中 國 醫 藥 大 學
癌症生物學研究所
碩士學位論文

探討白藜蘆醇抑制肺癌細胞對表皮生長因子受體之酪胺酸激酶抑制劑的抗藥性

Resveratrol Decreases the Resistance of Lung Cancer Cells to EGFR Tyrosine Kinase Inhibitor

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致 謝：

時光飛梭，一轉眼兩年的碩士生涯就這樣即將進入尾聲。想當初，在剛進入到蘇振良老師實驗室學習時，我還是這領域的超級門外漢，如今我卻能做出一篇論文，真的是堪稱奇蹟啊！

短暫的兩年中，我遇到了許許多多、大大小小的瓶頸，也很慶幸在最後都能堅持到底並化危機為轉機的找出一條路。這樣的經歷讓我在面對失敗、面對壓力學會如何去調適，真的讓我不論在知識與心靈上皆成長許多。

在這期間，最感謝許多曾經幫助過我的貴人們。首先，謝謝蘇振良老師願意給予我機會在實驗室學習，提供豐厚的資源讓我無後過之憂的進行研究，在學習過程中，也很謝謝老師的耐心指導與包容。不僅如此，更謝謝老師在平常生活上關心與照顧。再者，感謝中山醫生化暨生物科技所謝逸憲老師與本校醫學系微生物學科賴志河老師擔任我的口試委員，給予我十分寶貴的指導。

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中文摘要:

肺癌在許多國家皆為一常見的惡性腫瘤，包括台灣在內，並且位居癌症死因之首。表皮生長因子受體（epidermal growth factor receptor, EGFR） 調控細胞許多的生理功能，包括細胞的增生、分化、移動以及代謝，其過度表現或活化與許多癌症的發生有所關聯。Gifitinib (Iressa, 艾瑞莎) 為表皮生長因子受體之酪胺酸激酶的抑制劑，已有效的運用在非小細胞肺癌患者的治療，但其功效卻往往受到爾後抗藥性發生的抑制。白藜蘆醇 (resveratrol) 是一種天然的植物抗菌素 (phytoalexin)，主要存在於葡萄、花生以及桑椹中，亦存在於中草藥厚朴、虎杖之中。在科學文獻的記載中具有抗癌活性，能防止細胞癌變，阻止惡性腫瘤擴散，對腫瘤發生的起始、促進和發展三個階段都有抑制作用，並且在許多的實驗模式下也被證實具有化學增敏與化學預防的功效，因此具有成為重要的抗癌藥物之潛力。為了研究白藜蘆醇對肺癌細胞的標靶治療之影響，我們建立 Iressa-resistance 的肺癌細胞株 (PC-9/IR)，當作我們的實驗模式。在功能分析上，我們發現在 PC-9/IR 細胞中，先給予無毒性劑量之白藜蘆醇 (1.25, 2.5, 5, 10 μM) 可敏感化艾瑞莎的毒殺效果。持續給予艾瑞莎 48 小時，在 PC-9/WT 細胞中可誘發 55.37% ± 0.37%的細胞死亡，但在 PC-9/IR 細胞中只有 8.76% ± 1.56%的比例。在合併治療上，我們觀察到在 PC-9/IR 中先給予白藜蘆醇可增強艾瑞莎所誘導的細胞死亡(單獨處理艾瑞莎的細胞死亡比例為 8.76% ± 1.56%，合併白藜蘆醇與艾瑞莎的處理，細胞死亡比例則為 48.71%±3.12%)。這些結果顯示，在具有抗藥性的肺癌細胞中，白藜蘆醇可能可以敏感化表皮生長因子受體之酪胺酸激酶的抑制劑。為了進一步了解其中所調控的分子機制，我們預測一些調控可能的下游目標，例如，上皮-間質轉化 (epithelial-mesenchymal transition, EMT) 相關分子與 microRNAs。藉由二維電泳分析法與 microRNA 矩陣分析法，我們發現一些可能參與在肺癌細胞對表皮生長因子受體之酪胺酸激酶的抑制劑之抗藥性。

關鍵字：肺癌、表皮生長因子受體、Gifitinib、酪胺酸激酶抑制劑、抗藥性、白藜蘆醇、敏感化

英文摘要 (Abstract):

Lung cancer is one of the most common malignancies in many countries, including Taiwan, and also is the first leading cause of cancer-induced death. EGFR (epidermal growth factor receptor) regulated a lot of biological functions of cells including proliferation, differentiation, motility and metabolism; its overexpression or activate mutation are associated with a number of cancers. Gefitinib (Iressa), a specific EGFR tyrosine kinase inhibitor, has been used in treatment of non-small lung cancer patients. However, its therapeutic activity is limited by the development of drug resistance. Resveratrol (trans-3, 4', 5-trihydroxystilbene) is a polyphenolic compound found in various plants and some Chinese herbs. Resveratrol has the ability to inhibit cancer formation at initiation, promotion and progression; also it has been shown to receive chemosensitive and chemopreventive effects in different experimental systems. To define the effects of resveratrol on target therapy of lung cancer cells, we used Iressa-resistance (PC-9/IR) lung cancer cells as our experimental models. In the functional assay, we found that pre-treatment with non-toxic dosages of resveratrol (1.25, 2.5, 5, 10 μM) sensitized the toxic effect of Iressa (2.5 μM) in Iressa-resistant PC-9/IR cells. Treatment with Iressa for 48 hrs induced 55.37% ± 0.37% cell death in PC-9/WT cells, but only 8.76% ± 1.56% in PC-9/IR cells. Pre-treatment with resveratrol increased Iressa-induced cell death in PC-9/IR cells (8.76% ± 1.56% cell death in vehicle + Iressa group; 48.71% ± 3.12% cell death in resveratrol + Iressa group). These results indicate that resveratrol may sensitize EGFR tyrosine kinase inhibitor in resistant lung cancer cells. To further evaluate the molecular mechanisms, we define the potential down-stream targets, such as epithelial-mesechymal transition (EMT)-related proteins and microRNAs, involved in resveratrol-induced de-resistance of EGFR tyrosine kinase inhibitor in lung cancer cells. By two-dimensional gel electrophoresis and microRNA microarray assay, we found some potential downstream targets which may involve in the resistance of lung cancer cells to EGFR tyrosine kinase inhibitor.

Key words：lung cancer、EGFR、Gefitinib、tyrosine kinase inhibitor、drug resistance、resveratrol、sensitize

1. Introduction

1.1 Lung cancer

Lung cancer is one of the most common malignancies in many countries, including Taiwan, and also is the first leading cause of cancer-induced death among men and women. It is responsible for over 1 million deaths worldwide annually (1). The most common type of lung cancer is non-small cell lung cancer (NSCLC) which accounts for almost 80% of such death (2, 3). According to the database of Department of Health in Taiwan, in 2008 lung cancer caused the casualty in Taiwan area to reach 7777 people (20.0%), for cancer cause of death 1st. Despite advances in surgical resection, radiotherapy and chemotherapy, <15% of patients with NSCLC survive beyond 5 years of initial diagnosis.

The medium survival of metastatic NSCLC is 8 to 10 months when treated with the most active combination of conventional chemotherapeutic agents (4, 5). For example, the gemcitabine-cisplatin combination chemotherapy is one of the most effective chemotherapy regimens against NSCLC in recent years (3). Although combination chemotherapy has improved the prognosis of NSCLC patients, there are still many patients who have initial resistance to chemotherapy or develop drug resistance after several courses of chemotherapy (6). Therefore, development of molecular targeted agents becomes the newly therapeutic strategies for NSCLC treatment.

1.2 EGFR tyrosine kinase inhibitor

Epidermal growth factor receptor (EGFR) is a member of a family of closely related growth factor receptor tyrosine kinases, which include EGFR (ErbB1), HER2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4) (7). EGFR is a key signal transduction component that is commonly altered in >60% of NSCLC (8). Also, it is overexpressed in other majority of solid tumors, including breast cancer, head-and-neck cancer, colon cancer, renal cancer, and ovarian cancer (9). Such overexpression produces intense signals and activation of downstream signaling pathways, resulting

in cells that have more aggressive growth and invasiveness characteristics (10). It is well known that genomic amplification, point mutations, and autocrine loop activation are responsible for the increased activity of EGFR. Almost 90% of these somatic activating mutations in EGFR consist of in-frame deletions in exon 19 and L858R point mutations in exon 21 (11, 12). These mutations are more frequently present in females than in males, in nonsmokers than in smokers, in East Asians than in other ethnic groups, and in adenocarcinomas than in other tumor types (13).

Recent years, EGFR has received a significant amount of attention, because of various strategies involving small-molecule inhibitors have also been developed to target EGFR and/or its family members, and these are in various stages of clinical trial (14). The specific tyrosine kinase inhibitors (TKIs), including gefitinib and erlotinib, have been developed as therapeutic agents for NSCLC treatment. Several clinical trials have shown that both agents cause responses in 70% to 75% of all NSCLC patients with tumors harboring EGFR mutations (13, 15). Despite the dramatic responses of cancers with sensitizing EGFR mutations to TKIs, these tumors invariably develop drug resistance within 9 to 12 months (16, 17, 18).

Gifitinib (Iressa) is an orally active, reversible, low-molecular-weight tyrosine kinase inhibitor which is highly selective for EGFR tyrosine kinase. It prevents the binding of ATP to the ATP-binding pocket of the EGFR in a competitive manner, thereby leading to the loss of catalytic activity and block the signal transduction pathways implicated in the proliferation and survival of cancer cells (19, 20, 21, 22). Significant antiproliferative effects of daily oral administration of Iressa have been observed in A549 NSCLC, HX62 ovarian, MCF-7 breast carcinoma, colorectal carcinoma, and several prostate carcinoma human xenograft models (23). Although it has provided dramatic clinical response and even survival benefits for a subset of lung cancer patients, its efficiency is limited by the development of drug resistance which is the nightmare of every cancer patients. Therefore, additional treatments for NSCLC patients who failed with treatment of Iressa are urgently needed.

1.3 Resveratrol

Resveratrol (*trans*-3, 5, 4'-trihydroxystilbene) is a polyphenols which was first isolated from white hellebore (*Veratrum grandiflorum O. Loes*) in 1940, and since then it has also been found in various plants including grapes, peanuts, and mulberries (24). In 1963, the root in *Polygonum caspidatum* used in traditional Chinese and Japanese medicine called Ko-jo-kon (25) was found the existence of resveratrol. The sources of resveratrol were red wine and red grape (26), and resveratrol was synthesized in the leaf epidermis and in the grape skins (27). Resveratrol can prevent or slow the progression of illnesses including cardiovascular disease (28), cancer (29) and ischemic injury (30, 31) . Several studies have shown that resveratrol could extend lifespan by silent information regulator 2 (Sir2)-dependent mechanisms in *Saccharomyces cerevisiae* (32), *Caenorhabditis elegans* (33) and *Dorsophila melanogaster* (34), and also extend lifespan of short-lived fish *Nothobrachius furzen* (35). Resveratrol is an activator of SIRT1 which has the ability to prolong survival of calorie restriction mice treated with high-calorie diet (36).

The effects of resveratrol are dependent on the cell type, cellular condition, and concentrations and may have opposing activities under different experimental conditions (27). The chemopreventive activity of resveratrol was associated with inhibition of cancer formation at initiation, promotion and progression in different types of cancer including liver cancer (37, 38) , colon cancer (39, 40), breast cancer (41), lung cancer (42, 43), melanoma (44, 45) , head and neck squamous cell carcinoma (46, 47), ovarian and cervical carcinoma (48-50). Resveratrol down-regulates the enzyme activity and transcriptional activity of cytochrome P-450 1A1 (CYP1A1) which is a carcinogen-activating enzyme, indicating that resveratrol can inhibit tumor progression at initiation stage (51). Resveratrol also inhibits tumor formation by down-regulating the expression of cyclooxygenase 2 (COX2) (52). Treatment with resveratrol induced cell apoptosis through various signaling pathways such as FasL pathway (53), mitochondria-dependent pathway (54), p53-dependent pathway (55-58) and Rb-E2F/DP pathway (59). Resveratrol mediates cell-cycle arrest at different stages in different types of cells (60-65) . Some transcription factors are inhibited

by resveratrol such as NF-κB (66-69), AP-1 (70), Egr-1 (71, 72), and AP-2α (73). Resveratrol also down-regulate protein kinases such as IκB (66), JNK, MAPK, ERK1/2 (57, 58, 74, 75), Akt (76, 77), PKC (78), PKD (79) and CKII (80). Pharmacokinetic studies show that in human, resveratrol has rapid metabolism (~8 to 14 mins) and converted to sulfate and glucuronide conjugate within ~30 mins in liver and kidney (81). Resveratrol inhibits vascular endothelial growth factor (VEGF)-induced angiogenesis in HUVECs (82) and also inhibits cancer cells invasion and migration in breast cancer (83). Recent studies shown that resveratrol inhibits the invasion of tumor cells through repression of MMP-2 (82) or MMP-9 (84). Resveratrol has the effect on prevention of tumor growth and lung colonization in Lewis Lung Carcinoma-Bearing Mice (85). Treatment with resveratrol in human colorectal cancer are under several clinical trials in phase I stage (34). Moreover, it was reported recently that resveratrol could sensitize a number of cancer cell lines to the anticancer actions of several other cancer drugs, including Paclitaxel (86).

1.4 Hypothesis and specific aim

EGFR is a key signal transduction component that is commonly altered in >60% of NSCLC (8). The specific tyrosine kinase inhibitors (TKIs), Iressa, have been developed as therapeutic agents for NSCLC treatment. Although it has provided dramatic clinical response and even survival benefits for lung cancer patients, its therapeutic activity is limited by the development of drug resistance. Resveratrol, a polyphenols, has received attention because it has been shown to receive chemosensitization effects *in vitro*. Therefore, we sought to examine the combined effects of resveratrol and Iressa on reversing the drug resistance of human NSCLC.

2. Materials and Methods

2.1 Reagents

Resveratrol (＞99% purify) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of resveratrol was made in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at a concentration of 5 mM and stored at -80℃.

Iressa® (gefitnib tablets) was obtained from AstraZeneca Pharmaceuticals. A stock solution of 25 mM Iressa was kept at -80℃ and a dilution of 5 mM was prepared in DMSO for each experiment.

RNase A and propidium iodide were also purchased from Sigma-Aldrich. The stock solution of RNase A at a concentration of 20 mg/ml was kept at -20℃. And 500 μg/ml propidium iodide was also kept at -20℃.

