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Original Article

^{125}I inhibited the NSCLC both in vivo and in vitro

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Abstract: Lung cancer is the main reason of cancer-linked death all over the world. Non-small cell lung cancer (NSCLC) patients always have an extremely poor prognosis. It is urgent to find novel treating methods. It was previously showed that ^{125}I brachytherapy had been applied to the lung cancer treatment. However, fundamental researches are limited. In the present study, we first explored the mechanism by which ^{125}I radiation induced arrest or apoptosis of the cell cycle and relevant protein expression. Furthermore, we explored its effect on the invasion. We found that ^{125}I significantly induced cell apoptosis through mitochondrial pathway, triggered S phase arrest via regulating cyclinA2, p21 and CDK6 expressions. Meanwhile, ^{125}I could inhibit invasion of NSCLC cells by altering the expression level of vimentin, N-cadherin and MMP-9. Furthermore, we confirmed the effects of ^{125}I on NSCLC cell growth in vivo. The results indicated that ^{125}I obviously inhibited the tumor growth. Thus, we determined that ^{125}I brachytherapy remarkably restrained NSCLC cellular growth and intrusion by inducing apoptosis, S phase arrest and corresponding protein expression.

Keywords: ^{125}I, NSCLC, apoptosis, radioresistance

Introduction

Lung cancer is well-known as one of the usual as well as aggressive malignancies worldwide. Histopathologically, more than 85% of lung cancers are categorized to be non-small cell lung cancer (NSCLC) [1, 2]. Being short of biomarkers, more than half of lung cancers can only be diagnosed at distant phase which contributes to the poor prognosis. Despite of significant progress made in surgery, radiotherapy as well as chemotherapy, the total survival rate of people with lung cancer was merely 16% for all stages during five years [3, 4]. In spite of the fact that surgery is the sole radical treatment of NSCLC, tumors which may be radically resected are less than 20%. The cure and palliation have made radiotherapy to be a major treatment for lung cancers. Brachytherapy sources of low-energy photons such as those emitted by ^{125}I commonly have been applied to the radiotherapy of prostate cancer.

Radioactive seed implantation is regarded as a popular mode of brachytherapy which shall be conducted many times. It has been employed to remedy inoperable solitary lung cancers with success, while averting peripheral tissue being exposed to overmuch radiation [5, 6]. Radioactive ^{125}I seed interstitial brachytherapy is another way to treat inoperable NSCLC, with its good efficacy, minimum operative wound, and minimum side effects.

Clinical cases of more than two hundred suffers receiving treatment in our institution have illustrated that while employing ^{125}I brachytherapy with sublobar resection, local relapse was 2.0% in stage I NSCLC sufferers and remarkably less than 18.6% for only sublobar resection [7].

Moreover, repeated ^{125}I seed implantations have been good for suffers with multiple relapses, resulting in much longer median as well as progression-free survival time, and much higher overall one-year and two-year survival rates.

The curative effect of ^{125}I seed implantations may be restricted by various elements as well, for example, protecting by bone architecture,

distinctions in brachytherapy projecting mechanisms, as well as personal differences. In general, ^{125}I seed implantation is a valid therapy to treat massive tumors.

In current researches, methods have been carried out in vitro and in vivo to explore the effect of ^{125}I on NSCLC cells. The outcomes of the current study may inform the development of ^{125}I brachytherapy in NSCLC.

Methods and materials

Cell culture

A549 and H1299 cell lines were bought from the Shanghai Institutes for Biological Sciences. Cells were developed in RPMI-1640, replenished with 10% fetal bovine serum (FBS), 100 U/mL penicillins as well as 100 μg/mL streptomycin at 37°C with 5% CO_2.

QPCR

Total RNA was leached by employing TRIzol reagent in accordance with the maker's specification. 1 μg of total RNA was reversely transcribed employing the PrimeScript RT Reagent kit. Small nuclear RNA U6 was regarded as a normalization control. Each test was redone three times. Quantification of miRNA or mRNA was conducted by employing Bestar™ QPCR Master Mix in accordance with the maker's specification. Cyclic situations were as below: pre-degeneration at 95°C for 60 s, 95°C for 5 s, 58°C for 20 s, 40 cycles. Analysis of data was conducted employing the $2^{-\Delta\Delta Ct}$ means.

