RESEARCH ARTICLE

Effect of 5-aza-2'-deoxycytidine on Cell Proliferation of Non-small Cell Lung Cancer Cell Line A549 Cells and Expression of the TFPI-2 Gene

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Abstract

Objective: The present study employed 5-aza-2'-deoxycytidine (5-Aza-CdR) to treat non-small cell lung cancer (NSCLC) cell line A549 to investigate the effects on proliferation and expression of the TFPI-2 gene. Methods: Proliferation was assessed by MTT assay after A549 cells were treated with 0, 1, 5, 10 μmol/L 5-Aza-CdR, a specific demethylating agent, for 24 ,48 and 72h. At the last time point cells were also analyzed by flow cytometry (FCM) to identify any change in their cell cycle profiles. Methylation-specific polymerase chain reaction (MSPCR), real time polymerase chain reaction(real-time PCR) and western blotting were carried out to determine TFPI-2 gene methylation status, mRNA expression and protein expression. Results: MTT assay showed that the growth of A549 cells which were treated with 5-Aza-CdR was significantly suppressed as compared with the control group (0 μmol/L 5-Aza-CdR). After treatment with 0, 1, 5, 10 μmol/L 5-Aza-CdR for 72h, FCM showed their proportion in G0/G1 was 69.7±0.99%, 76.1±0.83%, 83.8±0.35%, 95.5±0.55% respectively ($P<0.05$), and the proportion in S was 29.8±0.43%, 23.7±0.96%, 15.7±0.75%, 1.73±0.45%, respectively ($P<0.05$), suggesting 5-Aza-CdR treatment induced G0/G1 phase arrest. MSPCR showed that hypermethylation in the promoter region of TFPI-2 gene was detected in control group (0 μmol/L 5-Aza-CdR), and demethylation appeared after treatment with 1, 5, 10 μmol/L 5-Aza-CdR for 72h. Real-time PCR showed that the expression levels of TFPI-2 gene mRNA were 1±0, 1.49±0.14, 1.86±0.09 and 5.80±0.15 ($P<0.05$) respectively. Western blotting analysis showed the relative expression levels of TFPI-2 protein were 0.12±0.01, 0.23±0.02, 0.31±0.02, 0.62±0.03 ($P<0.05$). TFPI-2 protein expression in A549 cells was gradually increased significantly with increase in the 5-Aza-CdR concentration. Conclusions: TFPI-2 gene promoter methylation results in the loss of TFPI-2 mRNA and protein expression in the non-small cell lung cancer cell line A549, and 5-Aza-CdR treatment could induce the demethylation of TFPI-2 gene promoter and restore TFPI-2 gene expression. These findings provide theoretic evidence for clinical treatment of advanced non-small cell lung cancer with the demethylation agent 5-Aza-CdR. TFPI-2 may be one molecular marker for effective treatment of advanced non-small cell lung cancer with 5-Aza-CdR.

Keywords: 5-Aza-CdR - TFPI-2 - DNA methylation - non-small cell lung cancer

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Introduction

Lung cancer remains the leading cause of cancer deaths worldwide, More than 80% of lung cancers are NSCLC (Jemal et al., 2009). Despite the intensive research carried out on this field and therapeutic advances, the overall prognosis of these patients remains unsatisfactory, with a 5-year overall survival rate of less than 15% (Sculier et al., 2008; Carvalho et al., 2009). In stage I to III NSCLC, the occurrence of extrathoracic metastasis also leads to a poor 5-year survival rate of 50% (Friedel et al., 2004). Surgery is the best curative therapeutic approach in the early stages. However, even in these patients, the mean 5-year overall survival rate is less than 70%. The scarcity of effective tools for early detection and therapy strategies is the reason of the poor 5-year overall survival rate. Consequently, developing molecular markers for early detection, predicting prognosis, and exploiting new therapy agents of lung cancer are urgently needed.