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was gained from Promega (Madison, WI, USA) and kept in dark at -20℃.

For RNA extraction, TRIzol® Reagent was used, obtained from Invitrogen (Carlsbad, CA, USA) and kept at 4℃.

2.2 Cell line and cell culture

Human NSCLC PC-9 cells and the iressa-resistant cell line PC-9/IR were cultured with RPMI-1640 (GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Invitrogen) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, GIBCO, Invitrogen). Cells were maintained at 37℃ in humidified 5% CO_2 atmosphere. For treatment, resveratrol and Iressa were diluted in medium and added to cultures to give the desired final concentrations. Untreated cultures received the same amount of the carrier solvent, DMSO.

2.3 Establishment of the Iressa-resistant PC-9 cells *in vitro*

In order to create a Iressa-resistant cell line, the PC-9 lung cancer cells were exposed to 1 μM Iressa for 48 hrs in PRMI-1640 plus 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin,

which was lower than the IC_{50} of PC-9/WT cells. After exposure to Iressa, cells were washed and cultured in drug-free medium until the surviving population of cells was grown to 80% confluence. The surviving cells were continuously exposed to increasing dosages and finally to a concentration of 10 μM. The established resistant cell line (PC-9/IR) was maintained by culture in a medium containing 7.5 μM Iressa. For all the *in vitro* studies, the resistant cells were cultured in drug-free medium for 1 day to eliminate the effects of Iressa.

2.4 Cell cycle analysis

Flow cytometric assay was performed to determine the cell cycle profiles of PC-9 lung cancer cells in response to Iressa treatment. PC-9/WT and PC-9/IR cells were plated in 10-cm tissue culture dishes and treated with various concentration of Iressa (1, 5, 10 μM) for 48 hrs, and then the cells were washed twice with PBS, trypsinized and fixed in 70% ethanol overnight at 20℃. Cells were resuspended in PBS containing 0.1% Triton X-100 and RNase A (0.2 mg/ml) (Sigma-Aldrich). The cell suspension (3×10^5 ~5×10^5 cells/ml) was incubated at 37℃ for 30 min. Propidium iodide (Sigma-Aldrich) was added at a final concentration of 20 μg/ml and the cell suspension was kept in the dark at 4℃ for 1 hr. The cells were filtered and the cell cycle was analyzed by flow cytometry with the FACSCalibur flow cytometer (Becton Dickinson). Cellular debris was excluded from the analysis by raising the forward scatter threshold, and the DNA content of the intact nuclei was recorded on a linear scale. A minimum of 10000 events was collected on each sample.

2.5 Cell proliferation by MTS assay

Growth and inhibition of growth were assessed by the MTS assay according to the manufacturer's instructions (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA). This assay, a colorimetric method for determining the number of viable cells, is based on the bioreduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium (MTS) by cells to a formazan product that is soluble in cell culture medium, can be detected spectrophotometrically. Briefly, the cells were seeded into 96-well plates at a density of 4×10^3 cells per well, and treated with Iressa (0-10 μM) or resveratrol (0-10 μM).

After incubation for 48 hrs, the MTS reagent were added to each well and then incubated for a further 2 hrs (37℃ and 5% CO_2). Absorbance was measured at 490 nm using a microplate reader (BioTek). Each combination of cell line and drug concentration was set up in 4 replicate wells and repeated at least 3 times. The percentage of cell viability is shown relative to untreated controls.

2.6 Western blot analysis

Western blot analysis was used for assaying the phospho-EGFR, EGFR, phosphor-AKT, AKT, phospho-extracellular signal-regulated kinase (ERK) 1/2, ERK, LDHA expression. Bio-Rad expression system was employed in this experiment. Briefly, the whole cells were harvested and washed by PBS. The process was first lysed in NETN lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.5 % NP 40 and 1 Mm EDTA) containing protease inhibitor cocktail (Sigma-Aldrich) and centrifuged at 13000 rpm for 30 min. The supernatant was extracted and transferred to the acrylamide gel under manufacture's recommendation (Amersharm, Arlington Heights, IL). Protein were heated in 4X sample buffer at 100℃ 10 min and then loaded into each well for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated protein was transferred to a PVDF member (Millipore) by all-wet method (Bio-Rad) under 300 mA for 3 hrs. Non-specific blocking was performed by immersing the membrane in 5 % milk in Tris-buffered saline-Tween (TBS-T) (10 ml of 2 M Tris-HCl pH 7.4, 100 ml of 5 M NaCl, 0.5 ml of 100 % Tween-20, distilled water 890 ml) for 1 hr at room temperature. Followed by various antibody; phospho-EGFR (Tyr 1173), EGFR, phospho-AKT (Ser 473; Ser 308), AKT, phosphor-ERK, ERK, LDHA (Cell signaling) and β-actin (Sigma-Aldrich). All antibodies were diluted to a recognition level (1:1000) and incubated at room temperature for 2 hrs or overnight as condition needed. Washing the membrane with TBST three times (10 min/time), and HRP labeled second antibody (Santa Cruz, CA, USA) for enhanced chemiluminescence (ECL) marker were diluted at a 1:5000 concentration. The membrane was incubated at room temperature for 1 hr, and wash with TBST three times (10 min/times) for removed exceed antibody. The ECL detection kit (Millipore) was added to the membrane and incubated for 1 min without agitation. The membrane was wrapped in

SaranWarp and placed in film cassette with film (Kodak) in the dark room for optimal exposure condition before final film development.

2.7 Oligo gene expression microarray analysis (Human Whole Genome OneArray ™ Microarray, Phalanx)

PC-9/WT and PC-9/IR cells were harvested and total RNA were purified using the TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. Assays were performed in triplicate and total RNA from triplicates were pooled. All RNA samples were of the high purity with 260/280 absorbant ratio of 1.8-2.2 measured by NanoDrop ND-1000 (NanoDrop), well intact with RIN score of above 7 determined by Agilent RNA 6000 Nano Assay (Agilent), and no DNA contamination checked by agarose gel electrophoresis. The aminoallyl-RNA (aRNA) probes were amplified using the MessageAmp aRNA kit (Ambion) and labeled with the NHS-Cy5 (GE). The Cy5-labeled aRNA probes were also purified and quantified. The labeled probes were hybridized at 50°C for 16 hrs to the human Oligo Microarray (Phalanx Human Whole Genome OneArray ™ Version 4.3, Phalanx Biotech), which contains 30,968 well-characterized genes. After washing 3 step (Wash Ⅰ, 42°C, 5 min；Wash Ⅱ, 42°C, 5 min；Wash Ⅲ, 25°C, 5 min), the array was exposed to a phosphorimaging cassette and then scanned with the Axon 4000B Scanner (Molecular Devices) as well as analyzed with Genepix software (Molecular Devices).

Tests were at least performed twice (including technical and biological replicates; reproducibility >0.95). The standard value to exclude “noise” or background was the signal intensity value of median-background >0, the GenePix flag score =0, and the signal/noise ratio (SNR) ≧3. The qualified probes must be present in at least 50% of the biological samples in order to be selected for normalization and fold change calculation. All data were processed by Global scaling normalization. Statistic P-value could be calculated using pair *t*-test of Microsoft Excel function.

2.8 MicroRNA microarray hybridization (NCode™ miRNA Labeling System, Invitrogen)

For NCode™ miRNA Labeling System experiments, RNA was prepared by TRIzol® (Invitrogen) extraction. Total RNA was poly(A) tailed using poly(A) polymerase. A DNA polymer with either Alexa Fluor® 3 or Alexa Fluor® 5 dye molecules was ligated to the poly(A)-tailed RNA via T4 DNA ligase and an oligo(dT) bridge consisting of bases complementary to the dye-labeled DNA Alexa Fluor® polymer and bases complementary to the poly(A) tail on each tailed RNA molecule. The ligation and subsequent steps were protected from light. Alexa Fluor® 3– and Alexa Fluor® 5–labeled samples were combined and their volume reduced to half by a SpeedVac® Concentrator (Thermo Savant). Bovine serum albumin and 2X hybridization buffer were added to each sample. Samples were heated to 65°C for 10 min. The hybridization mix was applied to an NCode™ Multispecies Microar-ray V2 and incubated at 52°C for 8–16 hrs. Microarrays were washed for 15 min in three successive wash buffers: 2X SSC and 0.2% SDS at 52°C, 2X SSC at room temperature, and 0.2X SSC at room temperature. Microarrays were dried by centrifugation in a tabletop centrifuge and scanned using a GenePix® 4000B Array Scanner (Molecular Devices). GenePix Pro 5.0 software will be used for image acquisition, normalization, and data analysis.

2.9 Two-dimensional gel electrophoresis analysis, liquid chromatography and tandem mass spectrometry (LC–MS/MS)

The different expression proteins between PC-9/WT and PC-9/IR were investigated by two-dimensional gel electrophoresis serviced by Mission Biotech. Briefly, the cells were harvested and each sample of 1 mg protein were placed in a rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 0.2% Bio-Lyte (pH 3 to 10), and a trace of bromophenol blue and applied to linear IPG Readystrips (24 cm; pH 3–10; Bio-Rad) by in-gel rehydration for 12 hrs at 20°C. Isoelectric focusing (IEF) was performed using a protein IEF cell (Bio-Rad) under the following conditions: 20°C, 250 Vhr for 30 min; 1 kVhr for 2 hrs; 10 kVhr for 5 hrs; and 10 kVhr until 70 kVhr was achieved. Once the IEF was completed, the individual strips were equilibrated for 15 min in an equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris–HCl (pH 8.8), 20% glycerol, and 2% DTT), and then for another 15 min in the same buffer except that the DTT was replaced by

2.5% iodoacetamide. After equilibration, the proteins were separated in the second dimension by vertical 12% SDS-PAGE with the IPG strips mounted on the top of the gels. SDS-PAGE was run with a constant voltage of 200 V at 16 °C until the bromophenol blue front reached the bottom of the gel. Gels were then stained using an improved commasie blue-staining method. The image analysis was by ImageMaster™ 2D Platinum (Swiss Institute of Bioinformatics). Differentially expressed protein spots were manually cut from the commasie blue-stained gels, transferred into eppendorf tubes and digested. Liquid chromatography and tandem mass spectrometry (LC–MS/MS) analysis was provided by GRC Mass Spectrometry facility (Genomics Research Center, Academia Sinica). Data processing for protein identification and quantification was performed using SWISS-PROT and IPI databases.

2.10 RNA isolation and reverse transcription

Cultured cells were washed twice with PBS, and total RNA was extracted using the TRIzol® Reagent (Invitrogen). Reverse transcription reactions contained 1 μl of RNA, 50 nM stem-stoop RT primer, 0.25 mM each dNTP, 50 units of moloney murine leukemia virus reverse transcriptase (MMLV-RT; Invitrogen), 1X reverse transcription buffer, 10 mM DTT, and 4 units of RNase inhibitor. The stem-loop RT primers were designed according to Chen C (87). The sequences of mature miRNAs were obtained from the Sanger Center miRNA Registry (http://microrna.sanger.ac.uk/sequences/) and were as follows: miR-20a RT primer (5'- GTT GGC TCT GGT GCA GGG TCC GAG GTA TTC GCA CCA GAG CCA ACC TAC CTG -3'); miR-21 RT primer (5'- GTT GGC TCT GGT GCA GGG TCC GAG GTA TTC GCA CCA GAG CCA ACT CAA CAT-3'); miR-23a RT primer (5'- GTT GGC TCT GGT GCA GGG TCC GAG GTA TTC GCA CCA GAG CCA ACG GAA AT-3'); miR-200c RT primer (5'- GGT TGG CTC TGG TGC AGG GTC CGA GGT ATT CGC ACC AGA GCC AAC TCC ATC A-3'); miR-574-5p RT primer (5'- GTT GGC TCT GGT GCA GGG TCC GAG GTA TTC GCA CCA GAG CCA ACA CAC ACT-3'); and miR-U47 RT primer (5'-GTT GGC TCT GGT GCA GGG TCC GAG GTA TTC GCA CCA GAG CCA ACA CCT CAG-3').

The reactions were incubated at 37℃ for 50 min, 16℃ for 30 min, followed by pulsed RT of 60 cycles at 20℃ for 30 sec, 42℃ for 30 sec and 50℃ for 1 sec. Reactions were terminated by incubating at 85℃ for 5 min to inactivate the reverse transcriptase.

2.11 Real-time PCR quantification

Real-time PCR will be performed using a Roche LightCycler 480 Real-Time PCR system. PCR reactions contained 0.5 μM of each forward and reverse primer, 0.1 μM Universal ProbeLibrary Probe #21 (Roche), 1X LightCycler TaqMan Master, and 2 μl of cDNA. Amplification curves will be generated with an initial denaturing step at 95℃ for 10 min, followed by 50 cycles of 95℃ for 5 sec, 60℃ for 10 sec, and 72℃ for 1 sec. The U47 small nuclear RNAs will be used as an internal control. The forward primers were for miR-20a (5'- CGG CGG ATA AAG TGC TTA TGT -3'), miR-21 (5'- CGG CGG TAG CTT ATC AGA CTG -3'), miR-23a (5'- CGG CGG ATC ACA TTG CCA GGG -3'), miR-200c (5'- CGG CGG TAA TAC TGC CGG GTA A -3'), miR-574-5p (5'- CGG CGG TGA GTG TGT GTG TGT -3') and U47 (5'-CGG CGG TAA TGA TTC TGC CAA A-3'). The reverse primer was 5'-GTG CAG GGT CCG AGG T-3'.

2.12 Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol® reagent (Invitrogen) and reverse transcribed into single-stranded cDNA with MMLV-RT (Invitrogen) as manufacturer's instructions. The primer sequences for IGFBP7, ASNS, SLC47A2, KISS1, NKX2-5, MAGEC2, EFEMP1, PSAT1, PCK2, GAS6, FN1, STC2, SLC1A4, MT1E, S100A4, S100A2, SPRRIB and GAPDH used were shown in Table 1. The reaction mixture was first denatured at 95℃ for 5 min. For these candidate genes, the PCR condition was 95℃ for 30 sec, 50℃ for 30 sec and 72℃ for 30 sec for 30 cycles; for GAPDH was 94℃ for 30 sec, 55℃ for 30 sec and 72℃ for 30 sec for 22 cycles followed by 72℃ for 10 min.

