

Original Article

Activation of AKT/ERK confers non-small cell lung cancer cells resistance to vinorelbine

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Abstract: Vinorelbine is a semi-synthetic vinca-alkaloid approved for the treatment of non-small cell lung cancer (NSCLC). However, the lower objective response rate and higher adverse effects of vinorelbine hinder its wide use in treatment of advanced NSCLC. Therefore, it is of great interest to uncover the biomarkers for sensitivity of NSCLC cells to vinorelbine to allow the identification of patients most likely to benefit from vinorelbine-based chemotherapy and to improve the therapy. In present work, four NSCLC cell lines were divided into vinorelbine-sensitive (VS) group and vinorelbine-resistant (VR) group according to their sensitivities to vinorelbine. And then the gene expression profiles of these two groups was compared, the differentially expressed genes (expression difference higher than 100% and $p < 0.05$, totally 496 genes) were applied to Ingenuity Pathway Analysis (IPA). IPA results showed that NF- κ B and PTEN signaling were predicted to be inactivated in VR cell lines, which was partially validated by quantitative PCR or western blotting experiments. The higher expression of RAF1 mRNA and the activation of AKT/ERK proteins in VR NSCLC cell lines may confer resistance to vinorelbine. Our work may provide potential pathway signature for vinorelbine sensitivity and some therapeutic targets for combined therapy.

Keywords: Non-small cell lung cancer, vinorelbine, NF- κ B signaling, PTEN signaling, AKT, ERK

Introduction

Vinorelbine is a semi-synthetic vinca-alkaloid approved for the treatment of non-small cell lung cancer (NSCLC), which also has demonstrated activity against breast cancer [1-4], ovarian cancer [5], Hodgkin Lymphoma [6] and nasopharyngeal carcinoma [7]. Vinorelbine has been evaluated in NSCLC in the adjuvant and advanced settings as a single agent and in combination with other agents (typically a platinum or gemcitabine) with modest success. The objective response rate (ORR) to vinorelbine is 15-23% for (locally) advanced NSCLC patients [8-10]; for advanced NSCLC patients treated with combination of vinorelbine and cisplatin, the ORR is 28-34% [11-13]. Meanwhile, the higher rates of adverse effects, including grade 3 to 4 neutropenia, anemia and nausea, have been demonstrated in the use of combination of vinorelbine and cisplatin for advanced NSCLC patients. Collectively, the lower ORR and higher adverse effects of vinorelbine hinder its wide use in treatment of advanced

NSCLC. Therefore, it is of great interest to uncover the biomarkers for sensitivity of NSCLC cells to vinorelbine to allow the identification of patients most likely to benefit from vinorelbine-based chemotherapy and to improve the therapy.

Vinorelbine is an antimitotic agent and its main mechanism of action is related to the inhibition of microtubule dynamics leading to a mitotic arrest and cell death [14]. Expression of several genes, either in protein or mRNA level, has been associated with the sensitivity of cancer cells to vinorelbine. For example, expression of excision repair cross-complementation group 1 (ERCC1) [15, 16], BRCA1 [17-19], ribonucleotide reductase subunit M (RRM1) [16, 20, 21], class III β -tubulin (TUBB3) [22-25], BCL-2 [26, 27], stathmin [21] and slug/SNAI2 [28] was reported to affect sensitivity of NSCLC or other cancer cells or patients to vinorelbine/cisplatin doublets, some of above molecules may serve as predictive or prognostic biomarkers. However, there are not yet large clinical trials in which the prog-

nostic effect of these molecules is validated. Moreover, it seems likely that single-molecule biomarker for drug sensitivity is basically not too solid in many cases. Recently, it is proposed that oncogenic pathway signature other than single-molecule biomarker may be more meaningful and accurate for sensitivity prediction [29, 30]. Hence, we also attempt to analyze the sensitivity signature to vinorelbine in NSCLC cells by this method.

In present work, four NSCLC cell lines, two are sensitive and two are resistant to vinorelbine, were used to analyze the differential gene expression profiles. The significantly expression-altered genes were clustered into canonical pathways to figure out the biomarkers for sensitivity prediction of vinorelbine.