It has shown that the occurrence of NSCLC is a multi-gene-involved, multi-stage pathological process under the action of both genetic and epigenetic factors including DNA methylation, and that epigenetic mechanism plays an important role in the occurrence and development of NSCLC (Fischer et al., 2006; Basseres et al., 2012; Dmitriev et al., 2012; Nelson et al., 2012). Tissue factor pathway inhibitor-2 (TFPI-2) is a potent inhibitor of plasmin which activates matrix metalloproteinases (MMPs) involved in degradation of the extracellular matrix. Its secretion in the tumor microenvironment

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makes TFPI-2 as potential inhibitor of tumor invasion and metastasis (Gaud et al., 2011). The gene that codes for TFPI-2 has been mapped to chromosome 7q22 by fluorescence in situ hybridization (Kempaiah et al., 2007). The expression of TFPI-2 gene in tumors is inversely related to an increasing degree of malignancy, which may suggest a role for TFPI-2 in the maintenance of tumor stability and inhibition of the growth of neoplasms, so, TFPI-2 is considered as a candidate tumor suppressor gene (Sierko et al., 2007). Aberrant methylation of TFPI-2 promoter cytosine-phosphorothioate-guanine (CpG) islands in human cancers was widely documented to be responsible for diminished expression of mRNA encoding TFPI-2 and decreased or inhibited synthesis of TFPI-2 protein during cancer progression and invasion (Rao et al., 2001; Hube et al., 2003; Jee et al., 2009; Ribarska et al., 2010; Takada et al., 2010; Tang et al., 2011). Abnormal methylation is reversible, demathylating agents could be used to induce re-expression of the inactivated genes due to methylation. 5-Aza-CdR is a specific inhibitor of DNA methyltransferase (DNMTs), which induces the re-expression of tumor suppressor genes by demethylating promoter CpG island (Ding et al., 2012; Jin et al., 2012).

In the present study, the lung cancer cell line A549 were treated in vitro with demethylating agent 5-Aza-CdR so as to investigate the changes in methylation status and expression of TFPI-2 gene after 5-Aza-CdR treatment, the results will provide the experimental evidences for the clinical therapy.

Materials and Methods

Materials

The main materials used included human non-small cell lung cancer cell line A549 (China Center for Type Culture Collection of Wuhan University), RPMI 1640 medium (Hyclone), 5-Aza-CdR (Sigma), sodium bisulfite (Sigma), Wizard DNA Clean-up (Promega), glycogen (New England Biolabs), SssI (New England Biolabs), Taq enzyme (Takara), dNTPs (Takara), DMSO (Sigma), real-time PCR Kit (TOYOBO), the sheep anti-human TFPI-2 polyclonal antibody (Santa Cruz) and the Trizol reagent for total RNA extraction (Invitrogen).

Cell culture

A549 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2. At the logarithmic phase of cell growth, with a percentage of viable cells over 95%, the cells were remained hungry for 24 h in the RPMI 1640 medium containing 1% fetal bovine serum, followed by the drug treatment. Cells were treated with 0, 1, 5, 10 μmol/L 5-Aza-CdR, a specific demethylating agent for 24 to 72 h. Cells were harvested for further analysis. The cells which were treated with 0 μmol/L 5-Aza-CdR were used as the control group.

MTT Assay

A549 cells were trypsinized and seeded in 96-well plates at a density of 1×10^4 cells/well and cultured for 24 h. And then, 0, 1, 5, 10 μmol/L 5-Aza-CdR was added and the plates were incubated at 37°C, 5% CO_2 for 24, 48, 72h. At the end of incubation, 20 μL of 5 mg/mL MTT was added into each well followed by incubation for another 4 h. The medium was then aspirated and 150 μL dimethylsulfoxide (DMSO) was added into each well. The plates were mixed gently by rocking back and forth until the blue sedimentation was completely dissolved. optical density (OD) was measured at 490 nm using an ELIASA and the growth inhibition rate was calculated using the following formula: Inhibition rate(%)=(Average OD value of experimental group-Average OD value of control group)/Average OD value of control groupx100%. Each experiment was repeated three times.

FIow Cytometer

Cell cycle distribution was analyzed using propidium iodide staining.CelIs were seeded at 1×10^6 cells/well in 6-well plates.and exposed to 0, 1, 5, 10×10^{-6} μmol/L 5-Aza-CdR for 72 h. The cells were harvested and washed twice with PBS, then fixed with ice-cold 70% ethanol. The sample was concentrated by removing ethanol and re-suspended in a PBS solution containing propidium iodide (0.05g/L) and RNaseA (100 mg/ml) for 3h at 37°C in the dark. The samples were then measured by flow cytometry.Cell division activity was measured by proliferation index(PI), PI=(S+G2/M)/(G0/G1+S+G2/M). Each sample was repeated three times.

