

RESEARCH ARTICLE

Effects of Intermittent Cisplatin Intervention on 6PGD Levels and Tumor Growth and Migration in A549 Lung Cancer Cells

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ABSTRACT

Aim: To investigate the changes of A549 protein 6PGD expression, tumor growth and migration, NADPH and ROS levels in lung cancer cells after intermittent intervention with DDP. **Methods:** The sensitivity of A549 cells to DDP was detected by cck-8 method, A549 cells were treated by DDP intermittent, 6PGD protein expression was detected by Western blot method, and the ROS level of A549 cells was determined by ROS kit. NADPH levels of A549 cells were determined by NADPH kit. **Result:** After DDP intermittent intervention, the expression of 6PGD protein in lung cancer cells A549 was increased, the growth and migration of A549 cells were significantly inhibited, and ROS and NADPH levels were significantly increased. **Conclusion:** 6PGD were upregulated in after DDP intermittent intervention and affected the migration ability in lung cancer cells.

KEYWORDS: Lung cancer, 6PGD, Drug resistance, Cisplatin, NADPH, ROS, Migration.

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INTRODUCTION

Lung cancer is one of the most common malignant tumors with the highest morbidity and mortality. Non-small cell lung cancer is one of the one of the main types of lung cancer, which has extremely high morbidity and mortality all over the world(1). The occurrence of cell migration in lung cancer tissue is one of the main reasons why it is difficult to cure and increase mortality(2-5). DDP is an effective chemical drug for the clinical treatment of lung cancer, but due to long-term DDP drug intervention, the emergence of drug resistance in lung cancer cells is the primary problem in the treatment of lung cancer (6, 7). Glucose 6-phosphate dehydrogenase (6PGD) is one of the main enzymes in the pentose phosphate pathway (PPP). It is reported that 6PGD is up-regulated in many tumors and is related to tumor growth. Its catalytic product NADPH is the metabolism of PPP. One of the important sources of products of the pathway, not only provides reducing energy for biosynthesis, but also can offset the toxicity of reactive oxygen species (ROS) to cells, so it is a very promising anti-cancer target(8). In this study, A549 cells were treated with DDP to produce drug resistance, and the 6PGD protein level, ROS and NADPH changes of A549 cells were observed, and their effects on cell growth and migration were observed.

MATERIALS AND METHODS

Cell lines and main reagents : The A549 cell line was provided by Dr. Sheng Hao of Jinan University, RPMI 1640 medium, fetal bovine serum, trypsin (Gibco, USA), 6PGD antibody (Santa Cruz Biotech), Transwell chamber (Thermo Fisher), 24-well plate

(Thermo Fisher), ROS kit (Beyotime), NADPH kit (Beyotime), DDP (Sigma-Aldrich), CCK8 (Beyotime).

Methods: The A549 cell line was cultured in RPMI 1640 complete medium containing 10% fetal bovine serum. The cells were cultured in a 5% CO₂, 37°C saturated humidity incubator.

IC₅₀ detection of drug DDP: Adjust the density of A549 cells to 2.5×10⁴ cells/ml with RPMI 1640 culture medium containing 10% fetal bovine serum, and inoculate them into a 96-well cell culture plate. Each group has 3 multiple wells. The experiment is repeated 3 times, 200μl/well After the cells adhere to the wall, gently aspirate the culture medium, and add DDP with a gradient of 200μl/well. The final concentration of DDP is 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 mg/L, respectively. After 48h, use CCK8 colorimetric method to detect cell survival, measure the absorbance (OD) value of each well at 450nm wavelength, and calculate cell survival rate according to the following formula: cell survival rate = OD drug group/OD control group × 100%. The half inhibiting concentration (IC₅₀) of the drug was calculated by linear fitting method.

Establishment of DDP-resistant A549 cells: By calculating the IC₅₀ concentration is 0.8 mg/L. Select 0 mg/L, 0.4 mg/L, 0.8 mg/L, 1.2 mg/L DDP to treat A549 cells in logarithmic growth phase. After 6 hours, discard the drug-containing culture medium and replace with normal 1640 medium. After 12 hours, Replace the drug-containing medium with the above concentration gradient, repeat this 6 times, and then continue to culture A549 cells with the drug-containing medium with the above

concentration gradient. So far, the cell groups are described as A549, DDP-0.4, DDP-0.8 and DDP-1.2. Subsequently, A549, DDP-0.4, DDP-0.8 and DDP-1.2 were treated with 0 mg/L, 0.4 mg/L, 0.8 mg/L, 1.6 mg/L, 3 mg/L, 4 mg/L, and 6 mg/L. 4 groups of cells. The CCK8 colorimetric method was used to detect cell survival to evaluate the established A549 cell resistance to cisplatin.