Materials and methods

Cell culture

Calu-6, SK-MES-1, NCI-H1395 and NCI-H1975 cells (American Type Culture Collection, Rockville, Md.) were cultured in DEMM or RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml) and Streptomycin (100 µg/ml) (Life Technologies) in a humidified atmosphere containing 5% CO₂ at 37°C. Cells in the exponential growth phase were used for all the experiments.

Vinorelbine sensitivity determination

Calu-6, SK-MES-1, NCI-H1395 and NCI-H1975 cells (500-1500 cells/each well) were grown in 100 µl of culture medium containing serum per well in a 96-well plate. After 24 h, the cells were treated with seven different doses (0, 0.4, 1.3, 4.0, 13, 40, 130, 400 nmol/L) of vinorelbine. 5 days later, 10 µl of AlamarBlue (CellTiter-Blue® Cell Viability Assay, Promega) was added to each well and incubated at 37°C for 1.5 h and the cell viability was assayed according to the manufacturer's instruction. Every treatment for each cell line was triplicate in the same experiment. The cell viability was calculated relative to the untreated cells and the IC₅₀ dose was calculated through Graphpad Prism 5.0 software.

DNA microarray analysis

The microarray data for basal expression of Calu-6, SK-MES-1, NCI-H1395 and NCI-H1975

cells were extracted from Sanger Institute (<http://www.cancerrxgene.org/downloads/>). The average expression of some gene in two sensitive cell lines and two resistant cell lines was compared. Those genes whose expression was markedly ($p < 0.05$) altered by higher than 100% were subjected to Ingenuity pathway analysis (IPA).

Quantitative PCR (qPCR)

Calu-6, SK-MES-1, NCI-H1395 and NCI-H1975 cells in the exponential growth phase were collected for RNA extraction. cDNA was synthesized using PrimeScript RT reagent kit with gDNA Eraser (Takara, RR074A) for RT-PCR with oligo-dT and random primer. Real-time qPCR was performed on CFX-96 (Bio-lab), with endogenous control Actb. Gene expression was calculated relative to expression of Actb endogenous control and adjusted relative to expression in SK-MES-1 cells. The primers for qPCR validation were as follows:

Actb: forward (F): 5'-GCATCCCCCAAAGTTCACAA-3', reverse (R): 5'-GGACTTCCTGTAACAACGCATCT-3'; CDH1: F:5'-GGCCAGGAAATCACATCCTA-3', R:5'-GGCAGTGTCTCTCAAATCC-3'; CD24: F:5'-TGGATTTGACATTGCATTGA-3', R:5'-TGGGGGTA-GATTCTCATTATC-3'; MYC: F:5'-GGCGAACACAC-AACGTCTTG-3', R:5'-TGGTCACGCAGGGCAAA-3'; MYCN: F:5'-TCCACCTCACCTCCATGACA-3', R:5'-AGGAATGACTTTCTGTTGTTGGAA-3'; RAF1: F:5'-ATTGGGAAATAGAAGCCAGTGA-3', R:5'-CAAAAG-AGCCTGACCCAATC-3'; HOXB6: F:5'-GCGAGACA-GAAGAGCAGAAGTG-3', R:5'-AATTCATCCGCTGC-ATCCA-3'; HDAC1: F:5'-TGACAAGCGCATCTCGAT-CT-3', R:5'-CTTCAGAATCGGAGAACTTCTCT-3'; IGF1: F:5'-TCAACAAGCCCACAGGGTATG-3', R:5'-ACATCTCCAGCCTCCTTAGATCAC-3'; FGFR3: F:5'-CCCAAATGGGAGCTGTCTCG-3', R:5'-CCCGGTC-CTTGCAATGCC-3'; TRAF3: F:5'-CTTGATGGCG-TCTGGTGGTA-3', R:5'-CTGCGGACAGAATGGTCG-TT-3'; CDK5R2: F:5'-CCCCATCTGCCCTTCTTGTT-3', R:5'-CAAGCTGACACAAGTGGAGGAA-3'; FG-F7: F:5'-AAGGGACCCAAGAGATGAAGA-3', R:5'-CCTTTGATTGCCACAATTCC-3'; HOXB5: F:5'-TCC-TTCCATGCTCCCAACTC-3', R:5'-CACAGACACAA-ACATTCAGAAACT-3'; TNFRSF11B: F:5'-GAAG-GGCGCTACCTTGAGAT-3', R:5'-TGCACCACTCCA-AATCCAG-3'; DKK3: F:5'-TAGAGCCTGATGGAGC-CTTG-3', R:5'-GGCTTGACACATACACCAG-3'; IG-F2: F:5'-AAGTCGAGAGGGACGTGT-3', R:5'-CCA-GGTGTCATATTGGAAGAACT-3'.

