

RESEARCH ARTICLE

Pemetrexed Induces G1 Phase Arrest and Apoptosis through Inhibiting Akt Activation in Human Non Small Lung Cancer Cell Line A549

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Abstract

Pemetrexed is an antifolate agent which has been used for treating malignant pleural mesothelioma and non small lung cancer in the clinic as a chemotherapeutic agent. In this study, pemetrexed inhibited cell growth and induced G1 phase arrest in the A549 cell line. To explore the molecular mechanisms of pemetrexed involved in cell growth, we used a two-dimensional polyacrylamide gel electrophoresis (2-DE) proteomics approach to analyze proteins changed in A549 cells treated with pemetrexed. As a result, twenty differentially expressed proteins were identified by ESI-Q-TOF MS/MS analysis in A549 cells incubated with pemetrexed compared with non-treated A549 cells. Three key proteins (GAPDH, HSPB1 and EIF4E) changed in pemetrexed treated A549 cells were validated by Western blotting. Accumulation of GAPDH and decrease of HSPB1 and EIF4E which induce apoptosis through inhibiting phosphorylation of Akt were noted. Expression of p-Akt in A549 cells treated with pemetrexed was reduced. Thus, pemetrexed induced apoptosis in A549 cells through inhibiting the Akt pathway.

Keywords: Pemetrexed - 2-DE - Akt - apoptosis - A549 non small cell lung cancer cells

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Introduction

As the leading cause of cancer death worldwide, lung cancer has the highest incidence and mortality among 27 kinds of cancers (Ferlay et al., 2010). And non small lung cancer (NSCLC) has a proportion of 80%-85% in all lung cancers (Stinchcombe et al., 2009; Yu Z et al., 2014). In the first- and second-line treatment of non small lung cancer, chemotherapy is one of the common methods using chemotherapeutics drugs, such as pemetrexed and docetaxel (Bareschino et al., 2011; Gettinger et al., 2011).

Pemetrexed (Alimta®) is an antifolate agent with a structure containing a core group of pyrrolo pyrimidine and inhibits the key enzymes (thymidylate synthase, dihydrofolate reductase and glycinamide ribonucleotide formyltransferase) which are necessary for the synthesis of folic acid, thereby can block biological resynthesis process of thymine nucleotides and purine nucleotides. Pemetrexed is firstly used for treatment of malignant pleural mesothelioma combined with cisplatin. On August 19, 2004, FDA gave official approval for pemetrexed for injection in treatment with local advanced or metastatic non small lung cancer (Cohen et al., 2005). And up to now, pemetrexed has demonstrated antitumor activity

for first- and second-line treatment in several cancers in clinical research (Dai et al., 2005; Raizer et al., 2012). In previous studies, pemetrexed has better therapeutic effect combined with other chemotherapy agents like cisplatin, vinorelbine, oxaliplatin, carboplatin, and gemcitabine (Socinski et al., 2009; Smit et al., 2012). Furthermore, in recent study, pemetrexed has induced promising efficacy outcomes combined with some molecular inhibitors (De Boer et al., 2011).

In previous studies of molecular mechanism, pemetrexed is first known as an inhibitor of some key enzymes of folic acid (Adjei et al., 2000; Smith et al., 2000; Molina et al., 2003; Adjei et al., 2004). Recent reports have demonstrated that pemetrexed inhibits cell cycle and induces apoptosis in cancer cell (Ramirez et al., 2007; Buque et al., 2011). Furthermore, pemetrexed induces DNA damage and intrinsic and extrinsic apoptosis through activating ataxia telangiectasia mutated/p53-dependent and -independent signaling pathways in A549 cells (Yang et al., 2011). And in A549 cells pemetrexed also induces S phase arrest and apoptosis by activation of ERK-mediated Cdk2/cyclin-A signaling pathways (Yang et al., 2011). In other reports, pemetrexed activates AMPK signaling pathway through accumulation of purine

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intermediate aminoimidazolecarboxamide ribonucleotide, and inhibits mTORC1-dependent and -independent processes in cancer cells (Rothbart et al., 2010). In addition, the Superarray cancer pathway gene array has been used for determining biomarkers in A549 cells treated with pemetrexed (Wu et al., 2010). Although the molecular mechanisms of pemetrexed in cancer cells including cell cycle and apoptosis have been reported, the more mechanisms of pemetrexed are not clearly.

