科技部補助專題研究計畫成果報告
期末報告

肺腺癌細胞對 Pemetrexed 愛寧達之抗藥性和癌症轉移的機制研究

計 畫 類 別 ： 個別型計畫
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執 行 期 間 ： 103 年 08 月 01 日至 104 年 07 月 31 日
執 行 單 位 ： 中山醫學大學醫學研究所

計畫主持人： 許國堂

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中 華 民 國 104 年 10 月 19 日

中文摘要： Pemetrexed 愛寧達(Alimta) 注射劑型是目前治療非小細胞肺癌的化療藥物。愛寧達似乎是對非小細胞肺癌中的非鱗狀上皮癌(non-squmous types)有較佳的療效，因此被推薦用來治療非鱗狀上皮類之肺癌。因為其治療肺癌的效果和一線藥物相同，因此自 2009 年二月起，衛生署已經核准愛寧達可用於肺腺癌的第一線化療用藥，讓肺癌病患在化療第一時間就可以選用愛寧達，不必像過去，得等到第一線化療失敗後才能使用。Pemetrexed (愛寧達)為葉酸拮抗劑，作用機轉是分別抑制 glycinamide ribonucleotide formyltransferase (GARFT)，dihydrofolate reductase (DHFR)與 thymidylate synthase (TS)三個酶，而使其作用更廣泛。這些酵素蛋白是癌細胞製造合成 DNA 及 RNA 所需 thymidine 及 purine nucleotides 的關鍵步驟，因此可以藉此抑制作用來殺死癌細胞。目前對於愛寧達之抗藥性產生之機轉一般皆認為是因為 TS 蛋白質高量所造成，對其餘的抗藥性基因並無深入的研究，基本上都是以舊一代的單一作用之葉酸拮抗劑抗癌藥物產生之抗藥性做為論點，這和愛寧達是多重作用之抗葉酸藥物的本質不相符。因此，研究肺癌對愛寧達之抗藥性產生之機轉就顯得非常需要。我們已經建立起兩株抗藥細胞株(A549/A400 和 CL1-0/A200)來做為此次研究的材料。從我們的數據顯示，愛寧達之抗藥基因可能依據細胞株而有異有同，但是兩株抗藥細胞株都對 vincristine 有比較高的敏感性。另外還觀察到 A549/A400 和 CL1-0 /A200 抗藥細胞株的癌症轉移能力會增強。為此，我們在原來的申請書提出三年的研究計畫包含下列三個目標：[壹]、找出重要的抗藥基因並且抑制其表現或將其過度表達來觀察其與抗藥性產生之關聯。[貳]、探討是否可以使用 vincristine 之化療用藥做為當愛寧達治療抗藥性產生後時之後續藥物，並且使用小鼠模式來驗證其可適用性。同時也要探討為何 vincristine 敏感性增加的相關機制。[叁]、研究為何愛寧達之抗藥細胞株的癌症轉移能力會增強的相關機制。雖然此申請書只得到壹年的經費補助，我們感謝國科會、科技部的審查委員對我們長期的鼓勵和支持，將此申請書的大部份目標皆已完成並且投稿正等待審查中(Title: Pemetrexed resistance enhances epithelial–mesenchymal transition in lung cancer through the ERK-ZEB1 pathway with suppression by vinca alkaloids)。預期從對愛寧達抗藥細胞株的研究結果來得到如何來反轉其抗藥性的方法，進而延長病人之存活。並且研究出適合使用愛寧達治療肺癌的參考基因來做為新的治療肺腺癌之策略依據。

中文關鍵詞： 肺腺癌，愛寧達，抗藥性，癌症轉移

英 文 摘 要 ： Pemetrexed (ALIMTA, LY231514, MTA, Eli Lilly and Company) is a novel multitargeted antifolate that inhibits one or several key folate-requiring enzymes of the thymidine and purine biosynthetic pathways, in particular, thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT). These enzymes are involved in the synthesis of nucleotides, ultimately hindering ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) synthesis. Recently, high TS expression has been demonstrated as major factor for pemetrexed resistance. Whether all of above mentioned alterations would result in pemetrexed-resistance is not well demonstrated. Therefore, a clear characterization of the mechanisms to overcome pemetrexed-resistance is required. We used two pemetrexed-resistant sublines (A549/A400 and CL1-0 /A200) sublines as materials and found that genes associated with pemetrexed resistance are similar but varied in these two sublines. Only the high sensitivity to vincristine is identical. We also observed that higher migration and invasion abilities have been obtained in both A549/A400 and CL1-0 /A200 sublines.

Therefore, we have proposed a three-year research plan as following: (1) Characterization of the pemetrexed-resistance genes (TS, FPGS and others). Apply in vivo animal model for pemetrexed resistance and vincristine sensitivity. (2) Target the NEFL gene as the gene mediates vincristine sensitivity. Further investigate the role of promoter hypermethylation in NEFL expression. (3) Application of in vivo animal model and in vitro studies for pemetrexed resistance and metastasis. Although only one-year funding has been granted, we highly appreciate to the reviewers for their long-term support. We have completed most of the proposed investigations and the manuscript has been submitted as the title of (Pemetrexed resistance enhances epithelial–mesenchymal transition in lung cancer through the ERK-ZEB1 pathway with suppression

by vinca alkaloids) which is currently under reviewing. By using the pemetrexed-resistant cell lines of A549 and CL1-0, we can further understand the mechanisms of pemetrexed resistance and associated metastasis.

英文關鍵詞： lung adenocarcinoma, antifolate, drug resistance, pemetrexed, metastasis

附件一

科技部補助專題研究計畫成果報告

（□期中進度報告/x期末報告）

肺腺癌細胞對 Pemetrexed 愛寧達之抗藥性和癌症轉移的機制研究

計畫類別：x個別型計畫　□整合型計畫

計畫編號：MOST　103-2320-B-040-015-

執行期間：　2014 年 08 月 01 日至　2015 年 07 月 31　日

執行機構及系所：中山醫學大學醫學研究所

計畫主持人：許國堂

共同主持人：

計畫參與人員：Ling-Yen Chiu, I-Lun Hsin, Tsung-Ying Yang, Wen-Wei Sung, Jinghua Tsai Chang, Jiunn-Liang Ko

本計畫除繳交成果報告外，另含下列出國報告，共 ___ 份：

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□出席國際學術會議心得報告

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研究計畫中文摘要：關鍵詞：**肺腺癌，愛寧達，抗藥性，癌症轉移**

肺腺癌細胞對 Pemetrexed 愛寧達之抗藥性和癌症轉移的機制研究

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研究計畫英文摘要：

關鍵詞: lung adenocarcinoma, antifolate, drug resistance, pemetrexed, metastasis

Mechanism investigation of pemerexed resistance and associated metastasis in human lung adenocarcinoma cells

Pemetrexed (ALIMTA, LY231514, MTA, Eli Lilly and Company) is a novel multitargeted antifolate that inhibits one or several key folate-requiring enzymes of the thymidine and purine biosynthetic pathways, in particular, thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT). These enzymes are involved in the synthesis of nucleotides, ultimately hindering ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) synthesis. Peviouly studies have demonstrated the cytotoxic activity of this agent in a broad range of tumor types including NSCLC. Pre-clinical and clinical studies have identified a plethora of mechanisms of antifolate resistance with classical single targeted antifolates such as methotrexate (MTX). These include downregulation of the reduced folate carrier (RFC) and various alterations in the target enzymes DHFR, TS and folylpolyglutamate synthase (FPGS). Recently, high TS expression has been demonstrated as major factor for pemetrexed resistance. Whether all of above mentioned alterations would result in pemetrexed-resistance is not well demonstrated. Therefore, a clear characterization of the mechanisms to overcome pemetrexed-resistance is required. We used two pemetrexed-resistant sublines (A549/A400 and CL1-0 /A200) sublines as materials and found that genes associated with pemetrexed resistance are similar but varied in these two sublines. Only the high sensitivity to vincristine is identical. We also observed that higher migration and invasion abilities have been obtained in both A549/A400 and CL1-0 /A200 sublines.

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Future application of our findings may benefit the management of lung adenocarcinoma therapy with pemetrexed and other drugs.

Manuscript:

Title: **Pemetrexed resistance enhances epithelial–mesenchymal transition in lung cancer through the ERK-ZEB1 pathway with suppression by vinca alkaloids**

Running title: ERK regulates EMT in pemetrexed resistance

Authors:

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Abstract: (244 words)

High thymidylate synthase (TS) level in cancer tissue is considered to result in resistance to pemetrexed therapy for advanced stages of nonsquamous non-small cell lung cancers. To further investigate the mechanism of pemetrexed resistance and potential prognostic outcomes in lung cancer, we established pemetrexed-resistant lung adenocarcinoma cell sublines from CL1 harboring a mutated Tp53 gene (R248W) and A549 harboring wild type Tp53. We found the TS expression is upregulated in both pemetrexed-resistant sublines and the reduced TS level achieved through shRNA inhibition resulted in higher pemetrexed

sensitivity. We also demonstrated that the acquisitions of pemetrexed resistance enhances epithelial–mesenchymal transition (EMT) *in vivo* with a mice animal model and *in vitro* with CL1 and A549 sublines, which was associated with upregulation of ZEB1 which, in turn, downregulates E-cadherin and upregulates fibronectin. When ERK1/2 phosphorylation was reduced by an inhibitor (U0126) or siRNA inhibition, both pemetrexed-resistant sublines reduced their migration and invasion abilities. Therefore, the ERK-mediated pathways induce apoptosis with pemetrexed treatment, and may in turn mediate EMT when cancer cells are resistant to pemetrexed. We further demonstrated that the growth of pemetrexed-resistant tumors could be inhibited by vinblastine *in vivo* and vincristine *in vitro*. Our data indicate that pemetrexed resistance could be relieved by non-cross-resistant chemotherapeutic drugs such as vinca alkaloids and might be independent to Tp53 status. Furthermore, the phosphorylation of ERK was reduced by vincristine. This finding provides a new insight for overcoming pemetrexed resistance and metastasis by application of vinca alkaloids.

Key words: Pemetrexed resistance; Thymidylate synthase; Epithelial–mesenchymal transition; ERK; ZEB1; Vinca alkaloids

Introduction: (4984 words)

Pemetrexed (LY231514, Eli Lilly and Co, Indianapolis, IN) is an antifolate that was developed to inhibit multiple enzyme targets involved in both pyrimidine and purine synthesis. Pemetrexed inhibits at least three key enzymes including thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT) (1, 2). Pemetrexed enters cells via the reduced folate carrier (RFC), with transport kinetics similar to those of methotrexate (MTX), and binds to folate receptor-α with a very high affinity, similar to folic acid (3). Inhibition of TS results in decreased turnover of dUMP to dTMP, which is necessary for DNA synthesis (4). These targets are related to the cytotoxicity of pemetrexed, because both thymidine and hypoxanthine are required to circumvent cellular death caused by pemetrexed (5).