2.13 Construction and production of shRNA in lentiviral vector

shHNRNPA2B1 (TRCN0000001058), shCPT2 (TRCN0000003091), shACAA1 (TRCN0000036072), shAPOBEC3C (TRCN0000052102), shLDHA (TRCN0000164922),

pLKO.1-shLuc vector (shRNA against luciferase, act as a control), pMD.G plasmid and pCMVdeltaR8.91 plasmid were obtained from National RNAi Core Facility at the Genomics Research Center (Academia Sinica, Taipei, Taiwan). Recombinant lentiviruses were produced by co-transfecting 293T cells with the lentivirus expression plasmid, the lentivirus packaging vector pCMVdeltaR8.91, and the vesicular stomatitis virus G glycoprotein (VSVG) expression vector pMD.G using the Lipofectamine ™ LTX according to manufacture's instructions. The viruses were collected from the culture supernatants on 2 days post-transfection and filtered by 0.45 μM filter. Cultured cells were incubated with lentivirus containing 8 μg/ml polybrene for 24 hrs, replaced medium and incubated for another 2 days. For stable clone, cells were then selected with 6 μg/ml puromycin for 1 week.

2.14 Statistical analysis

Data are presented as mean ± SE. Two-tailed Student's *t* test was used to analyze the difference between the means of the treatment and the control groups. Differences with a *p* value of less than 0.05 were considered statistically significant.

3. 結果 (Results)

3.1 The acquisition of Iressa resistance in NSCLC PC-9 cells

Lung cancer cell that have acquired resistance to Iressa may complicate future treatment. In order to define the effects of resveratrol on target therapy of lung cancer, we set up a EGFR tyrosine kinase inhibitor-resistant cell model. The Iressa-resistant cells were established and that were derived from the parental sensitive PC-9 cell line. These Iressa-resistant cells were selected by stepwise increasing the concentrations of Iressa. The concentrations of Iressa were started at 1 μM and achieved 10 μM. Several reports have been shown that the antitumor activity of EGFR tyrosine kinase inhibitor was associated with G_1 phase arrest in cancer cells (88-90). In order to investigate the characters of Iress- resistant cells different from parental PC-9/WT cells, PC-9/WT and PC-9/IR cells were cultured in the absence (control) or presence of various concentration (1, 5, 10 μM) of Iressa for 48 hrs. After treatment with Iressa, cells were stained with propidium iodide (PI) and subjected to DNA profile analysis by flow cytometry. Treatment with Iressa induced G_1 phase arrest of PC-9/WT cells, and increased the percentage of cells with sub-G_1 DNA content, which means the cell death. However, we did not find alteration of cell cycle or increasing of sub-G_1 cell population in PC-9/IR cells under the same experimental condition (Figure 1). To further confirm the EGFR tyrosine kinase inhibitor resistance, we performed MTS assay to analysis the inhibition of cell viability by Iressa on PC-9 lung cancer cells. Results showed that PC-9/IR cells were significantly resistant to Iressa than PC-9/WT cells in a concentration-dependent manner (Figure 2). The IC_{50} of Iressa in PC-9/IR cells (>10 μM) was at least 10-fold higher then parental PC-9/WT cells (IC_{50}, <1 μM). After that, we further evaluated the different expression of EGFR and downstream signaling proteins in PC-9/WT and PC-9/IR cells. Figure 3a showed that the expression of *EGFR* had no difference between PC-9/WT and PC-9/IR cells. Nevertheless, the protein expression of EGFR and downstream molecules were similar in parental PC-9 cells and Iressa-resistant cells (Figure 3B). In order to investigate the effects of resveratrol on cell viability in

lung cancer cells, PC-9/IR and PC-9/WT cells were treated with resveratrol for 48 hrs in indicated dosages (1.25, 2.5, 5, 10 μM). We found that resveratrol shows similar toxicity in both PC-9/WT and PC-9/IR cells (Figure 4). Treatment with resveratrol, even to 10 μM, did not cause significant reduction of cell viability in PC-9/WT and PC-9/IR cells. According to above data, we successfully established an Iressa-resistant lung cancer cell line, PC-9/IR, as the later study model.

3.2 Resveratrol-induced de-resistance of EGFR tyrosine kinase inhibitor in lung cancer cells

Recently, considerable attention has been also focused on resveratrol (3,5,4′-*trans*-trihydroxystilbene), a well-known natural polyphenol found in large amount in grapes (91), that has been reported to exert multiple biological activities (92) including anti-inflammatory (93), anti-oxidant (94), inhibition of platelet aggregation (95), antitumor (96), and induction of apoptosis (97). Remarkably, the cancer chemopreventive activity; Aggarwal *et al.* and Jang *et al.* of 2 represents an important add value and it seems to be strictly connected to the antitumor, and the proapoptotic effects (98-100). In 2010, Fukui and his research team reported that resveratrol sensitizes a number of cancer cell lines to several anti-cancer drugs, including paclitaxel (85). EGFR tyrosine kinase inhibitor, such as Iressa, has provided dramatic clinical response for EGFR mutation lung cancer patients. However, its efficiency is limited by the development of drug resistance.

Above literatures lead us come out a hypothesis: whether resveratrol sensitize EGFR tyrosine kinase inhibitor and has the de-resistance effect on Iressa-resistance PC-9/IR cells. To this end, we selected the Iressa dose (2.5 μM) base on the finding from figure 2. Both of PC-9/WT and PC-9/IR cells were pretreated with vehicle or various dosages of resveratrol (1.25, 2.5, 5, 10 μM) for 4 hrs in serum-free medium. Thereafter, the cells were incubated with 2.5 μM of Iressa for another 48 hrs. At the end of the incubation period, cell viability was analyzed by MTS assay. The results show that pre-treatment with non-toxic dosages of resveratrol increased Iressa-induced cell death in PC-9/IR cells (8.76% ± 1.56% cell death in vehicle + Iressa group; 48.71% ± 3.12% cell death in resveratrol

+ Iressa group, $p<0.0001$). The cell death of combined treatment with reveratrol and Iressa was increased at less 5-fold than Iressa treatment only in PC-9/IR cells (Figure 5).

These data suggested that resveratrol may sensitize and de-resistance of EGFR tyrosine kinase inhibitor in Iressa-resistant lung cancer cells, also provide a potential treatment strategy of EGFR tyrosine kinase inhibitor-resistant cancer patients.

3.3 The potential target molecular may involve in the resistance of lung cancer cells to Iressa

Resistance to anticancer drugs, not only chemotherapy drugs but also target therapeutic drugs is widely observed in lung cancer patients. This limitation of therapeutic potential provides a powerful stimulus for developing new therapeutic approaches. In our previous results, we showed that pre-treatment with resveratrol increased Iressa-induced cell death in Iressa-resistant lung cnacer cells. It suggested that resveratrol may sensitize EGFR tyrosine kinase inhibitor in Iressa-resistant lung cancer cells. Therefore, it is critical and timely to further evaluate the molecular mechanisms involved in resveratrol-induced de-resistance of EGFR tyrosine kinase inhibitor in lung cancer cells. To this end, we used non-biased experiments to evaluate the potential targets may involves in resveratrol-mediated de-resistance of EGFR tyrosine kinase inhibitor in Iressa-resistant lung cancer cells. We performed two-dimensional gel electrophoresis analysis, oligo gene expression microarray analysis and microRNA microarray assay to analyze the differential expression of proteins, genes and microRNAs in PC-9/WT and PC-9/IR cells. We found some potential downstream targets may involve in the resistance of Iressa of lung cancer cells.

The different expression of proteins between PC-9/WT and PC-9/IR were shown in figure 6, table 2 and table 3. In figure 6, it showed the two-dimensional protein maps of the PC-9/WT cells and PC-9/IR cells. The proteins are separated by different isoelectric point and molecular weight. To identify the differential expression proteins from two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), we punch out the target spots and then send to liquid chromatography and tandem mass spectrometry (LC–MS/MS) assay (cooperate with Genomics Research Center,

Academia Sinica). There were 15 candidate proteins significantly increase in PC-9/IR cells compared with PC-9/WT cells (Table 2). We also found 9 candidate proteins were dramatically decreased in PC-9/IR cells compared with PC-9/WT cells (Table 3). Through the prediction of these candidate proteins by IPI and SWISS-PROT databases, we selected several candidate proteins, such as CPT2 (Carnitine O-palmitoyltransferase 2), ACAA1 (Acetyl-CoA acetyltransferase), LDHA (L-lactate dehydrogenase A), HNRNPA2B1 (Heterogeneous nuclear ribonucleoproteins A2/B1) and APBEC3C (Probable DNA dC->dU-editing enzyme APOBEC-3C), to investigate whether these candidate proteins involve in Iressa resistance and resveratrol-mediated de-resistance of Iressa of lung cancer cells. PC-9/IR cells were infected with shLuc, shLDHA, shHNRNPA2B1, shACAA1, shAPOBOC3C and shCPT2 as described under "materials and methods" and were subjected to analysis the knockdown efficiency by real-time PCR quantification (Figure 7A). Data from western blot confirmed the decreased the expression of LDHA by shLDHA infection (Figure 7B). To further confirm the 2D prediction data, we examined the mRNA level and protein level of LDHA in PC-9/WT and PC-9/IR cells. As the results, the mRNA level of LDHA had no difference between PC-9/WT and PC-9/IR cells, but the expression of LDHA was higher in PC-9/IR cells then in PC-9/WT cells (Figure 8). To further evaluate the role of LDHA, HNRNPA2B1, ACAA1, APOBEC3C and CPT2, we used the stable cells of knockdown of 5 candidate proteins from figure 7a. These cells were treated with or without Iressa for 48 hrs. In the end of incubation period, cells were collected and analyzed the population of sub-G_1 phase by flow cytometric assay. Figure 9 showed that knockdown of LDHA significantly increase the sub-G_1 phase after treatment with Iressa in PC-9/IR cells. These data indicated that LDHA regulated the resistance of PC-9/IR to Iressa.

According to the oligo gene expression microarray analysis, there are some differential expressing genes between PC-9/WT and PC-9/IR cells. There were 14 candidate target genes significantly up-regulated in PC-9/IR cells (Ratio greater than 4 times, Table 4) and 3 candidate target genes were down-regulated in PC-9/IR cells (Ratio less than 0.25 times, Table 5). To further

confirm the expression of these candidate genes in PC-9/WT and PC-9/IR cells, we used RT-PCR to analysis the expression of these candidate genes in mRNA level. The mRNA level of IGFBP7 (Insulin-like growth factor binding protein7), SLC47A2 (Multidrug and toxin extrusion protein 2), FN1 (Fibronectin1) and GAS6 (Growth arrest-specific protein 6 Precursor) were significantly upregulated in PC-9/IR cells (Figure 11A). SPRR1B (Cornifin-B) was dramatically decreased in PC-9/IR cells (Figure 11B).

To further look for microRNAs potentially regulated the Iressa-resistance of lung cancer cells. We analyzed the global microRNA expression in PC-9/WT cells and PC-9/IR cells using microRNA microarray. We only found 5 candidate microRNAs, miR-20a, miR-21, miR-23a, miR-200c and miR-574-5p, were upregulated (≧2 times) in PC-9/IR cells compared with PC-9/WT cells (Table 6). Through real-time PCR analysis, the differential expression of these up-regulated miRNAs in PC-9/IR cells was confirmed (Figure 12).

Summarized above results, we exposed the evident that resveratrol may sensitize Iressa and de-resistance of EGFR tyrosine kinase inhibitor in lung cancer cells those acquired drug-resistance. Moreover, we found LDHA regulated the resistance of PC-9/IR to Iressa and might have other potential dowmstream molecules such as proteins, genes and microRNAs involve in Iressa-resistance.

4. 討論 (Discussion)

Our current study provided evidence for the first time that treatment with resveratrol sensitize EGFR tyrosine kinase, Iressa. We found pre-treatment resveratrol with non-toxic dosages, increased Iressa-induced cell death in drug-resistant lung cancer cells. Lung cancer is one of the most prevalent malignancies worldwide and remains the leading cause of cancer death (86). The EGFR tyrosine kinase inhibitors gefitinib and erlotinib are effective therapies for NSCLC patients. However, the vast majority of gefitinib-responsive NSCLC ultimately progresses to a resistant state, and the emergence of this resistance severely limits the clinical efficiency of this drug. Therefore, it is necessary to develope a new therapeutic approach.

Resveratrol, a nature agent, has been shown to potentiate the apoptotic effects of cytokines, chemotherapeutic agents, and γ－radiation (101). Several studies found that cancer cells exposed to resveratrol were sensitized to Paclitaxel-induced apoptosis, and it was by way of modifying the expression of apoptotic regulator proteins (102). In present study, we therefore examined the combined-treatment effects of resveratrol and Iressa in Iressa-resistant cells. The present work has led to identify the molecular mechanisms of the resveratrol-induced de-resistance of EGFR tyrosine kinase inhibitor in lung cancer cells. To understand the signaling profiles will help us to know better about how resveratrol to sensitize EGFR tyrosine kinase inhibitor in resistant lung cancer and provide new anti-cancer therapeutic strategies for EGFR tyrosine kinase inhibitor-resistant lung cancer patients.

It is notably that the effects of resveratrol on chemosensitization are dynamic. Previous study revealed that, trans-resveratrol reduced cellular death in SH-SY5Y neuroblastoma cells exposed to Paclitaxel by inhibiting Paclitaxel-induced activation of caspase 7 and the degradation of poly (ADP-ribose) polymerase (103). The contrasting effects of resveratrol may be dose-dependent. It potentiates the effects of cytokines and chemotherapeutic agents at higher concentrations and inhibits their effects at lower concentrations. In the future, in animal model experiments, the

dosages of resveratrol should be more concerned.

There are many factors has been report that regulate the chemosensitization of resveratrol. Resveratrol exerts its sensitizing effects by interruption with the cellular signaling pathways, induction of cell cycle arrest, and selective modification of apoptosis regulatory proteins (104). In our experiment, we discovered the novel molecular mechanisms involved in the regulating the chemosensitization of resveratrol to EGFR tyrosine kinase inhibitors. To this end, we performed non-bias experiments, such as two-dimensional gel electrophoresis analysis, oligo gene expression microarray analysis and microRNA microarray assay. Several targets which might regulate the resistance of lung cancer cells to Iressa were also be evaluated.

