RESEARCH ARTICLE

Effects of Vinorelbine on Cisplatin Resistance Reversal in Human Lung Cancer A549/DDP Cells

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Abstract

Multi-drug resistance (MDR) is an essential aspect of human lung cancer chemotherapy failure. Recent studies have shown that vinorelbine is involved in underlying processes in human tumors, reversing the MDR inseveral types of cancer cells. However, the roles and potential mechanism are not fully clear. In this study, we explored effects of vinorelbine in multi-drug resistance reversal of human lung cancer A549/DDP cells. We found that vinorelbine increased drug sensitivity to cisplatin and intracellular accumulation of rhodamine-123, while decreasing expression of P-glycoprotein (P-gp), multi-drug resistance-associated protein (MRP1) and glutathione-S-transferase π (GST-π) in A549/DDP cells. At the same time, we also established downregulation of p-Akt and decreased transcriptional activation of NF-ϰB and twist after vinorelbine treatment. The results indicated that vinorelbine might be used as a potential therapeutic strategy in human lung cancer.

Keywords: Vinorelbine - human lung cancer - multidrug resistance

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Introduction

Lung cancer is a kind of malignant tumor with the highest morbidity and mortality. There are more than 1.5 million patients detected with lung cancer each year all over the world. China is one of the countries with a high incidence rate of lung cancer. Chemotherapy occupies an important position in the comprehensive treatment of lung cancer, but its efficacy is still not satisfactory. Such situation is mainly caused by the drug resistance of lung cancer cells. Lung cancer cells have developed the cross drug tolerance to a variety of chemotherapy drugs, which is also known as Multiple Drug Resistance (MDR) (Lu et al., 2013). The studies of pharmacogenetics and pharmacogenomics have shown that the formation of lung cancer drug resistance is closely associated with the genes related to lung cancer MDR and its abnormal signaling pathway. Therefore, it is necessary to search for new chemotherapy drug schemes, in order to improve the efficacy of chemotherapy, minimize the adverse effects of chemotherapy and prolong the survival cycle of the patients.

Vinorelbine (NVB), belonging to Vinca Alkaloids, is a specific anti-tumor drug which can block the cell cycle of cell division. Recently, the effect of NVB on reversing the MDR of tumor cells has drawn wide attention from the academic circle (Shukuya et al., 2012). This paper aimed to explore the effect and mechanism of NVB on reversing the MDR of lung cancer A549/DDP cells which are resistant to cisplatin.

Materials and Methods

Cell culture

The lung cancer cell lines A549 and the drug-resistant lung cancer cell line A549/DDP are purchased from American Type Culture Collection (ATCC), which was cultured in RPMI-1640 medium which contains 10% fetal bovine serum, 100 U/mL of penicillin and 100 U/mL of streptomycin at the conditions of 37 °C, saturated humidity and 5% (volume fraction) CO_2.

Detect the inhibition effect of NVB on A549/DPP cell proliferation by MTT assay

A549/DPP cells were regulated the density to 2×10^5/mL and cultured in 96-well plate with 100 μL in each well for 24 h. Dilute NVB in accordance with the gradient concentration of 0, 0.1, 1, 5, 10, 50 and 100 μM, respectively, and cultured the cell at the conditions of 37 °C and 5% CO_2 for 24 h. Then, add MTT (5 mg/mL) into each hole and culture the samples for another 4 h. Discard the supernatant and add in 200 ul DMSO, vibrated the samples for 10 min. Detect the OA value at the wavelength of 490 nm by enzyme-labeled reader and prepare the blank control group which is not treated by any drug and the negative control group which is treated by DMSO only. Plot the cell inhibition curve and calculate the proliferation inhibition rate of A549/DDP under the effect of NVB.

