Cell Cycle and Apoptosis Alteration of Human Cervix Adenocarcinoma Cells Induced by Low-dose $^{12}C^{6+}$ Beam and γ-Ray

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Abstract

Objective: The aim of this study is to explore the effect of $^{12}C^{6+}$ beam irradiation on cell cycle and apoptosis in cervix adenocarcinoma.
Materials and Methods: The Hela cells were exposed to $^{12}C^{6+}$ beam/γ-ray. Cell viability was determined by clonogenic assay. Cell cycle was detected by flow cytometric analysis. The apoptosis was detected by fluorence microscope with DAPI staining.
Results: The G_0/G_1 cells in the irradiated groups were significantly more than those in the control ($p<0.05$), the G_0/G_1 cells in the $^{12}C^{6+}$-irradiated groups were significantly 5-15% more than those in the γ-irradiated ones ($p<0.05$); the G_2/M arrest in $^{12}C^{6+}$-irradiated groups occurs later than those in the γ-irradiated groups ($p<0.05$). The apoptotic cells induced by $^{12}C^{6+}$ beam were significantly more than those by γ-ray ($p<0.05$); the survival fractions for cells irradiated by $^{12}C^{6+}$ beam were significantly less than those by γ-radiation ($p<0.05$).
Discussions: The low-dose $^{12}C^{6+}$ beam irradiation significantly suppresses Hela cells through the induction of cell cycle arrest and cell apoptosis as contrast to the low-dose γ-ray.

Keywords: carbon-ion, γ-ray, cell cycle, cell apoptosis, cervix adenocarcinoma

Özet

Servikal Adenokarsinom Hücrelerinde Düşük Doz $^{12}C^{6+}$ Işını ve γ-Işını ile İndüklenen Hücre Siklusu ve Apoptozis Değişiklikleri

Amaç: Bu çalışmada servikal adenokarsinom hücrelerinde düşük doz $^{12}C^{6+}$ ışınlamanın hücre siklusu ve apoptozise etkileri incelenmiştir.
Materyal ve Metot: Hela hücreleri $^{12}C^{6+}$ ve γ-ışınına maruz bırakıldı. Hücrelerin canlılığı klonojenik testlerle değerlendirildi. Hücre siklusu akım sitometrisi ile analiz edildi. Apoptozis DAPI boyasıyla floresan mikroskopi ile değerlendirildi.
Sonuçlar: Işına maruz bırakılan grupta G_0/G_1 hücreler, kontrol grubuna göre anlamlı derecede fazla idi ($p<0.05$). G_0/G_1 hücreler, $^{12}C^{6+}$ ile ışınlanan grupta γ-ışınlanan gruba göre %5-15 daha fazla idi ($p<0.05$); G_2/M arresti $^{12}C^{6+}$ ile ışınlanan grupta γ-ışınlanan gruba göre daha geç olmaktaydı ($p<0.05$). Apoptotik hücreler $^{12}C^{6+}$ ile ışınlanan grupta γ-ışınlanan gruba göre daha fazla ($p<0.05$); canlılığını devam ettiren hücrelerin oranı $^{12}C^{6+}$ ile ışınlanan grupta γ-ışınlanan gruba göre anlamlı derecede daha az idi ($p<0.05$).
Tartışma: Düşük doz $^{12}C^{6+}$ ışınlaması, γ-ışınlaması ile karşılaştırıldığında hücre siklusu duraklaması ve hücre apoptozisi indüksiyonu yolu ile Hela hücrelerini anlamlı derecede fazla baskılamaktadır.

Anahtar sözcükler: karbon-iyon ışınlama, γ-ışını, hücre siklusu, apoptozis, servikal adenokarsinom

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Introduction

Cervix cancer is the seventh, in overall frequency and the second most common cancer among women worldwide (1). In some countries, the cervix cancer is the leading cause of cancer in women (2). The overall 5-year survival rate is 40% (3). Advanced disease is mostly treated by radiotherapy, sometimes in combination with other therapies, but the conventional radiotherapy often leads to severe side-effect. The most desirable therapy should efficiently eradicate tumor cells with minimal deleterious effects to the surrounding normal tissues and minimal consequent damage to the whole organism.