In this study, we found pemetrexed inducing G1 phase arrest which is not consistent with previous report (Yang et al., 2011), thus we used 2-DE-based proteomics approach to analyze proteins differentially expressed in treated with or without pemetrexed in human non small lung cancer cell line A549. The key differentially expressed proteins and expression of *p*-Akt were determined using western blot analysis. Subsequently, we examined cell apoptosis in A549 cells treated with pemetrexed. Overall, these studies provided that pemetrexed inhibited cell growth and induced cell cycle arrest through inhibiting Akt pathway.

Materials and Methods

Cell culture

Human lung cancer cell line A549 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI medium 1640 supplement with 10% fetal bovine serum containing 100U/ml penicillin and 100µg/ml streptomycin. Cells were maintained at 37°C with 5% CO₂ atmosphere in humidified chamber. Pemetrexed (Lilly, France) was dissolved by Sodium Chloride and stored at -20°C.

Cell cycle analysis

Cells were plated in 6 well plates with a density of 105 cells per well and treated with 0, 1µM, 2µM and 4µM pemetrexed tomorrow. After incubated with pemetrexed for 24h and 48h, cells were collected and washed with PBS for twice. Then cells were re-suspended in 0.5ml propidium iodide (PI) solution (50µg/ml PI, 20µg/ml RNase A, 1mg/ml sodium citrate, 5% Triton-X 100) and incubated for 30min at room temperature. Cell cycle was analyzed by flow cytometry system and 104 cells per sample were collected by CellQuest (Becton-Dickinson, San Jose, CA) software. The proportion of cells in each phase was analyzed using ModFit software.

2-DE

2-DE was performed as described previously with minor modifications (Tong et al., 2008). Briefly, Cells were treated with 4µM pemetrexed and collected into a 1.5mL centrifuge tube after incubated for 48h. Then cells were re-suspended with lysis buffer (7M urea, 2M thiourea, 4%CHAPS, 50mM DTT, 0.2% Bio-lyte (w/v), 50ug/ml RNase and 200ug/ml Dnase). After mixed and placed at 4°C for 15min, the samples were centrifuged for 60min at 4°C, 15, 000rpm, the supernatants were harvested and the concentration of protein was measured by a Bio-Rad protein assay kit. The proteins were collected and stored at -80°C. Subsequently IPG strips were taken from fridge and maintained at 4°C for 10min. The protein samples (2mg)

were loaded onto IPG strips 17cm, pH3-10, non-liner, Bio-Rad and held for 45min. After IPG strips rehydrated for 12-16h, IPG strips were transformed into IEF cell (Bio-Rad). Composition of rehydration buffer was 7M urea, 2M thiourea, 4% CHAPS, 50mM DTT, 0.2% Bio-lyte (w/v). Once IEF was done, the strips were equilibrated with equilibration buffer containing 50mM Tris-Hcl pH8.8, 6M Urea, 30% glycerol, 2% SDS, 10mM DTT for twice. And then strips transferred to SDS-polyacrylamide gel electrophoresis system with 12% gels. The spots of protein were visualized by Coomassie Brilliant Blue (CBB) R-250. Three independent experiments were repeated.

Image analysis

The images were scanned with Bio-Rad GS-800 scanner and proteins expressed differentially in images were analyzed by PDQuest software (Bio-Rad). The quantity of each spot in a gel was normalized in a percentage of the total quantity and performed automatically by calculation of spot volume. The spot with two-fold (t-test, $p < 0.05$) and more changes in expression were chosen for MS/MS analysis.

In-gel Digestion

The spots of In-gel were digested by Trypsin Gold with Trypsin protocol (Promega, Wadison, WI). Briefly the spots excised from gel were destained twice for 45min at 37°C by 100mM NH₄HCO₃, 50% ACN and dehydrated for 5 min with 100% ACN. After dried at room temperature for 15min to remove ACN, the gels were preincubated at room temperature for 1h with 10µl Trypsin solution (1µg/µl) and then incubated overnight at 37°C with digestion buffer (40mM NH₄HCO₃, 10% ACN). The Tryptic digests were removed and saved in a new microcentrifuge tube after incubated with Milli-Q water for 10min and then extracted twice with 50µl of 50% ACN, 5% TFA for 60min each time at room temperature. All extracts were pooled and then dried in a SpeedVac at room temperature for 2–4 h. After purified and concentrated with Zip Tip pipette tips (Millipore Corporation), the peptides were ready for mass spectrometric analysis.