^{125}I seed irradiation

^{125}I radioactive seeds (BT-125-I) were bought from the Shanghai Xinke Ltd. Obvious radioactivity was 1.00 mCi/seed and half time was 59.4 days. Dosage fractionation was counted by employing a treatment planning system (TPS, Kelinzhong Institute of Atomic Energy, Beijing, China, No. YZB/1466-70-2004) On the basis of the American Association of Physicists in Medicine Task Group No. 43 (AAPM TG-43) formalism [8].

Gray's model of ^{125}I radiation has been carried out [9]. It contains a lower plaque layer which was a seed plaque, with 14 seeds equivalently spaced in recesses situated nearby a 35 mm circumference. The upper cell plaque layer was a cell culture layer. In the cell culture plaque, same recesses were established, around a 35 mm circumference as well. Primary dose rate was 12.13 cGy/h and total doses were 2, 4, 6, and 8 Gy.

MTT

Cell hyperplasia was quantified by measuring of absorbance of hydrosoluble triphenyltetrazolium chloride at 450 nm, employing the Cell Counter Kit-8 as depicted before. Leptocyte (500 cells) were seeded into six-well plates, hatched with DMEM with 10% FBS lasted 15 days. Cells were afterwards fixed with 4% formaldehyde lasted 20 min, stained with crystal violet for approximately 20 min at room temperature, and photographed. The colony-forming ability of every group of cells was estimated through calculating the colonies formed.

Flow cytometry

As to the analysis of cell cycle, cells were collected, rinsed by PBS for two times, afterwards, fixed with 70% ethanol (v/v) all the night at -20°C. Fixed cells were rinsed with PBS for two times, after that resuspended in PBS including PI (50 μg/ml)/RNase A (50 μg/ml) lasted ten min.

As to analyzing cell apoptosis, cells had been harvested and washed like in the cell cycle part, and annexin V-FITC/PI dyeing solution was pippited into 100 μL of cell suspension, which was blended and hatched in the dark for 15 min at ambient temperature. At last, not only cell cycle but also apoptosis were analyzed by employing a FACScan instrument armed with CellQuest software.

TUNEL

After therapy, cells were gathered and apoptosis was decided by employing TdT-mediated dUTP nick end labeling (TUNEL) assay kit (Roche, Basel, Switzerland) in accordance with the maker's instructions.

Transwell

Cells were plated onto the upper well of the chamber in serum free RPMI-1640 medium. The lower chamber was full of RPMI-1640 medium including 10% FBS. Being cultured 48 h later, cells which pierced the membrane were fixed with 4% paraformaldehyde lasted 20 min and dyeing by violet crystalline solution according to the protocol.

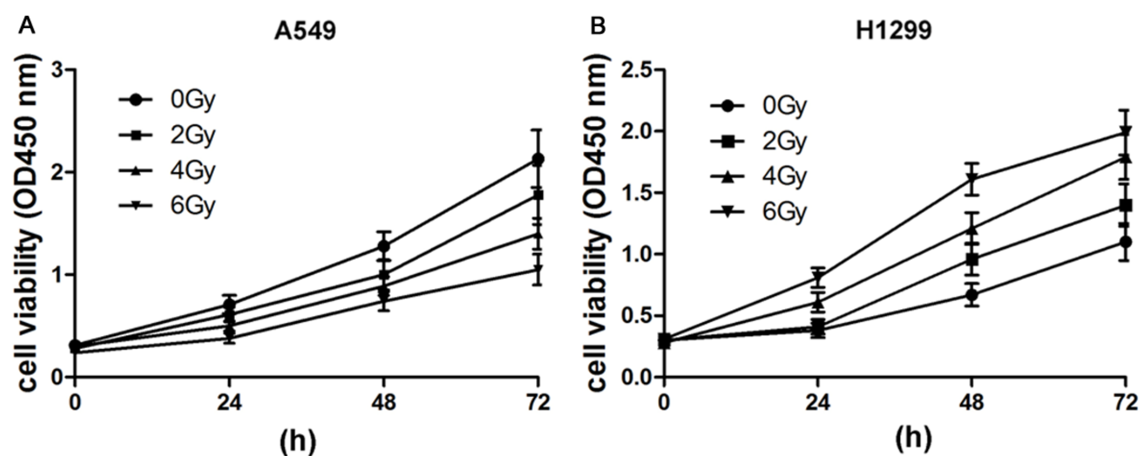

Figure 1. Cell viability of A549 and H1299 cells radiated by different dose of ^{125}I were detected by MTT assay. A. Cell viability of A549 cells radiated by different dose of ^{125}I. B. Cell viability of H1299 cells radiated by different dose of ^{125}I.