By two-dimensional gel electrophoresis analysis, the results show 5 candidate proteins may involved. Carnitine o-palmitoyltransferase 2 (CPT2), is a nuclear protein which is transported to the mitochondrial inner membrane. CPT2 together with carnitine palmitoyltransferase I oxidizes long-chain fatty acids in the mitochondria. Although the CPT2 involved in resveratrol-mediated de-resistance of EGFR-TKI is investigated yet, several papers were published that long-chain fatty acids suppress the development of major cancers (105-111). Acetyl-CoA acetyltransferase (ACAA1 or thiolase) is an enzyme which converts two units of acetyl-CoA to acetoacetyl CoA in the mevalonate pathway. Mevalonate metabolites play an essential role in transducing EGFR-mediated signaling. Mantha *et al.* reported that targeting the mevalonate pathway can inhibit EGFR function (112). Therefore, ACAA1 should be further investigating. L-lactate dehydrogenase A (LDHA) catalyzes the conversion of L-lactate and NAD^+ to pyruvate and NADH in the final step of anaerobic glycolysis. According to the Warburg effect, cancer cells derive most of their energy from anaerobic glycolysis and that is contributed to the malignancy of cancer cells. Hence, the mechanism of drug-resistant was through LDHA proteins. Heterogeneous nuclear ribonucleoproteins A2/B1 (HNRNPA2B1) belongs to hnRNPs. HnRNPs are RNA binding proteins and they complex with heterogeneous nuclear RNA (hnRNA). These proteins are associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA processing and other aspects of

mRNA metabolism and transport. It has been reported, increased HNRNPA2B1 expression was found in lung cancer cells, indicated that HNRNPA2B1 are involved in cancer progression. According to above literatures discussions, these proteins might regulate the resveratrol-mediated de-resistance of EGFR-TKI.

MicroRNAs (miRNAs) are endogenous small noncoding RNAs (20-23 nucleotides) that negatively regulate the gene expressions at the post-transcriptional level by base pairing to the 3' untranslated region (3'UTR) of target messenger RNAs. Evidence is emerging that particular microRNAs (miRNA) alterations are involved in the initiation and progression of human cancer. More recently, accumulating evidence is revealing an important role of miRNAs in anticancer drug resistance and miRNA expression profiling can be correlated with the development of anticancer drug resistance.

It has been reported resveratrol could regulate the expression of miRNA. A study reported by Lukiw *et al*. has shown that resveratrol analog CAY10512 regulated the expression of miR-146a (113). They found that miR-146a was up-regulated and complement factor H (CFH), an important repressor of the inflammatory response in the brain, was down-regulated in the brain of Alzheimer disease. The sequence of miR-146a was highly complementary to the 3'UTR of CFH, suggesting that CFH is a target of miR-146a. Further experiments showed that transfection of human neural cells with pre-miRNA-146a promoter-luciferase reporter construct in stressed human neural cells showed significant up-regulation of luciferase activity and low level of CFH gene expression, consistent with the observations on the brain of Alzheimer disease. Importantly, treatment of stressed human neural cells with resveratrol analog CAY10512 or an anti-sense oligonucleotide to miRNA-146a could inhibit miR-146a and restore CFH expression levels (113). These data indicate that resveratrol could regulate the expression of specific miRNA and alter the signaling transduction, leading to the alterations in cell physiological behavior. Therefore, we found miR-21, miR-23a, miR-200c and miR574-5p were up-regulated in PC-9/IR, imply that those microRNAs have significantly potentials in regulation of Iressa-resistance.

In conclusion, we present the first evidence demonstrating that resveratrol, a polyphenols, may sensitize EGFR tyrosine kinase inhibitor, Iressa, in resistant lung cancer cells. It is necessary to understand the specific molecular mechanism, to improve the current anti-cancer treatment and to develop novel therapeutic strategies against cancer.

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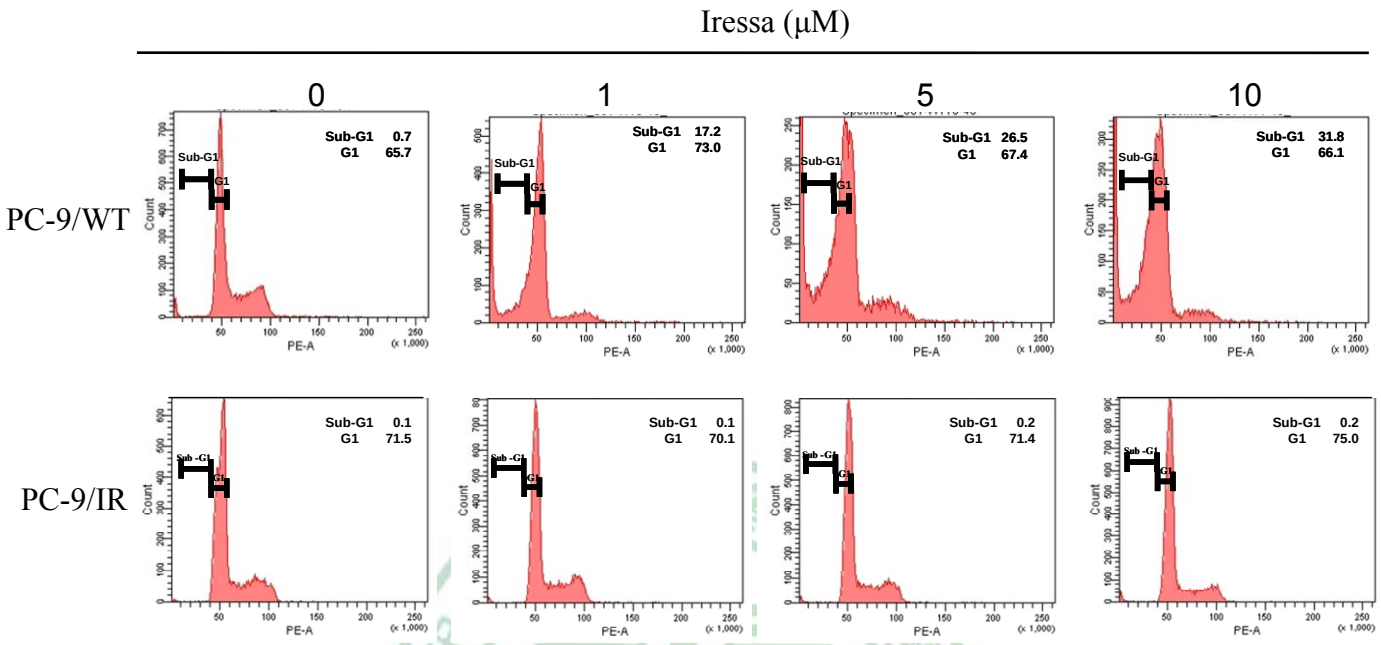
6. 圖片 (Figures)

A.



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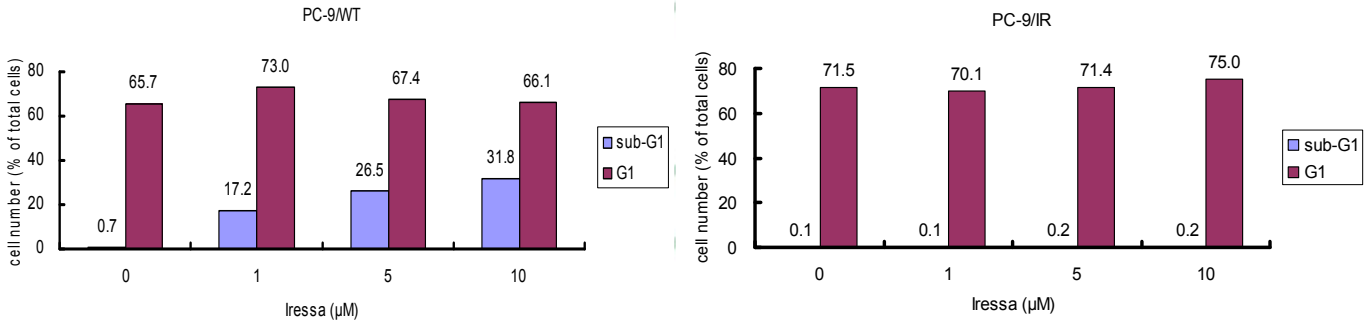


Figure 1. Cell cycle profiles of PC-9/WT cells and PC-9/IR cells after treatment with or without Iressa. *A*, PC-9/WT and PC-9/IR cells were treated with indicated 0, 1, 5, 10 μM of Iressa, respectively. After 48 hrs, these cells were trypsinized, fixed by 70% of ethanol, stained with propidium iodide and analyzed by flow cytometric assay. *B*, quantitative analysis of the ratio of phase (Sub-G_1, G_1) were shown.

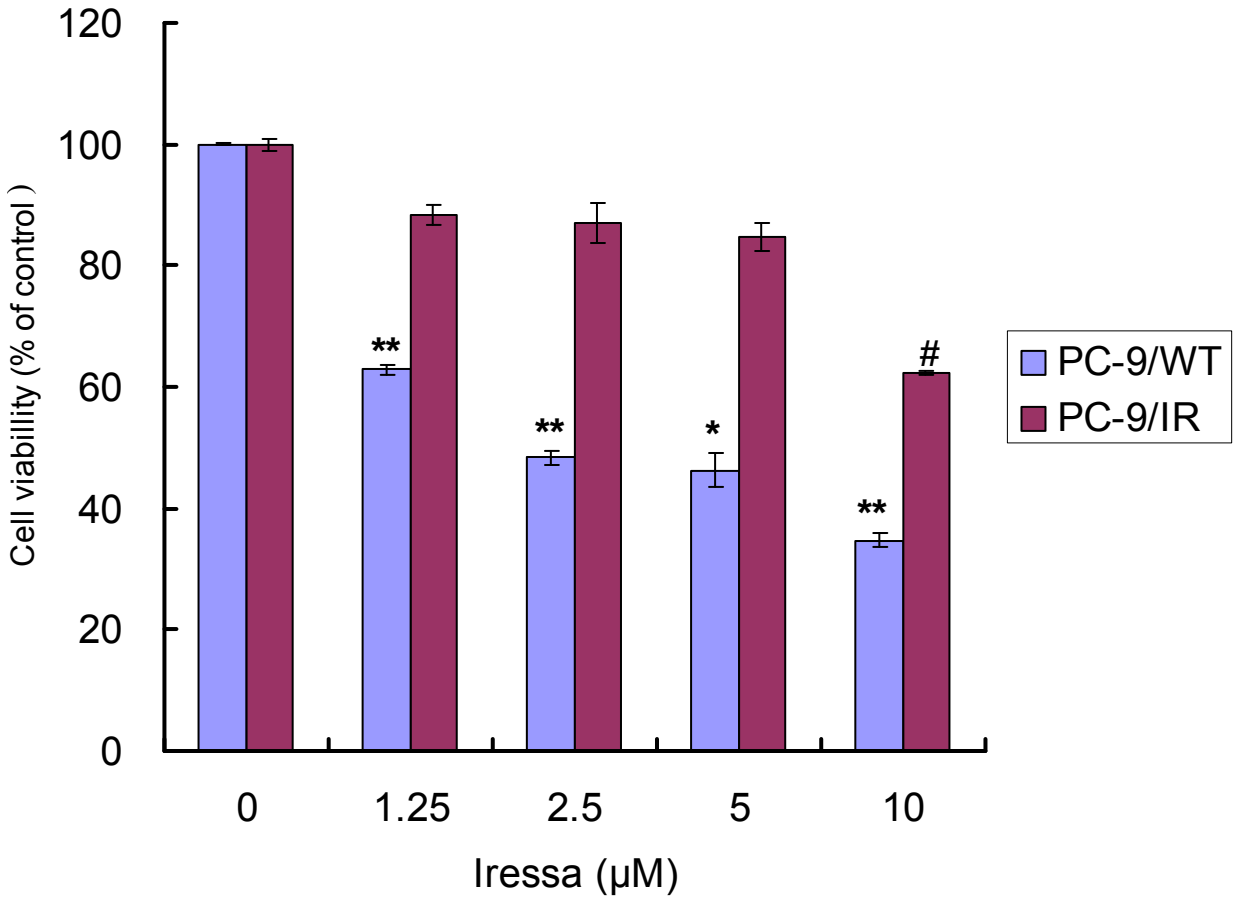
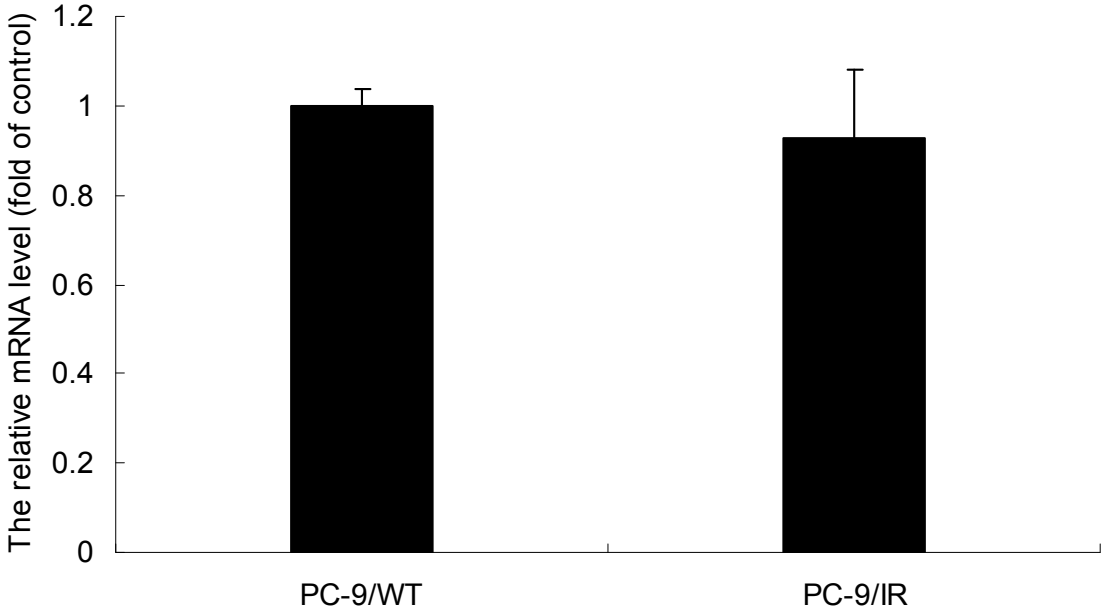


Figure 2. Concentration-response activity of Iressa on cell viability of PC-9/WT and PC-9/IR lung cancer cells. PC-9/WT and PC-9/IR cells were treated with various concentration of Iressa (1.25, 2.5, 5, 10 μM) and then incubated for 48 hrs. After that, MTS reagent was added to each plate and cells were incubated for a further 2 hrs. Absorbance was measured at 490 nm. Bars represent means ± SE from three independent experiments in triplicates. Asterisks denote a significant difference compared with values for untreated control of PC-9/WT. Hash denote a significant difference compared with values for untreated control of PC-9/IR. Control: 0 μM; * $p<0.05$; ** $p<0.01$; # $p<0.01$

A.