Mass spectrometry analysis

Mass spectrometry analysis and the identification of protein were performed as described before with minor modifications (Tong et al., 2008). Briefly, mass spectra were acquired using a Q-TOF mass spectrometer (Micromass, Manchester, UK) coupled with ESI ion source (Micromass). MassLynx software (Micromass) and MASCOT were used for acquiring and searching the database, respectively. The parameters of MSMS data search including: database, MSDB; Taxonomy, Homo sapiens; enzyme, Trypsin; Peptide Mass Tolerance, ± 0.2 Da; Fragment Mass Tolerance, ± 0.1 Da; Variable modifications, Carbamidomethyl (C); Max Missed Cleavages, 1. Proteins with individual ions scores more than 40 ($p < 0.05$) were selected for positively identified.

Western blot analysis

After cells treated with 0, 1µM, 2µM and 4µM pemetrexed for 48 h, proteins were extracted using

RIPA buffer purchased in Beyotime and supplemented with a cocktail of protease inhibitors. Protein samples were separated with 12% SDS-PAGE and transferred to PVDF membranes. After blocked with 5% skimmed milk dissolved with TBS contained 0.1% Tween 20 (TBST) for 2h at room temperature, membranes were incubated with primary antibodies at 4°C overnight. The following primary antibodies were used for western blot analysis: Rabbit anti-Bcl-2 (1:1000, Abcam), anti-Bax (1:1000, Abcam), anti-Akt (1:1000, Cell Signaling Technology), anti-Phospho-Akt (Ser473) (1:1000, Cell Signaling Technology), anti-GAPDH (1:1000, Cell Signaling Technology), anti-caspase-3 (1:1000, Cell Signaling Technology), anti-p21 (1:500, Proteintech), anti-EIF4E (1:1000, Proteintech), mouse anti-HSPB1 (1:1000, Abcam), anti-PCNA (1:1000, BOSTER), anti-cyclinD1 (1:1000, Proteintech), anti- β -actin (1:1000, Santa Cruz). After washing three times with TBST, the membranes were incubated with HRP-conjugated secondary antibodies (1:5000, Santa Cruz) for 1h at 37°C and visualized using enhanced chemiluminescence reagents (Millipore Corporation, Billerica MA).

Cell apoptosis analysis

Cells were plated in 6 well plates with a density of 105 cells per well and treated with 4 μ M pemetrexed for 48h. The cell death bodies were observed using light microscope. Hoechst staining was used to determine apoptosis cells. A549 cells were collected and washed gently with PBS for twice, and then cells were fixed with 4% paraformaldehyde for 15min and stained with Hoechst 33258 for 15min. Apoptosis cells were observed by fluorescence microscope.

Cells were plated in 6 well plates with a density of 105 cells per well and treated with 4 μ M pemetrexed for 48h. The cells were harvested and washed twice with cold PBS, suspended in a binding buffer and then stained with PI and annexin V for 15 min in dark. The stained cells were analysed directly by flow cytometry.

Statistical analysis

A paired student t-test was used for statistical analysis, $p < 0.05$ was considered significant.

Results

Induction of G1-phase cell cycle arrest by pemetrexed

To explore the effect on cell growth induced by pemetrexed, cells were treated with 0, 1 μ M, 2 μ M and 4 μ M pemetrexed for 24 and 48 h. The cell cycle was analyzed using CellQuest software in cells incubated with pemetrexed for 24 and 48h, respectively. Data shown that pemetrexed induced G1 phase arrest with dose- and time-dependent in A549 cells (Figure 1A). We subsequently examined the levels of some key regulators in cell cycle. We found that pemetrexed reduced the levels of PCNA and inhibited cell growth in A549 cells (Figure 1B). The p21 cyclin-dependent kinase inhibitor protein was strongly increased and the levels of cyclinD1 were reduced with dose-dependent upon treatment of pemetrexed (Figure 1C).