Methylation-specific polymerase chain reaction(MSPCR). Genomic DNA was obtained from cultured cells using standard phenol-chloroform protocols. The DNAs were treated with sodium bisulfite. Total reaction mixture volume of 25 μL, containing 2 μL DNA, 2.5 μL10 × PCR buffer, 0.5 μL each of the sense and antisense primers, 2 μL dNTPs, 0.2 μL Taq enzyme, and 17.3 μL double-distilled water. Reaction condition: pre-denatured at 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and with a final extension at 72°C for 5 min. The methyltransferase SssI-treated and untreated placenta DNA were used as the positive and negative controls respectively, while the double-distilled water as the blank control. The 1000 bp DL2000 was used as the molecular weight markers. The primer set used for methylation analysis of TFPI-2 was 5'-TTTATGTTTTTAAGAGGTGGATTTC-3' (sense) and 5'-TCTATCCTCCAGCAAGCATCGT-3' (antisense).The PCR products were separated by electrophoresis on the 2% agarosegel at 4V for 1min. The gels were photographed using a laser density scanner (Pharmacia LKB Ultroscan) for analysis. Each experiment was repeated three times.

Real-time polymerase chain reaction (real-time PCR). The TFPI-2 mRNA expression was determined with real-time PCR. The total RNA was extracted from A549 cells using Trizol method according to the manufacturer`s instruction. The cDNAs were synthesized from the templates in presence of reverse transcriptase and oligo (dT) 20 primers, As the internal control, β-actin transcripts were amplified from the same cDNA samples. The Primer sequences and condi¬tions for the real-time PCR product were previously described (forward primer: 5'-TTCTCCGTTACTACTACGACAGG-3'; reverse

primer: 5'-TCTATCCTCCAGCAAGCATCGT-3'. The housekeeping gene β-actin was used as an internal control for the real-time PCR (forward primer: 5'-GTCCACCGCAAATGCTTCTA-3'; reverse primer: 5'-TGCTGTCACCTTCACCGTTC-3'). 25 μL PCR reaction mixture was pre-denatured at 95°C for 1 min, and 40 cycles of 94°C for 15 s, 58°C for 20 s, and 72°C for 20s were performed with a final extension at 72°C for 5 min. SYBR green was used for the and each sample was repeated for 3 times. Comparative delta-delta Ct method was used for final result calculation: $\triangle Ct=Ct_{TFPI-2}-Ct_{\beta\text{-actin}}$, $\triangle\triangle Ct=\triangle Ct_{experiment\ group}-\triangle Ct_{control\ group}$, the relative mRNA level $=2^{-\triangle\triangle Ct}$. Each experiment was repeated three times.

Western blotting

100 μL of cell lysis buffer was mixed with 40 μL harvested cells to isolate the total cellular protein, and then quantitated by the Bradford method, 100 μL of the total protein was denaturated for 10 min, and 25 μL was taken for the 10% polyacrylamide gel electrophoresis. The proteins separated by the electrophoresis were then transferred onto the PVDF membrane, and the 10% skim milk used to block the PVDF membrane at room temperature for 1 h. The PVDF membranes were incubated in the 1:200 dilution of the primary antibody at 4°C overnight, and then in the 1:2000 diluted alkaline phosphatase-labeled secondary antibody solution at 37°C for 2 h, followed by the DAB coloration. The PVDF membranes were photographed using a ChemiImager 5500 AlPhaInn Ch, and the subsequent data acquisition and mining were conducted with the Fluorchem V2.0 system. Taking the expression level of β-actin protein as the internal control, the ratio of scanning density of TFPI-2 protein to that of β-actin was finally calculated and used as the analyzing index to further investigate the changes in protein expression of TFPI-2 and compare them between different experimental groups. Each experiment was repeated three times.