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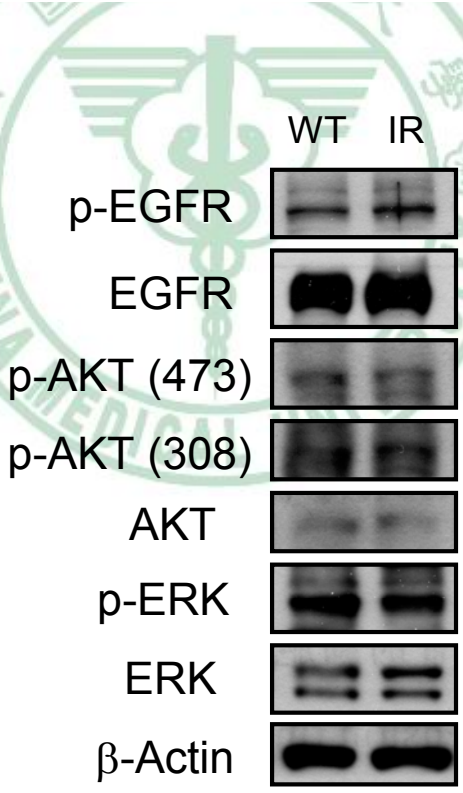


Figure 3.The expression of EGFR and downstream proteins were similar in PC-9/WT and PC-9/IR cells. *A*, the mRNA level of EGFR in PC-9/WT and PC-9/IR cells were analyzed by real-time PCR. *B*, western blot analyzed the expression of EGFR and downstream proteins, AKT and ERK. Findings were representative of at least three separate experiments.

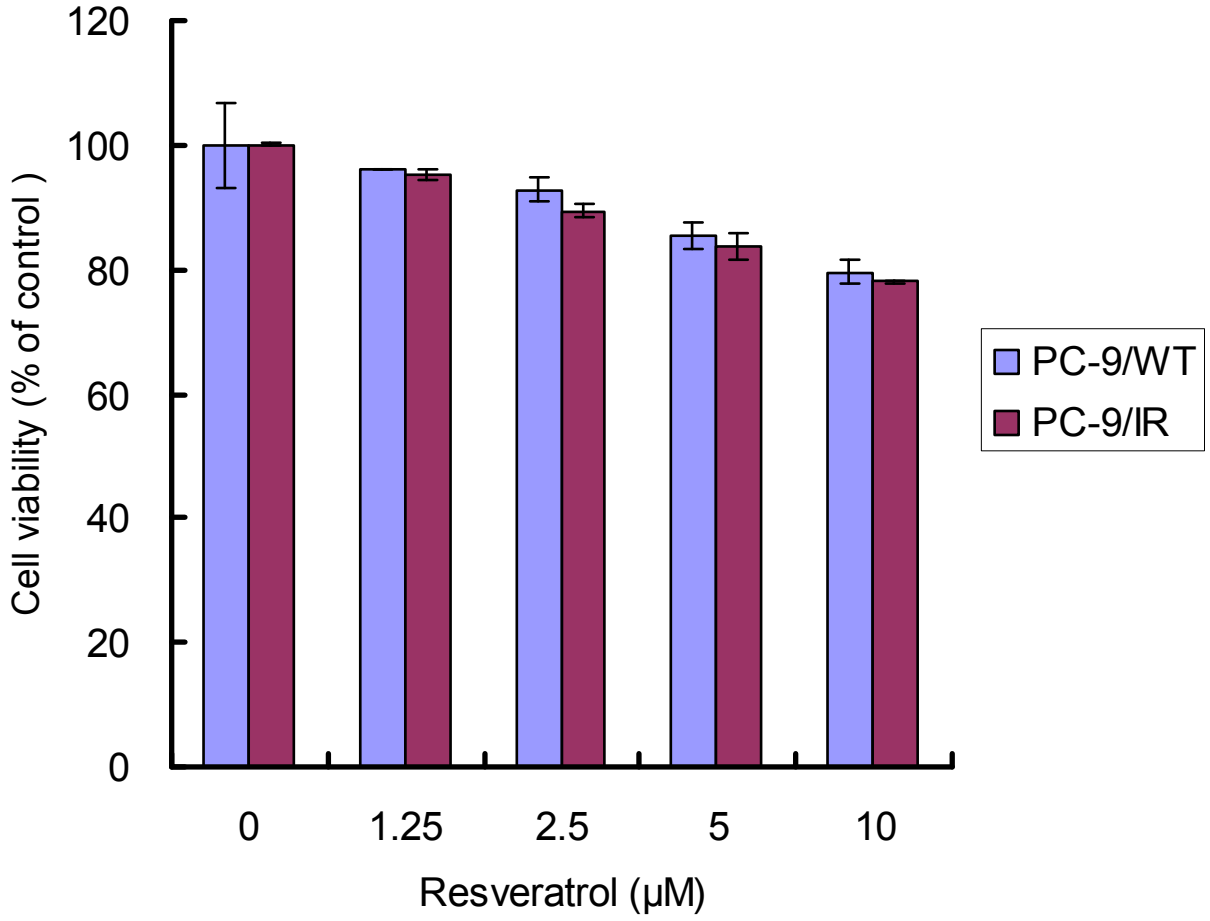


Figure 4. Concentration-response activity of resveratrol on cell viability of PC-9/WT and PC-9/IR lung cancer cells. PC-9/WT and PC-9/IR cells were treated with various concentration of Iressa (1.25, 2.5, 5, 10 μM) and then incubated for 48 hrs. After that, MTS reagent was added to each plate and cells were incubated for a further 2 hrs. Absorbance was measured at 490 nm. Bars represent means ± SE from three independent experiments in triplicates. Control: 0 μM

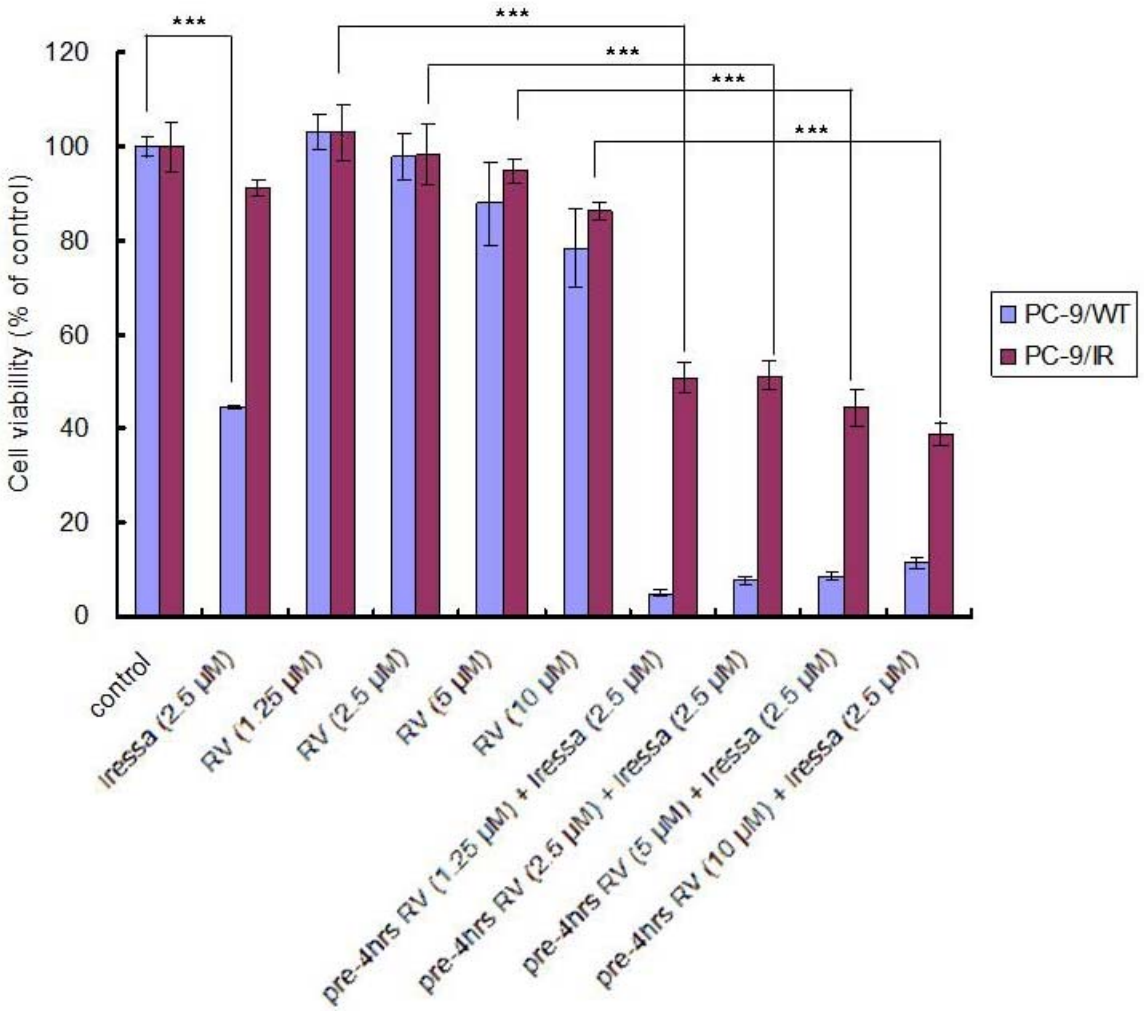
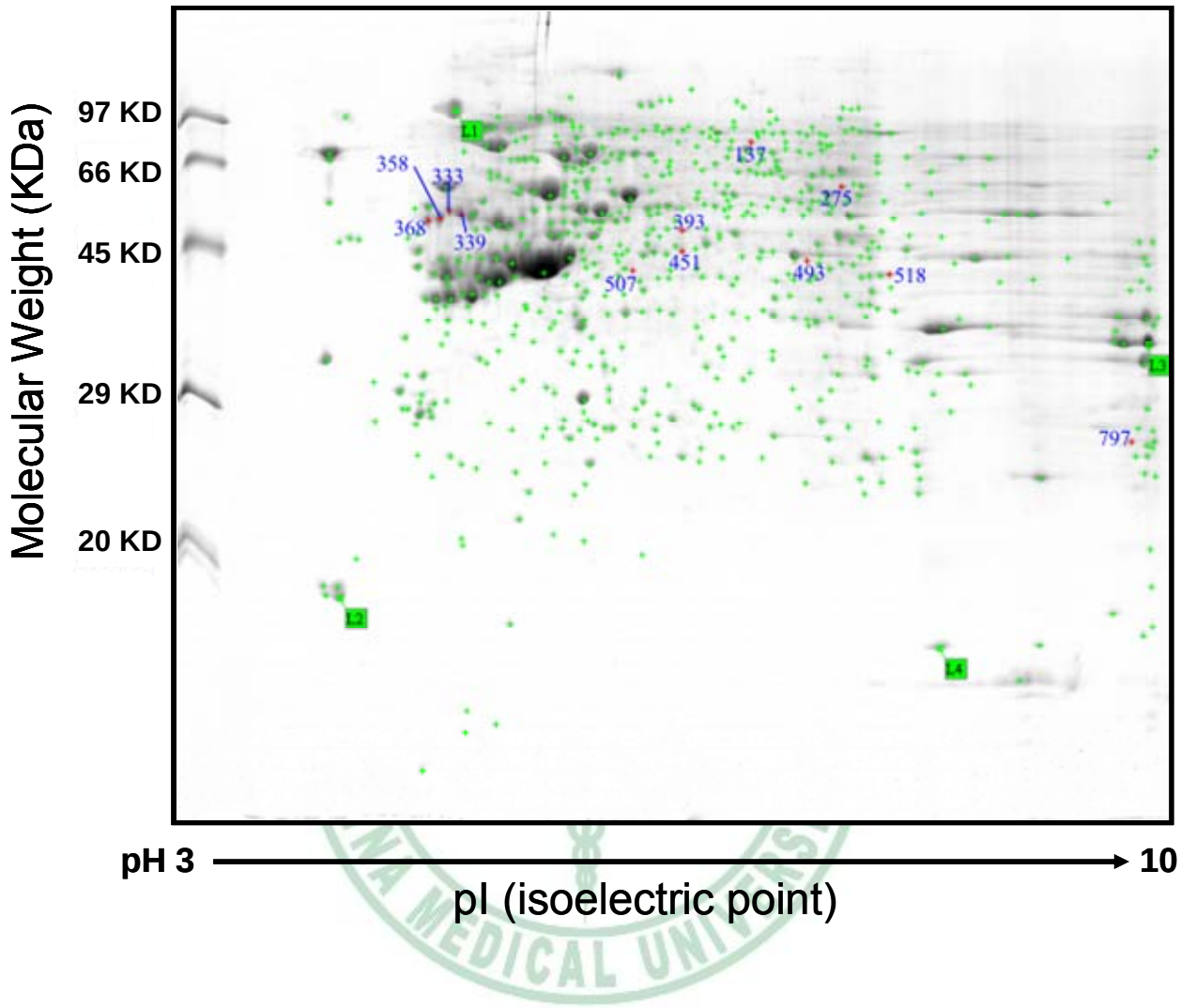


Figure 5. Effects of co-treatment of resveratrol and Iressa on cell viability in PC-9/WT, PC-9/IR human lung cancer cells. Both cells were pre-treated with 1.25, 2.5, 5, 10 μM resveratrol for 4 hrs and then treated with Iressa (2.5 μM). After 48 hrs incubation, MTS reagent was added, and cells were incubated for further 2 hrs. Absorbance was measured by ELISA reader at 490 nm. Bars represent means ± SE from three independent experiments in triplicates. Control: untreated; *** p<0.001

A.