Western blot to detect the expression of 6PGD protein level: A549 cells treated with intermittent intervention with DDP concentration of 0 mg/L, 0.4 mg/L, 0.8 mg/L, and 1.2 mg/L were collected, and protein content was determined by BCA method. A 12% separating gel and a 5% concentrated gel are prepared routinely. The proteins are separated by SDS-PAGE electrophoresis and transferred to the PVDF membrane. Block with TBST containing 5% skimmed milk powder for 2 hours at room temperature, add rabbit anti-human 6PGD primary antibody and 1:1000 β -actin monoclonal antibody at 1:1000, and incubate overnight at 4°C. ECL chemiluminescence kit (Beyotime) shows the results. Use Image J bundled with 64-bit Java 1.8.0_112 software to quantitatively analyze the integrated absorbance value (IOD) of protein bands, use β -actin as an internal reference to calculate the ratio of β -actin, and represent the relative protein level as a percentage of the control group.

Tumor migration experiment : Adjust the cell density to 50×10^4 cells/ml with serum-free 1640 medium. Aspirate 100 μ l and spread it in the upper chamber of Transwell. Set 3 multiple wells in each group and add 600 μ l of RPMI1640 medium containing 1% fetal calf serum to the lower chamber. After culturing in a 5% CO₂, 37°C saturated humidity incubator for 16 hours, wash with PBS twice, fix the cells with 10% paraformaldehyde for 15 minutes, then wash with PBS 3 times, stain with 0.1% crystal violet for 25 minutes and then wash with PBS 2 times, and wipe the upper chamber cells with absorbent cotton. Observe the number of migrated cells under an inverted microscope.

ROS kit to detect ROS level : Adjust the density of A549 cells to 15×10^4 cells/ml with RPMI 1640 culture medium containing 10% fetal bovine serum, and inoculate them into 6-well cell culture plates. Each group has 3 multiple wells. The experiment is repeated 3 times, 2ml/well After the cells adhere to the wall, gently aspirate the culture medium and add serially diluted DDP to the final concentration of 0 mg/L, 0.4 mg/L, 0.48 mg/L, 1.2 mg/L. After adding the DCFH-DA probe to the concentration indicated by the manufacturer and incubating for 1 hour, the result was analyzed with a highly sensitive intelligent flow analyzer BD FACS VERSE (BD Company, USA).

Detect the level of NADPH/NADP⁺ ratio: The ratio of NADPH/NADP⁺ was determined by the colorimetric assay kit (Beyotime). In short, 2×10^6 cells were trypsinized, washed with PBS, and 200 μ l NADP⁺/NADPH extract was added. Then centrifuge at 12,000g at 4°C for 5-10 minutes, and take the supernatant as the sample to be tested. The supernatant was tested for the ratio of NADPH/NADP⁺ according to the manufacturer's protocol.

Statistical Analysis: The statistical data was processed by GraphPad 6.0.1 analysis software, and the comparison of the means between the two groups was processed by t test, $P < 0.05$ means the difference is statistically significant.

RESULT

Cell growth after intermittent DDP treatment: After A549 cells were treated with DDP with a concentration gradient of 0.4 mg/L, 0.8 mg/L, 1.6 mg/L, 3 mg/L, 4 mg/L and 6 mg/L, the IC₅₀ of DDP for A549 cells was calculated by linear fitting It is 0.8mg/L. Compared with the control group, it was observed that the cell survival rate was significantly suppressed. (figure 1).

Establishment of drug-resistant cells after intermittent DDP treatment : After DDP concentration of 0.4mg/L, 0.8mg/L, 1.2mg/L long-term intermittent intervention induction, compared with the control group, A549 cells have obviously developed resistance to DDP. (figure 2)

6PGD protein is upregulated in cisplatin-resistant A549 cells: In A549 cells intermittently intervened with 0.4mg/L, 0.8mg/L, and 1.2mg/L DDP, Western blot results showed that the 6PGD protein level of DDP-resistant A549 cells was increased in a dose-dependent manner (Figure 3).

Changes of ROS in A549 cells resistant to cisplatin : At the same time, the changes of ROS levels in A549 cells were observed under the intervention of the above concentrations. Compared with the control group A549 cells, the ROS levels increased significantly in a concentration-dependent manner (Figure 4).