Identification of differentially expressed proteins in A549 cells treated with pemetrexed

The proteins differentially expressed in A549 cells treated with 4 μ M pemetrexed compare with control cells were analysis by 2-DE. And the images were stained by CBB R250 and analyzed using PDQuest software. The protein spots differentially expressed in two groups with intensity alterations at least two-fold changes and p -value less than 0.05 were selected for identification. According to these criteria, 49 spots were chosen for MS/MS analysis, and we successfully identified 20 protein spots from the 49 protein spots, including 14 upregulated and 6 downregulated protein spots as shown in Figure 2A. The protein information including accession number, Mascot score, mass, PI, match peptides, sequence coverage and fold change were listed in Table 1. Subsequently, those 20 proteins were grouped by their subcellular localization and functions. Proteins were mostly localized in cytoplasm (64%), and about 16% in nucleus, 8% in membrane and secreted, respectively, only 4% in mitochondrion. Cell functions were classified as follows: metabolism (16%), energy metabolism (28%), signal transduction (24%), cytoskeleton (16%), epigenetics and nuclear signaling (8%), others (8%) (Figure 2B).

Validation of protein spots with western blot analysis

To validate the proteins identified using mass spectrometry, the following 3 proteins which is related to cell cycle or apoptosis were selected for western blot analysis: GAPDH, HSPB1, EIF4E. As shown in Fig. 3A and B, the expression levels of proteins analyzed by

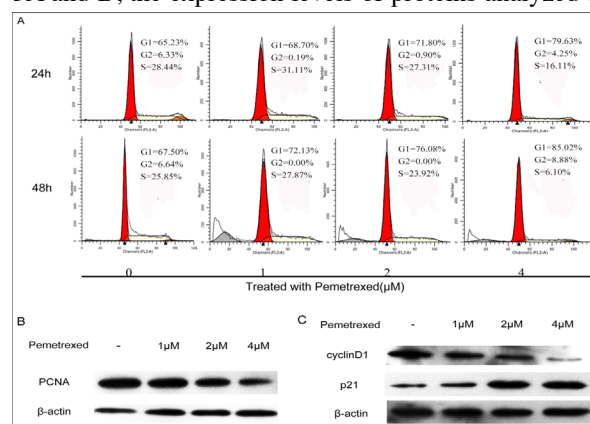


Figure 1. Induction of G1-phase Cell Cycle Arrest by Pemetrexed

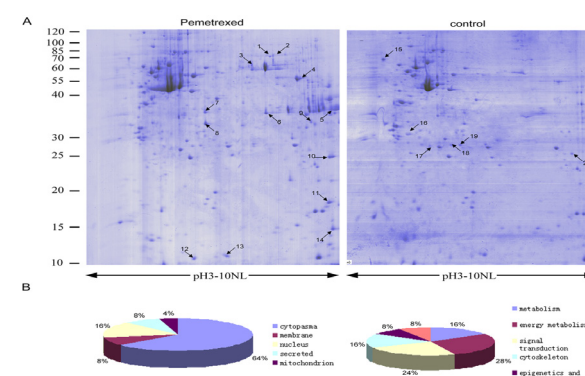


Figure 2. Identification of Differentially Expressed Proteins in A549 Cells Treated with Pemetrexed

Table 1. Identification of Differentially Expressed Proteins in A549 Cells Treated with Pemetrexed

