Int J Clin Exp Med 2015;8(11):20516-20523
www.ijcem.com /ISSN:1940-5901/IJCEM0013597

Original Article

In vitro effects of mitomycin C on the proliferation of the non-small-cell lung cancer line A549

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Received July 26, 2015; Accepted October 28, 2015; Epub November 15, 2015; Published November 30, 2015

Abstract: Non-small-cell lung cancer (NSCLC) is the leading cause of death from cancer in the United States. Chemotherapy prolongs survival among patients with advanced disease, but at the cost of clinically significant adverse effects. As a novel promising oncotherapy method, induced differentiation by mitomycin C has been applied for NSCLC therapy at recent year. In this study, the molecular mechanism of differentiation interruption by mitomycin C in the NSCLC line A549 was investigated. High dosage of mitomycin C (300 μM) could significantly inhibit cell proliferation ($P < 0.05$) by 48.39 ± 3.32% ($P < 0.05$), under which cell shrinkage and disruption were observed. Flow cytometry assay showed that the proportion of G1/G0 cells significantly increased, while that of S and G2/M cells significantly decreased after treatment of mitomycin C (10 or 300 μM) for 24 h. These results indicated that cell arrest by mitomycin C appeared. Additionally, up-regulation of retinoblastoma (*Rb*) gene by low concentration of mitomycin C (10 μM) was detected using immunohistochemistry (IHC) and Western blot assay, indicating a role in the regulation of cell cycle inhibition of this cell line.

Keywords: Mitomycin C, non-small-cell lung cancer (NSCLC), differentiation, cell cycle, retinoblastoma (*Rb*) gene

Introduction

Lung cancer is one of the leading causes of cancer-related deaths in the world with one million new cases annually in terms of incidence and mortality [1-4]. It accounts for 13% (1.6 million) of the total cases and 18% (1.8 million) of the deaths in 2008 [1]. Tobacco use is believed to be the main cause of lung cancer, accounting for 80-90% [5, 6], among which cigarette smoking consists of approximately 95% tobacco use [7]. Non-small-cell lung cancer (NSCLC) accounts for approximate 80% of all lung cancer cases. The management of patients with NSCLC is based on systemic chemotherapy. However, the survival improvement remains probably low. Additionally, though chemotherapy could prolong survival among patients with advanced disease, clinically significant adverse effects reduces its usefulness since excessive toxicity is often reported [8].

Induced differentiation has been widely use in human acute promyelocytic leukemia (APL) [9-12], and is promising for cancer therapy [13, 14]. One of the notable advantages of induced differentiation for cancer therapy is that it can induce cancer cells to reversely differentiate into normal or near-normal cells, rather than kill cells [14]. When exposed to differentiation inducers, a series of responses could be occurred in cancer cells, including morphological changes, decline of proliferation ability, recovery of normal functions, and changes of cell cycle, etc. Therefore, the combination of induced differentiation with surgical treatment might be a potential novel method for NSCLC therapy.

The sequential cell life includes G1, S, GZ, and M phrase, among which G1/S and GZ/M are the two key regulatory points. The process of cell cycle is regulated by many related genes

including oncogenes and anti-oncogenes [15]. The disorder of cell cycle is an important indicator in tumor development and progression [15]. Researches showed that cell cycle normalization were observed in cancer cells using differentiation inducers, in which the cell ratio of G0/G1 phrase elevates, while that of G2/M phrase declines [15-17]. At present, suppression of cell proliferation to regulate cell cycle through induced differentiation in tumor cell has gained more and more concern [18-20].

The human non-small-cell lung cancer (NSCLC) line A549 was first developed by D. J. Giard, et al in 1972 [21], which can be cultured *in vitro* easily and are widely used as an *in vitro* model for drug metabolism and function assessment [22]. Although the use of induced differentiation for lung cancer therapy were reported [23-25], there are few direct clues that report on the molecular mechanism of differentiation interruption by mitomycin C of different doses in the NSCLC line A549. In this study, we study the *in vitro* effects of mitomycin C on the proliferation of the NSCLC line A549, which may provide a better understanding of the molecular mechanism of mitomycin C-induced differentiation for lung cancer and also allow us to develop new drugs that can inhibit and eliminate lung tumor cells in the near future.