Detect the effect of NVB on reversing the drug resistance

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of A549/DPP cells by MTT assay

A549/DPP cells were regulated the density to 2×10^5/mL and cultured in 96-well plate with 100 μL in each well for 24h. Dilute DDP in accordance with the gradient concentration of 0, 0.1, 1, 5, 10, 50 and 100 μM, respectively, added the NVB solution with concentration of 1 and 5 μM into culture medium. Culture the cell samples at the conditions of 37 °C and 5% (volume fraction) CO_2 for 24 h. Then, add MTT (5 mg/mL) into each hole and culture the samples for another 4 h. Discard the supernatant and add in 200 ul DMSO and vibrated the samples for 10 min. Detect the OA value at the wavelength of 490 nm by enzyme-labeled reader and prepare the blank control group which is not treated by any drug and the negative control group which is treated by DMSO only. Plot the cell inhibition curve and calculate IC_{50} of A549/DDP cells under the effect of NVB at the concentration of 1 and 5 μM, respectively. Then, calculate the change of drug resistance fold of A549/DDP cells under the effect of NVB.

Rhodamine-123 accumulation assay

Cells were treated by 1 and 5 μM NVB for 24h, digested by trypsin, suspended, and centrifugated. Discard the supernatant and suspend the samples in the culture medium containing 5 mmol•L^{-1} Rhodamine-123. Then, culture the samples at the conditions of 37 °C and 5% (volume fraction) CO_2 for 30 min. Wash the cells for 3 times with culture medium and cultured the samples at the conditions of 37 °C and 5% (volume fraction) CO_2 for another 10 min. Wash and suspend the cells in 1mL pre-cooling culture medium and detect the fluorescence intensity of the cells by flow cytometer at 488 nm.

P-glycoprotein expression assay

Cells were treated by 1 and 5 μM NVB for 24 h, digested by trypsin, suspended, and centrifugated. Discard the supernatant and suspend the samples in the culture medium containing 20 μl anti-P-glycoprotein-PE and incubated for 30 minutes in the dark at room temperature. Wash and suspend the cells in 1mL pre-cooling culture medium and detect the fluorescence intensity of the cells by flow cytometer at 488 nm.

Determine of glutathione

Cells were treated by 1 and 5 μM NVB for 24h, digested by trypsin, suspended, and centrifugated. Cell lysates were prepared, and reacted with assay solution for 5 min at 25°C. DTNB were used to measure the intracellular glutathione (GSH) by Total Glutathione Assay Kit (Beyotime Institute of Biotech, Jiangsu, China). Analysis was performed according to the manufacture's instruction and the absorbance was detected by enzyme-labeled reader.

Detect the expression of mRNA of the MDR-related genes by real time PCR

Cells were treated by 1 and 5 μM NVB for 24 h, digested by trypsin, suspended, and centrifugated. Extract the total RNA of cells by Trizol kit. The mRNA expression of MRP1 and GST-π were detected by real-time PCR. And the condition was as following: decontamination at 50 °C for 2 min, denaturation at 95 °C for 2 min, followed by n cycles at 95 °C for 20 sec and at hybridization 95°C for 40 sec. RT-PCR was performed by standard methods using the following primers: MRP1, Forward: 5'-ACTTCCACATCTGCTTCGTCAGTG-3', Reverse: 5'-ATTCAGCCACAGGAGGTAGAGAGC-3'; GST-π, Forward: 5'-TGGGCATCTGAAGCCTTTTG-3', Reverse: 5'-GATCTGGTCACCCACGATGAA-3'; GAPDH(internal reference), Forward: 5'-GCAGGGGGGA GCCAAAAGGG-3', Reverse: 5'-TGCCAGCCCCAGC GTCAAAG-3'.