PC-9/WT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45 KD
29 KD
20 KD
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333
368
339
137
275
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507
493
518
797
L1
L2
L3
L4
pH 3
10
pI (isoelectric point)

B.

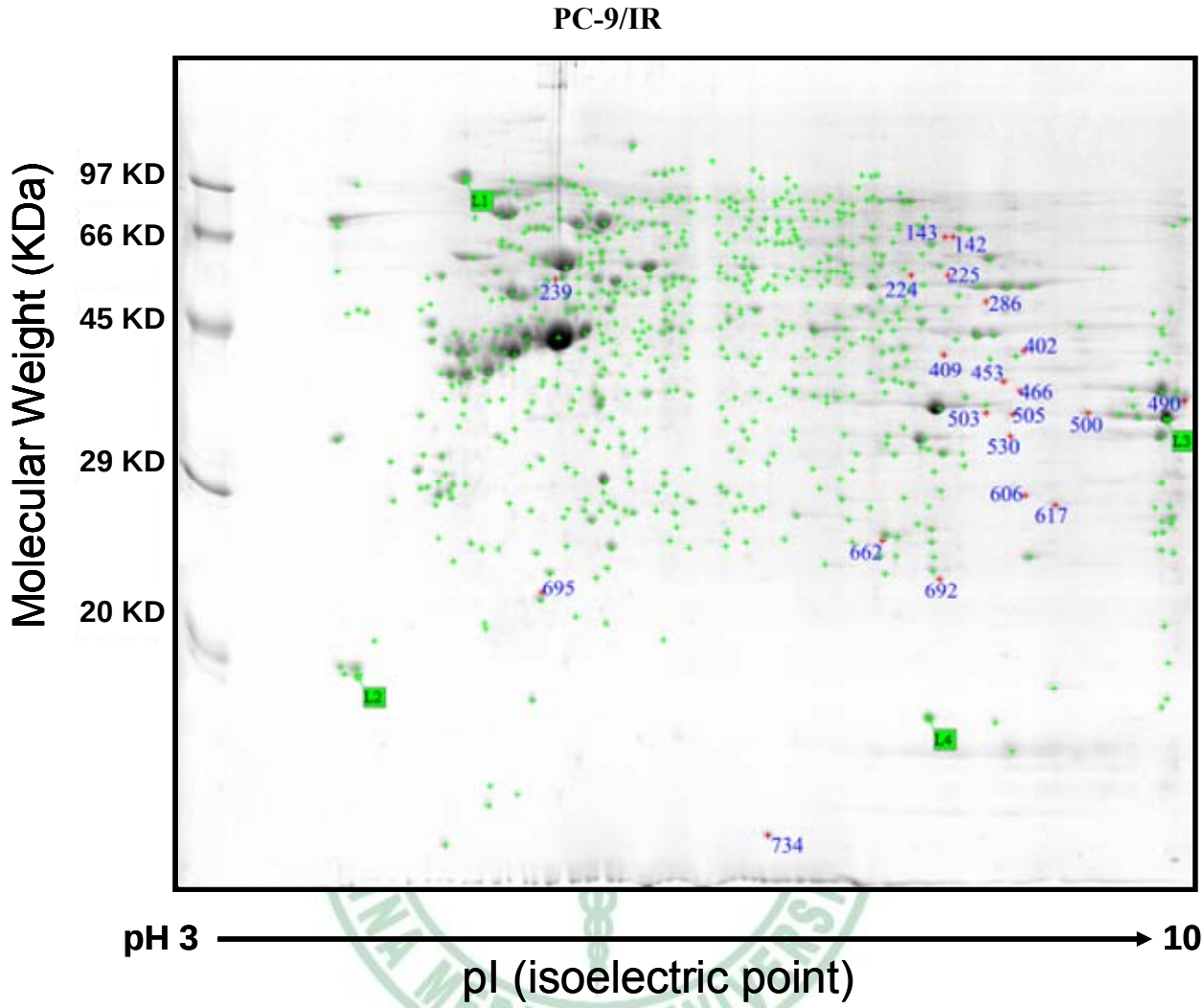
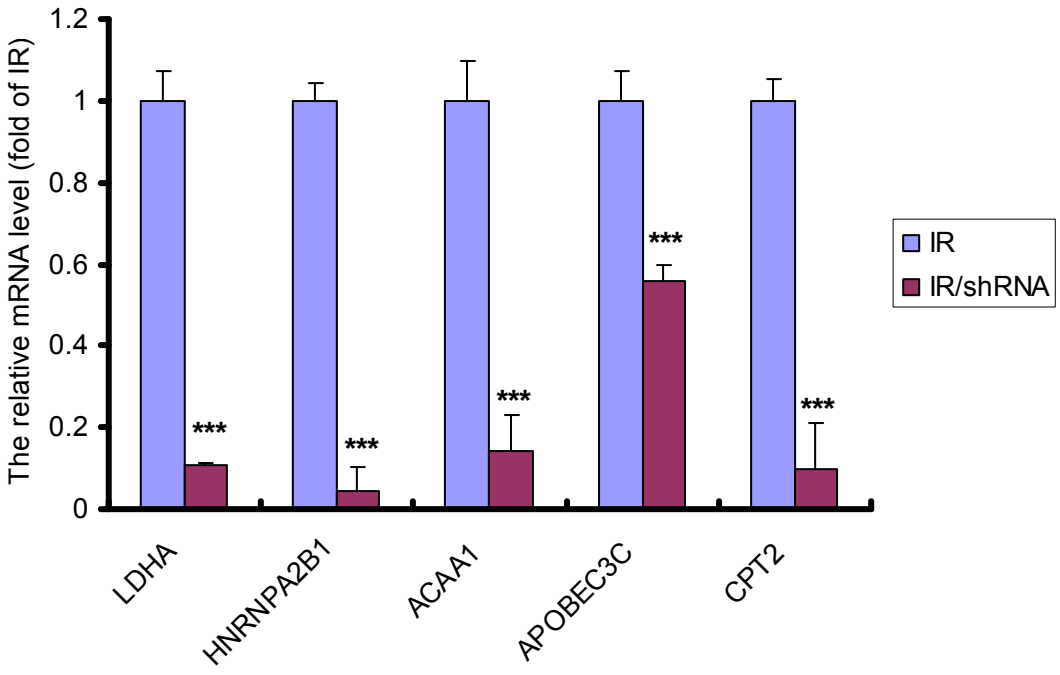


Figure 6. The differential protein expressions between PC-9 wild-type cells and Iressa-resistant cells were identified by two-dimensional gel analysis. *A*, the upregulation protein expressions in PC-9/WT compared with PC-9/IR cells were shown by coomassie blue gel staining. *B*, the upregulation protein expressions in PC-9/IR compared with PC-9/WT cells were also shown by coomassie blue gel staining.

A.



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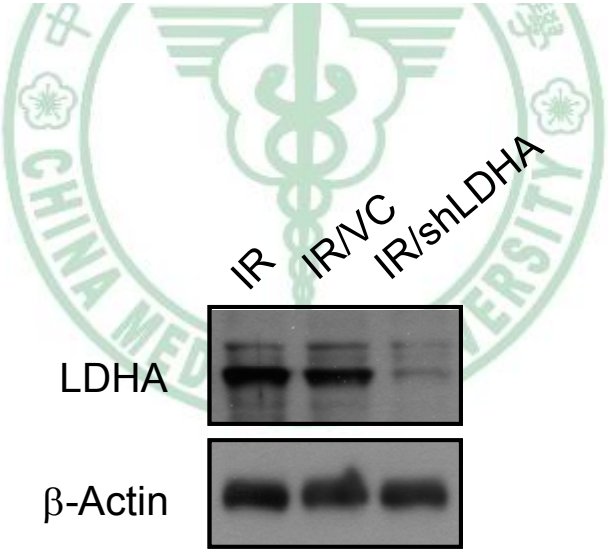
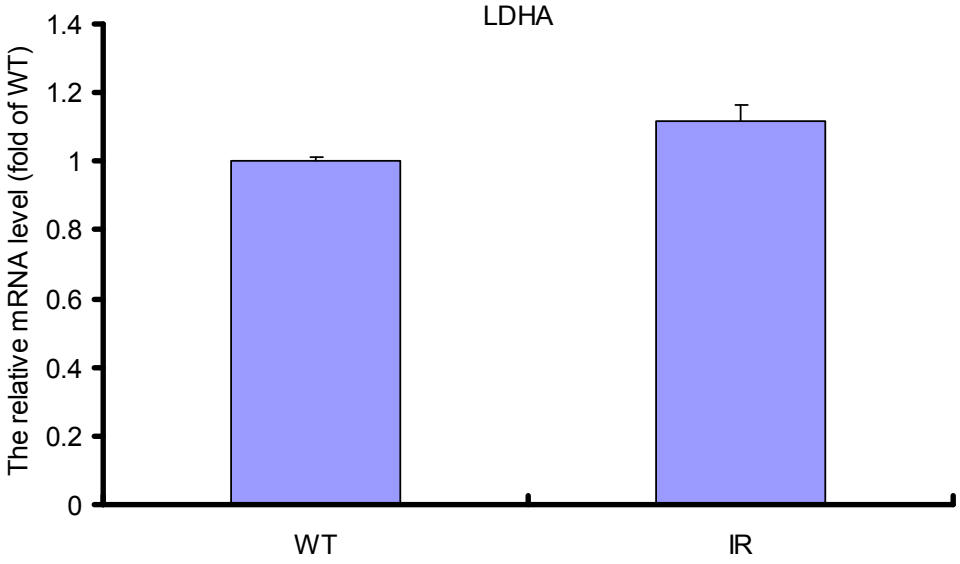


Figure 7. Stable knockdown indicated target genes in PC-9/IR cells. *A*, lung cancer cells were infected with shLDHA, shHNRNPA2B1, shACAA1, shAPOBOC3C and shCPT2 as described under "Materials and Methods" and were subjected to analysis the knockdown efficiency by real-time PCR quantification. *B*, the expression of LDHA in IR, IR/vector control and stable silenced-LDHA cells were analysis by western bolt. Bars represent means ± SE from three independent experiments in triplicates. Asterisks denote a significant difference compared with values for uninfected control. *** p<0.001

A.



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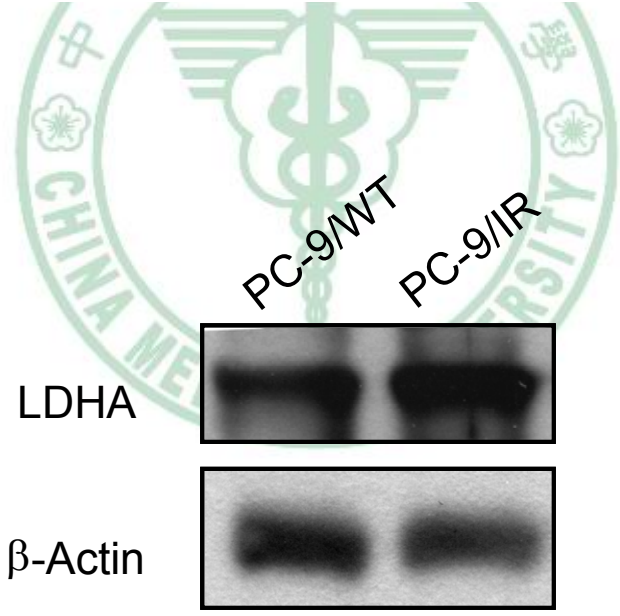


Figure 8. The different expression of LDHA between PC-9/WT and PC-9/IR cells. *A*, the mRNA level of LDHA were analysis by real-time PCR quantification. Data were representative of three separate experiments. *B*, western blot was performed with anti-LDHA antibody of total cell extract form PC-9/WT and PC-9/IR cells. The β-actin protein was used as a loading control. Findings were reproduced on three separate occasions.