Materials and methods

Cell line and mitomycin C treatment

The human NSCLC cancer cell lines A549 was purchased from Cancer Research Institute of China Medical University (Shenyang, China). The cells were cultured in DMEM (Gibco) supplemented with 10% (m/v) fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin on culture plates at 37°C in a 5% CO_2 atmosphere with stable humidity. The density of cells was 1×10^5 cells/ml before starting the culture. The A549 cells were treated using mitomycin C for 24 h for further experiments.

Agents

Doxorubicin (DOX), paclitaxel, vincristine, cisplatine and 3-(4,5-dimethylthiazol-yl)-2,5-diphenyllapatinibrazolium bromide (MTT) were products of Sigma Chemical Co. from Genewindows Co. (Guangzhou, China). Dulbecco's modified Eagle's medium (DMEM) and RPMI medium 1640 were products of Gibco BRL from Genewindows Co. (Guangzhou, China). All antibodies were purchased from Santa Cruz Biotechnology Inc from Genetime Co. (Guangzhou, China). Other routine laboratory reagents were obtained from commercial sources of analytical (Guangzhou, China).

MTT assay

The effects of mitomycin C on cell viability was assessed by MTT assay as described previously [26]. Briefly, cells were plated at a density of 3000 cells per well into 96-well plates. At the end of treatment, the supernatant was removed, and 20 μl of the tetrazolium compound, MTT, and 270 ml of fresh DMEM medium were added. After incubation for 4 h at 37°C, 120 μl of DMSO was placed in each well to dissolve the tetrazolium crystals. Finally, the absorbance at a wavelength of 570 nm was recorded using a multi-well plate reader (Tecan, Maennedorf, Switzerland). Each experiment was performed four times. Results are expressed as the percentage growth inhibition with respect to the untreated cells.

Microscopic inspection

Digested cell culture (3×10^5 cells/ml) was added to a 24-well plate (0.9 ml for each well) and incubated for 12 h. Then 0.1 ml mitomycin C of low (10 μM) or high concentration (300 μM) per well was added. Cells were incubated for 24 h before observation.

Flow cytometry (FCM) analysis

A549 cells at log phrase were collected at a final concentration of 2×10^5 cells/ml, and were incubated in 6-well plate for 12 h (2.7 ml for each well). Then 0.3 ml mitomycin C of low (10 μM) or high concentration (300 μM) per well was used to induce the cells for 24 h. Simultaneously, 0.3 ml cell culture, as negative control, was cultured for 24 h, collected, washed with PBS, and fixed with 70% ethanol, in sequence. Cells were centrifuged to eliminate ethanol, washed with PBS, and stained with propidium iodide (PI) in dark for 30 min before FCM analysis. Finally, BD FACSCalibur (BD, USA) was used to detect cell cycle. Cells were sampled using sampling software Cell Quest 3.0. The proportion of cells in different phrases were quantified by ModFitLT 3.0 [27]. Each experiment was performed four times.