Detect the expression of protein of the MDR-related genes by Western blotting

Cells were treated by 1 and 5 μM NVB for 24h, digested by trypsin, suspended, and centrifugated. Pre-cooling cell lysate was added and performed ice bath lysis for 30 min, then, performed centrifugation at 12000 r•min^{-1} and 4 °C for 20 min. Extract and transfer the supernatant to a new EP tube. Detect the protein concentration by Bradford method. Extract 40 μg of total protein to perform the polyacrylamide gel [PAGE, 4% (mass fraction) concentrated gel, 10% (mass fraction) separating gel] electrophoresis at 80 volts for 3 h. Transfer the samples electrically to the PVDF membrane and incubated in 5% skimmed milk for 1 h at room temperature. Then, add in the MRP1 (1:800), GST-π (1:800), p-Akt (1:600), total-Akt (1:600) and β-actin (1:5000) antibody and incubated for overnight at 4 °C. Wash the membrane with TBS-T for 3 times. Add in the second antibody labeled by horseradish peroxidase (HRP) (1:5000) and incubate the samples at room temperature for 30 to 45 min. Wash the membrane with TBS-T for 3 times. Use the ECL chemiluminescence agent to develop color for 1~2 min, and then, expose the X-ray film.

Detect the promoter activity by dual luciferase reporter system

Transfect 1 μg of NF-κB and Twist fluorescent reporter plasmid and 0.02 μg of control plasmid into the cell samples of both the control group and the testing group for 6 h. Then, cells were treated by 1 and 5 μM NVB for 24 h, detect the luciferase activity in accordance with the operation instruction.

Statistics method

SPSS 13.0 software is used to perform the statistical analysis in this study. The experimental data is expressed in the form of Mean ± SD. According to the requirements of different data processing and statistical analysis methods, variance or t-test is applied to analyze the data. Each experiment is repeated for three times. $P < 0.05$ is considered as the standard of statistically significant.

Results

NVB increases the sensitivity of A549/DDP cells to cisplatin

The MTT results (Figure 1A) indicated that the toxic effect of NVB on A549/DDP cells was increased

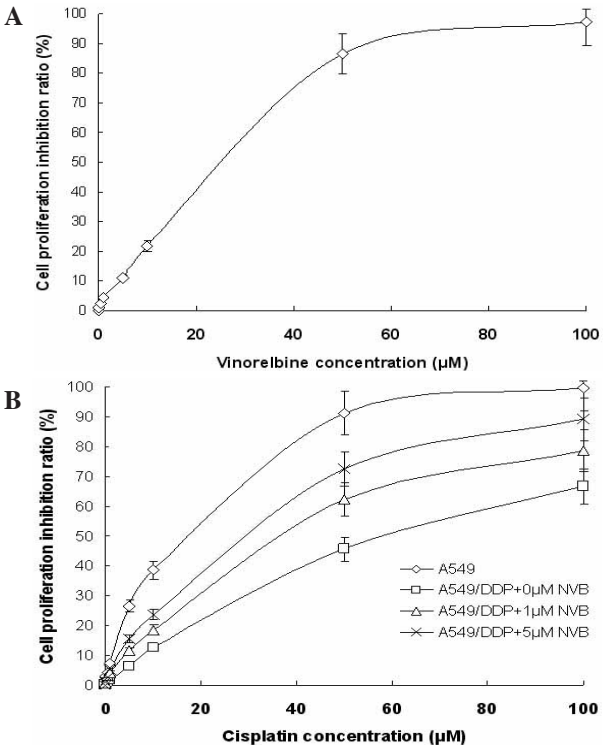

Figure 1. The Effect of Vinorelbine on Proliferation Inhibition and Cisplatin Drug Sensitivity of Human Lung Cancer Cells. (A) The effect of Vinorelbine on proliferation inhibition of A549/DDP cells. Values were presented as the means±SD, n=5. (B) The effect of Vinorelbine on cisplatin drug sensitivity of A549 and A549/DDP cells. Values were presented as the means±SD, n=5

significantly with the increase of NVB concentration; meanwhile, the phenomenon of dose-dependent was observed. 1 and 5 μM were the non-toxic (<5% inhibition ratio) and low-toxic (<15% inhibition ratio) concentration, respectively, so these two concentrations were used in the experiment. With the effect of NVB at the concentration of 1 and 5 μM, the sensitivity of A549/DDP cells to cisplatin was significantly increased. The value of IC_{50} was 31.62 and 22.47μM (Figure 1B), and the Reversal Fold (RF) was 1.75 and 2.46, respectively, indicating that NVB is able to enhance the sensitivity of lung cancer A549/DDP cells to cisplatin.