Intracellularly, pemetrexed is polyglutamated to the active pentaglutamide by a reaction catalyzed by folylpolyglutamate synthase (FPGS). Pemetrexed is one of the best substrates for FPGS when compared to other antifolates such as MTX (6). The enzyme counteracting antifolate polyglutamylation is γ-glutamyl hydrolase (GGH). The rate of monoglutamyl glutamylation is equal to the rate of hydrolysis mediated by GGH. Pemetrexed can be considered a prodrug, because its pentaglutamate form is the predominant intracellular form, and it is over 60-fold more potent in its inhibition of TS than the parent compound (7). The increased cellular retention of polyglutamated pemetrexed forms may explain the success of the 3-week administration schedule. Previously, we have reported that pemetrexed-induced S-phase arrest and apoptosis is associated with an increase in Cdk2 and cyclin-A expression and activation, which is ERK-dependent and upstream of caspase-3. Our findings suggested that the ERK-mediated Cdk2/cyclin-A signaling pathway is an important regulator of pemetrexed-induced S-phase arrest and apoptotic cell death (8).

Recently, it has been reported that TS gene expression is significantly increased in pemetrexed-resistant small cell lung cancer cell lines (9). Knockdown of TS expression using siRNA has enhanced pemetrexed cytotoxicity in PC6/MTA-4.0 cells. Zhang *et al.* established two adenocarcinoma cell lines (PC-9 and A549) with pemetrexed resistance. TS and DHFR were significantly increased in the four pemetrexed-resistant A549 sublines (10). From the clinical data, Chen *et al*. evaluated the association of TS and DHFR expressions and the treatment efficacy of pemetrexed in non-small cell lung cancer (NSCLC) patients from

Taiwan (11). The associations of the DHFR expression level and median progression-free survival (PFS) as well as overall survival (OS) were not statistically significant. TS expression, rather than DHFR, may be an important predictive factor of the treatment efficacy of pemetrexed in NSCLC patients. Another report concluded that better response usually appears in patients with a lower expression of TS by meta-analysis (12) with a significant association between TS expression and outcomes of pemetrexed-based chemotherapy for NSCLC. Therefore, it can be concluded that upregulation of TS gene expression may play an important role in pemetrexed resistance. In addition, the TS protein level may be a predictive marker for pemetrexed sensitivity in lung cancer.

Multiple studies have revealed that chemoresistance cells often acquire an epithelial-mesenchymal transition (EMT)-like phenotype (13). During the acquisition of EMT characteristics, epithelial cancer cells lose the expression of proteins that promote cell–cell contact, such as E-cadherin and β-catenin, and gain the expression of mesenchymal markers, such as fibronectin, vimentin, and N-cadherin, leading to remodeling of the cytoskeleton and enhancement of cancer cell migration and invasion. Recently, an EMT phenotype was observed in gemcitabine-resistant pancreatic cancer cells (14, 15), gefitinib-resistant non-small cell lung cancer (16), oxaliplatin-resistant colorectal cancer cells (17), paclitaxel-resistant ovarian cancer cells (18) and tamoxifen-resistant breast cancer cells (19). The association of pemetrexed resistance with EMT alteration has not been reported yet and the question of how EMT is mechanistically involved in pemetrexed resistance has not been answered.

TGF-β triggers diverse cellular processes including growth arrest, tissue fibrosis and EMT (20, 21). As a result, TGF-β activates R-Smads (Smad2 and Smad3) via phosphorylation at their C-terminal serine residues. R-Smads form a heterocomplex with Smad4 and translocate into the nucleus to regulate gene expression (22-26). These pathways are referred to as Smad-dependent pathways. Snail and Slug, key regulators of TGF-β-induced EMT, are sufficient for the induction of single-cell invasion (27). In addition to the Smad signaling pathways, TGF-β also elicits diverse types of non-Smad signaling pathways. Among them, activation of Ras, mitogen-activated protein kinases (MAPKs) such as ERK and p38 MAPK, Rho GTPases, and PI3K/Akt signaling has been linked to TGF-β-induced EMT (28-30). Recent studies have identified the crucial role of TGF-β signaling pathways in inducing EMT through the Smad-dependent and Smad-independent pathways (21, 31-33).

Transcription factors involved in EMT such as Snail, Slug, TWIST, and the ZEB families mainly repress expression of E-cadherin during EMT (34-37). The transcription factor ZEB1 can be activated by the TGF-β, TNF-α and IGF1 signaling pathways. A correlation of ZEB1 expression and loss of E-cadherin has been demonstrated in tumor cell lines of lung adenocarcinomas (38). Therefore, ZEB1 is also a crucial mediator of EMT, exerting its effects on induction of EMT by inhibiting expression of E-cadherin.

Finding a way to control the growth of pemetrexed-induced resistance in lung cancer cells is clinically important. Cross-resistance or multidrug-resistance of chemotherapeutic drugs severely reduces the prognosis of cancer patients. Most of the cross-resistance originates from the ABC-related genes that are overexpressed in cancer cells selectively (39). However, it has been reported that pemetrexed-resistant lung cancer sublines show cross-resistance to cisplatin, but not to docetaxel, vinorelbine, and 5-fluorouracil (10) which points to the possibility of reversing pemetrexed resistance by using another clinically chemotheraputic drug. In this study, we have identified the signaling pathway that controls pemetrexed-induced EMT. Furthermore, we also provide evidence that vinca alkaloids, a group of clinically used anti-cancer drugs, reversed the pemetrexed resistance and EMT. These findings may be applied immediately to overcome pemetrexed resistance.

Materials and methods

Cell Culture

Human lung adenocarcinoma cells CL1 harboring a mutated Tp53 gene (mut-Tp53, R248W) were cultured in RPMI-1640 medium (Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS; Gibco) at 37 ℃ in a humidified atmosphere of 5% CO_2. Human adenocarcinoma A549 cells (ATCC) harboring wild type Tp53 (wt-Tp53) were maintained as previously described (40). Both cell lines were tested (Jun, 2015) by Mission Biotech (Taipei, Taiwan) using the PromegaGenePrint®10 System and analyzed by ABI PRISM 3730 GENETIC ANALYZER and GeneMapper® Software V3.7.

Drugs and chemicals

Pemetrexed was provided by Eli Lilly Corporation (Indianapolis, IN, USA). Methotrexates (MTX), 5-fluorouracil (5-FU), and vincristine were purchased from Sigma-Aldrich (St Louis, MO, USA). The drugs were dissolved in dimethyl sulfoxide (MTX, 5-FU) and ddH_2O (pemetrexed, vincristine) respectively, stored at -20℃, diluted in culture medium immediately before use. U0126, PD98059, SB253580 were purchased from Cell Signaling (Danvers, MA). Vinblastine was obtained from Teva Pharmaceutical Industries Limited.

Establishment of the pemetrexed resistant CL1 and A549 sublines

The pemetrexed resistant sublines were established from parental cells in a stepwise manner by exposure to increasing concentrations of pemetrexed. For example, CL1 cells of low cellular density were seeded onto a 10-cm Petri dish and treated with 5 nM pemetrexed until the surviving cells grew into an obvious colony. The selected colony was amplified in the presence of 20 nM pemetrexed until confluence before the drug dose was increased in multiples of two for the next round of selection. The pemetrexed resistant sublines maintained at 200 nM pemetrexed are denoted as CL1/A200. A similar approach was applied to obtain the A549/A400 subline.

Cytotoxicity assay (MTT assay)

Chemosensitivity to pemetrexed was determined using MTT assay. Approximately 2×10^4 cells per well were seeded onto 24-well plates. After 24 h incubation, the cells were exposed to various concentrations of pemetrexed in fresh medium for 72 h. At the end of the exposure period, the supernatant was removed and the cells were washed with PBS. Then, 300 μl MTT (1 mg/ml; Sigma) was added to each well followed by incubation at 37 ℃ for 2.5 h. Then the supernatant was removed and the cells were washed with PBS. To dissolve the water-insoluble formazan salt, 2-propanol solution was added (300 μl/well) with shaking at 70 rpm for 10 min at room temperature. Finally, the absorbance was measured at 570 nm using an ELISA plate reader (Molecular Devices SPECTRA max 340 PC). Mean values were calculated from three independent experiments. Chemosensitivity is expressed as the drug concentration for 50% cell survival (IC_{50}).

Protein extraction and Western blot analysis

Protein extracts were prepared from exponentially growing cells as described previously. Briefly, cells were collected and the cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate and 150 mM NaCl) plus protease inhibitors (Roche). Protein of 10-30 μg was used for SDS polyacrylamide gel electrophoresis. After transfer to the

PVDF membrane, the proteins were reacted with polyclonal anti-TS (Santa Cruz Biotechnology), anti-DHFR, FPGS, GGH (Genetex; Irvine, CA), anti-β-actin (NeoMarker), anti-p38, -pErk, -pJNK, -Zeb1, -E-cadherin, -Fibronectin, -Smad2/3 (Cell signaling) and anti-p53 (DAKO) separately, followed by conjugation of anti-rabbit (Santa Cruz Biotechnology) or anti-mouse (Calbiochem) IgG to horseradish peroxidase. A chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech) was used to determine the levels of protein expression.

TS silencing by the VZV-G pseudotyped lentivirus-shRNA system

Lentiviral infection of CL1 sublines was used to stably integrate and express short hairpin RNA (shRNA) targeting of TS mRNA sequences. Individual clones are identified by their unique TRC number: shGFP (TRCN0000072178) (responding sequence: CAACAGCCACAACGTC TATAT) for vector control targeted to GFP, shTS-1 (TRCN0000291720) (responding sequence: GCTGACAACCAAACGTGTGTT), shTS-2 (TRCN0000291655) (responding sequence: GCAAAGAGTGATTGACACCAT) and shTS-3 (TRCN0000045665) (responding sequence: GCTGACAACCAAACGTGTGTT) targeted to TS. Recombinant shTS lentivirus vectors were constructed and the viral titer was adjusted according to manufacturer instructions. To inhibit TS expression, the CL1 sublines ($5x10^4$/well) were sub-cultured onto 60-mm plates. After 16 h of incubation at 37℃, cells were infected with recombinant lentivirus vectors at a multiplicity of infection (MOI) of 1 with protamine sulfate added (0.8 μg/μl) to help infection. The next day, the medium was removed and the cells were selected by 2 μg/ml puromycin (Sigma, P8833) at 37℃ for 48 h, followed by MTT assay or protein detection.

Inhibition of ERK, ZEB1 and fibronectin by siRNA transfection

The cells of A549/A400 and CL1/A200 ($8x10^4$ cells) were seeded in 6-well plates for 16 h. The siRNA (30 pmol) for ERK (Sigma, siErk＃1 SASI_Hs01_00104111; siErk＃2 SASI_Hs01_00104113) were transfected with Lipofectamine RNAiMAX reagent (Invitrogen) for 4 h and then replaced with fresh culture medium and incubated for an additional 48 h. The cells were dissociated by trypsin/EDTA solution and reseeded for migration assay, or harvested with RIPA buffer for Western blots analysis. Similar procedures were applied to inhibition of ZEB1 with a mixed siRNA for ZEB1 (SASI_Hs02_00330526, 00330527, 00330528, 00330529) and fibronectin (SASI_Hs01_00203291, Hs02_00203292, Hs02_00203294, Hs02_00333045) with siC (universal NC control siRNA).