High linear energy transfer (LET) charged particle radiation offers several potential merits over conventional radiotherapy, such as a reduction in the oxygen enhancement ratio with proportionately greater killing of hypoxic cells, less variation in cell cycle-related radiosensitivity, and less capability for radiation-induced deoxyribonucleic acid (DNA) damage repair. It has been proved that carbon ion therapy is safe with respect to toxicity and offers high, local control rate (4-7).

Cell cycle checkpoints play a critical role in the damaged response system. The radiation-induced cell cycle arrest provides an opportunity for cells to repair DNA damage before entering the mitotic phase. It still remains to be elucidated whether the cell cycle and cell apoptosis induced by heavy-ion irradiation is different from that induced by ionizing irradiation, and whether the low-dose carbon-ion radiation has a better radiosensibilizing effect on cervix adenocarcinoma than the low-dose ionizing radiation does. In this study, we will elucidate the alteration of cell cycle and apoptosis in cervix adenocarcinoma through comparisons between groups irradiated with $^{12}C^{6+}$ ions or γ-ray.

Materials and Methods

Cell culture

Human cervix adenocarcinoma (Hela cell line with $^{wt}p53$, low level p53 expression, ATCC CCL-02) was grown as monolayers in Dulbecco's Modified Eagle Medium (DMEM) media with 10% fetal calf serum (FCS), 0.1% L-glutamine in 5% CO_2 at 37°C.

Irradiation procedure

Hela cells were inoculated at densities of $5x10^5$ into 25 cm^2 plastic flasks (Corning, USA), 24 hrs later, irradiated with $^{12}C^{6+}$ beam at the initial energy of 80.55 MeV/n and LET of 32.2 keV/μm in the water generated from Heavy Ion Research Facility in Lanzhou (HIRFL, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China), with a dose rate of 0.5 Gy/min. The acquisition of data (preset numbers converted by doses of irradiation) was automatically accomplished using a microcomputer during irradiation. Doses of the beams were determined with air ionization chamber. The cells irradiated by ^{60}Co γ-ray were similarly given by a FTC-50H model ^{60}Co teletherapy machine (Shanghai Nuclear Equipment Factory, China) at a source to cell surface distance (SSD) of 75 cm, with a dose rate 0.5 Gy/min. Irradiated cells were incubated for an additional period and thereafter analyzed for cell cycle and cell apoptosis (t=24, 72, and 120 h). The doses used for each kind of irradiation ($^{12}C^{6+}$/^{60}Co γ-ray) were 0, 0.5, 1.0 or 2.0 Gy.

Flow cytometric analysis of cell cycle

Following incubation, the cells were trypsinized, washed twice with cold phosphate buffered solution (PBS), fixed with 70% ethanol in PBS for 30 min, and then stained with a solution containing 5 μg/ml propidium iodide (PI), 10 kU/ml RNase (Sigma), and 0.005% Triton-100 in the dark for 30 min at room temperature before flow cytometric analysis. The samples were detected with FASC Calibur (Becton, Dickinson and Company, USA). A minimum of 10 000 cells analyzed in each sample served to determine the percentages of cells in each phase of the cell cycle using WinDMI 2.9 and Cylchred 1.0 softwares. Three independent experiments were performed.

Microscopical detection of apoptosis

Hela cells from exponential phase cultures were seeded on 6 mm slide in tissue culture dishes, 24 h later, exposed to $^{12}C^{6+}$-irradiation or γ-irradiation and further cultured for an additional 24, 72, and 120 h. The cells grown on 6 mm slide were washed twice with PBS and fixed for 30 min in 4% buffered paraformadelhyde at room temperature, washed with PBS, and then stained with DAPI (4', 6-diamidine-2-phenylindole dihydrochloride) (Cat No: D212-10, Dojindo, China) (0.1 μg/ml), incubated for 10 min, and washed again with PBS for 5 min. Cells were examined by fluorescence micros-

Table 1. The cell cycle alteration induced by $^{12}C^{6+}$ beam or γ-ray on day 1 (%)

