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Original Article

5-Aza-CdR can reverse gefitinib resistance caused by DAPK gene promoter methylation in lung adenocarcinoma cells

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Abstract: To explore the relationship between death associated protein kinase (DAPK) gene promoter methylation and gefitinib resistance in Lung adenocarcinoma cell lines. EGFR-mutation lung adenocarcinoma cell lines PC9 and the gefitinib-resistant with T790M Mutation cell lines PC9/GR were chosen as cell models, and PC9/GR were treated with 5-aza-CdR (1 μmol/L). The experiments were divided into three groups: PC9 group, PC9/GR group and PC9/GR with 5-Aza-CdR pretreatment group. Treat three groups cell with different concentrations gefitinib, the cell proliferation was determined by MTT assay. The apoptotic rates were detected by flow cytometry. The methylation of DAPK gene promoter region was examined by methylation-specific PCR (MSP). The expressions of DAPK protein were detected by Western blot. MTT results showed that the half maximal inhibitory concentration (IC50) of PC9 and PC9/GR cell lines increase from 0.12 μmol/L to 8.52 μmol/L. But after treated with 5-aza-CdR, the IC50 of PC9/GR cell lines decrease to 4.35 μmol/L, and the resistance index (RI) decrease from 71 to 36 ($P<0.05$). Flow cytometry results showed that the apoptosis rate were 24.80%±0.28%, 12.70%±0.31%, 19.8%±0.15% respectively. MSP results showed that DAPK gene promoter region was un-methylated in PC9 cells and methylated in PC9/GR cells, when treated with 5-aza-CdR, DAPK gene promoter region was partly methylated in PC9/GR cells ($P<0.05$). Western blot results showed that the levels of DAPK protein were reduced significantly in PC9/GR cell lines compared with PC9, and after treated with 5-aza-CdR, the expression levels of DAPK protein in PC9/GR were increased ($P<0.05$). In conclusion, DAPK gene promoter methylation may contribute to the downregulation of DAPK gene and protein, and consequently affect the sensitivity of gefitinib in lung adenocarcinoma lines, induced gefitinib resistance. But 5-Aza-CdR can reverse gefitinib resistance by demethylation of DAPK gene promoter.

Keywords: Lung adenocarcinoma, gefitinib, drug resistance, DAPK gene, methylation

Introduction

Epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) were successfully treatment for lung adenocarcinoma with EGFR sensitive mutation, but almost all of the patients for whom the initial therapy was effective will present progress after less than 8-10 months, and produce the acquired drug-resistance [1]. DAPK gene is a tumor suppressor gene located in the human chromosome 9q34.1 locus, which inhibits tumor development and tumor drug resistance [2]. DNA methylation can change the conformation of DNA region and lead to the inactivation of gene, which is the important method of epigenetic modification [3]. The present research find that DAPK gene expression deficiency can promote tumor growth and drug resistance, and the abnormal methylation of DAPK gene promoter is the important reason for DAPK gene expression deletion. This study was to explore the relationship between DAPK gene promoter methylation and gefitinib resistance in lung adenocarcinoma, and the mechanism of gefitinib resistance reversal by 5-Aza-CdR.

Materials

PC9 and PC9/GR cell lines were present by Guang-zhou Medical College. Gefitinib offered by AstraZeneca, and dissolved in DMSO solution. RPMI-1640 medium, fetal calf serum (FBS) and 5-Aza-CdR purchased from the Sigma company U.S. PCR reverse transcription kit purchased from the U.S. Invitrogen company. DAPK

Table 1. IC50 values and resistance index of three groups of cell lines to gefitinib

	PC9 cells	PC9/GR cells	PC9/GR (5-Aza-CdR pretreatment)
IC50 values	0.12 μmol/L	8.52 μmol/L	4.35 μmol/L
RI		71	36

primary antibody, secondary antibody and β-actin polyclonal antibody were provided by American Cell Signal Technology Company.