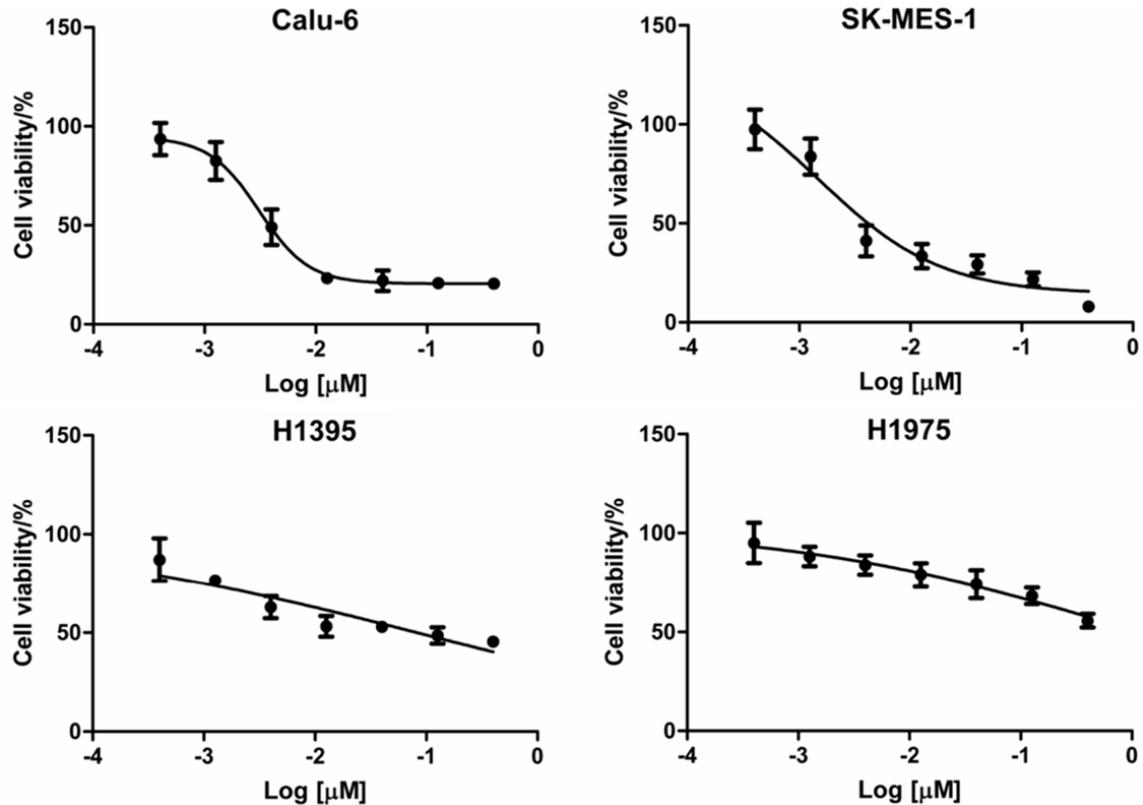


Figure 1. Determination of IC₅₀ doses of four NSCLC cell lines to vinorelbine. Calu-6, SK-MES-1, NCI-H1395 and NCI-H1975 cell lines were treated with seven doses of vinorelbine for 5 days, and then the cell viability was detected by AlamarBlue assay. IC₅₀ doses were calculated by Graphpad Prism 5.0 software. Every treatment was triplicate and the error bar represents the standard deviation (SD).

Protein isolation and western blotting

Cell pellets were resuspended in 1×SDS loading buffer (1 mmol/L Na₃VO₄, 10 mmol/L NaF, 1 mmol/L PMSF) containing protease inhibitors. Lysates (20 μg each lane) were applied to SDS-PAGE. Immunoblotting of Abs specific for GAPDH (Abmart, 080922), AKT (Santa Cruz, sc-8312), p-AKT (Santa Cruz, SC-7985-R), ERK (Abclonal, A0228) and p-ERK (Cell signaling, #9106S, pT202/204) were detected using HRP-conjugated anti-mouse (Promega) or anti-rabbit (Promega) and visualized by chemiluminescence detection system (Millipore, WBKLS0500).