NVB could increase the concentration of Rhodamine-123 and decrease expression of P-glycoprotein and GSH in A549/DDP cells

The results of flow cytometer showed that 1 and 5 μM NVB could increase the fluorescence intensity of Rhodamine-123 by 3.2 and 7.3 fold, respectively, compared with the control group, indicating that NVB is able to increase the concentration of Rhodamine-123 in A549/DDP cells. Meanwhile, 1 and 5 μM NVB could decrease the fluorescence intensity of P-glycoprotein by 52.3 % and 35.7 %, respectively, compared with the control group, indicating that NVB is able to decrease expression of P-glycoprotein in A549/DDP cells. Additionally, the results showed that the expression of GSH was 71.6% and 48.9% of the control group after 1 and 5 μM NVB treatment for 24 h, respectively (Figure 2).

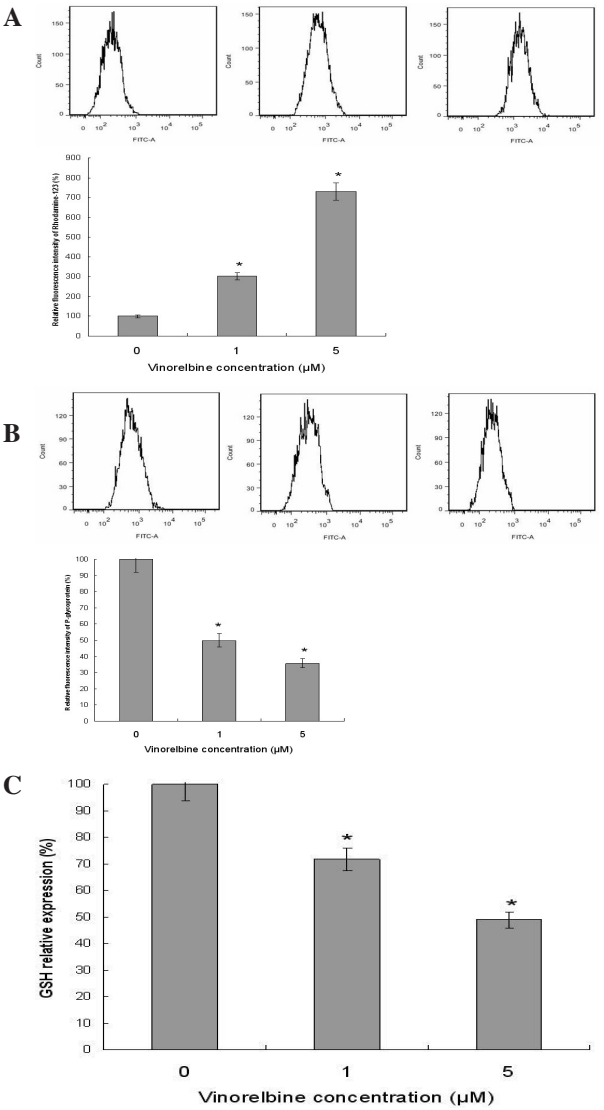

Figure 2. The Effect of Vinorelbine on Drug Excretion of Human Lung Cancer Cells. (A) The flow cytometry results in the effect of Vinorelbine on intra-cellular Rh-123 content of A549/DDP cells. Values were presented as the means±SD, n=3; *compared to the control group, $P<0.05$. (B) The flow cytometry results in the effect of Vinorelbine on P-glycoprotein expression of A549/DDP cells. Values were presented as the means±SD, n=3; *compared to the control group, $P<0.05$. (C) The effect of Vinorelbine on GSH expression of A549/DDP cells. Values were presented as the means±SD, n=5; *compared to the control group, $P<0.05$

The effect of NVB on expression of the MDR-related genes of A549/DDP cells

The total RNA concentration of the extracted cell samples detected by the UV spectrophotometer was ranged from 1.15 to 1.25 μg/μL. The ratio of A260/A280 was between 1.8 and 2.0, indicating that the concentration and purity of RNA was compliant with the experimental requirements. GAPDH was taken as the internal reference. The RT-PCR test results showed that the expression of MRP1 mRNA was 28.1% and 11.3%, and GST-π mRNA was 44.7% and 17.1% of the control group after 1 and 5 μM NVB treatment for 24 h, respectively (Figure 3A).