Materials and methods

Cell culture

The PC9 and PC9/GR cell line placed in incubator with RPMI-1640 culture fluid (containing 10% FBS), and 37°C, 5% CO_2 saturated humidity, and PC9/GR were treated with 5-aza-CdR (1 μmol/L). The experiments were divided into three groups: A: PC9 group, B: PC9/GR group and C: PC9/GR with 5-Aza-CdR pretreatment group.

The cell proliferation was determined by MTT assay

The PC9/GR cell lines were pretreated for 72 hours by 5-Aza-CdR, then fetched the cell lines of PC9, PC9/GR and PC9/GR (5-Aza-CdR pretreatment) at the logarithmic phase respectively, digested them to be the cell suspension containing 3×10^4 cells per mL, After the adherence of the cell, the PC9 and PC9/GR cell lines were added Gefitinib at different concentrations of 0.00 μmol/L, 0.001 μmol/L, 0.01 μmol/L, 0.1 μmol/L, 1.0 μmol/L, 5.0 μmol/L, 10.0 μmol/L and 0.00 μmol/L, 0.1 μmol/L, 1.0 μmol/L, 5.0 μmol/L, 10.0 μmol/L, 20.0 μmol/L, 40.0 μmol/L respectively. Placed in the 37°C, 5% CO_2 incubator and culturing for 48 h, then added 20 mL of MTT into each holes (5 mg/mL), added 150 ml of DMSO into each hole, detected the absorbance value of each well (A) at wave length of 492 nm and calculated the average value. IC50 values of each group were calculated by SPSS16.0 software. Calculated the resistance index (RI) = IC50 of the drug-resistant cell line/IC50 of the parental generation cell line. Three repeats were tested.

Assaying the apoptosis rate of the cell by means of the flow cytometry

Collected the cell at logarithmic phase. The experimental grouping and medication were as following: the experiment consisted of PC9, PC9/GR and PC9/GR (5-Aza-CdR pretreatment). After 24 h, digested the cells with pancreatin and collected the cells, added the combined buffer solution to suspend the cells again to prepare the cell suspension, adjusted the cell density to 1×10^6 cells/ml. Drewg 100 μL of cell suspension, added 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) to the suspension, blended these components, incubated the mixture away from light at the room temperature, added the combination 400 μL of buffer solution, analyzed them on the FCM immediately. Set the zero value using the unstained cell, tested the data for each tube loaded the sample for the percentage of the apoptotic cell using the Annexin V-FITC simple staining tube and the PI simple staining tube as the standard control. Three repeats were tested.

The methylation of DAPK gene promoter region was examined by methylation- specific PCR (MSP)

DNA were extracted by Phenol chloroform-isoamyl alcohol method, the DNA were modified (methylation) of DNA by sulfite Kit. And PCR amplification was carried out with this template. Three repeats were tested. Methylation primer sequence: upstream: 5'-GGATAGTCGGATCGAGTTAACGT-C-3', downstream: 5'-CCCTCCC AAACGCCGA-3'. Non-Methylation primer sequence: upstream: 5'-GGAGGATA GT TGGATTGAGTTAAGTT-3', downstream: 5'-CAAATCCCTCCCAAACACCAA-3'. PCR reaction conditions: Firstly pre-denaturation for 5 min at 95°C, then to 95°C 30 s, 56°C 30 s, 72°C 30 s, 40 cycles, the last extend to 7 min at 72°C. PCR product was photographed after 2% agarose gel electrophoresis. And, the positive reference of PCR was the peripheral blood DNA in normal person modified by the methyl transferase, and the negative reference was the normal peripheral blood DNA.