Results

4 NSCLC cell lines showed dramatically different sensitivities to vinorelbine

Four NSCLC cell lines, Calu-6, SK-MES-1, NCI-H1395 and NCI-H1975, were selected to investigate their sensitivities to vinorelbine. 7 differ-

ent doses of vinorelbine were subjected to these four cell lines for 5 days and the IC₅₀ doses were calculated (**Figure 1**). The IC₅₀ doses of Calu-6, SK-MES-1, NCI-H1395 and NCI-H1975 cell lines to vinorelbine at 5 days were 3.1 (R²=0.999), 1.5 (R²=0.957), 82.3 (R²=0.864) and 882 (R²=0.981) nmol/L, respectively. Calu-6 and SK-MES-1 were very sensitive to vinorelbine, while NCI-H1395 and NCI-H1975 were resistant to vinorelbine. Although the IC₅₀ values were different from the data from Sanger Institute (IC₅₀ doses of Calu-6, SK-MES-1 and NCI-H1395 to vinorelbine were 5.7, 5.7 and 53.5 nmol/L, respectively), the trends of sensitivity to vinorelbine were in accordance.

DNA microarray analysis showed that NF-κB and PTEN signaling were in different status between the sensitive and the resistant cell lines

And then, the DNA microarray data of basal expression of cancer cells were downloaded from Sanger Institute. The average expression