The results of Western blotting showed that NVB could decrease the expression of MRP1 and GST-π compared with the control group, indicating that NVB is able to

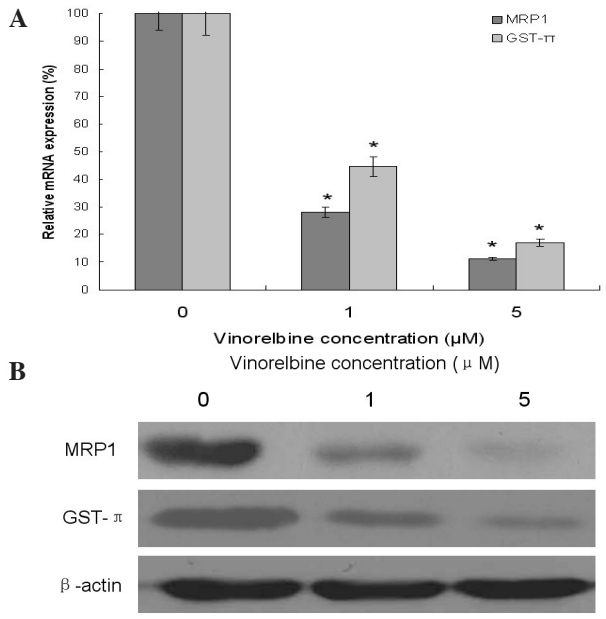

Figure 3. The Effect of Vinorelbine on Expression of MRP1 and GST-π of Human Lung Cancer Cells. (A) The real time PCR results showed that the mRNA levels of MRP1 and GST-π were down-regulated by Vinorelbine treatment. GAPDH was used for normalization. Values were presented as the means±SD, n=5; *compared to the control group, $P<0.05$. (B) The Western blot results showed that the protein levels of MRP1 and GST-π were down-regulated by Vinorelbine treatment. β-actin was used for normalization

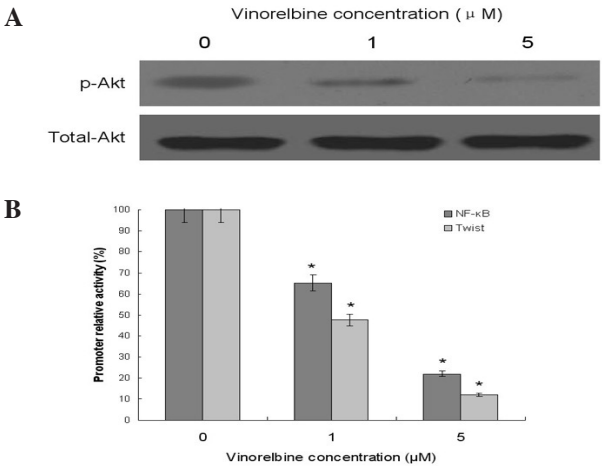

Figure 4. The Effect of Vinorelbine on MDR-Related Signaling Molecule of Human Lung Cancer Cells. (A) The Western blot results showed that the phosphorylation levels of Akt was down-regulated by Vinorelbine treatment. Total Akt was used for normalization. (B) The dual luciferase report results showed that the activation of the NF-κB and Twist transcription factor was down-regulated by Vinorelbine treatment. Values were presented as the means±SD, n=5; *compared to the control group, $P<0.05$

reduce the expression of the MDR-related proteins in the tumor cells, including MDR1 and GST-π after 1 and 5 μM NVB treatment for 24 h (Figure 3B).