Statistical analysis

Statistical analyses were performed using the SPSS13.0 software package. The results were expressed as mean ± standard deviation (SD). Continuous variables were compared by the analysis of variance (ANOVA), and multiple comparisons were analyzed by the LSD. A *P*-value of 0.05 or less was considered significant.

Results

Effects of 5-Aza-CdR on the Proliferation of A549 Cells. As shown in Figure 1 and Table 1, a dose and time dependent inhibition of cell growth was observed after A549 cells were treated with 0, 1, 5, 10 μmol/L 5-Aza-CdR for 24 ,48 and 72 h. For example, When A549 cells were treated with 0, 1, 5, 10 μmol/L 5-Aza-CdR for 72 h, the inhibition rate of growth of A549 Cells was 1.0±0.5%, 13.4±0.5%, 16.6±1.0%, 21.4±0.8% respectively ($P<0.05$). When A549 cells were treated with 10 μmol/L 5-Aza-CdR for 24h,48h,72h, the inhibition rate of growth of A549 Cells was 15.7±0.7%, 17.1±0.7%, 21.4±0.8% respectively ($P<0.05$).These data suggest that 5-Aza-CdR inhibited the proliferation of A549 cells, and significantly increased inhibition rate of growth of A549 cells with increasing 5-Aza-CdR concentration and delaying treatment time.

Table 1. The Inhibition Rate of Growth of A549 Cells after A549 Cells were Treated with Various 5-Aza-CdR Concentrations for 24h, 48h and 72h ($\bar{\chi}$±S, %)

5-Aza-CdR concentrations	24h	48h	72h
0×10–6mol/L	1.5±0.8	1.0±0.5	1.0±0.5
1×10–6mol/L	11.7±0.8*	11.6±0.5*	13.4±0.5*
5×10–6mol/L	13.2±1.0*	14.2±0.8*	16.6±1.0*
10×10–6mol/L	15.7±0.7*	17.1±0.7*	21.4±0.8*

*$P<0.05$ vs control group (0×10^{-6} mol/L)

Table 2. The Changes of the Cells Cycles after A549 Cells were Treated with Various 5-Aza-CdR Concentrations for 72h ($\bar{\chi}$±S, %)

5-Aza-CdR concentrations	G0/G1	S	G2/M	PI
0×10^{-6} mol/L	69.57±0.99	29.77±0.43	0.67±0.59	30.43±0.99
1×10^{-6} mol/L	76.11±0.83*	23.65±0.96*	0.23±0.34	23.89±0.83*
5×10^{-6} mol/L	83.80±0.35*	15.67±0.75*	0.52±0.50	16.19±0.34*
10×10^{-6} mol/L	95.51±0.55*	1.73±0.45*	4.75±0.11*	6.49±0.55*

*$P<0.05$ vs control group (0×10^{-6} mol/L)

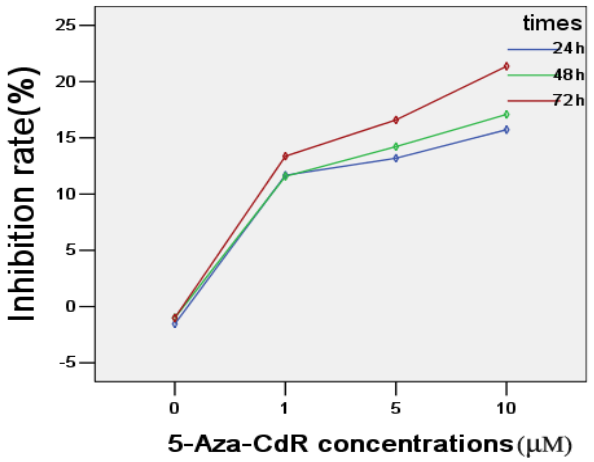

Figure 1. Inhibition Rate Curve of A549 Cells after Treatments with 0, 1, 5, 10 μmol/L 5-Aza-CdR for 24, 48 and 72h. The result showed that 5-Aza-CdR significantly increased Inhibition rate of growth of A549 cells with increasing 5-Aza-CdR concentration and delaying treatment time. The inhibition rate of growth of A549 Cells were analyzed by MTT. Data are representative results of 3 individual experiment

