

## Regulatory mechanism of extracellular signal-regulated kinase signal pathway for DNA methylation in lung carcinoma cells.

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### Abstract

**Objective:** This study aimed to discuss the regulatory mechanism of Extracellular Signal-Regulated Kinase (ERK) signal pathway for DNA methylation in lung carcinoma cells.

**Methodology:** Human lung carcinoma cells were cultured, and the cells in logarithmic phase were inoculated in 96-hole plates and divided into three groups A-C. Six complex holes were present in each group. Phosphoric buffer salt solution, agonist epidermal growth factor, and blocker U1026 solution of ERK pathway were added into groups A-C, with a total amount of 25 µmol/L. Then, they were continuously treated for 48 h. Methyl thiazol tetrazolium method was used to determine cell proliferation rate. Fluorescent quantitative Polymerase Chain Reaction (qPCR) method was used to test the expression quantities of DNA methylase 1 (Dnmt1), DNA methylase 3a (Dnmt3a), DNA methylase 3b (Dnmt3b), tumor suppressor gene (*p16*), and Ras association domain family gene (*RASSF1A*) mRNA.

**Results:** Significant differences exist among groups A-C in cell proliferation rate, as well as in Dnmt1, Dnmt3a, Dnmt3b, *p16*, and *RASSF1A* mRNA, and their protein expression levels (P<0.05). Cell proliferation rate and the expression quantities of Dnmt1, Dnmt3a, and Dnmt3b mRNA and their protein expression levels in group B were significantly higher than those in groups A and C (P<0.05). By contrast, the expression quantities of *p16* and *RASSF1A* mRNA and their protein expression levels were significantly lower than those in groups A and C (P<0.05). Cell proliferation rate and the expression quantities of Dnmt1, Dnmt3a, and Dnmt3b mRNA and their protein expression levels in group A were significantly higher than those in group C (P<0.05).

**Conclusion:** ERK signal pathway blocked both proliferation rate of lung carcinoma cells and DNA methylation level because of the down-regulation of Dnmts mRNA and their protein expression levels and the up-regulation of cancer suppressor genes *p16* and *RASSF1A* mRNA and their protein expression levels.

**Keywords:** Lung carcinoma, Extracellular regulation of protein kinase signal pathway, DNA methylation.

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### Introduction

Lung carcinoma, the most common malignant tumor, has become a leading cause for human death with morbidity still being on the rise [1]. Epigenetics has exerted regulatory effect on the genesis and development of various cancers, including DNA methylation modification, histone modification, chromosome modification, and non-coding RNA [2,3]. DNA methylation modification plays a significant role in the genesis of lung carcinoma. DNA methylation and extracellular regulated protein kinase-motogen-activated protein kinases signal transduction pathway mediate or regulate relevant genetic expressions in the genesis and development of lung carcinoma [4]. However, further study is needed on the

regulatory mechanism of ERK signal pathway for the DNA methylation of lung carcinoma cells [5]. In the current study, this was carried out by applying ERK signal pathway agonist and blocker in cells *in vitro*.

### Materials and Methodology

#### Materials

Human carcinoma cell stain A549 (Shanghai Institute of Biochemistry and Cell Biology, CAS); RPMI 1640, DMEM culture medium, fetal calf serum, and Trizol total RNA extraction kits (Life Technologies, USA); agonist epidermal growth factor (EGF) and blocker U1026 of ERK pathway

(Sigma, USA); real-time fluorescent quantitative polymerase chain reaction (qPCR) and reverse transcription kits (Takara, China); cell lysis buffer IP, Dnmt1, Dnmt3a, and Dnmt3b mice anti-human McAb (monoclonal antibodies) (Santa Cruz, USA); and horse radish peroxidase and goat anti-rabbit polyclonal antibodies (Chembiochem, USA) were used for the experiments.

### Methodology

**Detection of cell proliferation rate through methyl thiazol tetrazolium (MTT) method:** After 48 h, 5 mg/mL 20  $\mu$ L MTT solution was added into each hole for 4 h of continuous culturing. After the culture solution was abandoned, 150  $\mu$ L dimethylsulfoxide was added into each hole for 10 min of decolorization and shaking reaction. Enzyme-linked immunosorbent assay was used to detect the absorbance (optical density value) at 570 nm. Holes containing only the culture medium solution constituted the control group, which was used to detect cell proliferation rate.

**Detection of mRNA expression through real-time fluorescent qPCR:** After 48 h, Trizol method was used to extract cellular total RNA. Agarose electrophoresis and ultraviolet (UV) spectrophotometer were used to determine RNA purity and concentration, respectively. RNA at 10  $\mu$ g was reversely transcribed into cRNA according to the instructions of the reverse transcription kit and put under reaction under real-time fluorescent qPCR. The primers of Dnmt1, Dnmt3a, Dnmt3b, *p16*, and *RASSF1A* were designed and synthesized by the Shanghai Bioengineering Co., Ltd.