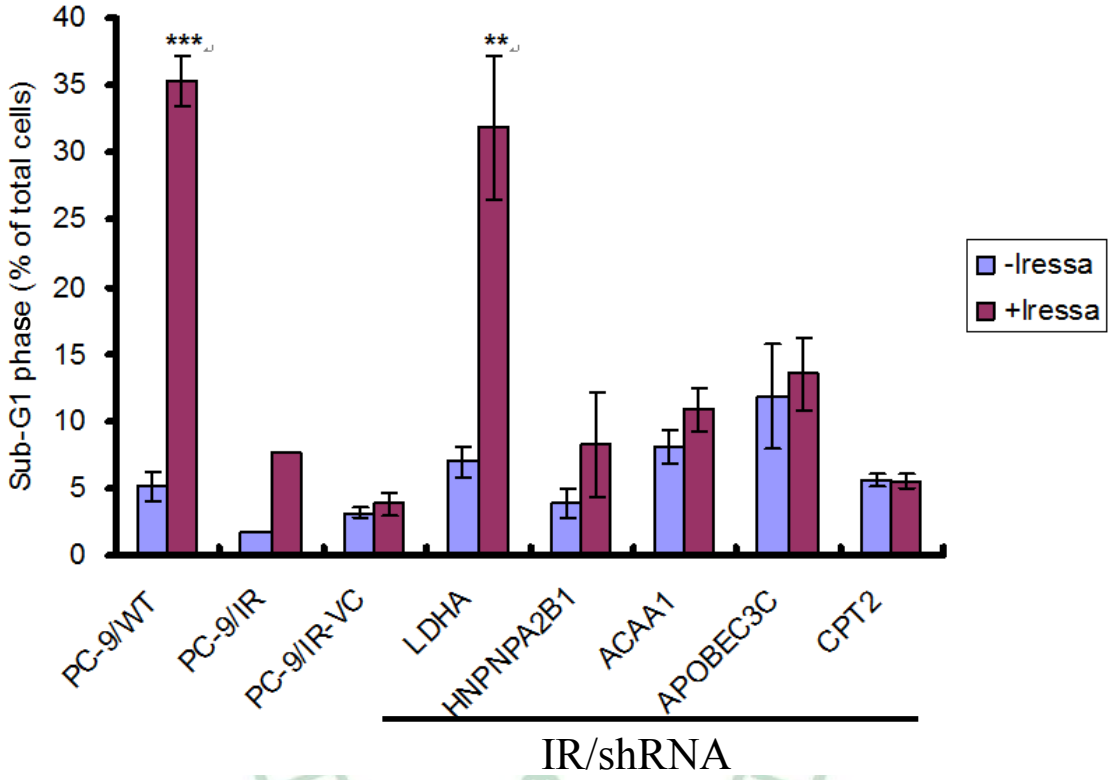
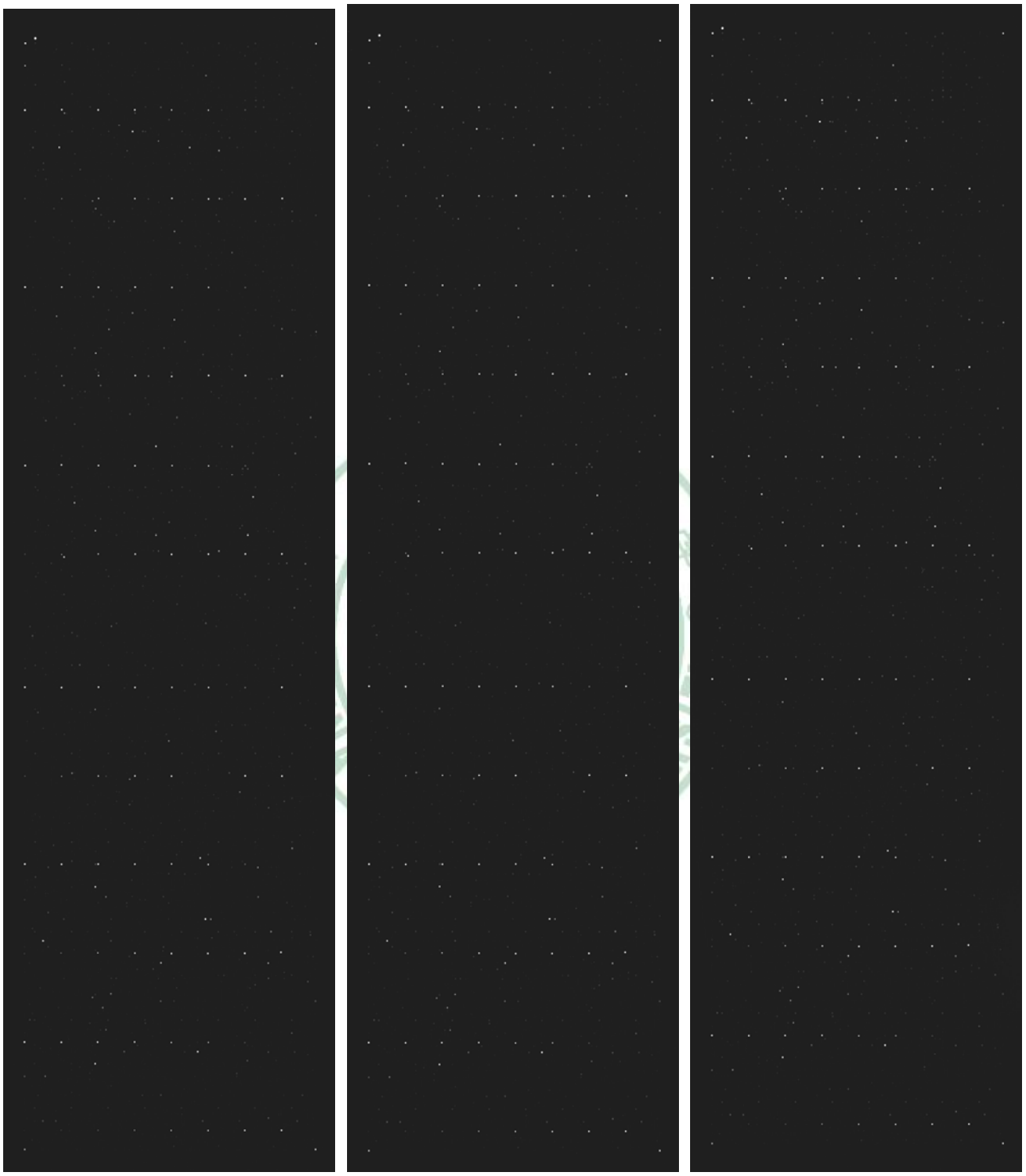


Figure 9. Knockdown of LDHA increase the sub-G_1 phase after treatment with Iressa in PC-9/IR cells. PC-9/WT, PC-9/IR, and stable knockdown indicated genes or control vector were treatment with or without Iressa (2.5 μM) for 48 hrs. In the end of treatment, cells were collected and analyzed the population of sub-G_1 phase by flow cytometric assay. Bars represent means ± SE from three independent experiments in triplicates. Asterisks denote a significant difference compared with values for untreated control. ** p<0.01; *** p<0.001

A.

PC-9/WT



B.

PC-9/IR

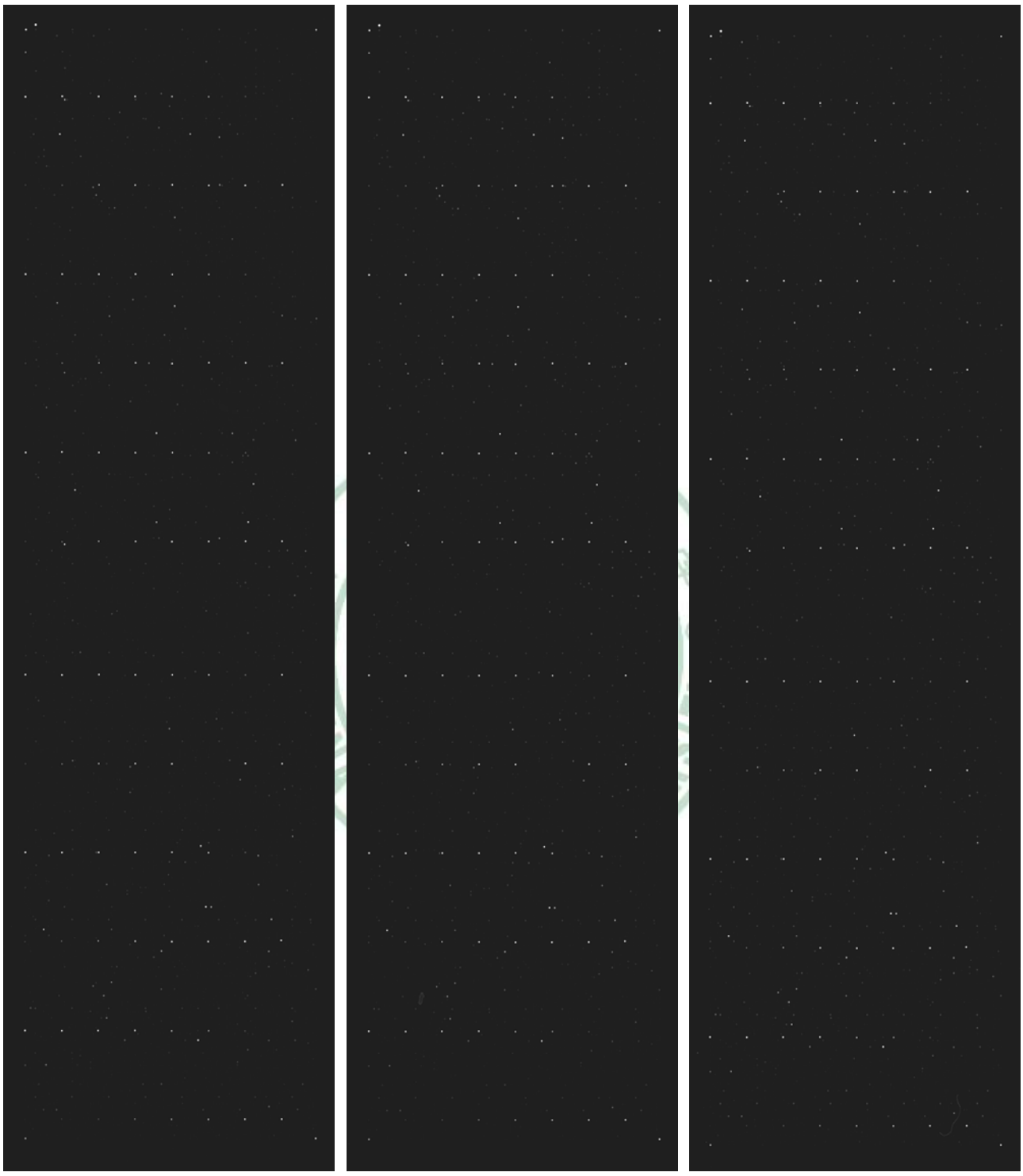
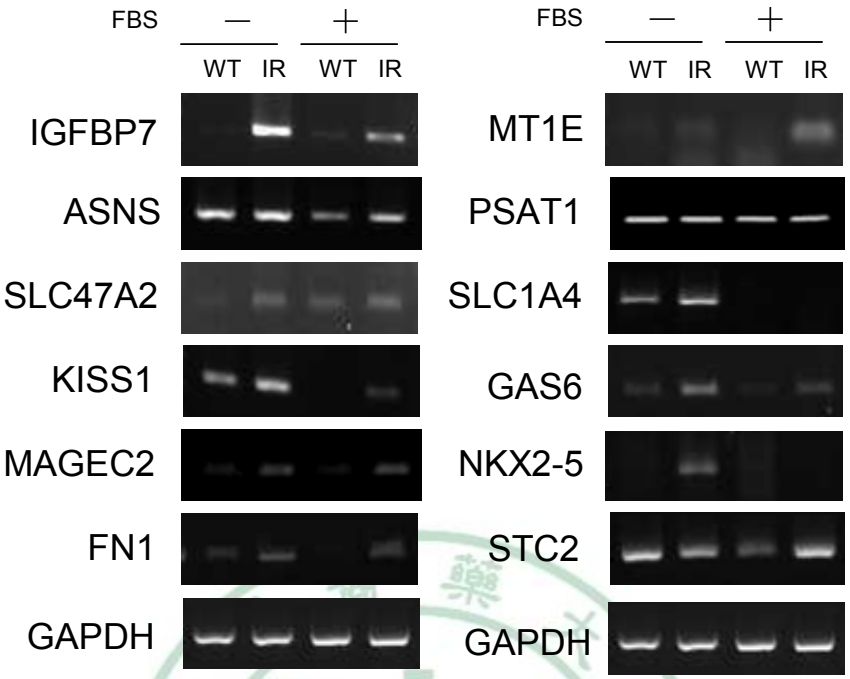


Figure 10. Differentially expressed genes between PC-9/WT cells and Iressa-resistant cells were analyzed by oligo gene expression microarray. The 3 repeats cDNA microarray chip images of PC-9/WT (*A*) and PC-9/IR (*B*).

A.



B.

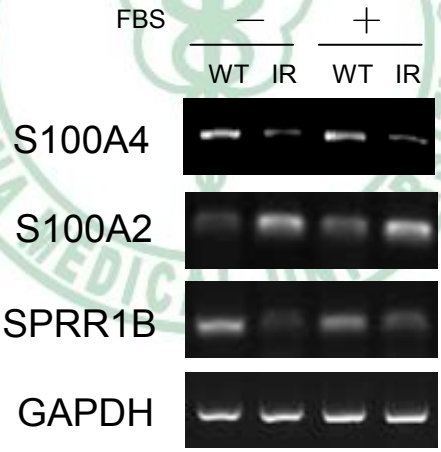
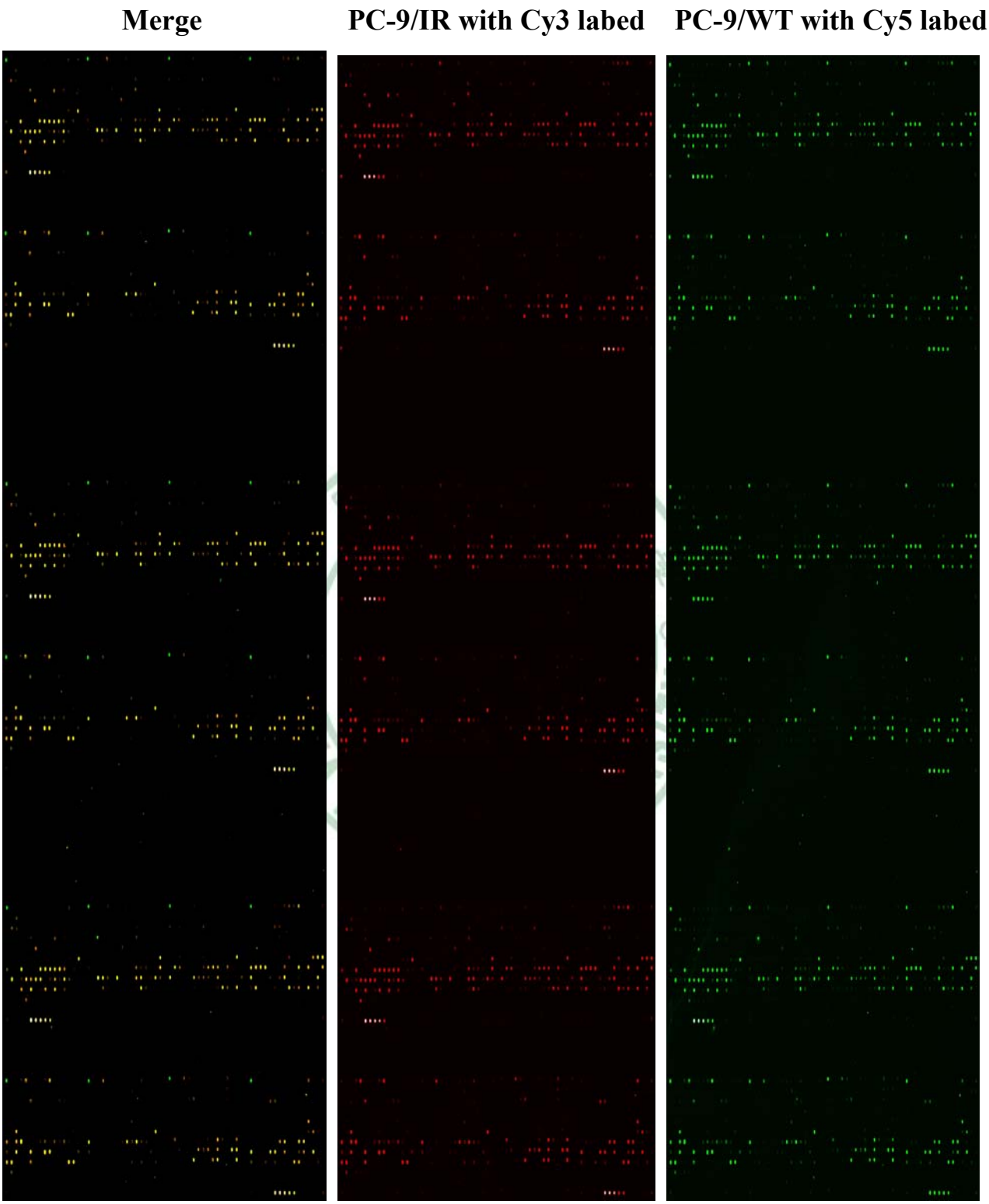


Figure 11. Endogenous expressions of candidate genes in PC-9/WT and PC-9/IR cells. The expression of candidate genes obtained from oligo gene expression microarray analysis, were confirmed by RT-PCR analysis. *A*, showed the mRNA level of upregulated candidate genes in PC-9/IR cells. *B*, presented the mRNA level of downregulated candidate genes in PC-9/IR cells. PC9/WT and PC-9/IR cells were incubated with or without FBS. After 24 hrs, RNAs were extracted from both cells and the expressions of candidate genes were determined by RT-PCR. Results were representative of three independent experiments.