Effects of 5-Aza-CdR on the Cell Cycle of A549 Cells. After A549 cells were treated with 0, 1, 5, 10 μmol/L 5-Aza-CdR concentrations for 72h, the percentage of G0/G1 was 69.57±0.99%, 76.11±0.83%, 83.80±0.35%, 95.51±0.55% respectively ($P<0.05$); the percentage of S was 29.77±0.43%, 23.65±0.96%, 15.67±0.75%, 1.73±0.45% respectively ($P<0.05$); the percentage of cycling cells was 30.43±0.99%, 23.89±0.83%, 16.19±0.34%, 6.49±0.55% respectively($P<0.05$), suggesting the G0/G1 arrest of cell cycle (Table 2). The results suggest that 5-Aza-CdR inhibited proliferation of A549 cells with increasing 5-Aza-CdR concentration. TableII showed the changes of the cells cycles after A549 cells were treated with various 5-Aza-CdR concentrations for 72 h.

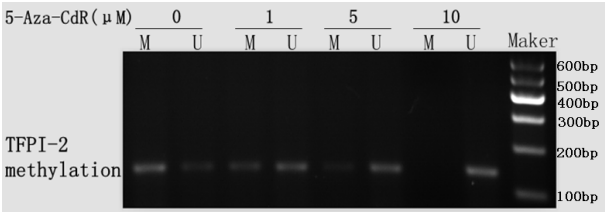

Figure 2. Effect of 5-Aza-CdR on the Demethylation of TFPI-2 Gene in A549 Cells were Treated with Various Concentrations (0, 1, 5, 10 μmol/L) of 5-Aza-CdR for 72h. Methylation status of TFPI-2 gene was measured by MSPCR (M,methylated PCR products; U, unmethylated PCR products)

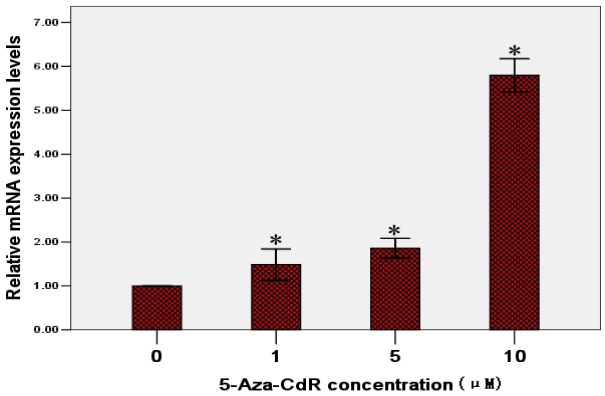

Figure 3. Effect of 5-Aza-CdR on the Expression of TFPI-2 Gene mRNA in A549 Cells were Treated with Various 5-Aza-CdR Concentrations (0, 1, 5, 10 μmol/L) for 72h. TFPI-2 gene mRNA expression was analyzed using real-time PCR. Data were express as the fold change from control and presented as means±SD from 3 independent experiments performed in triplicate (**P*<0.05, one-way ANOVA was used to determine *P* values)

The methylation status of TFPI-2 gene in A549 cells. MSPCR showed that hypermethylation in the promoter region of TFPI-2 gene was detected in A549 cells which were not treated with 5-Aza-CdR, and only partial demethylation of the TFPI-2 gene promoter was detected in A549 cells after 1, 5 μmol/L 5-Aza-CdR treatment, and unmethylation of the TFPI-2 gene promoter was detected in A549 cells after 10μmol/L 5-Aza-CdR treatment (Figure 2). The results suggested 5-Aza-CdR induced further decrease in the methylation of TFPI-2 gene promoter.

The expression of TFPI-2 mRNA in A549 cells. Real-time PCR showed that the expression of TFPI-2 gene mRNA levels was 1±0, 1.49±0.14, 1.86±0.09, 5.80±0.15 (*P*<0.05), after A549 cells were treated with 0, 1, 5, 10 μmol/L 5-Aza-CdR concentrations for 72 h (Figure 3). The results suggest that 5-Aza-CdR increased the expression of TFPI-2 gene mRNA levels in A549 cells with increasing 5-Aza-CdR concentration.