Mice metastasis and xenograft models

Cells were washed and resuspended in PBS. Subsequently, 4- to 6-week-old SCID mice (n=5/group) were injected in the lateral tail vein with a single-cell suspension containing $5x10^5$ cells of A549 and subline in 50 μl PBS buffer or $2x10^6$ cells of CL1 and subline. Mice were scarified after 55 days (A549) and 75 days (CL1). All organs were examined for metastasis formation. The lungs were removed and fixed in 10% formalin fixative. The tumor weights were measured on a microbalance. The representative lung tumors were removed, fixed, and embedded in paraffin, which was then sectioned into 4μm layers and stained with hematoxylin and eosin (H&E) for histologic analysis and immunohistochemistry (IHC) with the indicated antibody. The current study was approved by the Chung Shan Medical University Animal Care Committee (Permit Number: 1542) and all efforts were made to minimize suffering. To establish A549/A400 tumor xenografts, Male BALB/c mice were injected subcutaneously with 5×10^6 cells (100 μl in serum-free

DMEM) plus 100 μl Matrigel (BD Biosciences, 354234). Twelve animals were then randomly divided into three groups consisting of four animals each. Thirty days after cell implantation, when the tumor size reached 120 mm^3, mice in each group were intraperitoneally injected with 100μl PBS and pemetrexed (100 mg/kg/mouse) per week to serve as controls. The vinblastine (VBL) group was injected with 4.8 mg/kg per mouse per week. Tumor sizes were measured every 3 days following drug injection and tumor volume was calculated by the following formula: 0.5 x larger diameter (mm) x small $diameter^2$ (mm). Due to tumor size variability, a non-parametric Mann-Whitney U test was applied for statistical analysis.

In vitro transwell migration assay

Cell migration assay was carried out in 24-well tissue culture plates filled with 1.5 ml DMEM (for A549 cells) or RPMI (for CL1 cells) containing 10% FBA with a 8 μm transwell filter membrane (Thermo Fisher Scientific Inc). The group of A549 cells ($2x10^4$ cells/100μl) in DMEM containing 0.5% FBS were added to transwell plates and incubated in 5% CO_2 at 37℃ for 4 h. We fixed the cells on the lower side of the insert filter quickly with ice-cold methanol for 30 min. The cells were dried at room temperature and then stained with 20% Giemsa solution (Merck KGaA, Darmstadt, Germany) overnight. We removed excess dye by quickly merging the insert in ddH_2O. The upper side of the filter membrane was then gently wiped with a cotton swab to remove the cell debris. Excess water was drained from the side of the insert using a cotton swab and the insert membrane was dried. Once stained, the cells were observed by using the optical microscope and photography. Similar procedures were applied to CL1 cells ($5x10^4$ cells/100μl) in RPMI containing 0.5% FBS which was incubated for 24 h.

In vitro transwell matrigel invasion assay

Invasion of tumor cells was evaluated using 24-well transwell cell plates with procedures similar to those of migration assay with matrigel (BD Biosciences cat. 354234) added. In medium-containing plates with a membrane, 0.5 μg/150μl of matrigel for A549 cells and 0.2 μg/150 μl of matrigel for CL1 cells were added and polymerized at 37℃ for 3 h. The groups of A549 cells ($2x10^4$ cells/100μl) were incubated for 16 h and CL1 cells ($5x10^4$ cells/100μl) were incubated for 48 h. After the indicated time, non-invasive cells were gently removed from the top of the matrigel with a cotton-tipped swab. Invasive cells at the bottom of the matrigel were fixed in ice-cold methanol, stained with 20% Giemsa solution and counted under a microscope. Results were averaged from three independent experiments.

Statistical analysis

All values are presented as mean ± SD. Data were compared among groups using a t-test and $*p<0.05$ is considered statistically significant.

Results

Establishment of pemetrexed resistant CL1 and A549 lung cancer sublines.

The A549 and CL1 cells were continuously exposed to an increasing concentration of pemetrexed as described in *Materials and Methods* by a selection process. According to MTT sensitivity assay, the established A549/A400, CL1/A100 and CL1/A200 sublines (Table 1) revealed their drug sensitivities in terms of IC_{50} (Inhibition concentration). The A549/A400, CL1/A100 and CL1/A200 sublines have 37.8-fold, 22.9-fold and 86.5-fold resistance to pemetrexed respectively when compared with parental cells. To

investigate whether pemetrexed resistance may result in cross-resistance to other antimetabolic chemotherapy, the sensitivities to MTX and 5-FU were also determined. Interestingly, only minimal resistances were detected. The A549/A400 is 2.3-fold resistant to MTX and 1.3-fold resistant to 5-FU. The CL1 /A100 subline is 1.1-fold resistant to MTX and 0.8-fold resistant to 5-FU. The CL1/A200 subline is 2.5-fold resistant to MTX and 0.6-fold resistant to 5-FU. The drug sensitivity profiles of the A549 and CL1 sublines in our study exhibit similar results to those previously reported for PC-9 and A549 pemetrexed resistant sublines (Zhang, 2011). Interestingly, all three pemetrexed-resistant sublines have only a low degree of cross-resistance to docetaxel (1.1 to 1.9-fold). Moreover, the pemetrexed-resistant sublines all showed a significant sensitivity to vincristine when compared to parental A549 (0.3-fold) and CL1 (0.6 to 0.8-fold) cells.

Characterization of the pemetrexed regulated genes

To investigate the mechanism of the pemetrexed resistance, we compared the expression of pemetrexed-target proteins TS and DHFR, as well as the expression of the poly/mono-glutamyl glutamylation enzymes FPGS and GGH by Western blot analysis. The protein levels of TS and DHFR significantly increased in A549/A400 (Fig. 1A), CL1/A100 and CL1/A200 sublines (Fig. 1B). The levels of FPGS were slightly increased in all three sublines. Upregulated GGH levels in A549/A400 cells and downregulated GGH levels in CL1 sublines were observed. It has been reported that the level of TS protein in cancer cells determines pemetrexed sensitivity in cell lines (10, 41) and lung cancer tissues (11). Therefore, to demonstrate the dominant role of TS overexpression in the acquired pemetrexed resistance, the sublines were infected with the shRNA expressing lentivirus that targets TS mRNA. Three shTS constructs significantly inhibited TS expression when compared with the shGFP control and the proteins of TS were detected by Western blotting. The pemetrexed sensitivities were enhanced in shTS-infected CL1/A100 (Fig. 1C) and CL1/A200 (Fig. 1D) sublines were measured by MTT cell survival assay. When TS protein expression was reduced, the viabilities of CL1/A100 and CL1/A200 cells were coordinately reduced with pemetrexed treatment. We also further investigated whether the 5-FU and MTX sensitivities could be affected by TS inhibition. From the results of MTT assay, the drug sensitivities of MTX (Fig. 1E) and 5-FU (Fig. 1F) in the TS-inhibited CL1/A200 subline were not altered significantly. Our data demonstrate that high levels of TS expression are associated with pemetrexed resistance. Inhibition of TS expression by shRNA enhances pemetrexed sensitivity, but not sensitivity to MTX and 5-FU.

Acquisition of pemetrexed resistance promotes metastasis in vivo

To answer whether pemetrexed resistance may result in lung cancer metastasis, we injected the A549/A400 and parental A549 cells through the tail vein of SCID mice and monitored the mice for 45 days. Although all A549-injected and A549/A400-injected animals developed lung tumors, a greater number and larger size of tumors were found in the animal group injected with A549/A400 cells and the weights of the tumors were significantly higher (Fig. 2A) in the pemetrexed-resistant A549/A400-injected animals. The lung tissues were further examined with H&E staining for tumor verification and higher TS expressions were detected in A549/A400 tumors by IHC (Fig. 2B).

The CL1 and CL1/A200 cells were also injected into SCID mice to test their metastatic potential. Since low metastasis of the CL1 cells has been demonstrated (42), it is not surprising that none of CL1-injected animals developed metastasis and only two of the CL1/A200-injected animals developed lung metastasis at 75 days when compared with the lungs of CL1-injected animals (Fig. 2C). The H&E staining and high TS

expression confirmed the CL1/A200 cells have metastatic potential. Although no solid tumors could be found in three of the CL1/A200-injected animals, evidences for inflammation of lung tissue were detected by an Mac3 antibody indicating the appearance of CL1/A200 in lung tissue without successful colonization (Fig. 2D).

Pemetrexed-resistant sublines show changed EMT molecular markers *in vitro*.

In order to further support the metastatic potential of pemetrexed-resistant sublines detected in the mice model, *in vitro* assays were performed using transwell cell culture chambers to compare the migration and invasion abilities of parental cells and sublines. The pemetrexed-resistant A549/A400 cells showed a 3.5-fold increase in migration with 4 h incubation (Fig. 3A) and a 3.2-fold increase in invasion with 16 h incubation (Fig. 3B) through the transwell membrane when compared with parental cells. Similar results also revealed that CL1/A100 and CL1/A200 have significant 2.7-fold increases in migration with 24 h incubation (Fig. 3C). Both CL1 sublines also have 2.8-fold and 3.7-fold increases in invasion abilities respectively with 48 h incubation (Fig. 3D). Therefore, we examined whether the induced EMT found in pemetrexed-resistant sublines came through smad-dependent or smad-independent pathways by Western blot analysis in all three sublines and compared the results with those of their parental cells with known EMT markers (Fig. 3E). According to the data, the expression of E-cadherin protein levels were downregulated but the phosphorylated smad2 and smad3 levels were not significantly increased in pemetrexed-resistant sublines, nor were the protein levels of Slug and Snail. Only phosphoryalted p38 and ERK levels were highly upregulated along with ZEB1 and fibronectin levels. The results indicate p38 and/or ERK signaling pathways may regulate EMT through a smad-independent pathway. Pemetrexed-resistant sublines that developed EMT were correlated with downregulated E-cadherin, and upregulated Fibronectin and ZEB1.

Regulation of EMT and MAPK signaling

The mechanisms of pemetrexed-resistant induced EMT were further investigated by inhibition of ERK1/2 phosphorylation with U0126 and p38MAPK phosphorylation with SB253580 followed by Western blot analysis. In the A549/A400 subline, the reduced levels of phosphorylated-ERK (pERK) by U0126 were correlated with reduced ZEB1 and fibronectin expression as well as upregulated E-cadherin expression. In contrast, inhibition of p38MAPK by SB253580 had no effect on ZEB1, fibronectin and E-cadherin expression. The kinase inhibitor PD98059 specifically inhibits MEK-1-mediated activation of MAPK which does not directly inhibit ERK. This also caused reduced p-ERK and ZEB1 levels combined with upregulated E-cadherin expression (Fig. 4A). Inhibition of ERK phosphorylation resulted in A549/A400 reduced migration (Fig. 4B) and invasion abilities (Fig. 4C) of 50%, whereas inhibition of p38MAPK only had a residual effect. We also examined the CL1/A200 subline to analyze the mechanisms of pemetrexed-resistant induced EMT. Similar to the results obtained from A549/A400 investigation, inhibition of ERK1/2 phosphorylation with U0126 and PD98059 reduced p-ERK, ZEB1 and fibronectin levels in combination with upregulated E-cadherin expression (Fig. 4D). Inhibition of ERK phosphorylation resulted in CL1/A200 reduced migration (Fig. 4E) and invasion abilities (Fig. 4F) of approximately 50%.