Spot no.	Protein name	Gene name	Accession no. ^a	Mascot	Theoretical	Theoretical pI ^b	Matched Sequence	Fold change peptides	coverage	(%)
1	Stress-induced-phosphoprotein 1		STIP1		P31948	141	62639	6.4	50	4.2
2	Glucose-6-phosphate 1-dehydrogenase		G6PD		Q2Q9H2	113	54825	6.66	10	5.62
3	Retinal dehydrogenase 1		ALDH1A1		P00352	299	54731	6.29	33	17.97
4	Alpha-enolase		ENO1		P06733	1023	47038	6.99	83	17.26
	Beta-enolase		ENO3		P13929	525	46801	7.73	100	
	Gamma-enolase		ENO2		P09104	516	47137	4.91	100	
5	Glyceraldehyde-3-phosphate dehydrogenase		GAPDH		Q2TSD0	488	36049	8.57	70	8.55
6	Annexin A1		ANXA1		P04083	682	38583	6.64	82	4.98
7	Annexin A4		ANXA4		P09525	105	35752	5.84	100	2.71
	L-lactate dehydrogenase B chain		LDHB		P07195	101	36507	5.72	40	
8	Annexin A4		ANXA4		P09525	755	35752	5.84	69	3.58
9	Voltage-dependent anion-selective channel protein 2		VDAC2		P45880	226	31435	7.66	71	7.11
10	Peroxiredoxin-1		PRDX1		Q06830	357	22110	8.27	18	12.42
11	Cofilin 1, non-muscle isoform		CFL1		P23528	476	18371	8.26	63	13.67
12	Unnamed protein					102	22604	5.28	50	2.95
13	Protein S100-A11					66	11740	6.56	100	3.94
14	Profilin-1				P31949	266	14923	8.47	75	18.72
15	Calreticulin variant				P07737	128	46919	4.3	67	-2.99
	Alpha-crystallin B chain		CRYAB		Q53G71	91	20159	6.76	50	
16	Prohibitin		PHB		P02511	77	29820	5.57	33	-2.14
17	Peptidyl-prolyl cis-trans isomerase A		PPIA		Q6PUJ7	157	17881	7.82	33	-2.16
18	Heat shock protein beta-1		HSPB1		P62937	134	22783	5.98	67	-2.94
19	Peroxiredoxin-4		PRDX4		P04792	126	30540	5.86	50	-3.54
20	3-hydroxyacyl-CoA dehydrogenase type-2		HSD17B10		Q13162	450	26792	7.87	78	-4.44
	Eukaryotic translation initiation factor 4E		EIF4E		Q99714	93	24966	5.8	100	

^aAccession numbers were obtained from the ExPASy database; ^bTheoretical molecular mass (Da) and pI were searched from the ExPASy database; ^cProtein scores were derived from ion scores which more than 40 is significant ($p < 0.05$)

western blot were consistent with image analysis.

Pemetrexed inhibited cell growth through inhibiting Akt pathway

Previous reports shown that inhibition of HSPB1 expression inhibits cell proliferation and induces cell apoptosis through regulating Akt activation (Rane et al., 2003; Wu et al., 2007; Hayashi et al., 2012), and Akt inhibition induces cell apoptosis by increasing the expression of GAPDH (Leisner et al., 2012). Furthermore, EIF4E is the downstream of Akt pathway and regulates cell growth and apoptosis (De et al., 2004) so we hypothesis that whether pemetrexed inhibits cell growth through inhibiting Akt pathway. We examined the expression of Akt and *p*-Akt. As shown in Fig.4A, data indicated that the levels of *p*-Akt were decreased treated with pemetrexed in A549 cells. The maps of AKT pathway were searched from KEGG PATHWAY database. It shown that AKT pathway controlled cell cycle, cell survival and protein synthesis (Figure 4B).

Pemetrexed induces apoptosis in A549 cell line

G1 phase arrest of A549 cells induced by pemetrexed may lead to cell apoptosis. We carried out a number of assays to measure pemetrexed induced apoptosis in A549 cells. Cells were treated with 4 μ M pemetrexed for 48 h. Dead cells (arrow) were observed using microscopic analysis in A549 cells treated with pemetrexed (Figure 5A). And data from Hoechst staining and PI-Annexin V analysis showed that cells treated with pemetrexed undergo apoptosis, compared with control (4.67%), pemetrexed strongly induced apoptosis (20.69%) (Figure 5B and C). As a key regulator of apoptosis, caspase-3 was detected using western blot analysis. Cleavage of caspase-3 was observed at the concentration of 2 μ M and indicated its activation. We also