Colony forming assay

Cells were seeded into each well of 6-well plate with a density of 500 cells each well. Culture medium was displaced every 3 days. 2 weeks subsequent to seeding, colonies could be counted using crystal violet staining (Weijia; Thermo Fisher Scientific, Inc.) Colonies containing >50 cells were counted.

Western blot

All the protein was leached with RIPA lysis buffer and the concentration of protein was tested by employing nanodrop 2000. 40 μg proteins was separated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then shifted to a PVDF membrane. Subsequently, blots were obstructed with 8% skimmed milk. Membranes were hatched all the night at the temperature of 4°C with first antibodies. Four-time rinsing later, the membranes were hatched with a horseradish peroxidase-conjugated secondary antibody at the temperature of 37°C for 2 h. After washing three times in TBST, protein bands were visualized using chemiluminescence detection.

In vivo study

Female BALB/c nude mice were bought from Charles River (Beijing, China). A549 cells were collected and hypodermically injected into the flanks of the rats. Six weeks subsequent to the injection, mice were then randomized into two groups: radiation group and control group (n = 10 in each group); the mice were anesthetized and one ^{125}I seed with the radio dosage of 0.9 mCi was implanted in the tumor. Every 5 days, the tumor volume was counted using modified ellipsoid formula 1/2 (Length × Width/2). Two weeks subsequent to ^{125}I seed implantation, the mice were sacrificed, tumors were harvested. Half of every tumor was frozen in liquid nitrogen and kept in storage at the temperature of -80°C and the other half was fixed in 4% paraformaldehyde.

Statistic analysis

Student's t-test (2-tailed), one-way ANOVA, and the Mann-Whitney U test had been employed to measure data, accompanied with SPSS 16.0 (IBM, IL, USA). *P*-values of not reaching 0.05 were regarded statistically meaningful.

Results

^{125}I inhibited the NSCLC cell proliferation

After different dose of ^{125}I irradiation, cells were seeded into 96 well plates. MTT was utilized to estimate the proliferation of NSCLC cells at various phases. As **Figure 1** showed, ^{125}I at the dose of 2, 4, 6 Gy all significantly inhibited the proliferation of NSCLC cells which were dose dependently.

^{125}I induced apoptosis of NSCLC cells

After different dosage of ^{125}I irradiation, cells were collected for the Annexin V/PI staining.