Interference of ERK, ZEB1 and fibronectin expressions resulted in EMT suppression

The role of ERK1/2 in pemetrexed resistance induced EMT was further characterized by inhibition of ERK1/2 expression with siRNA interference. In A549/A400 cells, when ERK1/2 expression was downregulated by

siRNA to less than 50%, E-cadherin expression was significantly increased with reduced ZEB1 and fibronectin expression (Fig. 5A). The migration ability of the ERK interfered A549/A400 cells was reduced to 30% (siERK#1) and 10% (siERK#2) when compared with the si-controlled cells (Fig. 5B). When invasion ability was assayed, the abilities of siERK#1 and siERK#2 cells were reduced to 40% and 10% respectively (Fig. 5C). Similar approaches were also applied to CL1/A200 cells and reduced ERK expression also resulted in reduced ZEB1 and fibronectin expression with E-cadherin upregulation (Supplementary Fig. 1a). Interference of ERK expression reduced CL1/A200 cells (siERK#1 and siERK#2) to 50% and 30% of their migration (Supplementary Fig. 1b) and invasion (Supplementary Fig. 1c) abilities respectively. As shown in the data in Fig. 5A and Supplementary Fig. 1a, the degrees of ERK inhibition were correlated with the levels of ZEB1 and fibronectin downregulation, and were also reflected in the degrees of migration and invasion inhibition. To determine the role that ZEB1 plays in pemetrexed-mediated EMT, siRNA inhibition of ZEB1 (siZEB1) was applied and the E-cadherin level was increased and the Fibronectin level was decreased significantly without affecting ERK phosphorylation (Fig. 5D). Inhibition of ZEB1 expression also resulted in migration (Fig. 5E) and invasion (Fig. 5F) abilities of less than 30%. Similar results were obtained from CL1/A200 cells showing that upregulated E-cadherin and downregulated fibronectin resulted in less migration and invasion under ZEB1 inhibition (Supplementary Fig. 1d, e and f). When fibronectin was inhibited by siRNA (siFN), ZEB1 and E-cadherin levels were not altered (Fig. 5G) but migration (Fig. 5H) and invasion (Fig. 5I) were significantly reduced to less than 50%. The Cl1/A200 cells were also examined and similar data were demonstrated (Supplementary Fig. 1g, h, and i). We conclude that pemetrexed-mediated EMT occurs via ERK-signaling activation of ZEB1, which downregulates E-cadherin and upregulates fibronectin to promote EMT in both A549/A400 and CL1/A200 pemetrexed-resistant cancer cells.

Vinblastine inhibits pemetrexed-resistant tumor growth in a mice xenograft model and may also inhibit EMT

According to the data of drug sensitivities assay listed in Table 1, pemetrexed-resistant sublines were more sensitive to vincristine than their parental cells. Therefore, we tested the efficacy of vinblastine in a mice xenograft model inoculated with A549/A400 cells. Compared with the PBS or pemetrexed treatment, vinblastine significantly inhibited the growth of A549/A400 tumors (Fig. 6A). We then examined if vincristine could also attenuate the phosphorylation of ERK1/2 and suppress EMT. When A549/A400 cells were treated with vincristine (5 nM) for 24 h, ERK and p-ERK levels were reduced to 70%. With 48 h treatment, ERK and p-ERK levels were reduced to 70% and 40% respectively, when correlated with fibronectin downregulation (Fig. 6B). Similar results were also obtained from CL1/A200 cells treated with vincristine (Supplementary Fig. 2). The results indicate that vincristine inhibits the ERK signaling pathway on ERK expression and activation. Surprisingly, E-cadherin levels were not correspondingly upregulated when ZEB1 was reduced. We speculate that vincristine induces A549/A400 cell death. This might result in E-cadherin degradation. Because pemetrexed-resistant cells are sensitive to vincristine treatment, it is difficult to recognize the possible effect on EMT inhibition; therefore, we used 5 nM of vincristine to treat A549/A400 cells for *in vitro* transwell assays. With 24 h of vincristine treatment, the viability of vincristine-treated cells was maintained at approximately 90% when compared with untreated cells (Fig. 6C). Under a non-lethal dose of vincristine treatment, the migration of A549/A400 cells was reduced to 50% (Fig. 6D), and the invasion ability was also reduced to 50% (Fig. 6E). These results indicate that vincristine and vinblastine not only overcome pemetrexed resistance but also may suppress ERK-ZEB1 regulated metastasis in NSCLC. In summary, as

shown in Fig. 6F, we have demonstrated that the enhanced pemetrexed resistance of EMT is mediated by ERK and ZEB1, and vinca alkaloids may resolve resistance and reduce metastasis.

Discussion:

The consequences of pemetrexed resistance strongly affect the prognosis of lung cancer patients and warrant more investigation to resolve the resistance. Previously, we have identified pemetrexed-mediated apoptosis associated with the activation of ataxia telangiectasia mutated (ATM)/Tp53-dependent and -independent signaling pathways, which promote intrinsic and extrinsic apoptosis and activate the caspase signaling cascade (43). We also have suggested that the ERK-mediated pathway is an important regulator of pemetrexed-induced apoptotic cell death (8). However, when lung cancer cells develop pemetrexed resistance, researchers have yet to study whether Tp53 status could affect the mechanism of pemetrexed resistance. Accumulated data obtained from *in vitro* cell lines and patient sample studies indicate that high TS levels in cancer cells play a major role in pemetrexed non-response. Up to date, there is no clear evidence showing how to resolve resistance occurring after pemetrexed treatment.

In this study, we have established two pemetrexed-resistant sublines containing wt-Tp53 (A549/A400) and mut-Tp53 (CL1/A200) to further investigate the molecular mechanisms of pemetrexed-selected resistance. From the results of drug sensitivities analysis listed in Table 1, both pemetrexed-resistant sublines showed non-cross-resistance to methotrexate, 5-FU and docetaxel treatment. Our data agrees with a previous report showing that PC-9 and A549 pemetrexed-resistant sublines remain sensitive to methotrexate, docetaxel, and 5-FU (10). According to these results, the choice of docetaxel to manage first-line pemetrexed resistance could be suggested and there is no obvious bias against the status of Tp53 in pemetrexed-resistant sublines of docetaxel sensitivities. Furthermore, both sublines with wt-Tp53 and mut-Tp53 showed TS upregulation (Fig. 1). When TS was inhibited in mut-p53 sublines, these cells were re-sensitized to pemetrexed treatment without affecting methotrexate and 5-FU sensitivities (Fig. 1). While TS is the target protein of 5-FU, inhibition of TS did not increase 5-FU sensitivity. This may explain why 5-FU alone is not effective in lung cancer treatment.

Chemoresistance is usually companied with metastasis that blocks the efficiency of cancer therapy. Our data demonstrated that wt-Tp53 and mut-Tp53 lung cancer cells increased their metastatic potential after pemetrexed-selection. In the mice metastasis model (Fig. 2), enhanced metastasis can be observed in A549/A400 cells, which developed more tumors in the lung tissue. In contrast to the highly mobile A549 cells, CL1 cells showed no metastatic abilities and did not establish any tumors in mice lungs. Therefore, when low percentage of CL1/A200 cells established tumors *in vivo,* it suggested that metastasis can be induced by pemetrexed resistance. When both the Smad-dependent and Smad-independent pathways that induce EMT were analyzed, the ERK phosphorylations were increased in these sublines (Fig. 3). The transwell migration and metastasis data from *in vitro* assays also support that pemetrexed-mediated EMT is associated with ERK/ZEB1 activation and E-cadherin inhibition as well as upregulation of fibronection (Fig. 4). Interestingly, when EKR protein expressions were reduced by siRNA-ERK, the E-cadherin levels were negatively correlated with EKR protein expression levels and the metastatic abilities were correspondingly reduced (Fig. 5). We provide evidence to show that ERK activation is associated with ZEB1 upregulation and this linkage has not been reported previously. Activation of ZEB1 to repress E-cadherin expression has been shown (44), but the association of ZEB1 and fibronectin upregulation is a novel finding.

Considering the need for a standard treatment for pemetrexed-resistant patients, this issue offers an ideal

area in which to test new drug application strategies. There are four major vinca alkaloids in clinical use: Vinblastine (VBL), vinorelbine (VRL), vincristine (VCR) and vindesine (VDS). VCR, VBL and VRL have been approved for use in the United States. (45). Vinca alkaloids are classic chemotherapeutic agents that induce disruption of microtubules by binding to tubulin and inhibiting tubulin polymerization/microtubules formation (46). However, these drugs are a substrate of the ABCB1 (P-gp) transporter, therefore overexpression of ABCB1 in cancer cells is considered the major phenotype of multidrug resistance to vinca alkaloids (47, 48). We have shown that vinca alkaloids are non-cross-resistant to pemetrexed and may be applied to reverse pemetrexed resistance of NSCLC. A retrospective study to evaluate the activity and toxicity of VRL administered to a consecutive series of pemetrexed-pretreated malignant pleural mesothelioma (MPM) patients (59 patients) has been reported (49). It seems that VRL is moderately active in pemetrexed-pretreated MPM patients, with an acceptable toxicity profile.

Our study demonstrated that pemetrexed resistance could be relieved by vinca alkaloids and might be independent to the Tp53 status. The ERK signaling pathway is activated by pemetrexed to induce cancer cell death. The surviving cancer cells with upregulated TS may enhance EMT via the ERK/ZEB1 signaling pathway and result in metastasis. Therefore, to consider a second-line treatment for pemetrexed-pretreated patients, we suggest that vinca alkaloids deserve further clinical investigation.