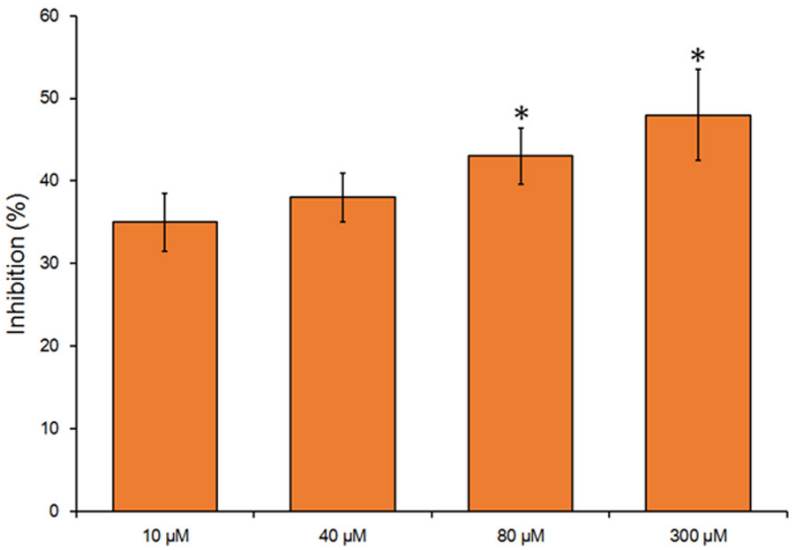

Figure 1. Growth inhibitory effect of mitomycin C (10, 40, 80 and 300 μM) on A549 cells. Data are expressed as means ± SD, n = 4. *P < 0.05 represents significant differences in comparison with the control group. Results are expressed as the percentage growth inhibition with respect to the untreated cells.

Immunohistochemical staining

Immunohistochemical staining was performed using the highly specific affinity-purified poly clonal anti-RB antibody, RB-WL-1, according to Xu et al [28]. Negative controls for each of the antibodies were performed using nonimmune serum instead of the primary anti body. Briefly, the sections were washed in phosphate-buffered saline followed by preincubation with 1.5% normal goat serum in phosphate buffer within a moist chamber for 4 h at room temperature. Those sections were then incubated overnight with RB-WL-1 antibody at a final concentration of 2 μg/ml. After being washed with 6 changes of phosphate-buffered saline containing 0.02% Triton X-100 over 15 min, the slides were processed for immunostaining with the avidin-biotinylated peroxidase complex method (Vector Laboratories, Burlingame, CA) according to the user manual. The tissue sections were briefly counterstained with Mayer's hematoxylin before mounting. Cultured cells were grown on sterile coverslips in tissue culture dishes overnight, fixed with 45% acetone/10% formaldehyde in 0.1 M phosphate buffer for 5 min, and then processed for immunohistochemicalassay as described above.

Western blot analysis

Cell lysates were prepared in RIPA buffer (50 mmol/l Tris-HCl buffer, pH 7.4, 150 mmol/l NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) supplemented with 1 × Halt protease inhibitor cocktail and 1 × Halt phosphatase inhibitor cocktail (Pierce, Rockford, IL). A Bio-Rad protein assay (Bio-Rad) was used to determine protein concentrations. Proteins were separated on 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Whatman, Boston, MA). Membranes were first hybridized with specific primary antibodies and then with HRP-conjugated secondary antibodies (Cell Signaling Technology). Protein bands were visualized using a commercial Immobilon Western Chemiluminescent HRP Substrate detection reagent (Millipore, Billerica, MA). The chemiluminescence of proteins transferred to PVDF membranes was detected with ECL Plus (GE Healthcare Amersham, Piscataway, NJ). Relative protein expression values were quantitatively determined via densitometry with ImageJ software.

Statistical analysis

All data were analyzed with statistical software package SPSS 16.0. The results were expressed as means ± SD. The statistical significance of the studies was determined by the parametric unpaired Student's *t* test. Differences with $P < 0.05$ are considered significant and $P < 0.01$ is considered highly significant.

Results

Growth inhibition of mitomycin C on A549 cells

To test the growth-modulatory effects of mitomycin C on A549 cells, a wide concentration range of mitomycin C (10, 40, 80 and 300 μM) was used. After 24 h treatment of mitomycin C, significant inhibition of cell growth was observed at high concentration of 80 and 300 μM compared with the control groups, with an average growth inhibition rate of 44.03 ± 4.18 and 48.39 ± 3.32%, respectively (**Figure 1**).