The expression of TFPI-2 proteins in A549 cells. Western blotting analysis showed the relative expression levels of TFPI-2 protein were 0.12±0.01, 0.23±0.02, 0.31±0.02, 0.62±0.03 (*P*<0.05) after treatment with 0, 1, 5, 10 μmol/L 5-Aza-CdR for 72h (Figure 4). TFPI-2 Protein expressions in A549 cells were gradually increased significantly, with the 5-Aza-CdR concentration increasing.

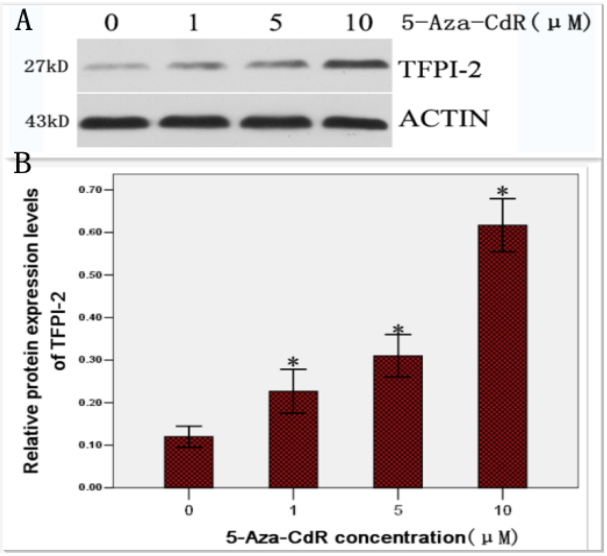

Figure 4. Effect of 5-Aza-CdR on the Expression of TFPI-2 Protein in A549 Cells were Treated with Various 5-Aza-CdR Concentrations (0, 1, 5, 10 μmol/L) for 72h. (A) Western blotting analysis for detection of TFPI-2 protein expression levels. (B) The relative expression levels of TFPI-2 protein before and after treatment of 5-aza-CdR. All results were presented as means±SD from 3 independent experiments performed in triplicate (**P*<0.05, one-way ANOVA was used to determine *P* values)

Discussion

Multiple genetic and/or epigenetic changes including DNA methylation, has been suggested to contribute to development and progression of human cancers including lung cancer. Epigenetic alterations which result in aberrant gene expression without any concomitant change in DNA sequence, is a rapidly expanding field that focuses on stable changes in gene expression (Boumber and Issa, 2011). Epigenetic changes have been shown to play important roles in determining when and where as well as whether a gene would express or silence. DNA methylation and the modification of histones including acetylation and deacetylation are important components of epigenetics, however, the most common modification is DNA methylation, which is considered as the best-characterized and most easily quantifiable epigenetic mechanism underlying gene expression or silencing (Li et al., 2012). DNA methylation is a type of covalent modification in which a methyl group is added to a cytosine in the genome via S-adenosylmethionine. This process occurs as an enzymatic reaction after DNA replication (Zuo et al., 2009). In mammalian cells, methylation of DNA is typically restricted to the 5-position of the pyrimidine ring of cytosine residues that are located in CpG dinucleotides (Goel, 2010). DNMTs are important for methylation, When DNMT1 is removed, the level of methylation of the whole genome is reduced by 3%, and when DNMT3 is removed, it is reduced by 4%. When they are both removed, the level is lowered by 98% (Gopalakrishnan et al., 2008).

TFPI-2 is a 32 kDa Kunitz-type serine proteinase inhibitor ,which is a potential inhibitor of the plasmin within the extracellular matrix, was reported to be

frequently methylated in various other cancers as well as in lung cancer (Glöckner et al., 2009; Takadaa et al., 2010; Wang et al., 2010). Silencing of the TFPI-2 gene by hypermethylation might contribute to tumor progression in NSCLC (Rollin et al., 2005). Methylation of TFPI-2 gene is an independent factor for an unfavorable prognosis in patients with NSCLC (Wu et al., 2012). Downregulation of TFPI-2 gene because of methylation could promote the migration and invasion abilities in lung cancer cell lines (Lakka et al., 2000). 5-Aza-CdR, a nucleoside analogue, could incorporate into DNA and exert direct cytotoxic and anti-proliferative effects on tumor cells. However, to our knowledge, there are few studies on effect of 5-aza-2'-deoxycytidine on TFPI-2 gene in non-small cell lung cancer A549 cell line.