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Table 1. Drug sensitivities of parental cells and drug resistant sublines

Drug	IC_{50} ± SD* (nmol/L)				
	A549	A549/A400	CL1	CL1/A100	CL1/A200
Pemetrexed	1304.7 ± 94.7 (1.0)	49333.3 ± 1755.9 (37.8)	32.1 ± 0.2 (1.0)	736.2 ± 49.1 (22.9)	2777.8 ± 481.1 (86.5)
Methotrexate	48.7 ± 0.6 (1.0)	113.3 ± 4.7 (2.3)	11.2 ± 0.2 (1.0)	12.0 ± 0.5 (1.1)	27.4 ± 1.6 (2.5)
5-FU	6000.0 ± 500.0 (1.0)	7900.0 ± 700.0 (1.3)	3980.0 ± 300.0 (1.0)	3330.0 ± 180.0 (0.8)	2460.0 ± 320.0 (0.6)
Docetaxel	6.4 ± 0.1 (1.0)	6.8 ± 0.4 (1.1)	3.1 ± 0.2 (1.0)	5.6 ± 0.8 (1.8)	6.0 ± 0.4 (1.9)
Vincristine	14.9 ± 0.1 (1.0)	4.2 ± 0.1 (0.3)	4.2 ± 0.3 (1.0)	3.3 ± 0.1 (0.8)	2.6 ± 0.4 (0.6)

NOTE:
A549/A400 is a pemetrexed resistant A549 subline; CL1/A100 and CL1/A200 are pemetrexed resistant CL1 sublines.
Cell survival was determined by MTT assay.
(Fold resistance) was relative to the parental cell line.
* Data are the mean ± SD of at least three independent experiments done in triplicate.

Figure Legends: (755 words)

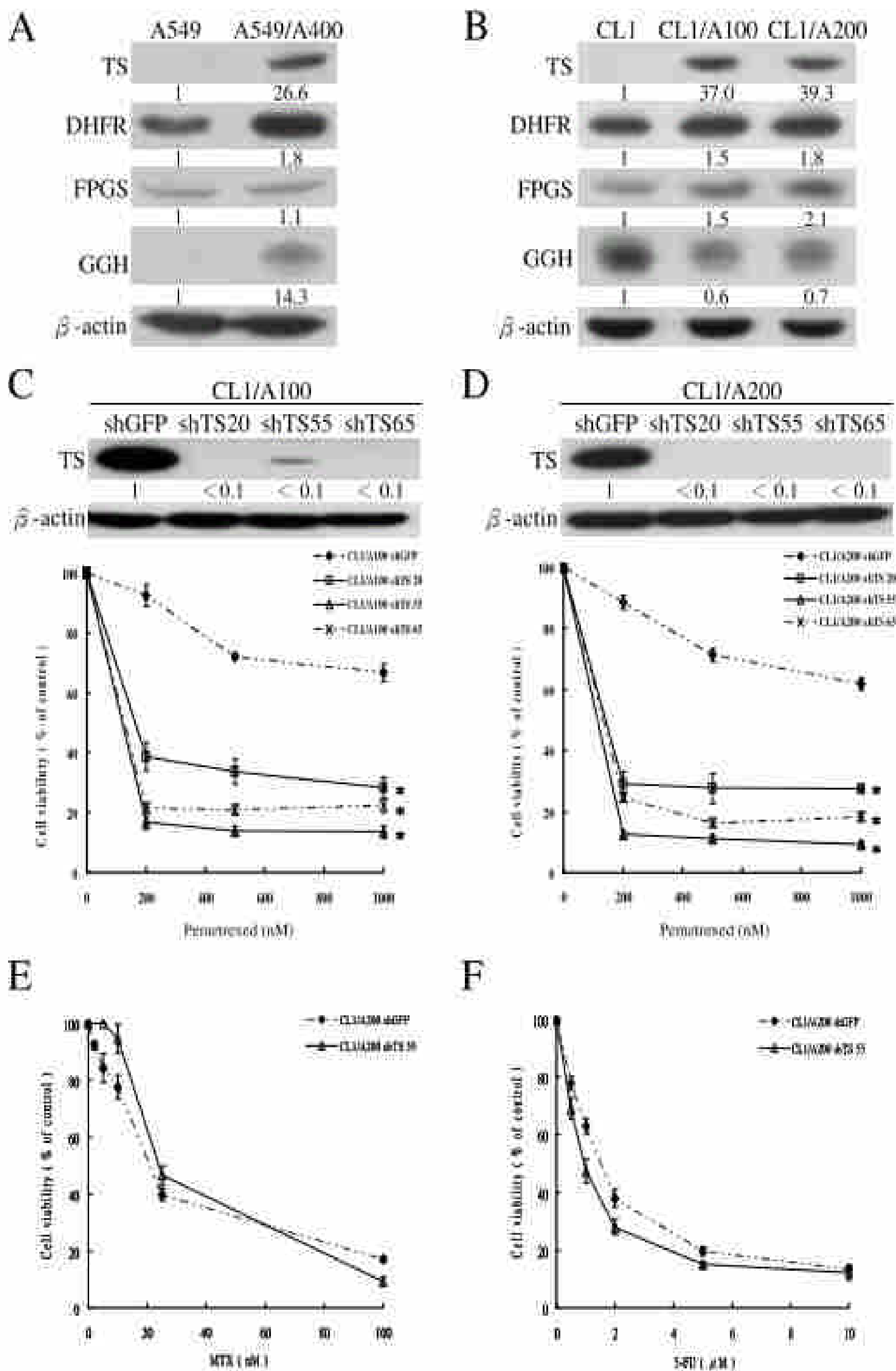


Figure 1. Characterization of the pemetrexed-resistant A549 and CL1 lung cancer cells.

The expression of TS, DHFR, FPGS and GGH were detected by Western blot assay in A549 (A) and CL1 (B) sublines. Inhibition of TS by shRNA in CL1/A100 (C) and CL1/A200 (D) sublines were followed by MTT assay. The sensitivities of TS-inhibited CL1 sublines to MTX treatment were examined by MTX (E) and 5-FU (F). *p<0.05 was considered statistically significant.

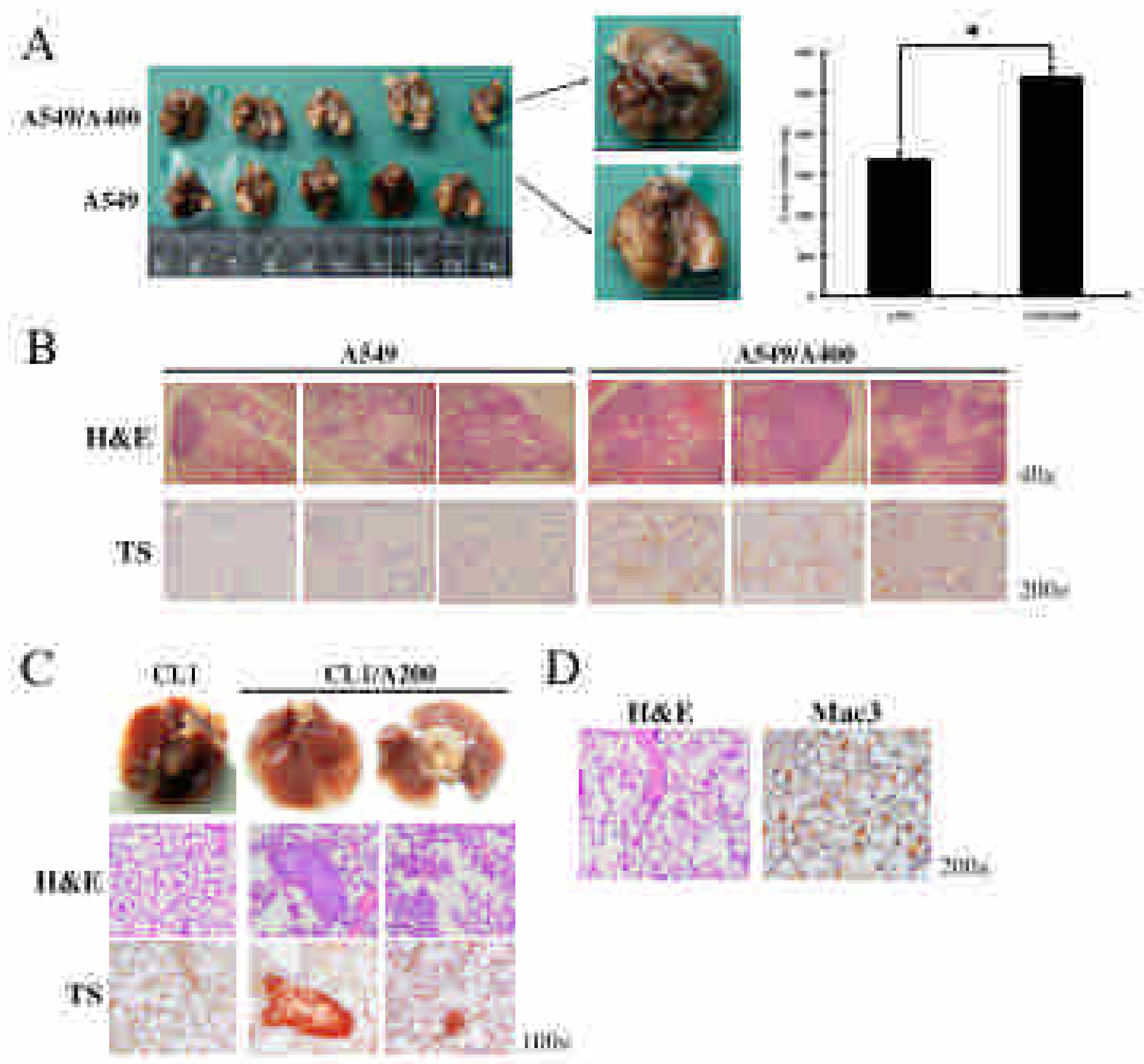


Figure 2. Investigation of the pemetrexed-induced metastasis by a mice metastasis model.

The cells of A549/A400 and A549 ($5x10^5$ cells/50 μl/mouse) were tail-vein injected into SCID mice (n=5) followed by monitoring for 55 days until sacrifice. The lung tissues were examined for tumor metastasis and the weights of the two groups were recorded and listed (A). H&E and IHC of TS staining were applied to verify the tumors and TS overexpression (B). The cells of CL1 and CL1/A200 ($2x10^6$ cells/ cells/50 μl/mouse) were tail-vein injected into SCID mice (n=5) and monitored for 75 days until sacrifice. All five CL1-injected mice were free of tumors and two of the CL1/A200 injected mice established metastatic tumors, which were stained with H&E and IHC with anti-TS antibody (C). Although three of the CL1/A200-injected mice did not establish metastasis, macrophages aggregated in the lung tissue were detected by IHC staining with anti-Mac3 antibody (D). *$p<0.05$ was considered statistically significant.