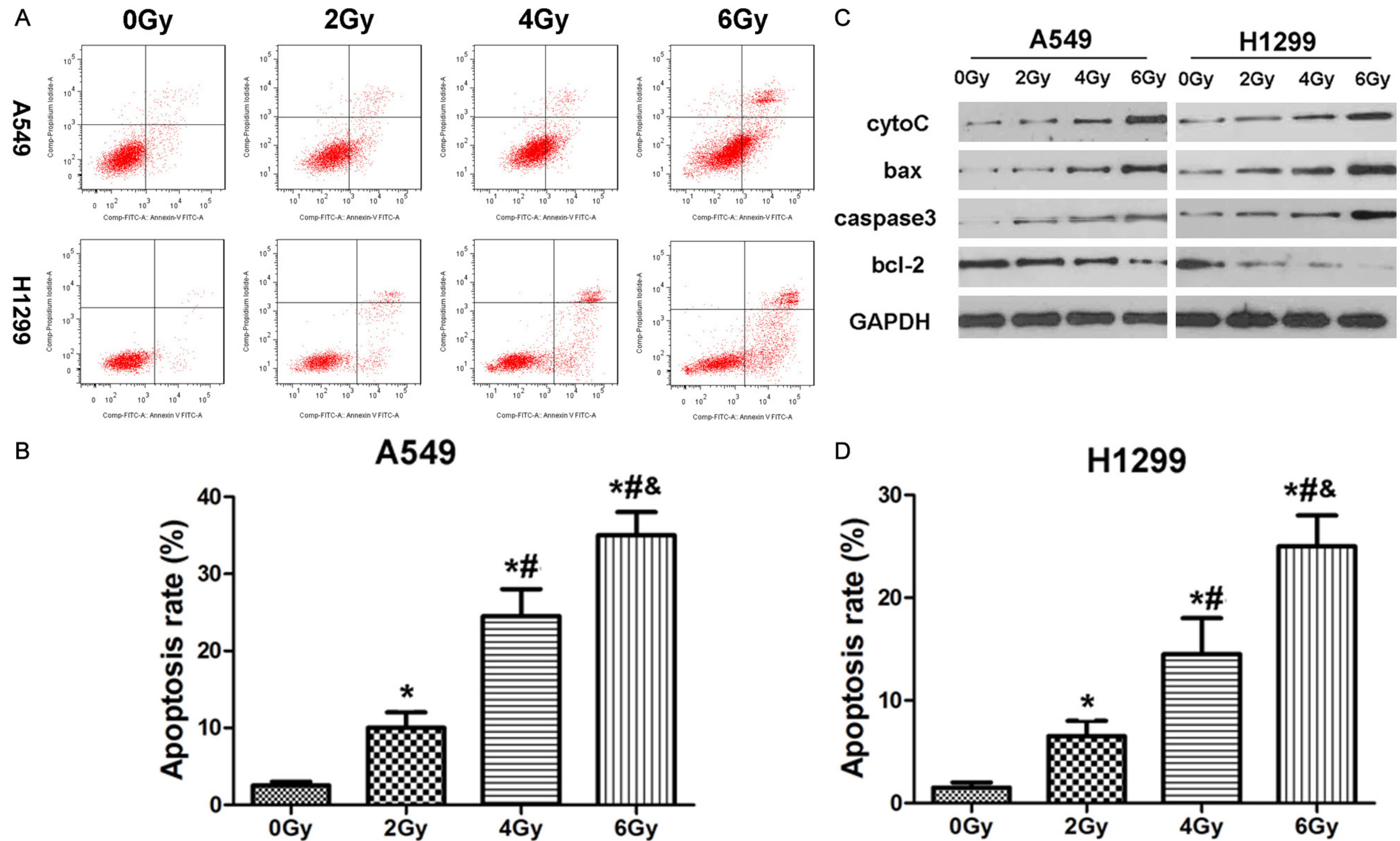

Figure 2. The apoptosis rates and expression of apoptosis relative proteins of NSCLC cells induced by ^{125}I were determined by flow cytometry and Western blotting. A, B, D. The apoptosis rates of A549 and H1299 cells induced by ^{125}I were determined by flow cytometry, respectively. C. The expression of apoptosis relative proteins of NSCLC cells induced by ^{125}I was determined by Western blotting. *P<0.05 vs 0 Gy, $^{\#}P$<0.05 vs 2 Gy, $^{\&}P$<0.05 vs 4 Gy.