Dose (Gy)	G_0/G_1		S		G_2/M	
	$^{12}C^{6+}$	γ-ray	$^{12}C^{6+}$	γ-ray	$^{12}C^{6+}$	γ-ray
0	29.1±0.98	29.1±0.98	56.3±2.12	56.3±2.12	14.6±0.45	14.6±0.45
0.5	51.2±1.57^{*,a}	44.6±1.64^{a}	34.2±1.15^{*,a}	37.3±1.24^{a}	14.6±0.87	13.6±0.76
1.0	52.6±2.01^{*,a}	45.4±1.56^{a}	28.4±1.26^{*,a,b}	36.8±2.15^{a}	19.0±0.54^{a,b}	17.8±0.48^{a,b}
2.0	58.4±1.76^{*,a,b,c}	46.4±1.36^{a}	28.4±1.16^{*,a,b}	38.2±1.42^{a}	13.2±0.68^{C}	17.8±1.03^{a,c}

Data represent mean ± SD from triplicate experiments. The significance of differences between radiated groups and controls was determined by Student's *t*-test. $^{*}p<0.05$, *vs* the same dose γ-ray irradiation; $^{a}p<0.05$, *vs* 0 Gy irradiation; $^{b}p<0.05$, *vs* 0.5 Gy irradiation; $^{c}p<0.05$, vs 1.0 Gy irradiation.

Table 2. The cell cycle alteration induced by $^{12}C^{6+}$ beam or γ-ray on day 3 (%)

Dose (Gy)	G_0/G_1		S		G_2/M	
	$^{12}C^{6+}$	γ-ray	$^{12}C^{6+}$	γ-ray	$^{12}C^{6+}$	γ-ray
0	44.1±0.78	44.1±0.78	46.5±1.12	46.5±1.12	9.4±0.36	9.4±0.36
0.5	53.3±1.89*,a	45.7±1.58	38.6±1.25*,a	44.4±1.15	8.1±0.58	9.4±0.46
1.0	54.8±1.95*,a	43.5±1.27	34.7±1.14*,a,b	46.5±1.32	10.5±0.65	9.9±0.39
2.0	56.2±1.24*,a	45.4±1.41	34.2±1.21*,a,b	49.4±1.36b	9.6±0.45*	5.2±0.81

Data represent mean ± SD from triplicate experiments. The significance of differences between radiated groups and controls was determined by Student's *t*-test. *p<0.05, *vs* the same dose γ-ray irradiation; ap<0.05, *vs* 0 Gy irradiation; bp<0.05, *vs* 0.5 Gy irradiation; cp<0.05, vs 1.0 Gy irradiation.

Table 3. The cell cycle alteration induced by $^{12}C^{6+}$ beam or γ-ray on day 5 (%)

Dose (Gy)	G_0/G_1		S		G_2/M	
	$^{12}C^{6+}$	γ-ray	$^{12}C^{6+}$	γ-ray	$^{12}C^{6+}$	γ-ray
0	44.1±0.68	44.1±0.68	47.5±1.23	47.5±1.23	8.4±0.46	8.4±0.46
0.5	45.8±0.89*,a	45.7±1.34	43.7±1.34*	46.8±1.35	10.5±0.47*,a	7.0±0.34
1.0	58.9±1.84*,a,b	43.5±1.17	24.9±0.98*,a,b	45.8±1.45	16.2±0.72*,a,b	11.9±0.59a,b
2.0	50.0±1.71*,a,b,c	45.4±1.24	34.0±1.09*,a,b,c	41.8±1.41a,b	16.0±0.56*,a,b	12.0±0.72a,b

Data represent mean ± SD from triplicate experiments. The significance of differences between radiated groups and controls was determined by Student's *t*-test. *p<0.05, *vs* the same dose γ-ray irradiation; ap<0.05, *vs* 0 Gy irradiation; bp<0.05, *vs* 0.5 Gy irradiation; cp<0.05, vs 1.0 Gy irradiation.

cope (Olympus) at 100x magnification using the appropriate DAPI filter. Apoptotic cells were morphologically defined by cytoplasmic and nuclear shrinkage and chromatin condensation or fragmentation. The percentage of apoptotic cells was calculated as a proportion of 250 total cells visualized in 10 different fields of each slide per experiment.