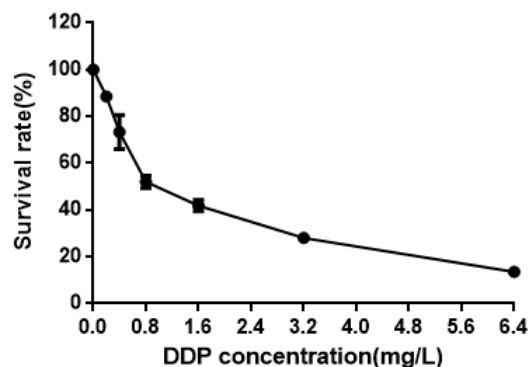


Figure 1: Survival curve of DDP treated A549 cell

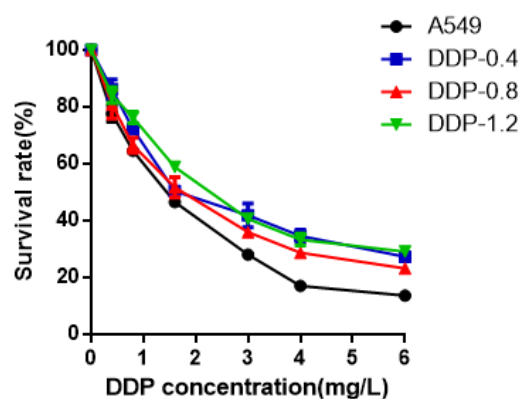


Figure 2: The establishment of DDP resistant A549 cell

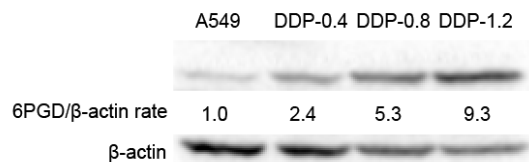


Figure 3: Expression of 6PGD protein in DDP resistant A549 cell

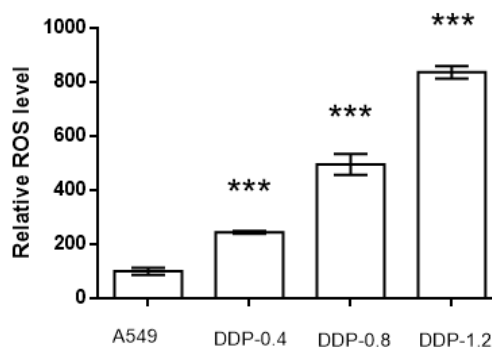


Figure 4: ROS levels of resistant A549 cell treated with DDP 0 mg/L, 0.4 mg/L, 0.8mg/L and 1.2 mg/L

Changes of NADPH in cisplatin-resistant A549 cells: Next, the changes in NADPH levels in DDP-resistant A549 cells was observed. Compared with the control group, 0.4mg/L DDP-induced NADPH levels in drug-resistant A549 cells did not change significantly, 0.8mg/L and 1.2mg/L NADPH was significantly increased in DDP-induced cells (Figure 5).

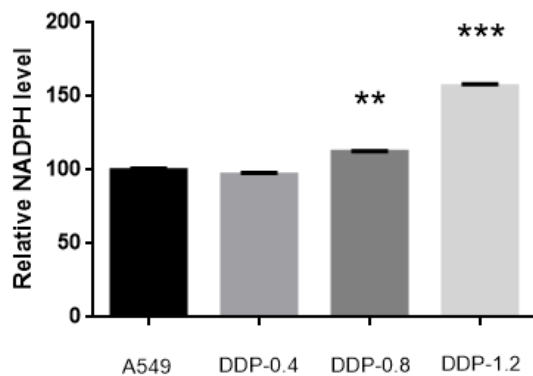


Fig.5: NADPH levels of resistant A549 cell treated with DDP 0 mg/L, 0.4 mg/L, 0.8mg/L and 1.2 mg/L

Effect of cisplatin-resistant A549 cells on cell migration: In order to verify the migration ability of the cells, the drug-resistant cells intermittently interfered by DDP were treated in a serum-free Transwell chamber for 16 hours. The cell body became larger, the edge protrusions increased, and the number of migrating cells was significantly reduced (Figure 6)

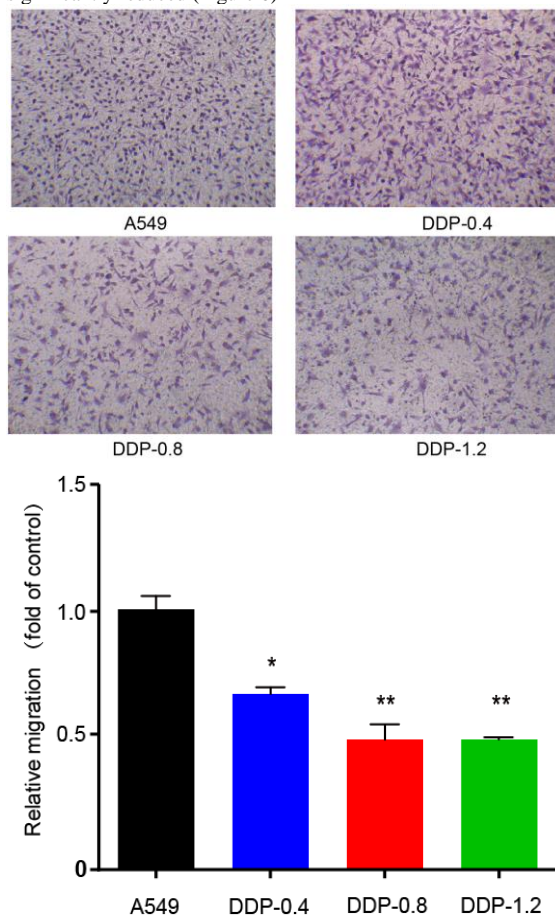


Figure 6: Transwell migration assay of 549 cell after intermittent intervention (Crystal Violet Stain×400).