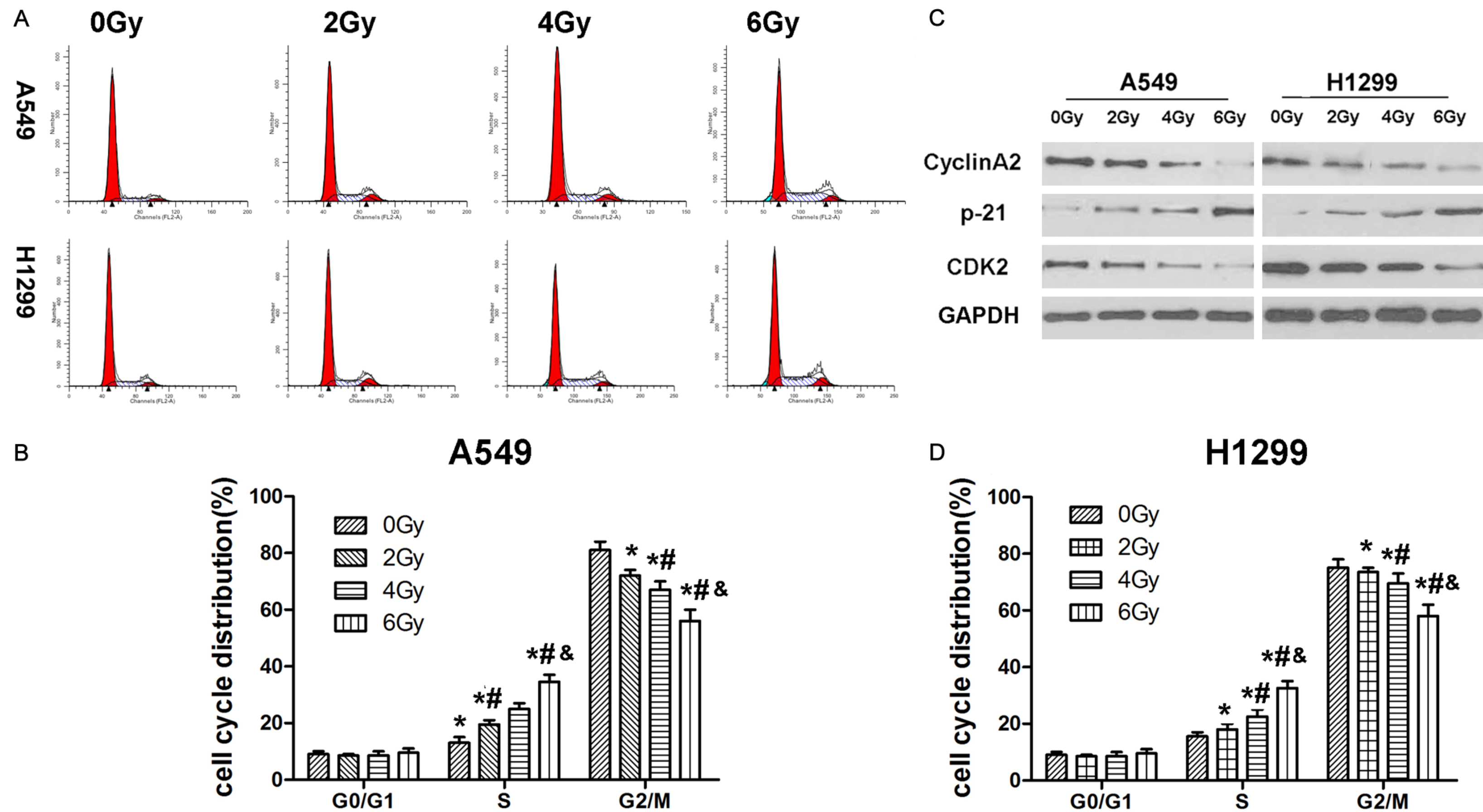

Figure 3. Cell cycle and apoptosis relative proteins of A549 and H1299 cells radiated by different dose of ^{125}I were detected by flow cytometry and Western blotting. A, B, D. The apoptosis rates of A549 and H1299 cells induced by ^{125}I were determined by flow cytometry, respectively. C. The expression of apoptosis relative proteins of NSCLC cells induced by ^{125}I was determined by Western blotting. $^{*}P<0.05$ vs 0 Gy, $^{\#}P<0.05$ vs 2 Gy, $^{\&}P<0.05$ vs 4 Gy.