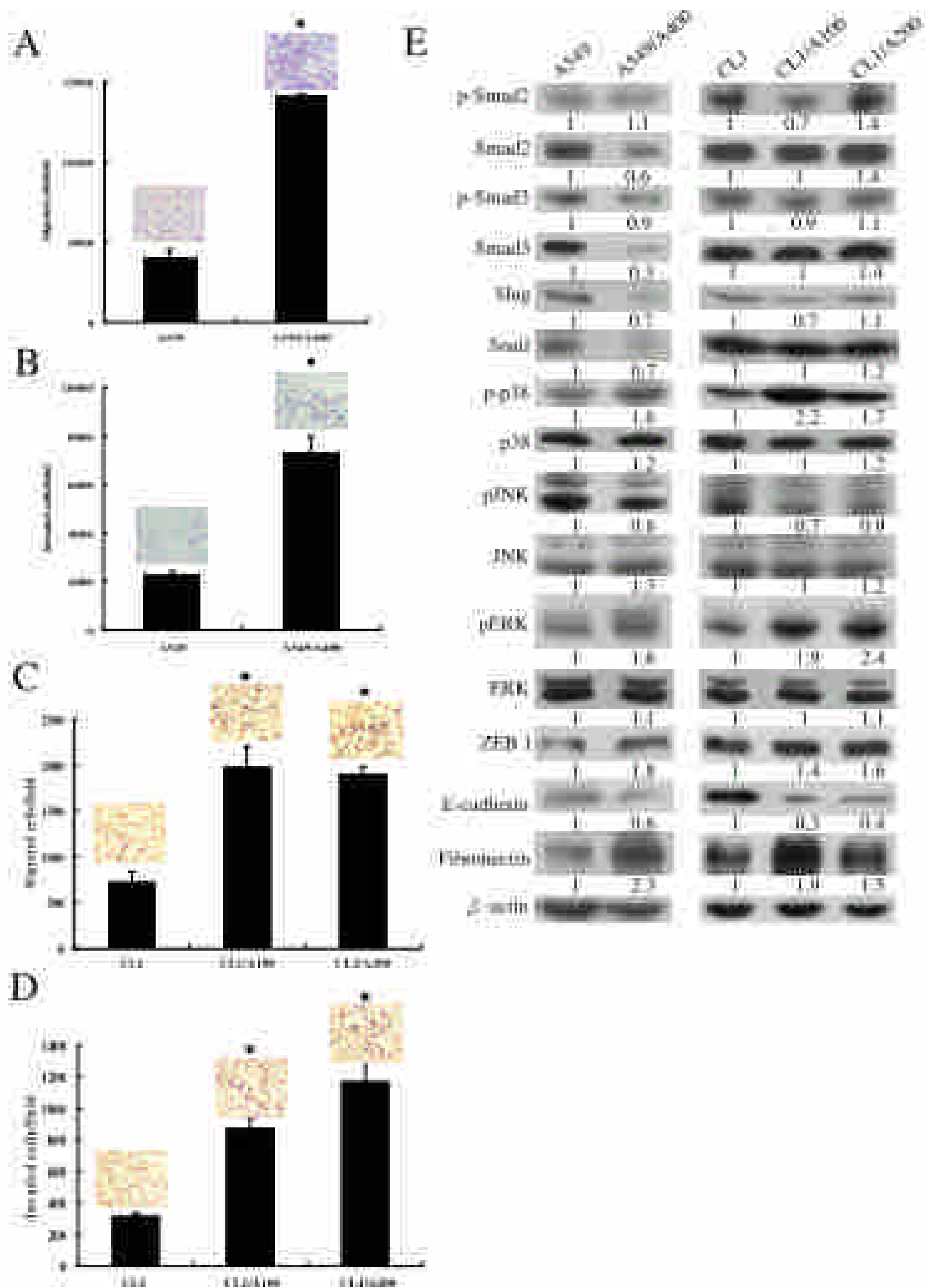


Figure 3. Analysis of the pemetrexed-induced metastasis in vitro.

The cells of A549 and A549/A400 ($2x10^4$ cells) were added into transwells for 4 h incubation to determine the migration ability (A) or for 16 h to measure invasion ability (B). The cells of CL1, CL1/A100 and CL1/A200 ($5x10^4$ cells) were added into transwells for 24 h incubation to determine the migration ability (C) or for 48 h to measure invasion ability (D). Western blot analyses were applied to examine the associated signaling pathways regulating EMT in A549 and CL1 sublines (E). *$p<0.05$ was considered statistically significant.

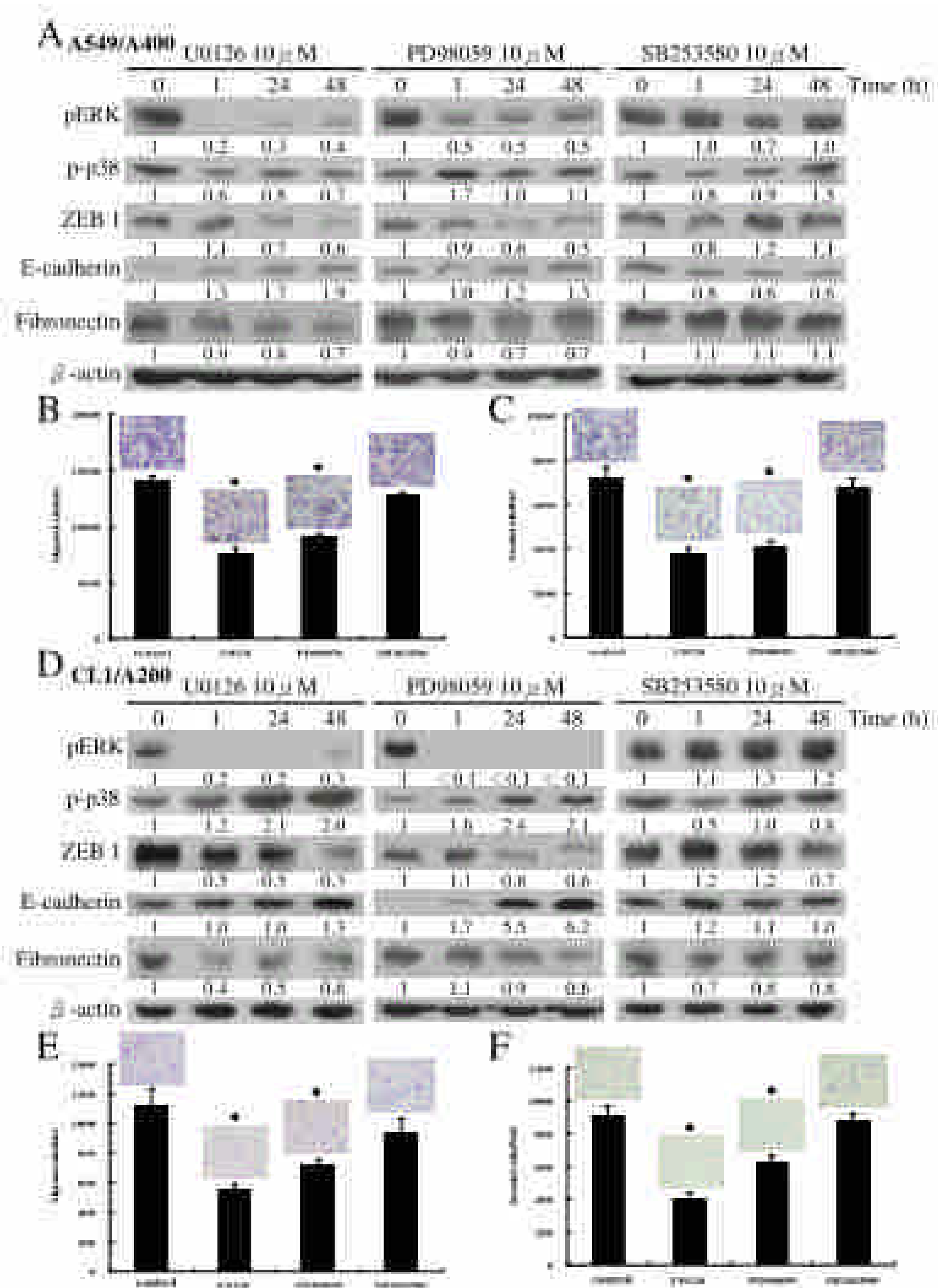


Figure 4. Determination of the ERK signaling pathway that regulates pemetrexed-induced EMT by kinase inhibitors.

The cells of A549/A400 were treated with U0126, PD98059 and SB253580 for the indicated time (1 to 48 h) and then harvested for Western blot analysis. The phosphorylated ERK and p38MAPK levels indicated the effects of kinase inhibition. The levels of ZEB1, E-cadherin and fibronectin represent the alteration of EMT upon phosphorylation inhibition (A). The A549/A400 cells were pretreated with the indicated kinase inhibitor for 24 h followed by transwell 4 h migration assay (B) or 16 h invasion assay (C). The CL1/A200 cells were treated with individual kinase inhibitors for the indicated time, followed by protein detection (D). The kinase inhibitor pretreated CL1/A200 cells were analyzed by transwell 24 h migration assay (E) or 48 invasion assay (F). *$p<0.05$ was considered statistically significant.

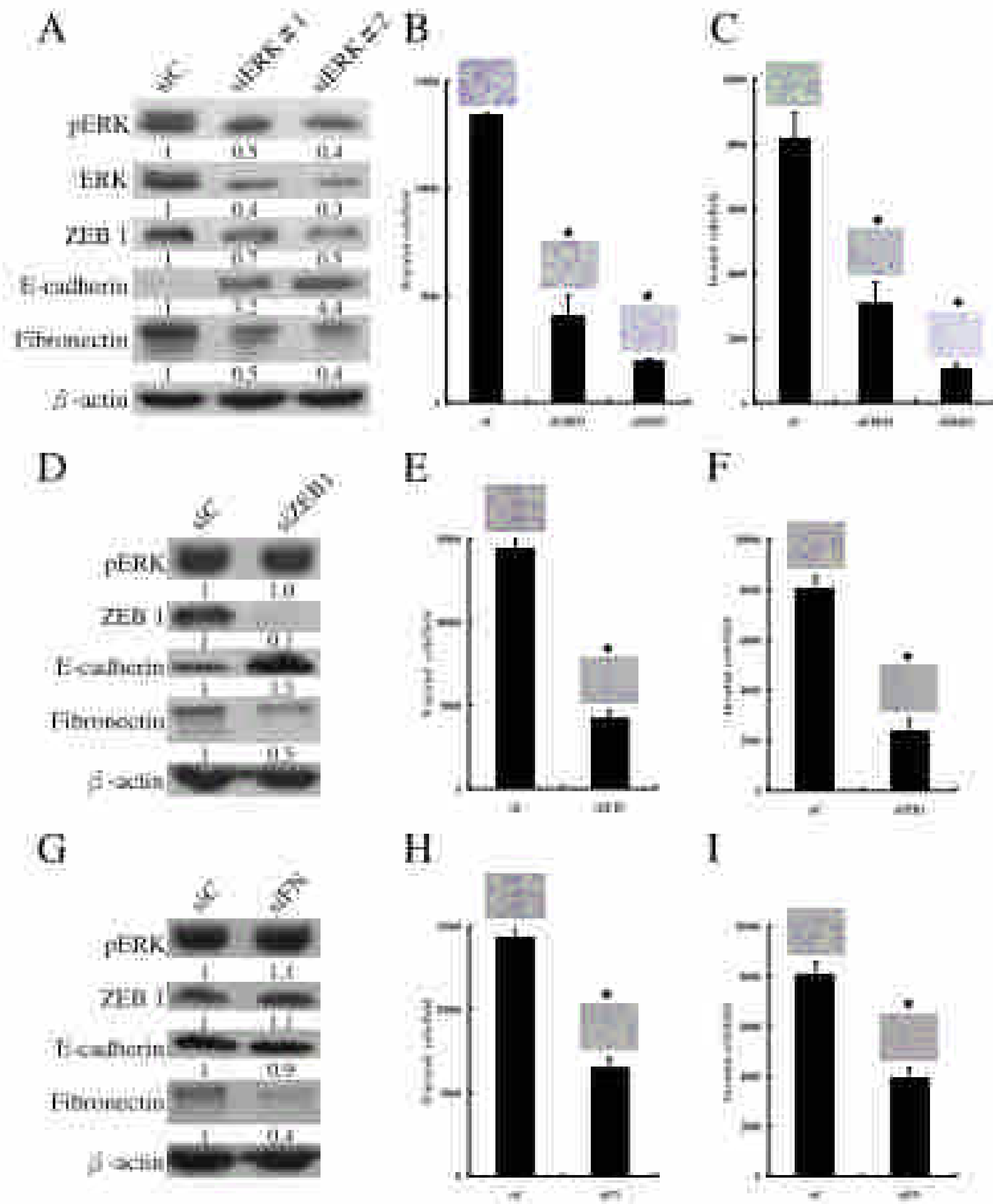


Figure 5. Inhibition of ERK by siRNA technology to correlate the EMT suppression in pemetrexed-resistant sublines.