Expression level of DAPK protein were detected by Western blot

Fetched the cell lines at the logarithmic phase. Extracted the total protein (TP) of the cells. Detected the expression of DAPK protein by Westhern blotting respectively: transferred the

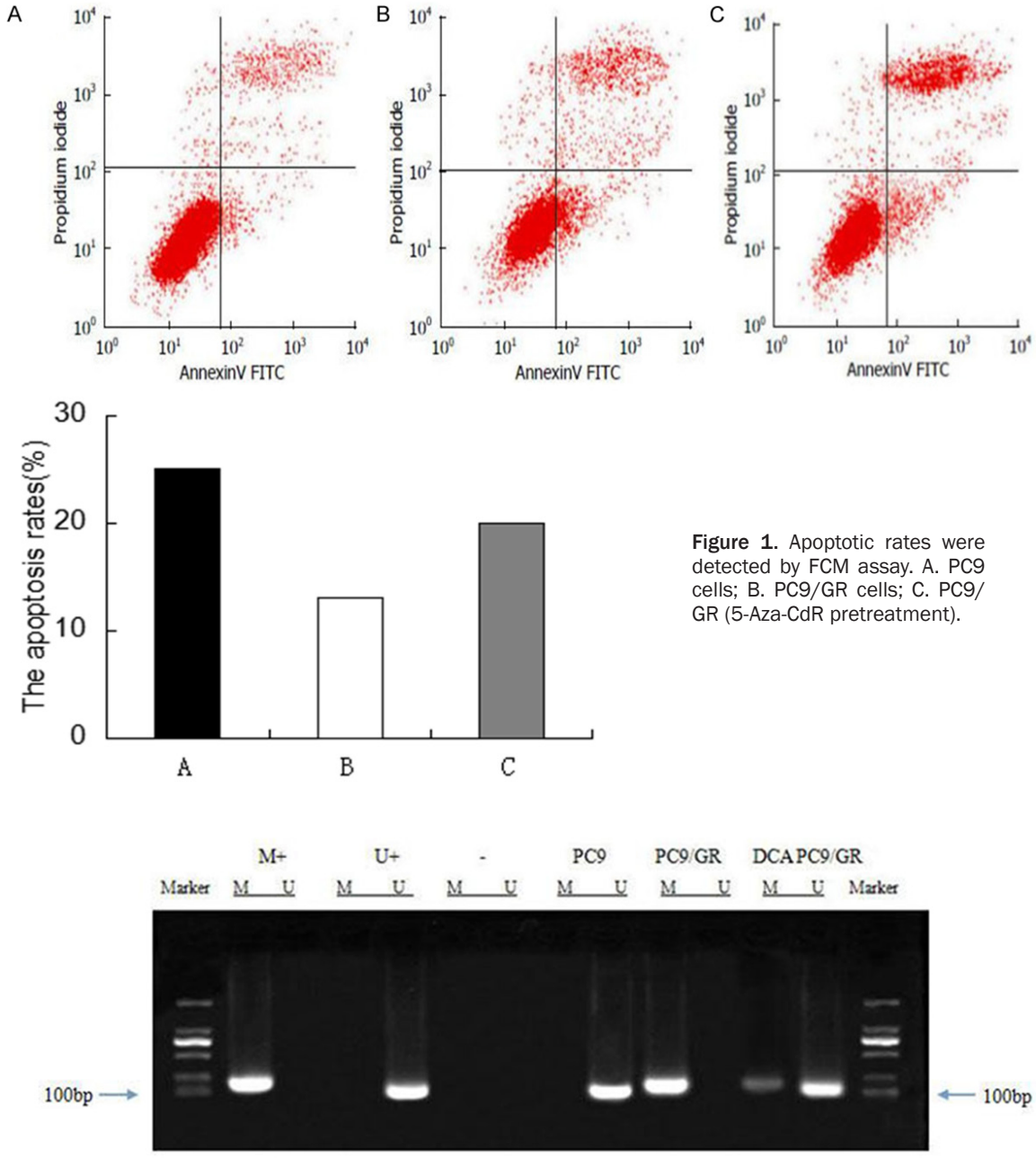

Figure 1. Apoptotic rates were detected by FCM assay. A. PC9 cells; B. PC9/GR cells; C. PC9/GR (5-Aza-CdR pretreatment).