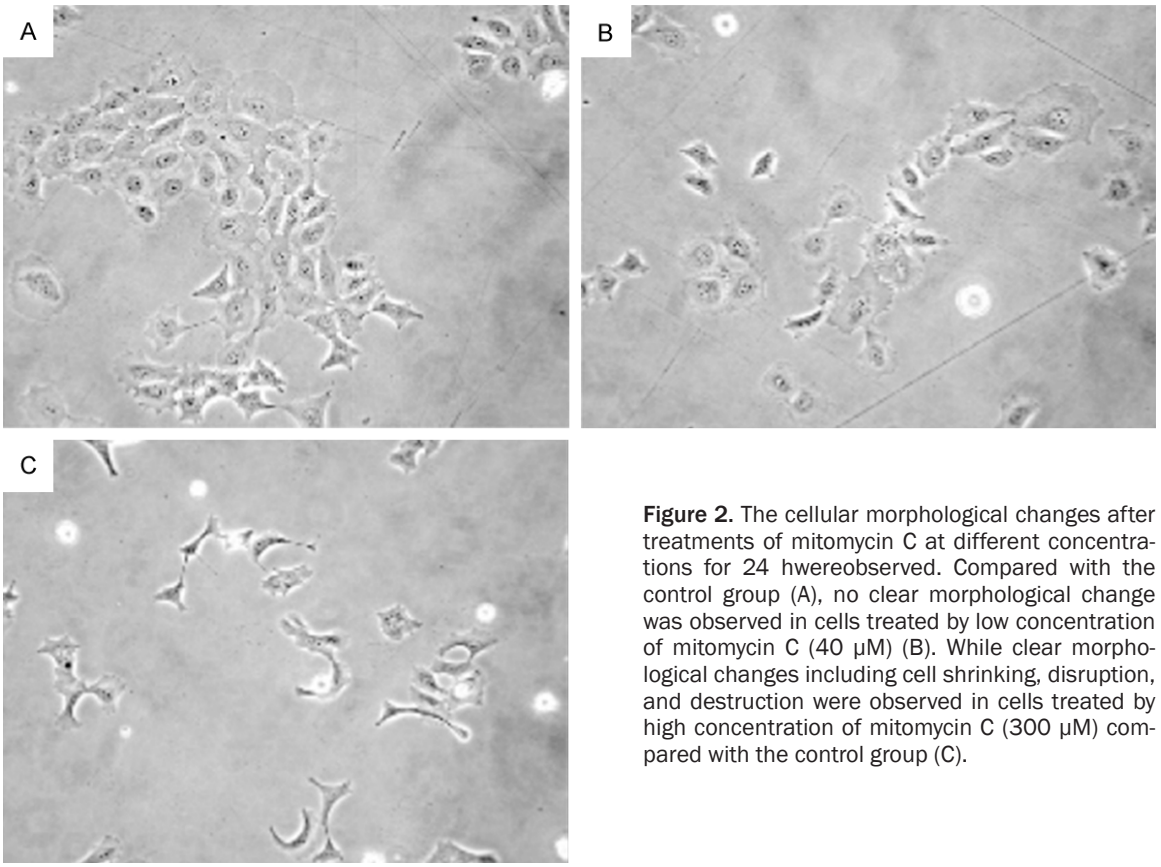

Figure 2. The cellular morphological changes after treatments of mitomycin C at different concentrations for 24 hwereobserved. Compared with the control group (A), no clear morphological change was observed in cells treated by low concentration of mitomycin C (40 μM) (B). While clear morphological changes including cell shrinking, disruption, and destruction were observed in cells treated by high concentration of mitomycin C (300 μM) compared with the control group (C).