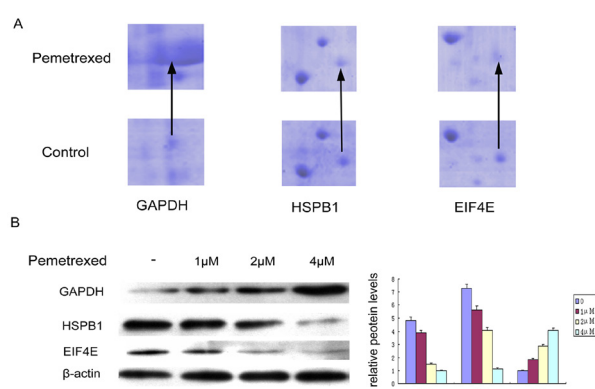
Pemetrexed Induces G1 Phase Arrest and Apoptosis through Inhibiting Akt in Human A549 Non Small Cell Lung Cancer Cells

Figure 3. Validation of Protein Spots with Western Blot Analysis

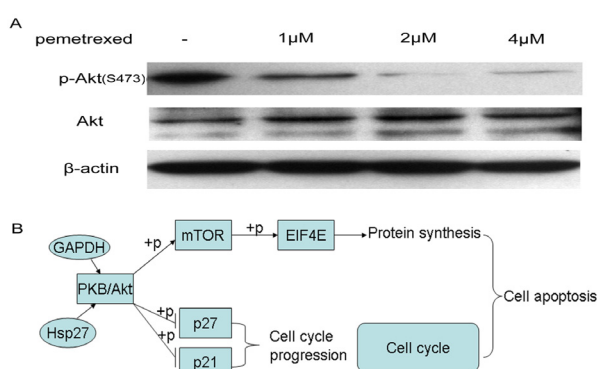


Figure 4. Pemetrexed Inhibited Cell Growth through Inhibiting Akt Pathway

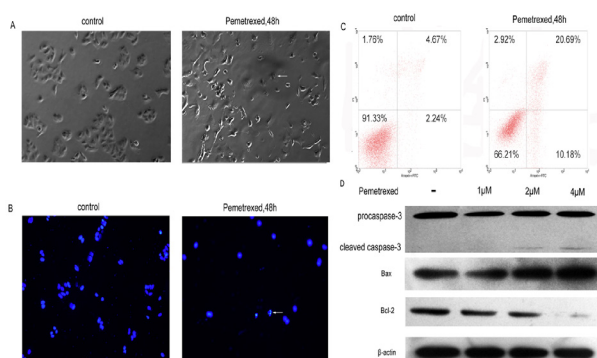


Figure 5. Pemetrexed Induces Apoptosis in A549 Cell Line

detected proapoptotic factor Bax and antiapoptotic factor Bcl-2, they increased or decreased with dose-dependent, respectively (Figure 5D).

Discussion

Lung cancer is the most common cancer worldwide, both in terms of incidence and mortality, accounting for 13% of the total cases and 18% of the deaths in 2008 (Luo et al., 2014). Proteomics is one of the most recent and rapidly growing areas in cancer molecular biology. Proteomics enables the scientists to interrogate a variety of biospecimens for their protein contents and measure the concentrations of these proteins. Current necessary equipment and technologies for cancer proteomics are mass spectrometry, protein microarrays, nanotechnology and bioinformatics (Parisa et al., 2014). In other words,

proteomics, provide scientists and clinicians with a powerful tool to understand the different processes involved in cancer development and progression, is widely used in cancer research (Deng et al., 2013; Li et al., 2013).

In the present study, we found pemetrexed inducing G1 phase arrest, and then we used 2-DE proteomics analysis approach to determine differentially expressed proteins in A549 treated with pemetrexed. We identified 20 proteins expressed differentially which participated in diverse processes including metabolism, energy metabolism, signal transduction, cytoskeleton and epigenetics and nuclear signaling. And three key proteins (GAPDH, HSPB1 and EIF4E) in these processes were validated using western blot analysis. Previous studies have indicated that these three key proteins induce apoptosis through inhibiting Akt pathway (Rane et al., 2003; Wendel et al., 2004; Tarze et al., 2007), so the expression of *p*-Akt was determined by western blot analysis. We found pemetrexed inhibiting phosphorylation of Akt and induced cell apoptosis in A549 cells.