The cells of A549/A400 (5×10^5 cells) were plated in 60-mm Petri-dishes followed by transfection with two different sequences of siRNA to inhibit ERK expression (siERK#1 and siERK#2). After 48 h incubation, the cells were harvested for pERK, ERK, ZEB1, E-cadherin and fibronectin protein detection (A) or seeded for transwell 4 h migration assay (B) and 16 h migration assay (C). A mixture of ZEB1 siRNAs were transfected into A549/A400 cells and the effects of inhibited ZEB1 were analyzed by Western blot (D), migration (E) and invasion (F) assays. Similarly, a mixture of fibronectin siRNAs were tranfected into cells and analyzed (G, H, and I) respectively. *p<0.05 was considered statistically significant.

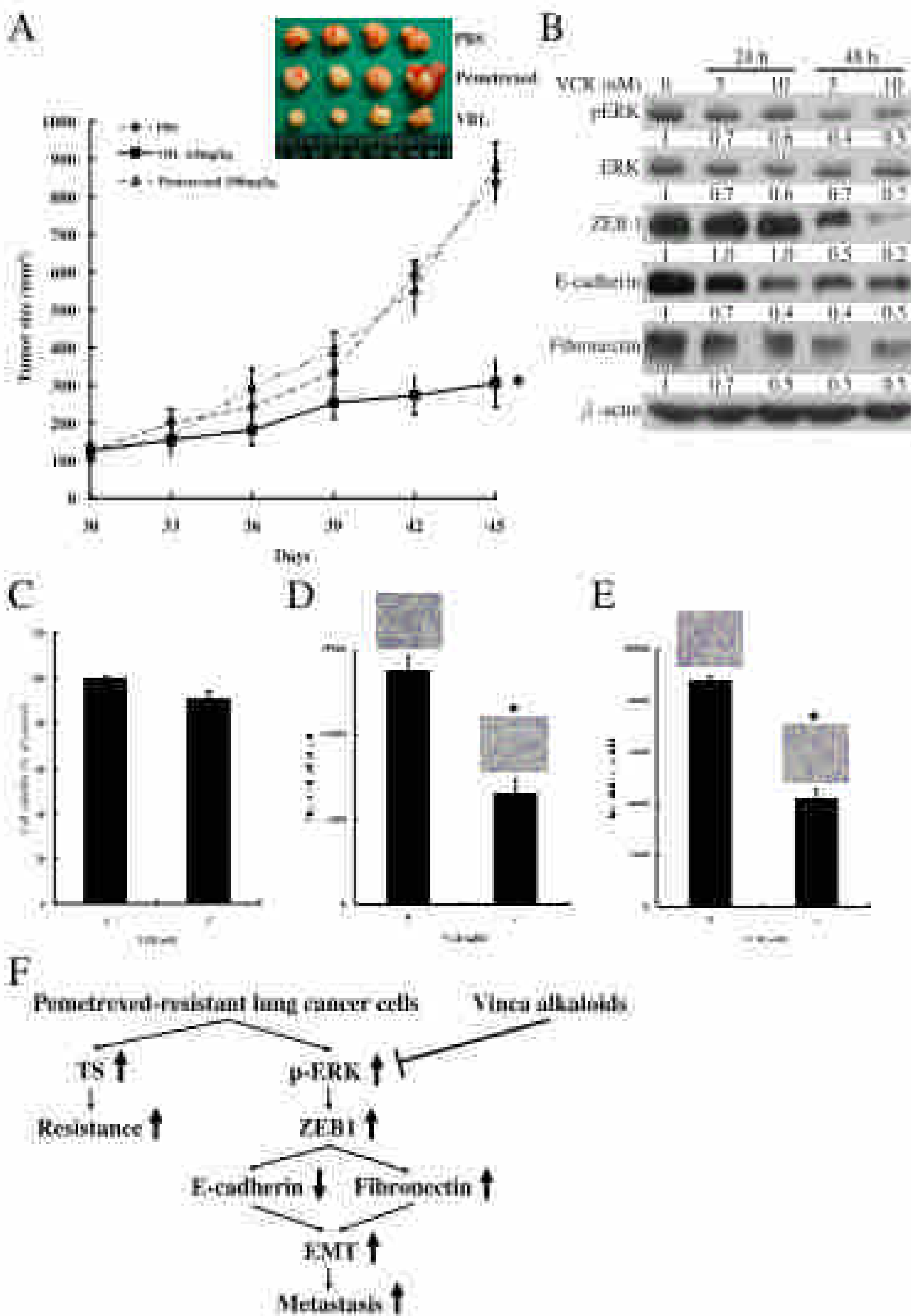
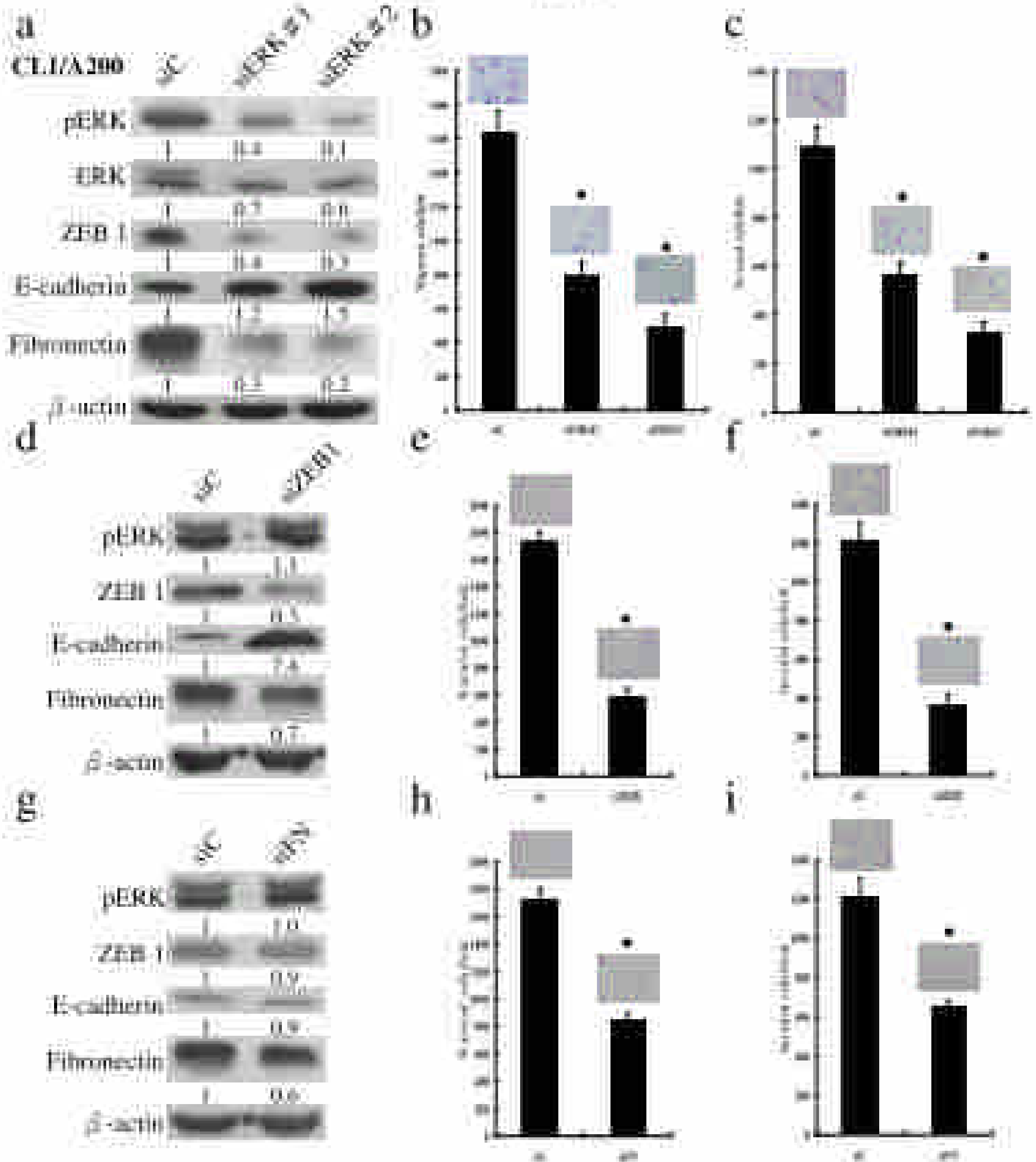


Figure 6. Vinblastine inhibits mice xenografted A549/A400 tumor growth and vincristine reduces phosphorylation of ERK.

The A549/A400 ($5x10^6$) cells were mixed with matrigel and injected subcutaneously. Twelve animals were then randomly divided into three groups. Thirty days after cell implantation when the tumor size reached 120 mm^3, mice in each group were intraperitoneally injected with PBS and pemetrexed (100 mg/kg/mouse) each week to serve as controls. The vinblastine (VBL) group was injected with 4.8 mg/kg per mouse per week. Tumor sizes were measured every 3 days following drug injection. A non-parametric Mann-Whitney U test was applied and *$p<0.05$ was considered statistically significant (A). The effects of vincristine (VCR) on A549/A400 cells were investigated by Western blot analysis (B). The cell viability with VCR (5 nM) was examined by MTT assay (C). The effects of VCR on migration (D) and invasion were determined (E). We have identified a signaling pathway that is regulated by vinca alkaloids in pemetrexed resistant lung cancer cells (F). *$p<0.05$ was considered statistically significant.