**Detection of methylation level through methylation-specific PCR:** After 48 h, trypsinization occurred in cells in the three groups, and these cells were collected into 1.5 ml centrifugal tube for 5 min of centrifugation at a speed of 13,000. The supernatant was discarded, and follow-up operation was implemented according to the procedure shown in the instructions of the DNA extraction kit. Agarose electrophoresis and UV spectrophotometer were used to detect DNA purity and concentration, respectively. The promoter sequences of tumor suppressor genes *p16* and *RASSF1A* were searched according to UCSU database. Methylated CpG islands in *p16* and *RASSF1A* promoter regions were predicted. Methylation-specific primers and non-methylated primers were then designed.

### Statistical analysis

SPSS17.0 software was used for analysis, and data were expressed by mean  $\pm$  standard deviation (SD). One-way ANOVA and Dunnett t-test were used for analysis.  $P < 0.05$  meant significant difference.

## Results

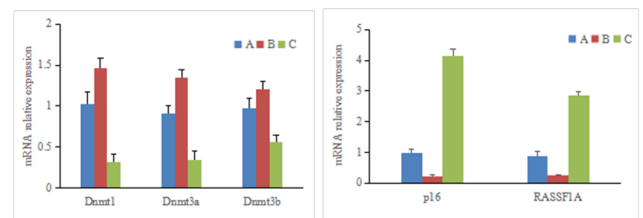
### Cell proliferation rate

Comparative differences among groups A-C in cell proliferation rate were significant ( $P < 0.05$ ), where cell

proliferation rate in group B was significantly higher than those in groups A and C ( $P < 0.05$ ), but that in group A was significantly higher than that in group C ( $P < 0.05$ ).

### Expressions of Dnmt1, Dnmt3a, Dnmt3b, p16, and RASSF1A mRNA

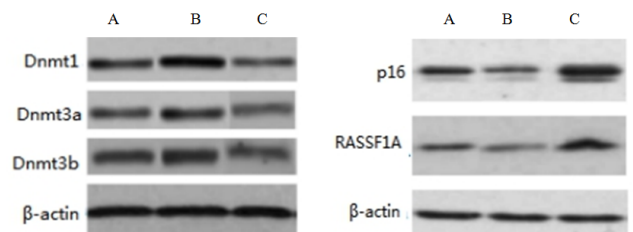
As shown in Figure 1, comparative differences among groups A-C in the expression levels of Dnmt1, Dnmt3a, Dnmt3b, *p16*, and *RASSF1A* mRNA were significant ( $P < 0.05$ ). The expression levels of Dnmt1, Dnmt3a, and Dnmt3b mRNA in group B were significantly higher than those in groups A and C ( $P < 0.05$ ), whereas the expression levels of *p16* and *RASSF1A* mRNA were significantly lower than those in groups A and C ( $P < 0.05$ ). The expression levels of Dnmt1, Dnmt3a, and Dnmt3b mRNA in group A were significantly higher than those in group C ( $P < 0.05$ ), whereas the expression levels of *p16* and *RASSF1A* mRNA were significantly lower than those in group C ( $P < 0.05$ ).



**Figure 1.** Expression levels of Dnmt mRNA, P16 and RASSF1A mRNA.

### Protein expression levels of Dnmt1, Dnmt3a, Dnmt3b, p16, and RASSF1A

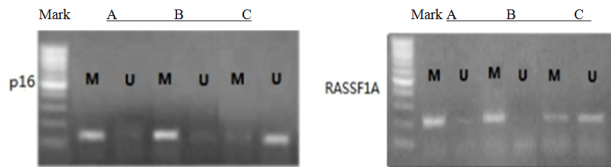
Comparative differences among groups A-C in the protein expression levels of Dnmt1, Dnmt3a, Dnmt3b, *p16*, and *RASSF1A* were significant ( $P < 0.05$ ). The protein expression levels of Dnmt1, Dnmt3a, and Dnmt3b in group B were significantly higher than those in groups A and C ( $P < 0.05$ ), but the protein expression levels of *p16* and *RASSF1A* were significantly lower than those in groups A and C ( $P < 0.05$ ). The protein expression levels of Dnmt1, Dnmt3a, and Dnmt3b in group A were significantly higher than those in group C ( $P < 0.05$ ), but the protein expression levels of *p16* and *RASSF1A* were significantly lower than those in group C ( $P < 0.05$ ), as shown in Figure 2.