AKT/ERK activation confers NSCLC cells resistance to vinorelbine

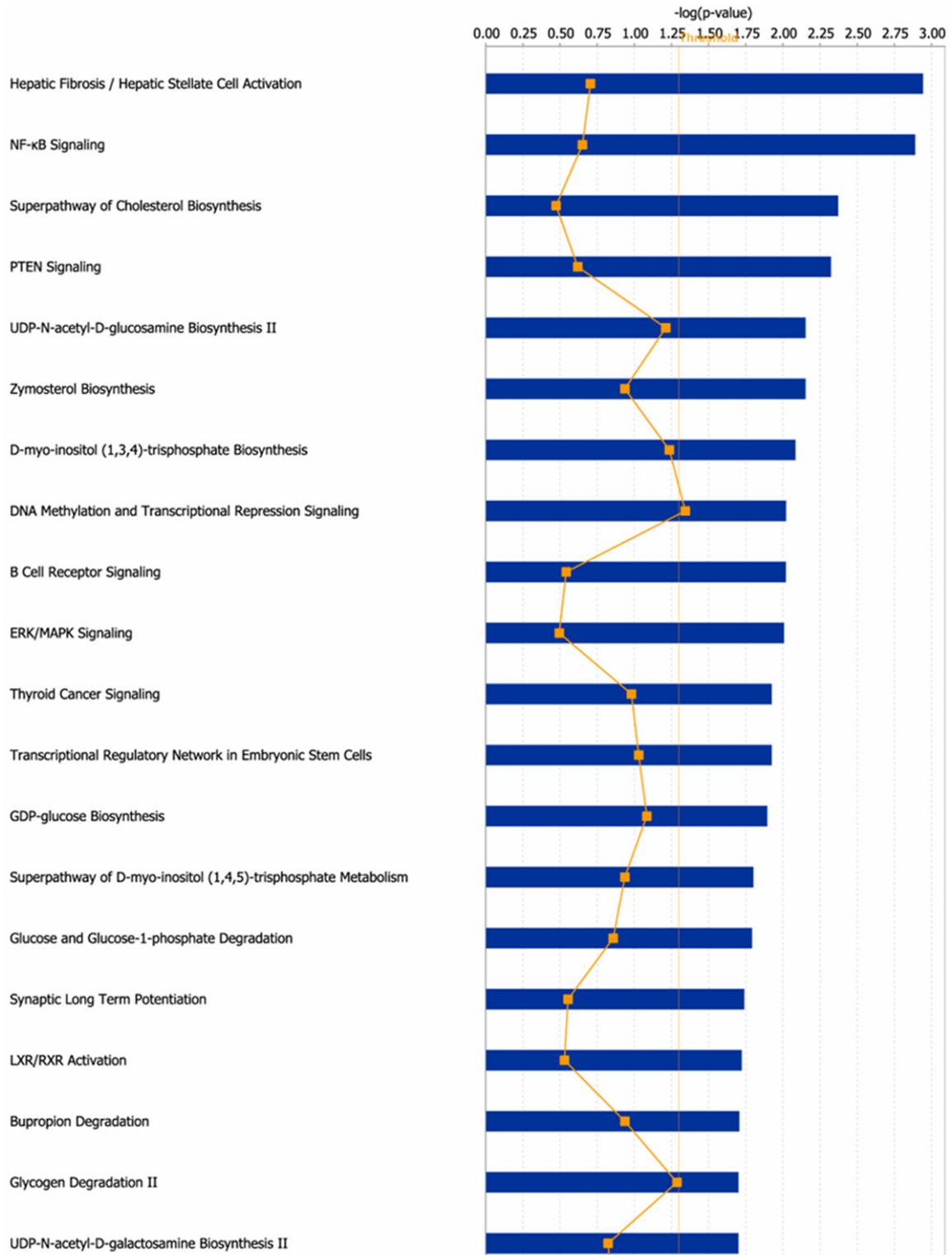


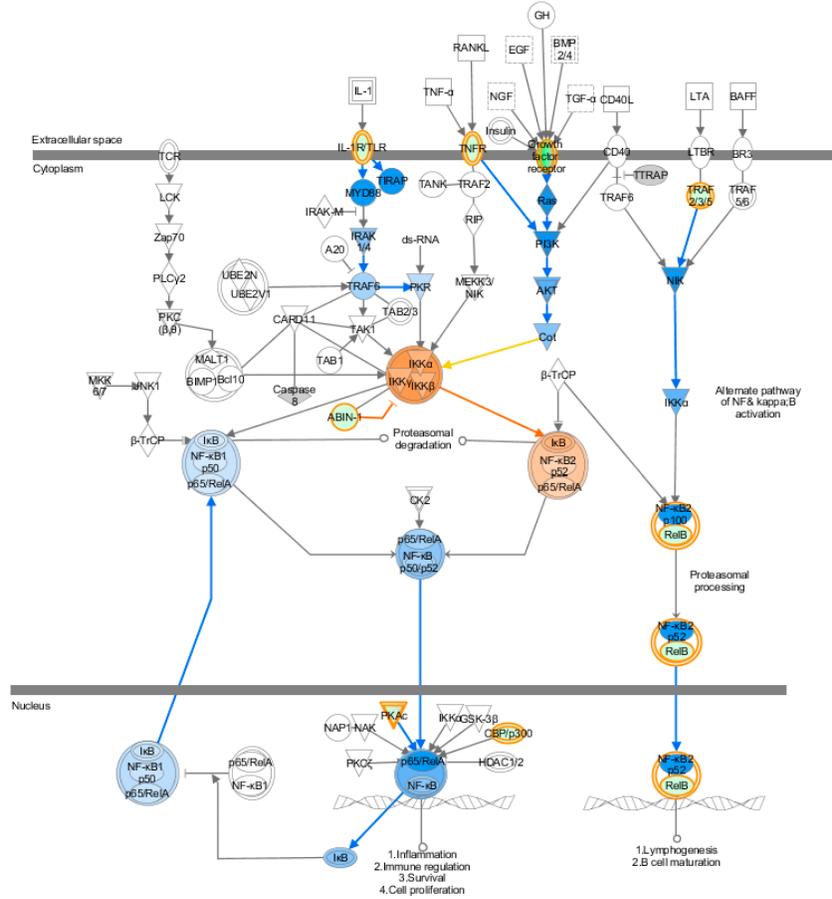
Figure 2. The most significant canonical pathways in which the differentially expressed genes were enriched. 496 differentially expressed genes were applied to Ingenuity Pathway analysis (IPA) software, and the most significant canonical pathways were shown.

of some gene in vinorelbine-resistant (VR) cell lines (NCI-H1395 and NCI-H1975) was com-