Cell viability

The cell viability was determined by clonogenic assay. After the irradiation, the cells were trypsinized, serial dilutions performed, and known numbers of cells were replanted in 60 mm culture dishes. In each of at least three independent experiments, three dilutions were made per radiation dose. Colonies were counted by staining with 0.4% Giemsa in PBS after 10 days and each point at survival curve was normalized against the plating efficiency of control cells. The survival fractions (SF) as a function of the radiation dose (D) were fitted to the linear quadratic (LQ) mathematical model described by the equation $SF=\exp(-\alpha D-\beta D^2)$.

Statistic Analysis

Results are expressed as means ± SEM for (n) experiments. The difference in response between different treated groups was evaluated using the Student's *t*-test with SPSS software. Statistical significance was defined as $p<0.05$.

Results and Discussion

The cell cycle alterations in human cervix adenocarcinoma cells induced by low-dose (0-2 Gy) $^{12}C^{6+}$ beam and γ-ray are shown in Table 1, 2, 3. The numbers of G_0/G_1 phase cells in the irradiated groups are significantly more than those in the control ($p<0.05$). There is a dose-related increase G_0/G_1 cells in all the treated groups compared to controls. Generally speaking, in normal cells, irradiation-induced DNA damage results in cell cycle arrest, allowing repair of DNA damage prior to cell division, during this process, the p53 protein is stabilized and induces cell cycle arrest at the G_1/S boundary through transcription of the cyclin-dependent kinase inhibitor CDKN1A (p21) and 14-3-3s (8-10), in heavily damaged cells, p53 can induce apoptosis. P53 has also been proposed to contribute to the maintenance of G_2 arrest after DNA damage (10-11). The gatekeeper gene p53 is the most commonly altered gene in human neoplasms. It inhibits G_1/S phase transition by accumulating p21, which inactivates the cyclin kinases necessary for initiating DNA synthesis (12). Despite the reports that p53 mutations have been found to be rare in cervical neoplasms (13-15), the oncogenic protein E6 of HPV binds to p53 so that the p53 is inactivated and degraded through the ubiquitin pathway (16-18). The inactivation of the p53 by HPV E6 probably plays a crucial role in the development of cervical cancer (19-21). Our data indicated that both $^{12}C^{6+}$ beam and ^{60}Co γ-ray could significantly lead cell cycle checkpoints (including G_1 arrest and G_2 arrest) in Hela cells. Moreover, the cell cycle alteration by $^{12}C^{6+}$ beam were more severe than that by ^{60}Co γ-ray (Table1, 2, 3), for example, on day 1, 3, there are 51.2-58.4% G_0/G_1 cells in the $^{12}C^{6+}$-irradiated groups and only 43.5-46.4% G_0/G_1 cells in the cells exposed to ^{60}Co γ-ray; on day 5, the numbers of G_0/G_1 cells in the 1.0, 2.0 Gy $^{12}C^{6+}$-irradiated groups were 5-15% more than those in the cells which received 1.0, 2.0 Gy γ-ray. The G_2/M arrest by $^{12}C^{6+}$ ion occurs later than those by γ-ray (Table1, 2, 3), for instance, on day 1, the G_2/M cells in 2.0 Gy $^{12}C^{6+}$-irradiated group is significantly less than that in the same dose γ-irradiated group ($p<0.05$), and on day 3, the G_2/M cells in 2.0 Gy $^{12}C^{6+}$-