DISCUSSION

The changes in cell metabolism which was one of the signs of tumorigenesis. Even under high oxygen stress, tumors have a tendency to use glycolysis to produce ATP. This phenomenon is called aerobic glycolysis or Warburg effect(9). Many studies have proven the importance of the Warburg effect in cancer. In addition to glycolysis, the pentose phosphate pathway (PPP) is also very active in tumors. The potential role of PPP oxidation branch in lung cancer has been widely reported. For example, knockout of many metabolic enzymes in the PPP pathway can inhibit cell growth and migration(10).

Cisplatin (DDP) is currently one of the most common effective drugs used to treat many solid tumors such as lung cancer, testicular cancer, head and neck cancer and ovarian cancer (11). However, due to long-term cisplatin intervention, many patients have developed severe drug resistance. Therefore, the therapeutic effect of cisplatin on tumors is greatly reduced (12). Therefore, exploring the molecular mechanism leading to cisplatin resistance to overcome the problem of cisplatin resistance is a problem that needs to be solved urgently. Many ways have been found to overcome cisplatin resistance. For example, overexpression of miR-194 is more likely to cause apoptosis in cisplatin-resistant lung cancer cells (13); Knockout of Long non-coding RNA H19 can reverse cisplatin resistance in ovarian cancer cells(14). However, the relationship between the metabolic enzyme 6PGD and the drug resistance of DDP, and the effect on tumor migration ability has not been reported.

6-gluconate phosphate dehydrogenase (6PGD) is one of the key enzymes in the pentose phosphate pathway, which is very important for the growth of cancer cells (15). Studies have shown that the expression of 6PGD is positively correlated with the progression of lung cancer. There are also reports that 6PGD is up-regulated in many cancers, so it is a promising anti-cancer target(16).

In this experiment, DDP was used to intermittently interfere with A549 lung cancer cells to make the cells resistant. Compared with normal A549 cells, the IC50 of drug-resistant cells was significantly increased under different concentrations of cisplatin. Firstly, it was found that 6PGD levels in DDP-resistant A549 cells showed high expression. In the experiment, it was found that the level of ROS in the cells increased significantly. It has been reported in the literature that reactive oxygen species (ROS), which are produced through various extracellular and intracellular interactions, have attracted wide attention. ROS levels involve growth, differentiation, progression and death of cells(17). For a long time, people have recognized that the increase in ROS levels can modify cell signaling proteins and have functional consequences. In addition, because glucose 6-phosphate generates ribulose-5-phosphate under the action of 6PGD, at the same time, 6PGD uses NAD⁺ as the electron acceptor, and the product is NADPH. The pentose phosphate pathway is the main source of NADPH in the body(18). And NADPH is necessary for fatty acid synthesis and removal of reactive oxygen species (ROS). Therefore, the pentose phosphate pathway plays a key role in helping tumor cells meet their anabolic needs and resist oxidative stress(19). In the experiment, it was observed that NADPH levels were indeed significantly increased in DDP-resistant A549 cells. Consistent with our experimental results, we speculate that in order to combat the damage caused by high levels of ROS, cells can upregulate 6PGD to produce a large amount of NADPH to combat high levels of ROS in cells. It was also observed in the experiment that the migration ability of cisplatin-resistant A549 cells was significantly reduced.

Therefore, the development of new drugs targeting 6PGD to combat cisplatin resistance is a promising direction. However, how to establish a detailed mechanism of the PPP metabolic pathway involved in the 6PGD target to overcome lung cancer resistance to cisplatin still requires a lot of in-depth research.

CONCLUSION

The level of 6PGD protein in A549 cell was upregulated after intermittent cisplatin intervention, and a large amount of NADPH was thus produced in response to the cell emergency to produce toxic ROS damaging effects on cell. Finally, inhibition of the growth and migration of drug-resistant A549 cell formed was observed.

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None.

DISCLOSURE STATEMENT

The authors declare that they have no conflicts of interest.

STATEMENT OF ETHICS

The experimental procedures were approved by the Committee for Animal Research of Jinan University, Guangzhou, China. All animal studies strictly conformed to the experimental guidelines of the Committee for Animal Care (Jinan University).

AUTHOR CONTRIBUTIONS

All authors made equal contributions in all parts of the study. All authors read and approved the final manuscript.

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