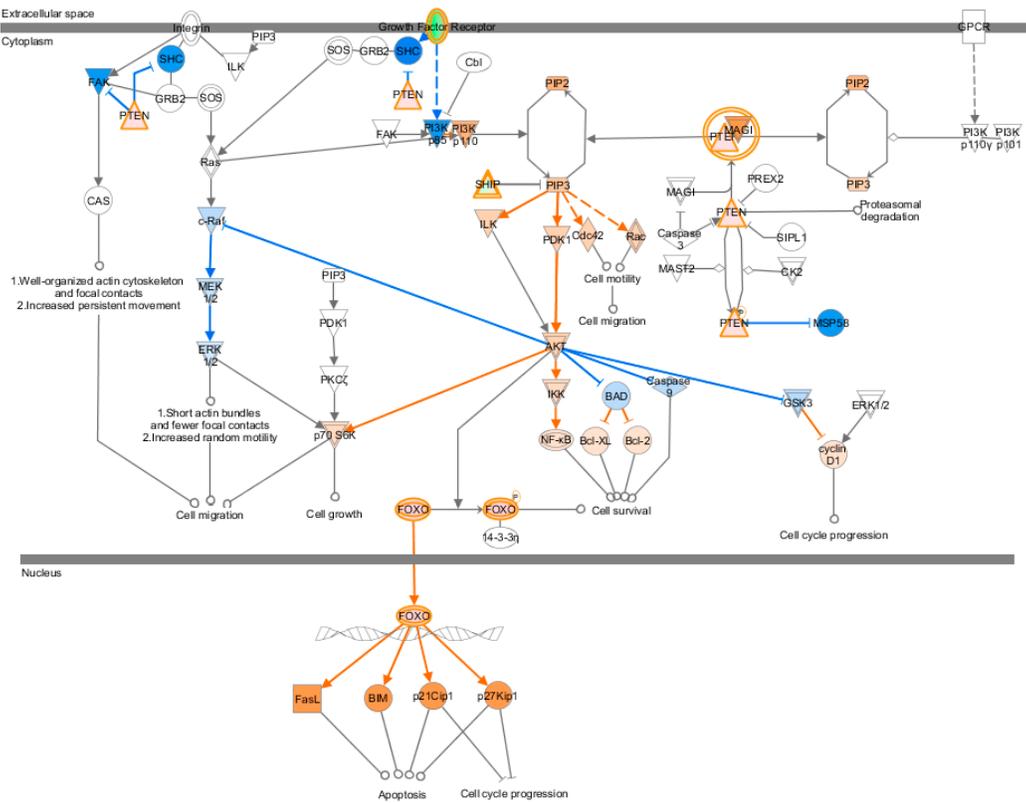
pared with that of the gene in vinorelbine-sensitive (VS) cell lines (Calu-6 and SK-MES-1).

