after all transient transfection were conducted using triplicate cultures.

*RT-PCR:*Total RNA was isolated from cultured cell lines using RNAzol B reagent (Biotecx Laboratories) according to the instructions of the manufacturer and then cDNA was prepared from 2ug of total RNA with random hexamer primers according to the cDNA synthesis ImProm-II protocol (promega). The specific oligonucleotide primer pairs for certain genes and the expected sizes of the PCR products were as follows: Edg-2, 5'-CAAAATGAGGCCTTACGACGCCA and 5'-TCCCATTCTGAAGTGCTGCGTTC, 621 bp; Edg-4, 5'-GCGCGCGGATCCACCATGGTCATCATGGGCCAGTGCT and 5'-GCGCGGTCGACTCAGTCCTGTTGGTTGGGTTGA, 1,236 bp; Edg-7, 5'-CTGATGTTTAACACAGGCCC and 5'-GACGTTGGTTTTCCTCTTGA, 402 bp; and beta-actin, 5'-CTTCTACAATGAGCTGCGTG and 5'-TCATGAGGTAGTCAGTCAGG, 305bp. IL-6,5`-CTTCGGTCCAGTTGCCTTCT and 5`-AGGAACTCCTTAAAGCTGCG, 609bp.

*Synthesis of NF-kB decoy ODN and treatment:*This study used synthetic double-stranded oligodeoxynucleotides (ODNs) as "decoy" cis elements to block the binding of nuclear factors to promoter regions of targeted genes, thus inhibiting gene transactivation. The following sequences of the phosphorothioate ODN were utilized: NF-kB decoy ODN: 5'-CCTTGAAGGGATTTCCCTCC-3' and 3'-GGAACTTCCCTAAAGGGAGG-5'; AP-1 decoy ODN: 5′-TGTCTGACTCATGTC-3′ and 3′-CAGACTGAGTACA-5′; scrambled decoy ODN: 5'-TTGCCGTACCTGACTTAGCC-3' and 3'-AACGGCATGGACTGAATCGG-5'. The ODN was mixed with an equal volume (10:l) of TransFastTM for 15 min and then incubated with the cells in serum-free medium.

*Western blotting:*Cells were lysed in a lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM

EGTA, 1% NP-40, 1 mM NaF, 1 mM Na3VO4, 2 mM phenylmethyl- sulfonylfluoride (PMSF), 1 μg/ml aprotinin and leupeptin in PBS). The lysates were centrifuged at 12 000 r.p.m. for 25 min at 4°C. The protein concentration then was measured using a Bio-Rad protein assay (Hercules, CA, USA). A 50ug protein sample was separated by SDSPAGE, transferred onto polyvinylidene difluoride (PVDF) membrane, and immunoblotted with various antibodies. Bound antibodies were detected using appropriate peroxidase-coupled secondary antibodies, followed by an enhanced chemiluminescent detection system (ECL, Boehringer Mannheim).

*Electrophoretic Mobility Shift Assay (EMSA):*Nuclear extracts were prepared using SK-OV-3 cells in extraction buffer (420 mM KCl, 20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 0.2 mM EDTA, and 20% glycerol) contained protease inhibitors (0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin). The nuclear extracts were incubated in binding buffer with double-stranded oligonucleotides for the classical consensus binding sites of NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3'), and the binding sites of Oct1 (5'-TGTCGAATGCAAATCACTAGAA-3') were labeled using DIG-11-dUTP by a DIG gel shift kit (Roche Diagnostics, Indianapolis, IN, USA). Samples were separated on non-denaturing 4% polyacrylamide gels in 0.5x TBE and then transferred onto nitrocellulose membranes. DIG-labeled probe-protein complexes were detected using horseradish peroxidase-conjugated anti-DIG antibodies, and visualized using an enhanced chemiluminescent system (Boehringer Mannheim).

*Statistics:*In this study, each experiment was performed in triplicate and all experiments were repeated at least three times on different occasions. A paired Student's *t* test was used to evaluate statistically significant differences in IL-6 protein levels between the LPA treatment groups and the vehicle control group. $P<0.05$ was selected as the statistically significant value.

五、結果與討論

LPA enhances IL-6 expression in human ovarian cancer cells, but not in normal ovarian surface epithelial (NOSE) cells

The Gi/PI3K/Akt pathway is required for LPA-augmented IL-6 expression in SK-OV-3 cells

Critical involvement of NF-κB activation in the induction of IL-6 by LPA

These results verified that LPA up-regulates IL-6 expression by Gi/PI3K/Akt/NF-κB signal transduction pathway in SK-OV-3 cells.

LPA is present in raised concentration in the ascites and plasma obtained from patients with ovarian cancer, previous reports have shown that the plasma LPA levels in ovarian cancer patients range from 1.0-43.1uM [28,29], therefore the concentration of LPA (20uM) utilized in this study was physiological and can achieved *in vivo*. LPA critically influences ovarian carcinogenesis, with LPA-related metabolites present in the ascites of ovarian cancer patients stimulating both anchorage-dependent and anchorage-independent growth of ovarian cancer cells, preventing apoptotic cell death induced by platinum, and increasing the production of factors involved in neovascularization and metastasis [1,9,30]. This study demonstrated that LPA significantly enhanced IL-6 expression in ovarian cancer cells but not NOSE cells, owing to the appearance of Edg4/Edg7 receptors in ovarian cancer cells. High amounts of IL-6 are present in the ascites of patients with ovarian cancer, and may confer resistance to apoptosis and anoikis via an autocrine mechanism. The experimental data presented here indicate that LPA is an essential microenviromental factor inducing IL-6 expression in ovarian cancer patients, and support previous works suggesting that Edg4/Edg7 may contribute to the deleterious effect of LPA in ovarian cancer [31,32].