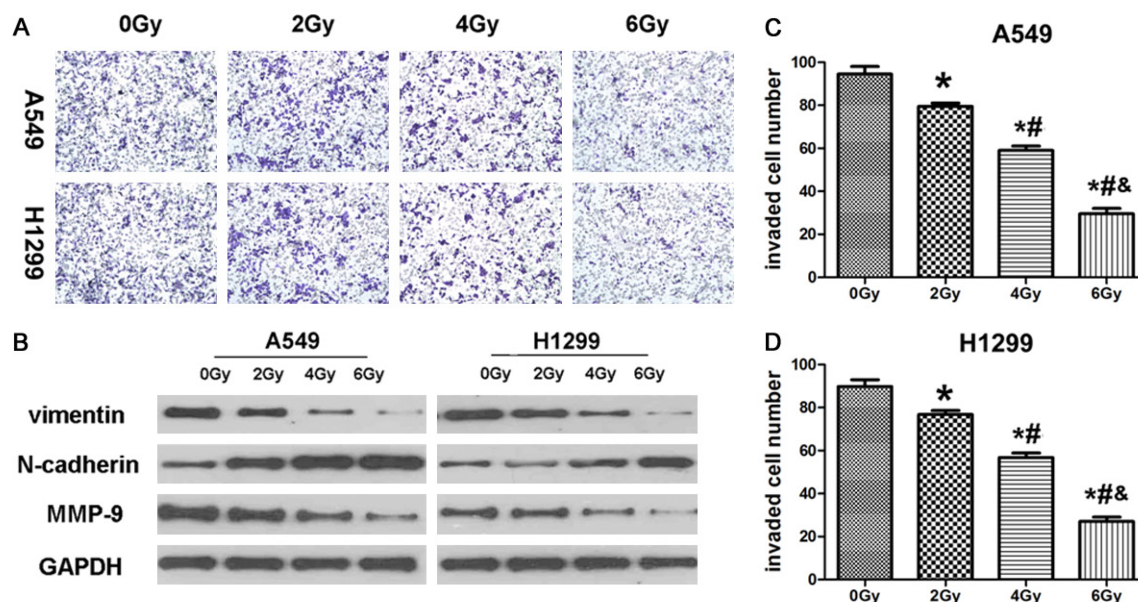

Figure 4. Cell invasion of A549 and H1299 cells radiated by different dose of ^{125}I were detected. A, C, D. Cell invasion of A549 and H1299 cells radiated by different dose of ^{125}I were photographed and calculated. B. The expression level of N-cadherin, vimentin and MMP-9 were detected by Western blotting. *P<0.05 vs 0 Gy, $^{\#}P$<0.05 vs 2 Gy, $^{\&}P$<0.05 vs 4 Gy.

Cell apoptosis were detected employing flow cytometry. The results showed that ^{125}I irradiation of 2, 4, 6 Gy all significantly induced apoptosis of NSCLC cells dose independently (**Figure 2A**, **2B**, **2D**). To further confirm it, we investigated the expression of apoptosis relative proteins. We found that ^{125}I irradiation obviously elevated the level of cytoC, bax, caspase3, but significantly inhibited the expression of bcl-2 (**Figure 2C**).

^{125}I attenuated the cell cycle

After different dose of ^{125}I irradiation, cells were collected for the PI staining. Cell cycle was detected using Flow cytometry. The results revealed that ^{125}I irradiation of 2, 4, 6 Gy all significantly induced S phase arrest of NSCLC cells dose independently (**Figure 3A**, **3B**, **3D**). Then we investigated the expression of apoptosis relative proteins. We found that ^{125}I irradiation obviously elevated the level of p21, but significantly inhibited the expression of cyclinA1 and CDK2 (**Figure 3C**).

^{125}I inhibited the invasion of NSCLC cells

After different dose of ^{125}I irradiation, cells were seeded in 12 well plates. Transwell assay were employed to determine the change on intrusion ability of NSCLC cells. The results indicated that ^{125}I irradiation inhibited the invasion ability significantly in NSCLC cells which was dose independently (**Figure 4A**, **4C**, **4D**). Meanwhile, ^{125}I irradiation elevated the expression level of N-cadherin, but reduced the level of vimentin and MMP-9 (**Figure 4B**).

^{125}I altered the growth of NSCLC cells

We further detected the effect of ^{125}I irradiation on the growth of NSCLC cells in vivo. In consistent with what we found in vitro, ^{125}I irradiation significantly attenuated the tumor growth (**Figure 5A-C**).

Discussion

NSCLC treatments are decided by the variety and stage of carcinoma, which include surgical operation, external beam radiation therapy (EBRT), chemotherapy, as well as high-dose-rate endobronchial brachytherapy (HDR-EBBT). Surgical operation is normally a kind of therapy for localized cancers, but merely nearly 20% of all suffers with NSCLC can be candidates for possibly curative excision.