AKT/ERK activation confers NSCLC cells resistance to vinorelbine

A

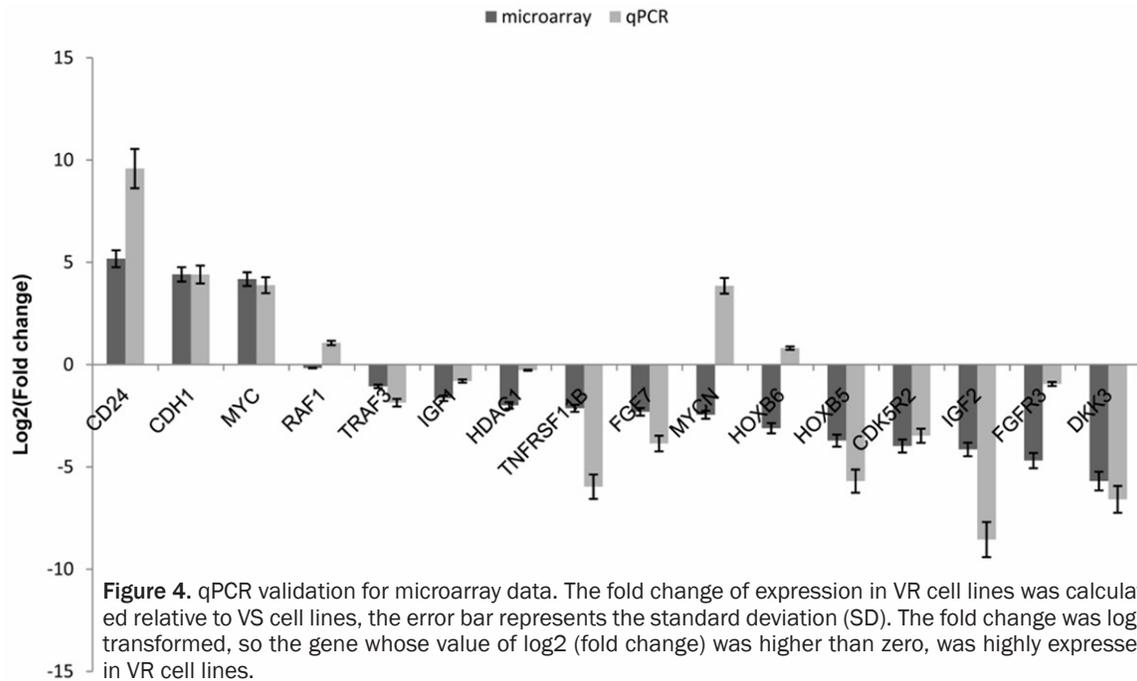


B



AKT/ERK activation confers NSCLC cells resistance to vinorelbine

Figure 3. NF- κ B signaling and PTEN signaling were predicted to be inactivated in VR NSCLC cell lines by IPA. The prediction was based on the expression of associated genes in DNA microarray data. The orange circle and arrow represent “induce”, while the blue circle and arrow represent “inhibit”. A: NF- κ B signaling in VR NSCLC cell lines. B: PTEN signaling in VR NSCLC cell lines.



Those genes whose expression was significantly ($p < 0.05$) altered by higher than 100% were selected. There were 204 highly expressed genes and 292 lower expressed genes in VR cell lines. These 496 differentially expressed genes were applied to Ingenuity Pathway analysis (IPA). IPA results showed that these genes were mainly enriched in NF- κ B signaling, superpathway of cholesterol biosynthesis, PTEN signaling and other pathways (**Figure 2**). NF- κ B signaling was predicted to be inhibited (**Figure 3A**) based on the downregulation of 11 associated genes, such as EP300, FGFR3, PRKACB, RELB and TRAF3, etc. PTEN mRNA itself was highly expressed in VR cell lines, however, 12 out of 16 associated genes (such as MBP, PDGFRB, MYC, BCL3 and TNFRSF11B, etc.) have expression direction consistent with inhibition of PTEN, and hence the AKT signaling was predicted to be activated in VR cell lines (**Figure 3B**).

qPCR validation

To validate the microarray data, 16 genes were selected to perform quantitative PCR in the four NSCLC cell lines. The fold changes of these

genes in VR/VS cell lines in both DNA microarray data set and qPCR data set were log₂ transformed and histogram in **Figure 4**. Although the change folds were varied between the two data sets, expression trends of most of genes were consistent between the two data sets except that of four genes, RAF1, HDAC1, HOXB6 and MYCN. The expression trends of these four genes were shown to be contrary between the two data sets. qPCR results showed that RAF1 was 2-fold highly expressed, HDAC1 was not much expression-altered, HOXB6 was 1.8-fold highly expressed, and MYCN was 14-fold highly expressed in VR cell lines. Expression trends of most of genes (12/16=75%) were consistent between the two data sets, suggesting that online data from Sanger Institute is reliable for gene expression profile analysis and that some important genes deserve further validation by qPCR or immunoblotting, such as oncogene RAF1 and MYCN.

Western blotting showed that AKT/ERK signaling was activated in VR cell lines

To further uncover the underlying mechanisms by which NSCLC cell lines confer resistance to

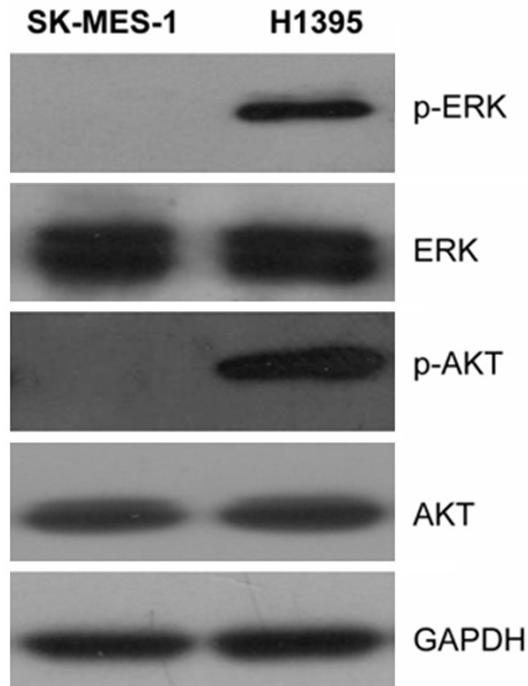


Figure 5. Immunoblotting of AKT/ERK for two NSCLC cell lines. SK-MES-1 and NCI-H1395 cells were sensitive and resistant, respectively, to vinorelbine. Total proteins from these two cell lines were subjected to SDS-PAGE and blotted onto PVDF membrane. And then the protein expression of AKT, p-AKT, ERK and p-ERK in these two cell lines was examined.