Table 1. The changes of cell cycle induced by mitomycin C (X ± S)

Group		Cell debris (%)	Apoptosis (%)	Diploid (%)	Aneuploid (%)
Control		1.88 ± 0.2167	0.0267 ± 0.0252	94.7867 ± 4.5027	5.2133 ± 4.5027
Mitomycin C	10 μM	18.8967 ± 3.9119	0.0400 ± 0.0693	98.7367 ± 1.4318	1.2401 ± 1.4673
	300 μM	46.9533 ± 5.0537	0.06934 ± 0.0723	99.9101 ± 0.1558	0.0901 ± 0.1558

However, low concentration of mitomycin C (10 and 40 μM) had no significant effect on cell growth in comparison with the control groups. These results indicated that the growth inhibitory effect of mitomycin C on A549 cells was concentration dependent (**Figure 1**).

Cellular morphological changes

After 24 h treatment of mitomycin C, the cell morphology was observed by FIMS. At low concentration of mitomycin C, the treated cells did not show clear difference in cellular morphology from the control groups (**Figuer 2B**); while clear morphological changes including cell shrinking, disruption, and destruction were observed in cells treated by high concentration of mitomycin C (300 μM) compared with the control group (**Figure 2C**). High concentration of mitomycin C could lead to the decrease of cell colony, which was also indicated by the degradation of cell diopter (**Figure 2C**).

Cell apoptosis induced by mitomycin C

Compared with the control groups, significant ($P < 0.05$) increase of the cell ratio of G1/G0 phrase, while significant ($P < 0.05$) decrease of that of S and G2/M phase, was observed; most cells stayed at G1 phase after treatment of mitomycin C at low and high concentrations (10 and 300 μM) (**Table 1** and **Figure 3**). The proportion of diploid slightly increased with a decrease of aneuploid; additionally, cell debris increased largely compared with the control group (**Table 2** and **Figure 3**). These results indicated that mitomycin C could induced cell apoptosis in the NSCLC line A549.

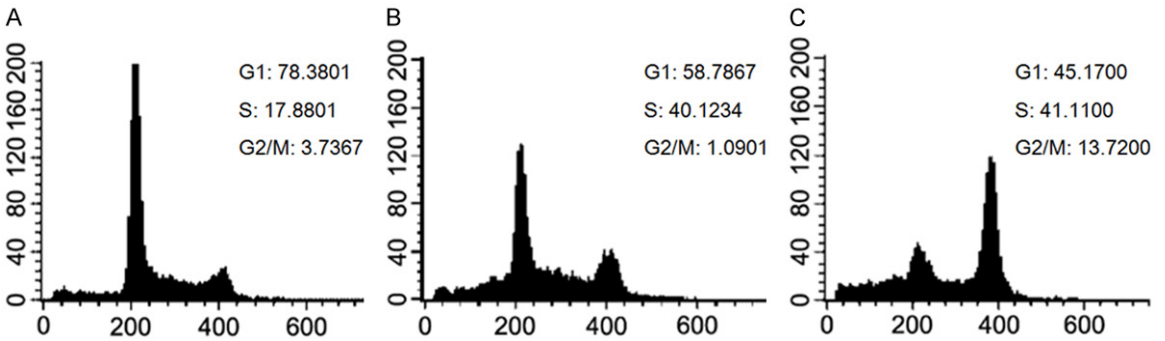

Figure 3. Cell apoptosis was induced by mitomycin C. Both low (10 μM) (B) and high (300 μM) (A) concentrations of mitomycin C could induced cell apoptosis compared with the control group (C). Dip: diploid; Anl: aneuploidy.