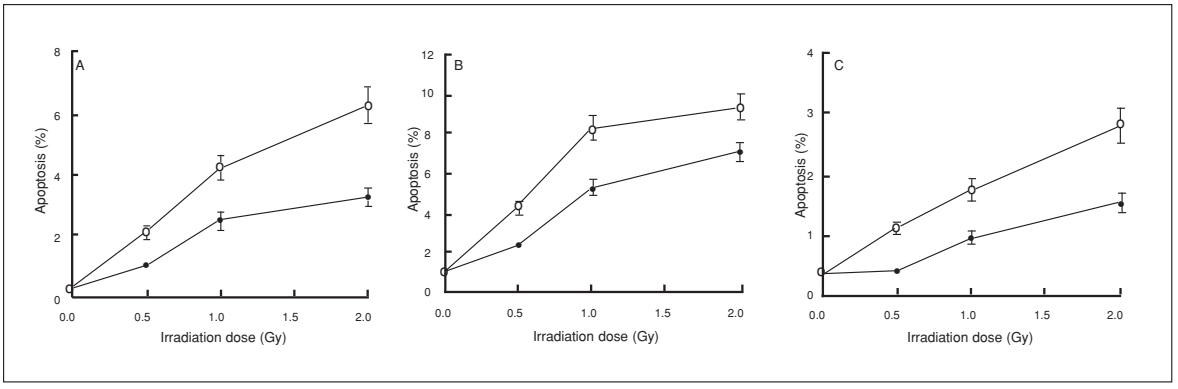

Figure 1. Dose-response time-course of low-dose $^{12}C^{6+}$ beam or γ-ray-induced apoptosis in Hela cell lines. Cell cultures were grown for 24 h (A), 72 h (B), and 120 h (C). ■ γ-ray irradiation; □$^{12}C^{6+}$ ion beam. Typical morphological changes associated with apoptosis were recognized using DAPI staining and visualized by fluorescence microscopy analysis. The number of apoptotic cells were quantiated at the indicated time points after radiation. Data represent mean ±SD from triplicate experiments.

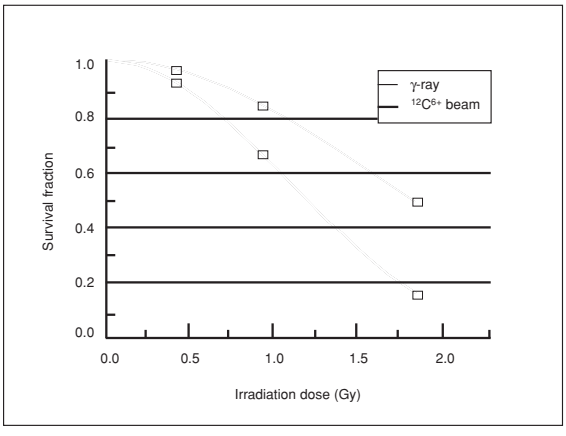

Figure 2. Clonogenic radiosensitivity of the Hela cell line. Survival data as a function of radiation treatment dose (0, 0.5, 1.0 and 2.0 Gy) were fitted to the LQ model described by the equation SF=exp (-αD-βD2).

irradiated group is significantly more than that in the same dose γ-irradiated group (p<0.05); on day 5, the number of G_2/M cells exposed to $^{12}C^{6+}$ beam were significant more than those exposed to γ-ray (p<0.05). It may be possible that there is an interaction between the DNA damage and a block in cell cycle, especially in irradiation of $^{12}C^{6+}$ beam which induced more irrepairable break in DNA than ^{60}Co γ-ray. The majority of heavily damaged cells was arrested in the G_2 phase and cannot proceed to mitosis or meiosis. This effect will be helpful for continuous radiotherapy because the cells in G_2 phase are sensitive to radiation.

Our results also showed that there are dose-dependent increases in the cell apoptosis induced by $^{12}C^{6+}$ ion and ^{60}Co γ-ray, this effect was low-dose dependent manner (Figure 1A-C). The cell apoptosis by $^{12}C^{6+}$ ion is significantly more than that by ^{60}Co γ-ray (p<0.05). The survival curves of Hela cells by $^{12}C^{6+}$ ion and by ^{60}Co γ-ray are shown in Figure 2. Our data suggested that the $^{12}C^{6+}$ ion irradiation has a greater effect on cell viability than the ^{60}Co γ-ray irradiation does (p<0.05). The mechanism of decrease in survival fraction may be through the cell cycle blocks and cell apoptosis by p53-independent mechanisms of p21 induction, for example, activation of Map kinase pathway and cytokines and inhibitory concentrations of growth factors like EGF (22,23).

In conclusion, $^{12}C^{6+}$ beam irradiation significantly leads to cell cycle checkpoint blocks, promotes cell apoptosis compared with ^{60}Co γ-ray. Our results may offer useful information for assessment of biological effects of heavy-ion radiation in tumor. Heavy ions with high LET have a greater RBE, and a greater effect on cells and biological molecules than the conventional radiation does. Hence, there is a need for further investigation of the mechanism of cell cycle and apoptosis alteration induced by heavy-ion.