vinorelbine, immunoblotting assay was carried out for AKT/ERK status in two NSCLC cell lines, SK-MES-1 and NCI-H1395. The results showed that AKT and ERK signaling were all activated in VR cell line (**Figure 5**), suggesting that these two signaling may play roles for vinorelbine resistance in NCI-H1395.

Discussion

Vinorelbine plus cisplatin is standard treatment in adjuvant therapy for locally advanced NSCLC patients. However, the lower ORR and higher adverse effects of this combination made it urgent to figure out the biomarkers for vinorelbine sensitivity to improve the therapy. As the first step, it is of great interest to find some pathway signatures for vinorelbine sensitivity in NSCLC cell lines.

In present study, four NSCLC cell lines with different sensitivities to vinorelbine were classed into two subgroups: vinorelbine-sensitive (VS) and vinorelbine-resistant (VR) cell lines, accord-

ing to their IC50 doses to vinorelbine. And then gene expression profiles were analyzed and those differentially expressed genes between VS and VR cell lines were applied to IPA. The microarray data was validated by qPCR and the underlying mechanisms were investigated through western blotting experiments.

With the improvement of high throughput technologies, more and more data of DNA microarray chips and sequencing are available online. However, so many different technologies and platforms make it difficult to use in one system. The DNA microarray data from Sanger Institute are reliable, which was validated by qPCR in our work (**Figure 4**). Hence, it is a convenient and economical way to use online data after some validation.

The differentially expressed genes were applied to IPA and the results showed that these genes were mainly enriched in NF- κ B signaling, superpathway of cholesterol biosynthesis, PTEN signaling and other pathways. NF- κ B signaling and PTEN signaling were predicted to be inactivated in VR NSCLC cell lines by IPA. Previous study has demonstrated that vinorelbine treatment (0.1 μ g/ml for 24 h) inhibited NF- κ B and hence induced apoptosis in NSCLC cell line, H520 [31]. Moreover, Tsai et al reported that vinorelbine can induce oxidative injury in human endothelial cells by mediating AMPK/PKC/NADPH/NF- κ B pathways [32]. Therefore, it is reasonable that NF- κ B signaling is too weak to be effectively inhibited by low dose of vinorelbine and hence confer cancer cells resistance to vinorelbine. As for PTEN, a famous tumor suppressor gene, genetic alterations targeting PTEN are among the most frequently noted somatic mutations in human cancers. Several clinical studies have suggested that expression of PTEN seemed to be a potential indicator of good prognosis, with patients whose tumors expressed PTEN having improved survival compared with those whose tumors did not [33, 34]. So, it is not surprised that PTEN signaling is inactivated in VR NSCLC cell lines. PTEN is the crucial negative regulator of PI3K-AKT-mTOR signaling, the absence of functional PTEN in cancer cells leads to constitutive activation of downstream components of the PI3K pathway including the AKT and mTOR kinases [35-37]. Furthermore, the highly expression of RAF1, which was validated by qPCR (**Figure 4**),

may activated its downstream ERK signaling. The hyperactivation of growth and survival signals, such as AKT and ERK, therefore confer resistance to chemotherapy like vinorelbine treatment, which was validated by our immunoblotting data (Figure 5). However, this hypothesis warrants further validation in more NSCLC cell lines and patients.

Taken together, we examined sensitivity of 4 NSCLC cell lines to vinorelbine and divided these cell lines into sensitive and resistant groups. The gene expression profiles between these two groups were compared and those differentially expressed genes were applied to IPA. IPA results showed that NF- κ B and PTEN signaling were inactivated in VR cell lines, and AKT/ERK was hence to be predicted to be activated, which was validated by qPCR or western blotting. Our work may provide potential pathway signatures for vinorelbine sensitivity and some therapeutic targets for combined therapy.

Disclosure of conflict of interest

We have no conflict of interest to declare.

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