Table 2. The changes of diploid content in different cell phrases induced by mitomycin C (X ± S)

Group		Diploid (%)		
		G1/G0	S	G2/M
Control		45.1700 ± 1.0101	41.1100 ± 1.2185	13.7200 ± 0.5801
Mitomycin C	10 μM	58.7867 ± 2.5613	40.1234 ± 1.5622	1.0901 ± 0.1277
	300 μM	78.3801 ± 0.1735	17.8801 ± 0.5456	3.7367 ± 0.3695

Enhanced expression of Rb gene in mitomycin C treated A549 cells

Immunohistochemical staining showed that almost all cell nuclei and brown cytoplasmic particles were sepia-colored after treatment of mitomycin C at low concentration (10 μM) for 24 h (**Figure 4B**). The mean OD of each group showed the expression level of the *Rb* gene. The mean OD of the control group was 0.3888 ± 0.0826; that of the mitomycin C-treated group was 0.5367 ± 0.0235. The mean OD of the mitomycin C-treated group was highly significant ($P < 0.01$) higher than that of the control group (**Figure 4C**). These results were further confirmed by Western blot assay (**Figure 4D**), suggesting that low concentration of mitomycin C was enough to induce *Rb* gene expression.

Discussion

For a long time, it was believed that carcinogenesis was an irreversible process during which cancer cells could not be turned back into normal healthy cells. Therefore, killing or damaging cancer cells is the crucial point for chemotherapy with drugs. However, the low specificity as well as distinct adverse effects of many chemotherapeutic drugs limits their massive use in cancer therapy [8]. As a novel treatment method, induced differentiation was first use in human APL [18]. Researches showed that many drugs could induce tumor cell differentiation at low concentration, and lead to cell apoptosis or death at high concentration [20, 29]. Among these differentiation inducers, mitomycin C is often used as cytotoxic agents in hematological malignant tumor and other cancers [30]. However, lack of direct clue of the inhibition effect on NSCLC proliferation hinders the illustration of the underlying molecular mechanism of this differentiation inducer.

Therefore, the aim of this study was to investigate the *in vitro* effect of mitomycin C on the NSCLC line A549 at the molecular level, with the purpose of disclosing the molecular mechanism of this differentiation inducer. We found that low concentration (10 μM) of mitomycin C was sufficient to inhibit A549 cell growth; this cell growth arrest was concentration dependent, i.e. elevated inhibitory effect was observed when dose was increased (**Figure 1**). However, the cell morphology did not significantly change at low concentration (10 μM) until dose was increased to 300 μM (high concentration) (**Figure 2**). In accompany with cell growth arrest, A549 cell apoptosis was induced after mitomycin C treatment for 24 h, as indicated by phrase changes compared with untreated control with respect to cell cycle