Pemetrexed provoked G1 phase arrest and inhibited cell growth. The expression of PCNA and p21 in A549 cells were decreased and increased respectively treated with pemetrexed which shown that pemetrexed inhibited cell growth. CyclinD1, which is a key regulator protein of G1 phase, was reduced. It was confirmed pemetrexed induced G1 phase arrest which is not consistent with previous report (Yang et al., 2011). Previous study has shown that EIF4E specifically enhance some key proteins expression (e.g. vascular endothelial growth factor (VEGF), cyclinD1, matrix metalloprotease 9 (MMP-9)) (De et al., 2004). And EIF4E levels were decreased treated with pemetrexed in A549 cells. So we infer that expression of cyclinD1 was controlled by EIF4E and as a key regulator of cell cycle.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is known as a housekeeping gene in many cellular processes in addition to glycolysis and participated in various processes including DNA repair, tRNA export, membrane fusion and transport, cytoskeletal dynamics, and cell death (Tristan et al., 2011). Recent reports have shown GAPDH has various functions dependent on its subcellular localization, especially in cytoplasm and mitochondria, GAPDH is a proapoptotic protein and regulator of cell death and apoptosis (Tarze et al., 2007; Colell et al., 2009). Moreover, recent study shows that aggregation of GAPDH resulted in oxidative stress-induced cell death through regulating Akt pathway (Leisner et al., 2012). In present study, GAPDH was increased in A549 cells after incubated with pemetrexed. Thus we suggested that pemetrexed increased the expression of GAPDH and induced cell apoptosis through inhibiting the activation of Akt in A549 cells.

HSPB1 is one member of small heat shock protein family and involved in many cellular processes. We know that HSPB1 have a protective function of cell death, but the mechanism is not clearly. And in some reports, inhibition of HSPB1 induces cell apoptosis by controlling Akt activation and further study shows that HSPB1 induces apoptosis through regulating interaction between Akt and its upstream activator MK2 (Rane et al., 2003; Wu et al.,

2007). So we confirmed that pemetrexed inhibited Akt pathway in A549 cells.

EIF4E is an essential regulator of protein synthesis. As a downstream of Akt pathway, EIF4E is controlled by the activation of Akt (Wendel et al., 2004). Previous studies have demonstrated that EIF4E plays a critical role in cellular transformation, tumor growth, tumor invasiveness and metastasis (De et al., 2004; Sun et al., 2005). In our study, the expression of EIF4E was reduced treated with pemetrexed in A549 cells, so it may be regulated by the inhibition of Akt which is consistent with the variation of GAPDH and HSPB1.

Furthermore, we found that the activation of Akt was inhibited by pemetrexed because the levels of *p*-Akt were decreased after incubated with pemetrexed in A549 cells. Akt also known as protein kinase B (PKB), a serine/ threonine protein kinase, plays a critical role in cell survival and apoptosis. As shown in Fig 4B, Akt regulates cell cycle, cell survival and metabolism through phosphorylation of Akt. Recent study has report that pemetrexed indirectly inhibiting Akt pathway in cancer cells (Ramirez et al., 2007; Rothbart et al., 2010). Thus pemetrexed may regulate the phosphorylation of Akt and inhibit cell growth and apoptosis.

As pemetrexed inhibited cell cycle and phosphorylation of Akt, A549 cells were entered in apoptosis. We determined cell apoptosis using Hoechst staining, flow cytometry. Caspase-3, a key regulator of apoptosis, antiapoptotic factor Bcl-2 and proapoptotic factor Bax was examined by western blot analysis. The levels of Bcl-2 and Bax were decreased and increased respectively. And cleaved caspase-3 was also observed in A549 cells, we determined cleaved caspase-3 at the concentration of 2μM. As we described before, accumulation of GAPDH, decrease of HSPB1 and EIF4E associated with Akt pathway resulted in cell apoptosis, we indicated that pemetrexed induced apoptosis in A549 cells through inhibiting Akt pathway.

In conclusion, we use 2-DE to analyze differentially expressed proteins in A549 cells treated with pemetrexed, and we found three key proteins associated with Akt pathway. Unfortunately, we don't determine how pemetrexed inhibit Akt pathway and the details of mechanism about GAPDH, HSPB1 and EIF4E interaction with Akt pathway. Notwithstanding its limitations, this study does suggest pemetrexed induce cell cycle and apoptosis through inhibit Akt pathway.

Acknowledgements

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