A.



B.

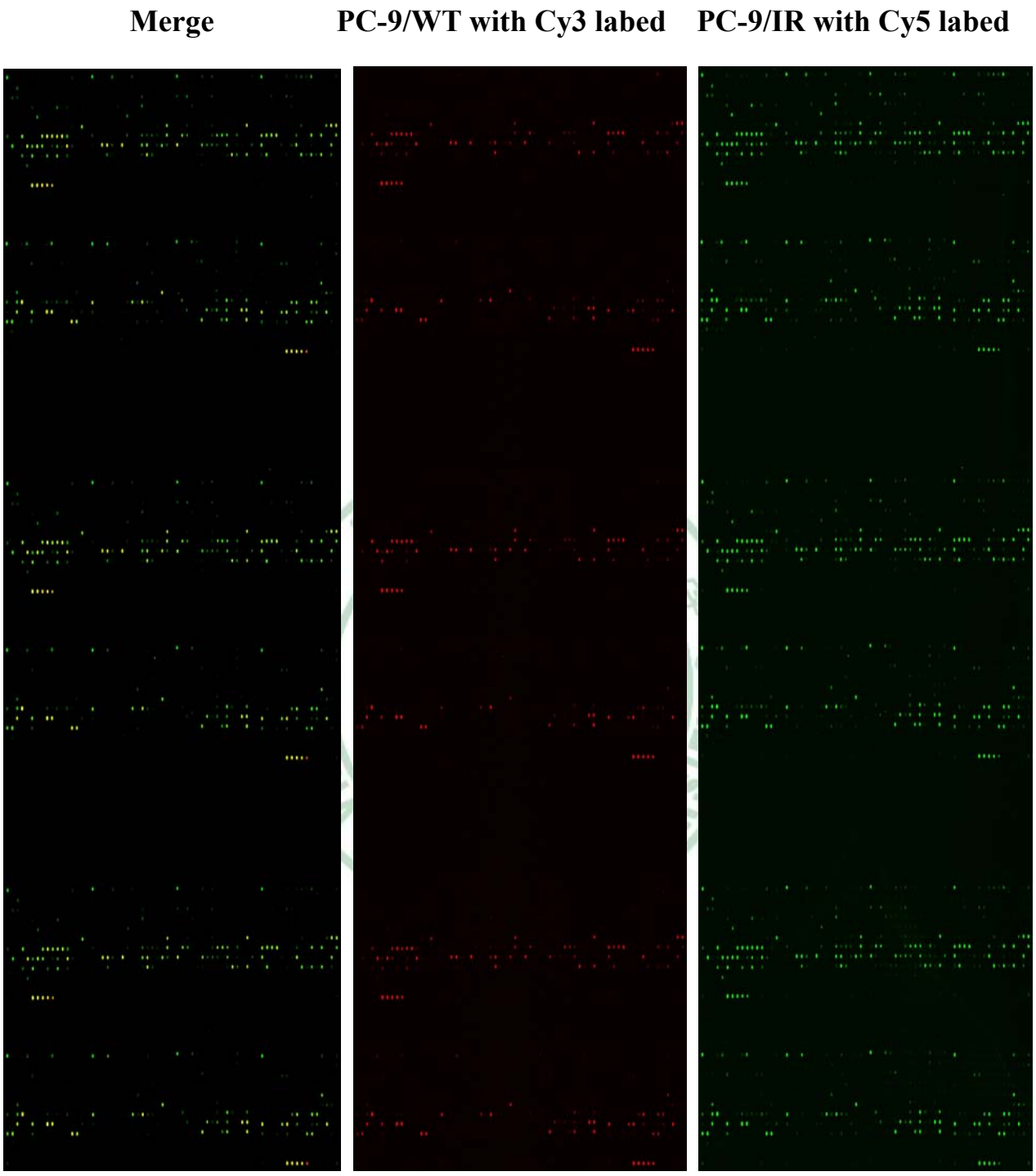


Figure 12. The differential microRNAs expression between PC-9 /WT and PC-9/IR cells. *A*, 3 repeats of microRNA microarray chip images of PC-9/WT (Cy5 labeled) and PC-9/IR (Cy3 labeled) cells. *B*, 3 repeats of microRNA microarray chip images of PC-9/WT (Cy3 labeled) and PC-9/IR (Cy5 labeled) cells.

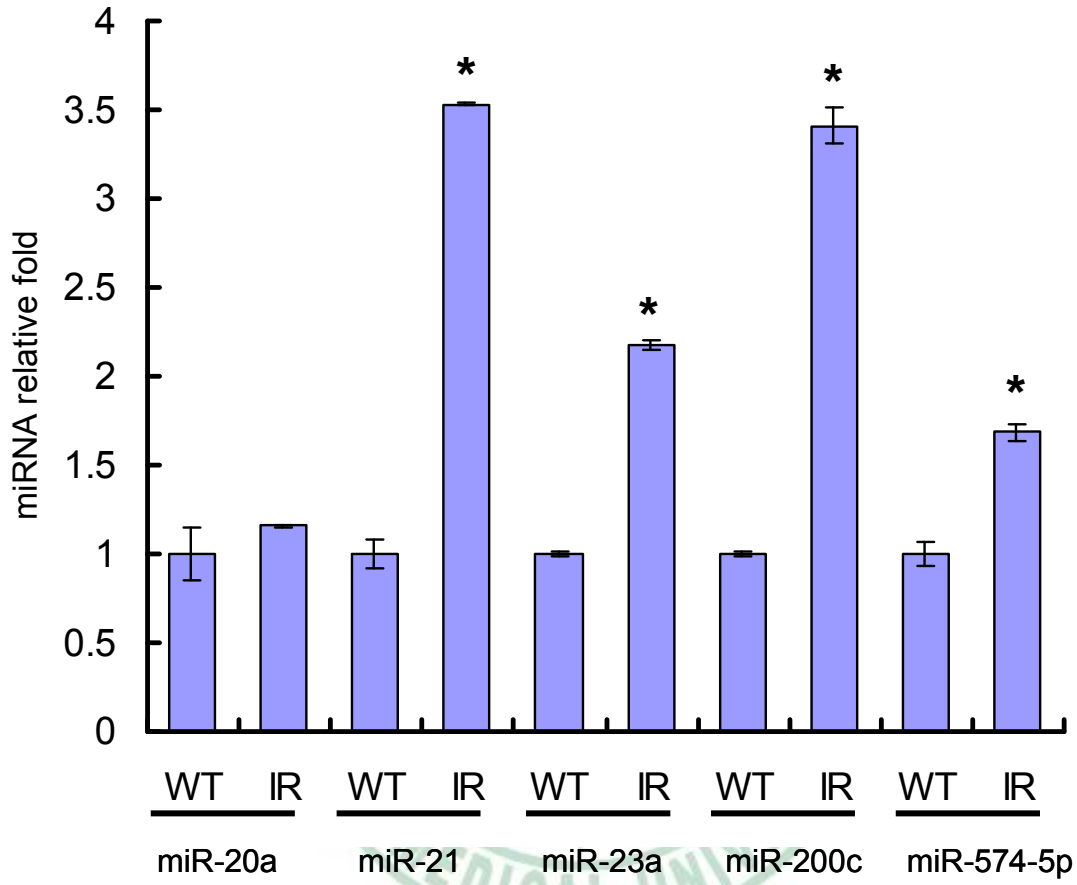


Figure 13. The expression of upregulated microRNAs was confirmed by real-time PCR analysis. Bars represent means ± SE from three independent experiments in triplicates. Asterisks denote a significant difference compared with values for each microRNA in PC-9/WT cells.* $p<0.05$

7. 表格 (Table)

Table 1. Primer sequences for RT-PCR

Ensembl number	Gene	Forward primer (5'→3')	Reverse primer (3'→5')	Tm
ENSG00000163453	IGFBP7	CAA GGT CCT TCC ATA GTG AC	GGC ATC AAC CAC TGT AAT TT	50℃
ENSG00000070669	ASNS	GGA GAA ACT CTT TCC AGG TT	CTG AAG CAC GAA CTG TTG TA	50℃
ENSG00000180638	SLC47A2	TAT TAC ATC ATC GGC CTA CC	GGC ACT CAG ACC TTG AAT AC	52℃
ENSG00000170498	KISS1	GCT ACT GCT TTT CCT CTG TG	CGA AGG AGT TCC AGT TGT AG	50℃
ENSG00000183072	NKX2-5	GAC ATC CTA AAC CTG GAA CA	CCT CTG TCT TCT CCA GCT C	54℃
ENSG00000046774	MAGEC2	TCT TCA TAA AGG GCA ACT GT	TGG ACT CTC TCT TCC ACA TC	50℃
ENSG00000115380	EFEMP1	AAG GTG GAA TGA AGT GTG TC	TGT GTT CAC TTT GCT CGT AG	50℃
ENSG00000135069	PSAT1	GAT GTT TCC AAG TTT GGT GT	CCA CTG GAC AAA CGT AGA AT	49℃
ENSG00000100889	PCK2	ATG AGG TTT GAC AGT GAA GG	TAG AAG ATG GTT GGA GGT TG	54℃
ENSG00000183087	GAS6	GAC ACC TGT GAG GAC ATC TT	GTT GAC CTT GAT GAC CAG AT	51℃
ENSG00000115414	FN1	TGA AGT TCA CTC AGG TCA CA	GTG ATC GTC TCA GTC TTG GT	51℃
ENSG00000113739	STC2	GGT TCA GTG TGA GCA GAA CT	ACT CAG ACT GTT CGT CTT CC	52℃
ENSG00000115902	SLC1A4	CAT CTA TAT TGG GCC ATG TT	GTG GCA GTC ACT AGA ATG GT	52℃
ENSG00000169715	MT1E	AGC TGC ACT TCT CCG ATG	CCT GCA AGT GCA AAG AGT	49℃
ENSG00000196154	S100A4	GTG TCC ACC TTC CAC AAG TA	CAG CTT CAT CTG TCC TTT TC	52℃
ENSG00000196754	S100A2	CGA CAA GTT CAA GCT GAG TA	ATA CTC CTG GAA GTC CAC CT	52℃
ENSG00000169469	SPRRIB	CTC CAC CTC AGG AAC CAT	GCT GGA GTG ACT ATT GAA GG	51℃
	GAPDH	ACC ACAGTC CAT GCC ATC AC	TCC ACC ACC CTG TTG CTG TA	58℃

Table 2. The proteins were upregulated in PC-9/IR cells compared with PC-9/WT cells.

Prot site	Prot describe
142	Carnitine O-palmitoyltransferase 2, mitochondrial
143	Carnitine O-palmitoyltransferase 2, mitochondrial
224	Succinate-semialdehyde dehydrogenase, mitochondrial
239	Actin, cytoplasmic 1
286	Probable ATP-dependent RNA helicase DDX47
402	Acetyl-CoA acetyltransferase, mitochondrial
500	L-lactate dehydrogenase A chain
503	Pyrroline-5-carboxylate reductase 2
505	Heterogeneous nuclear ribonucleoproteins A2/B1
530	Heterogeneous nuclear ribonucleoproteins A2/B1
606	Adenylate kinase isoenzyme 2, mitochondrial
617	Electron transfer flavoprotein subunit beta
662	ES1 protein homolog, mitochondrial
692	Probable DNA dC->dU-editing enzyme APOBEC-3C
659	Ferritin heavy chain

Table 3. The proteins were downregulated in PC-9/IR cells compared with PC-9/WT cells.

Prot site	Prot describe
137	Moesin
275	Alpha-enolase
339	Histone-binding protein RBBP7
358	Importin subunit alpha-2
393	Mitochondrial-processing peptidase subunit beta
451	Isoform 1 of Calcium-binding mitochondrial carrier protein SCaMC-1
493	Tu translation elongation factor, mitochon
507	Macrophage-capping protein
518	28s ribosomal protein S35, mitochonrial

Table 4. Candidate genes upregulation in PC-9/IR cells compared with PC-9/WT cells

Ensembl number	Ratio (log2)	P-value
IGFBP7	3.832937	2.88E-21
ASNS	2.966654	4.10E-41
SLC47A2	2.564668	6.30E-14
KISS1	2.414624	1.15E-22
NKX2-5	2.35415	2.09E-10
MAGEC2	2.308638	2.27E-08
EFEMP1	2.237491	5.21E-09
PSAT1	2.175749	0.00E+00
PCK2	2.117333	5.48E-10
GAS6	2.103822	1.89E-09
FN1	2.102836	1.10E-25
STC2	2.063752	5.36E-17
SLC1A4	2.030843	3.98E-14
MT1E	2.030176	1.53E-08

Table 5. Candidate genes downregulation in PC-9/IR cells compared with PC-9/WT cells

Ensembl number	Ratio (log2)	P-value
S100A4	-2.000276	2.38E-13
S100A2	-2.120414	5.13E-04
SPRRIB	-2.223453	3.68E-07

Table 6. Upregulated miRNAs in PC-9/IR cells compared with PC-9/WT cells

miRNA ID	microarray	Q-PCR
	Fold-change	Fold-change±SE
has-miR-20a	2.6	1.2±0.01
has-miR-21	2.3	3.5±0.01
has-miR-23a	2.6	2.2±0.03
has-miR-200c	2.0	3.4±0.09
has-miR-574-5p	2.2	1.7±0.05