In our study, hypermethylation in the promoter region of TFPI-2 gene was detected in A549 cells which were not treated with 5-Aza-CdR, and only partial demethylation of the TFPI-2 gene promoter was detected in A549 cells after 1, 5 μmol/L 5-Aza-CdR treatment, and unmethylation of the TFPI-2 gene promoter was detected in A549 cells after 10 μmol/L 5-Aza-CdR treatment. A549 cells treated with 5-Aza-CdR revealed demethylation of the TFPI-2 gene. The expression of TFPI-2 gene mRNA and protein were very weak in A549 cells which were not treated with 5-Aza-CdR, however, it were enhanced considerably after 5-Aza-CdR treatment.This result suggested that the weak expression of TFPI-2 gene in A549 cells may be due to the hypermethylation of CpG islands in the promoter of TFPI-2 gene. It was found the proliferation of A549 cells was inhibited significantly after various 5-Aza-CdR concentration treatment, 5-Aza-CdR significantly increased inhibition rate of proliferation of A549 cells with increasing 5-Aza-CdR concentration and delaying treatment time. The cell cycle was arrested in the G0/G1. The results indicated the anti-tumor role of 5-Aza-CdR. According to the mechanism of 5-Aza-CdR, this drug could induce the re-expression of certain silencing genes by demethylation, therefore, recover the function of some tumor suppressor genes. This suggested that the inhibitory role of 5-Aza-CdR in A549 cells had a close correlation with the re-expression of TFPI-2 gene.

So, aberrant hypermethylation of promoter regions is an alternative mechanism for inactivation of tumor suppressor genes. 5-Aza-CdR could efficiently reverse the expression of tumor suppressor gene silencing by DNA hypermethylation, which provided a new pathway for the early diagnosis and treatment of the lung cancer (Dolle et al., 2006). These findings provide theoretic evidence for clinical treatment of advanced non-small cell lung cancer with demethylation agents 5-Aza-CdR. TFPI-2 will be one of the molecular markers for effective treatment of advanced non-small cell lung cancer with 5-Aza-CdR.

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References

Basseres DS, D'Al F, Yeap BY, et al (2012). Frequent downregulation of the transcription factor Foxa2 in lung cancer through epigenetic silencing. *Lung cancer*, **77**, 31-7.

Boumber Y, Issa JP (2011). Epigenetics in cancer: what s the future? *Oncology (Williston Park)*, **25**, 220-6.

Carvalho L, Cardoso E, Nunes H, et al (2009). The IASLC lung cancer staging project. Comparing the current 6(th) TNM edition with the proposed 7(th) edition. *Rev Port Pneumol*, **15**, 67-76.

Ding Y, Long C, Liu X, et al (2012). 5-aza-2'-deoxycytidine leads to reduced embryo implantation and reduced expression of DNA methyltransferases and essential endometrial genes. *PLoS One*, **7**, e45364.

Dmitriev AA, Kashuba VI, Haraldson K, et al (2012). Genetic and epigenetic analysis of non-small cell lung cancer with NotI-microarrays. *Epigenetics*, **7**, 502-13.

Dolle L, Depypere H T, Bracke M E (2006). Anti-invasive/anti-metastasis strategies: new roads, new tools and new hopes. *Curr Cancer Drug Targets*, **8**, 729-51.

Fischer JR, Ohnmacht U, Rieger N, et al (2006). Promoter methylation of RASSF1A, RARbeta and DAPK predict poor prognosis of patient, with malignant mesothelioma. *Lung Cancer*, **54**, 109-16.

Friedel G, Steger V, Kyriss T, et al (2004). Prognosis in N2 NSCLC. *Lung Cancer*, **45**, S45-53.

Gaud G, Iochmann S, Guillon-Munos A, et al (2011). TFPI-2 silencing i ncreases tumor progression and promotes metalloproteinase 1 and 3 induction through tumor-stromal cell interactions. *J Cell Mol Medl Fe*, **15**, 196-208.

Gl ckner SC, Dhir M, Yi JM, et al (2009). Methylation of TFPI2 in stool DNA: a potential novel biomarker for the detection of colorectal cancer. *Cancer Res*, **69**, 4691-9.