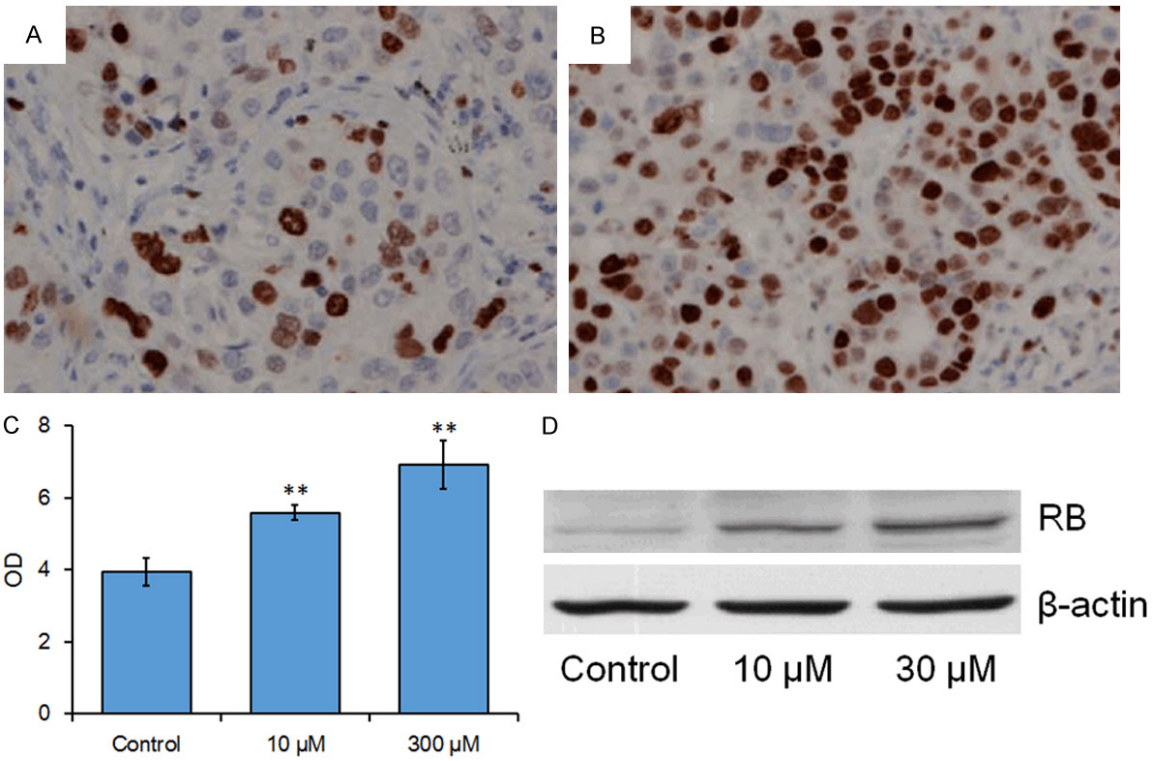

Figure 4. The expression of *Rb* gene in the A549 cells. Low concentration (10 μM) of mitomycin C could significantly ($P < 0.01$) enhanced the expression of *Rb* genes (A) compared with the control group (B), as indicated by immunohistochemical staining (400 ×), the mean OD (C) and Western blot assay (D). $^{**}P < 0.01$ represents highly significant differences in comparison with the control group.

detected by FCM assay (**Tables 1** and **2**; **Figure 3**). Also, low concentration (10 μM) was sufficient to induced cell apoptosis. These results indicated that low concentration of mitomycin C inhibited A549 cell proliferation by retarding cell growth to prolong cell cycle.

These clues were further enhanced by the expression analysis of *Rb* gene. The *Rb* gene is an anti-oncogene identified in retinoblastoma [31, 32]. *Rb* gene plays an important role in the regulation of tumor cell growth, proliferation, and apoptosis [32]. Its inactivity could lead to cell cycle disorder and thus activate many oncogenes and carcinogenesis [33, 34]. Low concentration of mitomycin C enhanced *Rb* expression in A549 cells (**Figure 4**), which suggested that *Rb* might participate in cell cycle regulation, especially G1 phrase (**Tables 1** and **2**; **Figure 3**), since the Plioab protein encoded by *Rb* gene was found to be involved in G1 phrase [35, 36].

The proportion of cell apoptosis in this study was slightly low (**Table 1**), FCM analysis did not found clear apoptosis peak (**Figure 3**), indicating that the inhibitory effect of mitomycin C on A549 cells was obtained mainly by cell cycle retardation (cell growth extension and proliferation deceleration) rather than cell apoptosis. Clear increase of G1/G0 cells and decrease of S and G2/M cells, as well as enhanced *Rb* expression in G1 phrase implied that the inhibitory effect of mitomycin C on A549 proliferation might be mainly attributed to cell arrest in G1 phrase. However, further studies are required to confirm the results and to investigate the underlying mechanism.

Conclusion

It can be concluded that low concentration of mitomycin C is sufficient to retard cell cycle and thus lead to proliferation inhibition in NSCLC line A549; *Rb* gene is activated by low concentration of mitomycin C to enhanced the proliferation inhibition; *Rb* gene is involved in the regulation of cell cycle retardation; the proliferation inhibition is concentration dependent, as high dose (300 μM) exerts more significant

effect on A549 cells. However, only high dose of mitomycin C clearly changes cell morphology. These findings may provide a better understanding of the molecular mechanism of mitomycin C-induced differentiation for lung cancer and also allow us to develop new drugs that can inhibit and eliminate lung tumor cells in the near future.

Disclosure of conflict of interest

None.

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