The biological responses of LPA signaling are determined by the spectrum of LPA receptors expressed on the cell surface. Ovarian cancer cells have variable Edg2 mRNA and protein level, and show no consistent change between normal and transformed ovarian cancer cells. Overexpression of Edg2 in ovarian cancer lines has been reported to induce apoptosis, suggesting that Edg2 may be a negative regulator of ovarian epithelial cell growth and metastasis [33]. In contrast, Edg4 and Edg7 mRNA expression appears to be elevated in

ovarian cancer cell lines compare to NOSE cells [31,32]. Previously, LPA has been shown found to regulate VEGF, and IL-8 expression by activating Edg4 and/or Edg7 mediated signaling [10,22]. In agreement with these studies, the experimental results presented here demonstrated that the generation of IL-6 by LPA is linked to the appearance of Edg4 and/or Edg7, highlighting the importance of Edg4/Edg7 in ovarian carcinogenesis.

The Edg family is G protein-coulpled receptors. Edg4/Edg7 receptors couple to Gi and Gq, which in turn feed into multiple effector systems [34-36]. This study demonstrated that LPA enhances IL-6 expression in ovarian cancer cells via PTX-sensitive G proteins, indicating that Gi-mediated signaling is critically involved in the induction of IL-6 by LPA. Activation of Gi by LPA may stimulate at least three distinct intracellular signals: inhibition of adenylyl cyclase; stimulation of the mitogenic RAS-MAPK cascade; and PI3K/Akt anti-apoptotic pathway [37-39]. The analytical results presented here have confirmed that a PI3K/Akt-, but not Erk or p38, dependent pathway leads to LPA-induced IL-6 up-regulation in SK-OV-3 ovarian cancer cells. This finding differs from previous reports showing that p38 MAPK and probably also JNK are important intracellular mediators of LPA-induced IL-6 production in ovarian cancer cells [22], implying that the signaling pathways leading to the IL-6 induction may vary according to the cell models or stimuli employed. Aberrant PI3K function is crucial in a wide range of important cellular processes associated with ovarian malignancies [40,41]. According to this study, the inhibition of PI3K activity would reduce LPA-mediated IL-6 expression and thus provide an effective approach for treating ovarian cancer patients.

PI3K/Akt has been reported to be a possible mediator of post-transcriptional mRNA stabilization [42,43]. However, the IL-6 promoter assay showed that LPA induces IL-6 mainly through transcriptional activation. Characterization of the upstream promoter sequences of IL-6 revealed that NF-kB and AP-1 are likely to mediate the effect of LPA. Pharmacological and genetic inhibition assays verified that NF-kB, but not AP-1, is critically involved in the transcriptional activation of IL-6 by LPA. Supporting evidence from previous studies demonstrated that activation of PI3K leads to phosphorylation and activation of the NF-kB, and NF-kB is targeted by Akt in anti-apoptotic signaling [44,45]. The development of ovarian cancer is associated with the acquisition of cellular resistance to anoikis, which is a form of apoptosis induced by detachment from the extracellular matrix [27].
Recently, Fang, X et al[22] found that the Edg4 and the p38 MAP kinase are important intracellular mediators of LPA-induced IL-6 in OVCAR-3 cells [22], and Qian, L et al found a new Edg7 receptor-selective agonist, metabolically stabilized LPA analogue (2S)-OMPT was shown to induce MAPK, Akt activation and IL-6 release in human OVCAR3 ovarian cancer cells [23]. Based on the above new findings, together with our finding here that Gi/PI3K-Akt/NF-kB signaling mediates the induction of IL-6 by LPA. We conclude that LPA is one of the important inducer of IL-6 in ovarian cancer cells. This information is valuable for developing novel molecular therapies for ovarian cancer.

六、計畫成果自評

上皮性卵巢癌是婦科癌症中致死率第一位的疾病.目前對上皮性卵巢癌的治療包括減積手術以及以 platinum 為主的化學治療然而,五年存活率仍小於百分之十. 我們利用 DNA 微陣列技術在卵巢癌組織中,試圖尋找與上皮性卵巢癌惡性化相關之基因,我們發現特定基因蛋白 Edg4/Edg7 會在癌組織中大量表現,由於 IL-6 在我們先前的許多研究亦顯示其在上皮性卵巢癌具重要角色,我們在本年度計畫中進行 Edg4/Edg7 ligan LPA 與 IL-6 的基礎功能研究.本計畫承蒙國科會支持以順利完成並進行論文發表.研究成果已經於 93 年六月投稿於 SCI 級期刊 Carcinogenesis,標題為 **Up-regulation of interleukin-6 in human ovarian cancer cell via a Gi/PI3K-Akt/NF-{kappa}B pathway by lysophosphatidic acid, an ovarian cancer activating factor.並於** 2004 Oct 7 為 Carcinogenesis 接受.本研究報告對

於上皮性卵巢癌的惡性話機轉提供一個新的方向,LPA 及其受體 Edg4/7 可成為治療的目標,值得更深入探討。

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