Goel A (2010). DNA methylation-based fecal biomarkers for the noninvasive screening of GI cancers. *Future Oncol*, **6**, 333-6.

Gopalakrishnan S, Van Emburgh BO, Robertson KD (2008). DNA methylation in development and human disease. *Mutat Res*, **647**, 30-8.

Hube F, Reverdiau P, Iochmann S, et al (2003). Transcriptional silencing of the TFPI-2 gene by promoter hypermethylation in choriocarcinoma cells. *Biol Chem*, **384**, 1029-34.

Jee CD, Kim MA, Jung EJ, et al (2009). Identification of genes epigenetically silenced by CpG methylation in human gastric carcinoma. *Eur J Cancer*, **45**, 1282-93.

Jemal A, Siegel R, Ward E, et al (2009). Cancer statistics, 2009. *CA Cancer J Clin*, **59**, 225-49.

Jin Q, Liu C, Yan C, et al (2012). 5-aza-CdR induces the demethylation of Syk promoter in nasopharyngeal carcinoma cell. *Gene*, **511**, 224-6.

Kempaiah P, Chand HS, Kisiel W (2007). Identification of a human TFPI-2 splice variant that is upregulated in human tumor tissues. *Mol Cancer*, **6**, 20.

Lakka SS, Konduri SD, Mohanam S, et al (2000). In vitro modulation of human lung cancer cell line invasiveness by antisense cDNA of tissue factor pathway inhibitor-2. *Clin Exp Metastasis*, **18**, 239-44.

Li XQ, Guo YY, De W (2012). DNA methylation and microRNAs in cancer. *World J Gastroenterol*, **18**, 882-8.

Nelson HH, Marsit CJ, Christensen BC, et al (2012). Key epigenetic changes associated with lung cancer development: results from dense methylation array profiling. *Epigenetics*, **7**, 559-66.

Rao CN, Lakka SS, Kin Y, et al (2001). Expression of tissue factor pathway inhibitor 2 inversely correlates during the progression of human gliomas. *Clin Cancer Res*, **7**, 570-6.

Ribarska T, Ingenwerth M, Goering W, et al (2010). Epigenetic inactivation of the placentally imprinted tumor suppressor gene TFPI2 in prostate carcinoma. *Cancer Genomics Proteomics*, **7**, 51-60.

Rollin J, Iochmann S, Bl chet C, et al (2005). Expression and methylation status of tissue factor pathway inhibitor-2 gene in non-smallcell lung cancer. *Br J Cancer*, **92**, 775-83.

Sculier JP, Chansky K, Crowley JJ, et al (2008). The impact of additional prognostic factors on survival and their relationship with the anatomical extent of disease expressed by the 6th Edition of the TNM Classification of Malignant Tumors and the proposals for the 7th Edition. *J Thorac Oncol*, **3**, 457-66.

Sierko E, Wojtukiewicz MZ, Kisiel W (2007). The role of tissue factor pathway inhibitor-2 in cancer biology. *Semin Thromb Hemost*, **33**, 653-9.

Takada H, Wakabayashi N, Dohi O (2010). Tissue factor pathway inhibitor 2 (TFPI2) is frequently silenced by aberrant promoter hypermethylation in gastric cancer. *Cancer Genetics and Cytogenetics*, **197**, 16-24

Tang Z, Geng G, Huang Q, et al (2011). Expression of tissue factor pathway inhibitor 2 in human pancreatic carcinoma and its effect on tumor growth, invasion, and migration in vitro and in vivo. *J Surg Res*, **167**, 62-9.

Wang S, Xiao X, Zhou X, et al (2010). TFPI-2 is a putative tumor suppressor gene frequently inactivated by promoter hypermethylation in nasopharyngeal carcinoma. *BMC Cancer*, **10**, 617.

Wu D, Xiong L, Wu S, et al (2012). TFPI-2 methylation predicts poor prognosis in non-small cell lung cancer. *Lung Cancer,* **76**, 106-11.

Zuo T, Tycko B, Liu TM, et al (2009). Methods in DNA methylation profiling. *Epigenomics*, **1**, 331-45.