The effect of NVB on the MDR-related signaling molecules of A549/DDP cells

The results of Western blotting showed that NVB could decrease the expression of p-Akt, but the expression of the total Akt protein in the tumor cells did not change significantly. The results of the dual luciferase reporter system showed that the activity of NF-κB promoter was 65.3% and 22.1% of the control group, respectively. The activity of Twist promoter was 47.7% and 12.1% of the control group after 1 and 5 μM NVB treatment for 24 h, respectively (Figure 4). It indicated that NVB could inhibit the phosphorylation of Akt and suppress the activation of the NF-κB and Twist transcription factor.

Discussion

Lung cancer is one of the cancers with a high incidence rate in China. The 5-year survival rate of radiotherapy treatment for lung cancer is only 50%, while the 5-year survival rate of the patients with advanced lung cancer (III, IV) is even lower, only about 10% to 40%. Chemotherapy is the major adjunctive therapy for the treatment of advanced lung cancer, but the formation of MDR often makes chemotherapy ineffective. Hence, it has become a hot topic in the anti-tumor study to search for effective MDR reversal agents.

The formation of MDR of tumor cells is a complicated process involving a variety of factors. Its mechanism mainly includes the increase of drug efflux, the change of activity of drug-metabolizing enzyme, and the change of drug affinity. Among these factors, drug efflux caused by the enhancement of drug transporter activity is an important reason for the formation of MDR in tumor cells. The transporter proteins include p-Glycoprotein (P-gp) encoded by gene MDR1 and the MDR-related protein (MRP), and glutathione-S-transferase-π (GST-π), etc. These proteins can pump cytotoxic drugs out of the cells relying on the energy provided by ATP. As a result, the intracellular drug concentration is greatly reduced, which thereby enables the cells to become resistant to many drugs (Wang et al., 2011; Ren et al., 2012; Wang et al., 2012).

This study observed that NVB can effectively improve the sensitivity of cells to cisplatin and reverse the drug resistance at the concentration of 1 and 5 μM. Meanwhile, the results of RT-PCR and Western blot indicated that the mRNA and protein level of MRP1 and GST-π were significantly reduced with the effect of NVB. The Rhodamine-123 accumulation and P-gp expression experiment can reflect the transportation capacity of the drug transporters. The results proved that NVB is able to significantly increase the Rhodamine 123 accumulation level inside A549/DDP drug-resistant cells, meanwhile, expression of P-gp was decreased, indicating that NVB may increase the intracellular concentration of cytotoxic drugs and reverse the drug resistance by inhibiting the expression of P-g (MDR1).

NF-κB and Twist can participate in many signaling pathways and play an important role in the formation and development process of tumors by promoting cell proliferation, inhibiting cell differentiation and apoptosis, and promoting the invasion and metastasis of tumor cells. At the same time, NF-κB and Twist can bond to the binding position at the promoter region of the MDR-related genes, so as to promote the transcription, translation and expression of drug resistance. It will then

result in the drug resistance of tumor cells (Al et al., 2011; Das et al., 2013; Wang et al., 2013). In this study, the Western blot and luciferase reporter experiment discovered that NVB can inhibit Akt phosphorylation and reduce the transcription activity of NF-κB and Twist, which will then lower the expression of the MDR-related genes and reverse the MDR of tumor cells.

In summary, this study observed that NVB can reduce the expression of P-gp, GSH, MRP1, and GST-π and increase the intracellular drug concentration of the tumor cells, which will thereby enhance the sensitivity of the lung cancer drug-resistant cells to cisplatin and reverse MDR. Its mechanism may be related to the inhibition of Akt phosphorylation and the reduction of NF-κB and Twist transcription activity. The results of this study provided an experimental basis for the clinical applications in the field of MDR reversal of lung tumor cells. However, the specific mechanism of NVB on reversing the MDR of tumor cells still needs to be further investigated.

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