Figure 2. Methylation status of DAPK gene promoter. M: Methylated bands, U: Non-methylated bands.

cell TP to the polyvinylidene difluoride (PVDF) film after 8% SDS-PAGE electrophoretic separation, blocked the protein with the TBST solution containing 0.5% skim milk powder, added the primary antibody diluted at the ratio of 1:1000 and β-actin (the internal reference) diluted at the ratio of 1:1000, incubated at 4°C for the night, rinsed three times with TBST, then added secondary antibody diluted at the ratio of 1:10000 incubate two hours, then added DAB for coloration; exposed under light for development. Three repeats were tested. Analyzed the gray value of the protein bands with the Image J software, showed the relative expression level of the aimed protein with the ratio of the gray value of the aimed protein bands to the gray value of internal reference β-actin protein.

Results

Effect of gefitinib to the cell proliferation

MTT results showed that the half maximal inhibitory concentration (IC50) of PC9 and PC9/

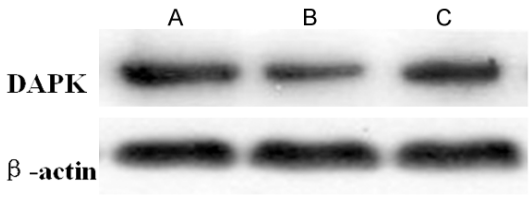

Figure 3. Expression level of DAPK protein in each groups (gel electrophoresis chart). A. PC9 group; B. PC9/GR group; C. PC9/GR with 5-Aza-CdR pretreatment group.

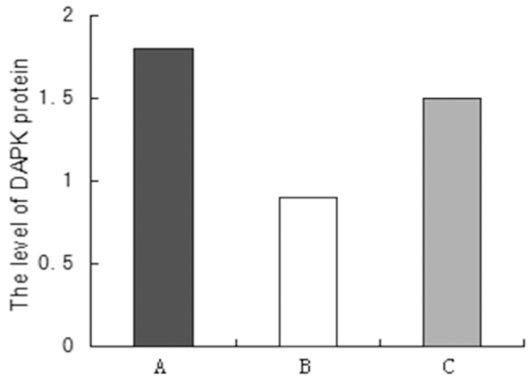

Figure 4. Relative expression level of DAPK protein in each groups. A: PC9 group; B: PC9/GR group; C: PC9/GR with 5-Aza-CdR pretreatment group.

GR cell lines increase from 0.12 μmol/L to 8.52 μmol/L. But after treated with 5-aza-CdR, the IC50 of PC9/GR cell lines decrease to 4.35 μmol/L, and the resistance index (RI) decrease from 71 to 36 ($P<0.05$) (**Table 1**).

The apoptosis rate of the cells treated with gefitinib

The assay result of the FCM method (**Figure 1**) showed that, the apoptosis rate of the PC9 cell and PC9/GR cell were 24.80%±0.28% and 12.70%±0.31% respectively, but after treated with 5-aza-CdR, the apoptosis rate of PC9/GR cell lines increased to 19.8%±0.15% ($P<0.05$).

Methylation status of DAPK gene promoter

MSP results showed that DAPK gene promoter region was un-methylated in PC9 cell lines and methylated in PC9/GR cells, when treated with 5-aza-CdR, DAPK gene promoter region was partly methylated in PC9/GR cells ($P<0.05$) (**Figure 2**).

Methylation (M) positive control only appear methylated bands and non methylated (U) positive control only non-methylated bands, negative control did not show any bands, indicating that the DNA were modified (methylation) completely, and the quality of MSP primer and the process of PCR were not contaminated.

Expression level of DAPK protein

Western blot results showed that the levels of DAPK protein were reduced significantly in PC9/GR cell lines compared with PC9, and after treated with 5-aza-CdR, the expression levels of DAPK protein in PC9/GR were increased ($P<0.05$) (**Figures 3**, **4**).