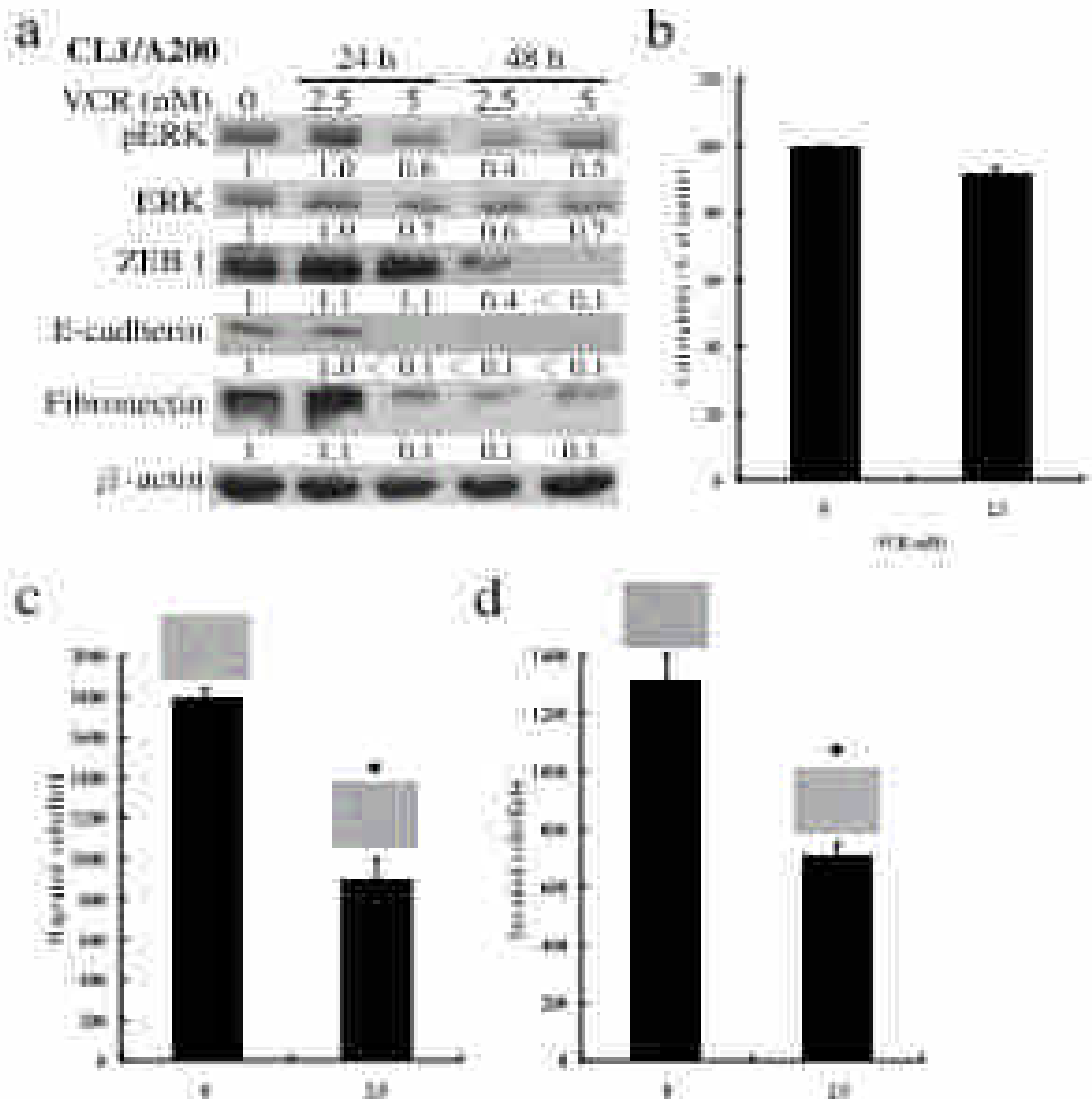
Supplementary figure 1



Inhibition of ERK by siRNA technology to correlate the EMT suppression in pemetrexed-resistant CL1/A200 cells.

The cells of CL1/A200 (5x105 cells) were plated in 60-mm Petri-dishes followed by transfection with two different sequences of siRNA to inhibit ERK expression (siERK#1 and siERK#2). After 48 h incubation, the cells were harvested for pERK, ERK, ZEB1, E-cadherin and fibronectin protein detection (a) or seeded for transwell 24 h migration assay (b) and 48 h migration assay (c) using 2x104 cells. A mixture of ZEB1 siRNAs were transfected into CL1/A200 cells and the effects of inhibited ZEB1 were analyzed by Western blot (d), migration (e) and invasion (f) assays. Similarly, a mixture of fibronectin siRNAs were transfected into cells and analyzed (g, h, i). *p<0.05 was considered statistically significant.

Supplementary figure 2



The effects of vincristine (VCR) on ERK phosphorylation.
Expression of ZEB1, E-cadherin and fibronectin in CL1/A200 cells treated with 2.5 and 5 nM of VCR were investigated by Western blot analysis (a). The cell viability with VCR (2.5 nM) was examined by MTT assay (b). The effects of VCR on migration (c) and invasion were determined (d). *p<0.05 was considered statistically significant.

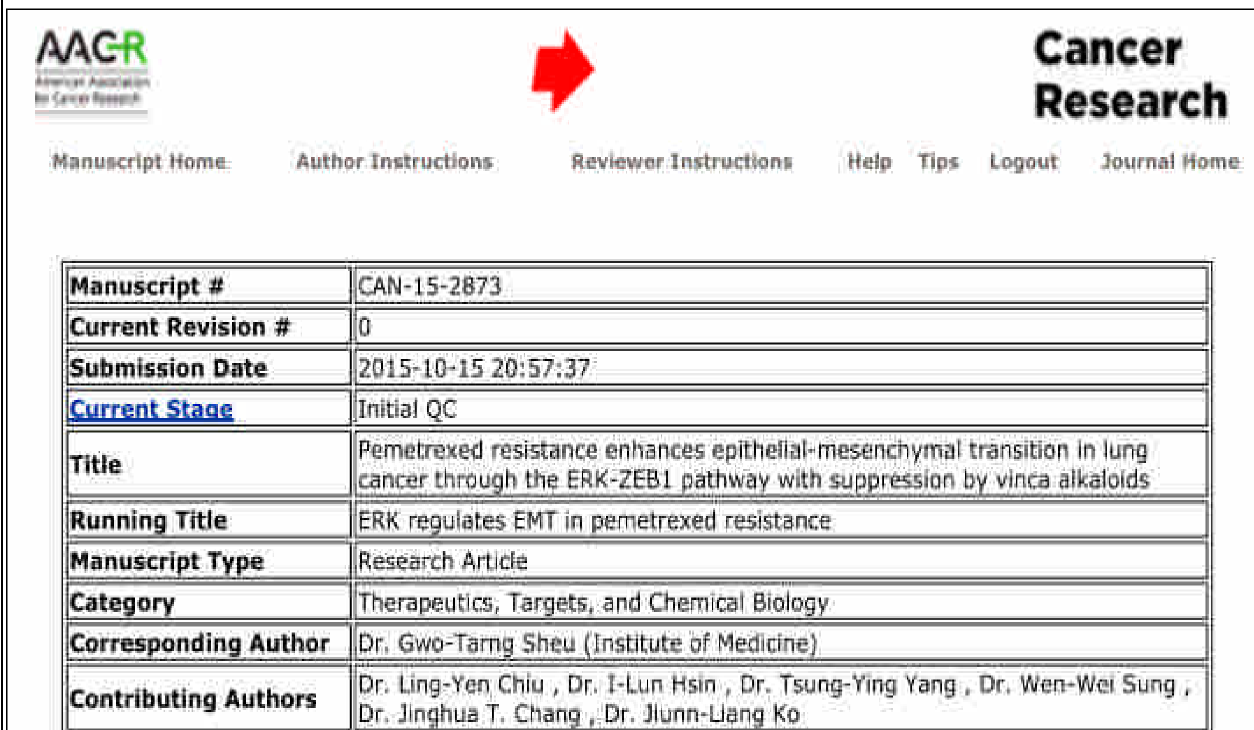
中　華　民　國 104 年　10 月

附件二

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否有嚴重損及公共利益之發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   □ x 成目標
2. 研究成果在學術期刊發表或申請專利等情形：
   論文： x 未發表之文稿，已經投稿



Manuscript #	CAN-15-2873
Current Revision #	0
Submission Date	2015-10-15 20:57:37
Current Stage	Initial QC
Title	Pemetrexed resistance enhances epithelial-mesenchymal transition in lung cancer through the ERK-ZEB1 pathway with suppression by vinca alkaloids
Running Title	ERK regulates EMT in pemetrexed resistance
Manuscript Type	Research Article
Category	Therapeutics, Targets, and Chemical Biology
Corresponding Author	Dr. Gwo-Tarng Sheu (Institute of Medicine)
Contributing Authors	Dr. Ling-Yen Chiu , Dr. I-Lun Hsin , Dr. Tsung-Ying Yang , Dr. Wen-Wei Sung , Dr. Jinghua T. Chang , Dr. Jiunn-Liang Ko

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性），如已有嚴重損及公共利益之發現，請簡述可能損及之相關程度（以 500 字為限）

Pemetrexed is commonly applied to first-line chemotherapy in nonsquamous non-small cell lung cancer patients; our findings suggest a new insight for overcoming pemetrexed resistance and associated metastasis in lung cancer by application of vinca alkaloids.

科技部補助計畫衍生研發成果推廣資料表

日期:2015/10/19

科技部補助計畫	計畫名稱: 肺腺癌細胞對Pemetrexed愛寧達之抗藥性和癌症轉移的機制研究
	計畫主持人: 許國堂
	計畫編號: 103-2320-B-040-015- 學門領域: 藥理及毒理

無研發成果推廣資料

103年度專題研究計畫研究成果彙整表

計畫主持人：許國堂			計畫編號：103-2320-B-040-015-				
計畫名稱：肺腺癌細胞對Pemetrexed愛寧達之抗藥性和癌症轉移的機制研究							
成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	1	100%	人次	
		博士生	0	1	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	1	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估 ■達成目標 □未達成目標（請說明，以 100 字為限） 　　□實驗失敗 　　□因故實驗中斷 　　□其他原因 說明：
2. 研究成果在學術期刊發表或申請專利等情形： 論文：□已發表 □未發表之文稿 ■撰寫中 □無 專利：□已獲得 □申請中 ■無 技轉：□已技轉 □洽談中 ■無 其他：（以 100 字為限） Manuscript # CAN-15-2873 Submission Date 2015-10-15 20:57:37 Current Stage Initial QC Title: Pemetrexed resistance enhances epithelial-mesenchymal transition in lung cancer through the ERK-ZEB1 pathway with suppression by vinca alkaloids Running Title: ERK regulates EMT in pemetrexed resistance Corresponding Author: Dr. Gwo-Tarng Sheu (Institute of Medicine)
3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限） Pemetrexed is commonly applied to first-line chemotherapy in nonsquamous non-small cell lung cancer patients； our findings suggest a new insight for overcoming pemetrexed resistance and associated metastasis in lung cancer by application of vinca alkaloids.