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References

1. Parkin DM, Freddie B, Ferlay J, Pisani P. Global Cancer Statistics, 2002. CA Cancer J Clin. 2005;55:74-108.
2. Ryu HS, Chang KH, Yang HW et al. High cyclooxygenase-2 expression in stage IB cervical cancer with lymph node metastasis or parametrial invasion. Gynecol Oncol. 2000;76:320-5.
3. Brinton LA. The epidemiology of human papillomavirus and cervical cancer, in Epidemiology of cervical cancer-Overview. Lyon, 1992;Vol.119, p3-23.
4. Kamada T, Tsujii H, Tsuji H et al. Efficacy and safety of carbon ion radiotherapy in bone and soft tissue sarcomas. J Clin Oncol. 2002;20:4466-477.
5. Nakano T, Suzuki M, Abe A et al. The phase I/II clinical study of carbon ion therapy for cancer of the uterine cervix Cancer. J Sci Am 1999;5:362-9.
6. Schulz-Ertner D, Nikoghosyan A, Thilmann C et al. Carbon ion radiotherapy for chordomas and low-grade chondrosarcomas of the skull base. Results in 67 patients. Strahlenther Onkol. 2003;179:598-605.

7. Schulz-Ertner D, Nikoghosyan A, Thilmann C et al. Results of carbon ion radiotherapy in 152 patients. Int J Radiat Oncol Biol Phys 2004;58:631-40.
8. Gudkov AV. and Komarova EA. The role of p53 in determining sensitivity to radiotherapy. Nat Rev Cancer. 2003;3:117-29.
9. Bunz F, Dutriaux A, Lengauer C et al. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science. 1998;282:1497-501.
10. Chan TA, Hermeking H, Lengauer C et al. 14-3-3 Sigma is required to prevent mitotic catastrophe after DNA damage. Nature. 1999;401:616-20.
11. Chan TA, Hwang PM, Hermeking H et al. Cooperative effects of genes controlling the G2/M checkpoint. Genes Dev. 2000;14:1584-8.
12. El-Deiry WS, Harper JW, O'Connor PM et al. WAF1/ClP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res. 1994;54:1169-74.
13. Busby-Earle RM, Steel CM, Williams AR et al. P53 point mutations in cervical carcinogenesis: low frequency and lack of correlation with human papillomavirus status. Br J Cancer. 1994;69:732-86.
14. Milde-Langosch K, Albrecht K, Joram S et al. Presence and persistence of HPV infection and p53 mutation in cancer of the cervix uteri and vulva. Int J Cancer. 1995;63:639-45.
15. Chen TM, Chen CA, Hsieh CY et al. The state of p53 in primary cervical carcinomas and its effects in human 245 cell cycle aberration in SCC of Cervix papilomavirus-immortalized human cervical cells. Oncogene. 1993;8:1511-8. 87.
16. Scheffner M, Werness BA, Huibregtse JM et al. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell. 1990;63:1129-36.
17. Werness BA, Levine AJ, Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science 1990;248:76-9. 90.
18. Cooper K, Herrington S, Evans MF et al. p53 antigen in cervical condylomata, intraepithelial neoplasia, and carcinoma: relationship to HPV infection and integration. J Pathol. 1993;171:27-34.
19. Innocente SA, Abrahamson JL, Cogswell JP, Lee JM. p53 regulates a G2 checkpoint through cyclin B1. Proc Natl Acad Sci USA. 1999;96:2147-52.
20. Agarwal ML, Agarwal A, Taylor WR, Stark GR. p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. Proc Natl Acad Sci USA. 1995;92:8493-7.
21. Turek LP, Smith EM. The genetic program of genital human papilomavirus in infection and cancer. Obstet Gynecol Clin North Am. 1996;23:735-58.
22. Jakus J, Yeudall WA. Growth inhibitory concentrations of EGF induce p21 (WAK1/Cip1) and alter cell cycle control in squamous carcinoma cells. Oncogene. 1996;12:2369-76.
23. Jiang H, Lin J, Su ZZ et al. Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21 WAF1/CIP1 expression in absence of p53. Oncogene. 1994;9:3397-406.