Discussion

Current study has showed that EGFR gene 20 exon T790M mutation was the main mechanism of EGFR-TKI acquired drug resistance [4]. In addition, EGFR-TKI resistance is also related to the amplification of c-Met gene, the down regulation of EMT and PTEN' pathway, the activation of IGF-1R, etc. But the mechanism has not yet been fully elucidated. PC9 cell lines with EGFR gene 19 exon 19E746~A750 deletion mutations, is highly sensitive to Gefitinib. And the gefitinib-resistance cell lines PC9/GR with T790M Mutation cell lines were induced in vitro by high-dose of gefitinib and gradually increasing concentration. PC9/GR cells not only exist exon-19 deletion mutations, but also exon-20 T790M mutation. Same with the main mechanism of acquired drug resistance of Gefitinib, it can be simulated the clinical resistance.

DNA methylation is a process of producing 5-methylcytosine by moving methyl to 5 carbon atoms of methylcytosine, under the action of DNA methylation transferase, and using S-adenosyl-L-methionine as a role of methyl donor. The feature of DNA methylation is that the genetic code and the DNA sequence are keep its' original order and the sequence is reversible [5]. In normal state, the CpG islands of the gene promoter region were generally nonmethylated. When the methylation occurs, the gene transcription is often quiet, and the expression of tumor related genes, such as anti-oncogene, DNA repair gene, etc. was down regulated or absent [6]. 5-Aza-2'-deo-xycytidine (5-Aza-CdR) is a methyltransferase inhibitor, can covalent binding with DNA methyl transferase, and also can reverse the methylation status of the promoter of tumor suppressor gene

by reducing DNA methyltransferase biological activity [7].

DAPK gene is a tumor suppressor gene, which located on 9q34.1 locus of human chromosome. DAPK protein, which is coded by DAPK gene, can promote the differentiation of tumor cells and inhibit tumor metastasis, and involved in apoptosis and drug resistance [8]. That DAPK gene was abnormal methylation in different degree has found in various of malignant tumors, such as gastric cancer, bladder cancer, cervical cancer, oral squamous cell carcinoma, breast cancer, prostate cancer, esophageal cancer, liver cancer, etc. That abnormal methylation can lead the absence of gene expression, lead to tumor, invasiveness and drug resistance [9-11]. The 5-Aza-CdR make human gastric cancer cell lines, bladder cancer, cervical cancer and other methylated genes DAPK removing methylation, re-expression or upregulation, meanwhile, it can inhibited the cells' proliferation, lead to apoptosis, or even to reverse the drug resistance [12-15].

In our study, we used the gefitinib sensitive PC9 cell lines and T790M mutant PC9/GR cell line to simulate clinical process. The results showed that, DAPK gene of PC9/GR cell line was totally in methylation status, the expression of DAPK protein was significantly reduced compared with PC9 cell lines, and it also highly resistant to Gefitinib. Using 5-Aza-CdR to pretreatment those PC9/GR cell lines, we found that, the methylation status of DAPK gene of PC9/GR cells were partly, the expression of DAPK protein was increased compared with before, and the PC9/GR cell lines which were pretreatment by 5-Aza-CdR were more sensitive to gefitinib compared with before. To some extent, it reversed the gefitinib resistance.

Conclusions

In summary, the DAPK gene promoter of PC9/GR cell lines was in methylation status, it can reduce the expression of DAPK gene, even cause the deletions, and down regulated the expression of DAPK protein, then lead to gefitinib resistance. So, we think the methylation status of DAPK gene promoter was closely related to gefitinib resistance in lung adenocarcinoma. And we also find 5-Aza-CdR can restore gene expression by reversing the highly methylated state of DAPK gene promoter, then to reverse the gefitinib drug resistant in some extent.

Disclosure of conflict of interest

None.

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