**Figure 2.** Detection through Western blot.

### Methylation analysis of *p16* and *RASSF1A* promoters

As shown in Figure 3, the detection showed that the promoter regions of tumor suppressor genes *p16* and *RASSF1A* in groups A and B presented methylation status, and the amplified bands of methylated primers in group B were more apparent. The *p16* promoter regions in group C presented un-methylated status. Some *RASSF1A* promoter regions presented methylation status, whereas others presented demethylation status.



**Figure 3.** Methylation-specific PCR amplification of *p16* and *RASSF1A* promoter (M: Methylation; U: Unmethylated).

### Discussion

Through the detection of cell proliferation rating among the three groups, we found the following results. Cell proliferation rate in group B was the highest, but that in group C was the lowest. Blocking ERK pathway could inhibit the proliferation of lung carcinoma cells. Based on a study on the expression levels of Dnmt1, Dnmt3a, Dnmt3b, *p16*, and *RASSF1A* mRNA through fluorescent qPCR, the expression levels of Dnmt1, Dnmt3a, and Dnmt3b mRNA in group B were the highest, and those in group C were the lowest. The expression levels of tumor suppressor genes *p16* and *RASSF1A* mRNA in group B were the lowest, and the highest were in group C. Blocking ERK pathway could inhibit Dnmts expressions with increasing expression levels of tumor suppressor genes *p16* and *RASSF1A*. The same conclusions were obtained through a study on the protein expression levels of Dnmt1, Dnmt3a, Dnmt3b, *p16*, and *RASSF1A* mRNA through Western blot. The Dnmts expression levels of tumor cells were significantly higher than those of normal cells and related to high methylation status of promoters in tumor suppressor genes. Through the detection of Dnmts mRNA levels in renal cell carcinoma through semi-quantitative PCR, Dnmt1 and Dnmt3b mRNA expression levels were found to significantly increase but eventually significantly descended and presented high methylation status through the detection of relevant tumor suppressor genes. In addition, Dnmt1 mRNA expression level had a correlation with prognosis. *P16* is a commonly seen tumor suppressor gene, and studies on its methylation status have been carried out in a large quantity. The studies found that *p16* was silenced due to the methylation of its CpG islands and the coding function of protein level declined or closed that led to tumor genesis. As a tumor suppressor gene, *RASSF1A* participates in ERK signal pathway, which results in the transduction pathway of growth effect signals. The following effects were observed. Tumor cell proliferation was inhibited. The apoptosis and senescence of tumor cells were facilitated by the inhibition of the activation of Ras kinases. Thus, the functional inactivation of *RASSF1A* results in failure of inhibiting effect on tumor cells. Researchers have detected the

expression levels of *p16* and *RASSF1A* mRNA in non-small cell lung carcinoma and found that their expression levels are distinctly down-regulated [6,7]. Therefore, blocking ERK signal pathway, down-regulating Dnmts and its protein expression levels, and up-regulating *p16* and Ras mRNA and its protein expression levels can exert an inhibitory effect on the proliferation and growth of tumor cells.

Through the detection of the methylation status of the promoter regions of *p16* and *RASSF1A*, the methylation status of tumor suppressor genes *p16* and Ras in group B was found to be distinct. *P16* in group C presented unmethylated status, and *RASSF1A* presented partial methylation status and partial demethylation status. This test indicated that inhibiting ERK pathway could facilitate the methylation of tumor suppressor genes *p16* and *RASSF1A* and activating ERK pathway could inhibit their methylation. Therefore, possible regulatory mechanism of ERK signal transduction pathway for DNA methylation level was Ras activated. Ras/ERK in tumor cells mediated the methylation of tumor suppressor genes CpG islands by up-regulating *Dnmts* gene expression. By contrast, if ERK signal transduction pathway activated by Ras was blocked, then Dnmts expression levels would be down-regulated to block the mediating effect of this signal transduction pathway on methylation. Due to the methylation of the promoter regions in multiple tumors, *p16* genes were silenced with inactivated expression. The study on *RASSF1A* also indicated that its main inactivation mechanism was the methylation of CpG locus in the promoter region and taken as an alternative tumor suppressor gene for lung carcinoma [8,9]. Tumor cells with significantly increasing protein expression levels of Dnmts show significant increase in methylation degrees in the promoter regions of tumor suppressor genes *p16* and *RASSF1A*. In addition, blocking the ERK signal transduction pathway can inhibit DNA methylation effect and activate tumor suppressor genes *p16* and *RASSF1A* to inhibit cancer cell proliferation [10].

### Conclusion

Results of our study on the regulatory mechanism of ERK signal pathway for DNA methylation of lung carcinoma cells showed that blocking ERK signal pathway leads to the inhibition of cell proliferation rate and DNA methylation level. It also inhibits proliferation and growth of cancer cells by down-regulating Dnmts mRNA and their protein expression levels and by up-regulating tumor suppressor genes *p16* and *RASSF1A* mRNA and their protein expression levels.

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