Moreover, chemotherapeutics and EBRT have not played an important role in results of suf-

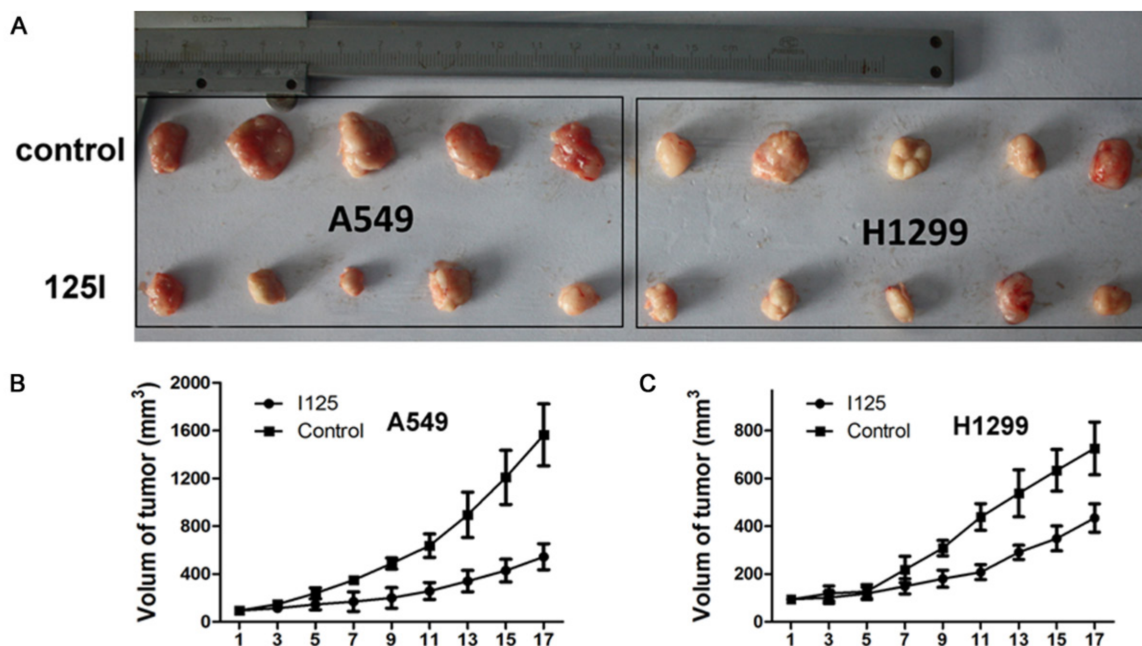

Figure 5. The tumor volume was measured in BALB/c nude mice with ^{125}I seed implantation. A. The picture of the tumor of A549 or H1299 cells from ^{125}I and control group. B, C. The tumor volume was measured in BALB/c nude mice injected with A549 or H1299 cells and implanted with ^{125}I.

fers with unresectable illness, and the gain often comes with a large number of toxicity, in particular for those who have other complicated diseases [10]. Employing external beam radiotherapy (EBRT) only, it is hard to send abundant radiation dosage to sufferers with massive or terminal tumors, for fear of injury to surrounding normal tissues. Repeated EBRT has restricted curative effect for these suffers and is connected with gradually enhanced complications due to low reliability of adjacent non-tumor normal tissue [11, 12].

A prospective study shows that 14 suffers with primary NSCLC received treatments with intraoperative ^{125}I implantation of the lung tumor through lateral incision or median sternotomy. Staging mediastinal node dissection was performed in every clinical case. Suffers were chosen while wedge or partial excisions had not been technically viable. The lobectomy or completion pneumonectomy shall be needed or lung function researches were limited. The range of doses is from 8,000 cGy in the surrounding area to 20,000 cGy centrally. Follow-up a minimal twelve months, median survival period was 16.7 and 15.1 months. Decentralized control was acquired from 10 of 14 suffers (71%) with partial faults happening in stage III suffers of pathology. When separated in accordance with the size of tumor, local control was obtained in 6 of 7 tumors of less than 3 cm and 4 of 5 tumors of 3-5 cm. Both cases with masses larger than 5 cm lose efficacy locally. There was one death and two complications after operation. The other suffers were discharged from hospital in one week of surgical operation. Not any radiation pneumonitis occurs. ^{125}I lung brachytherapy has been a remarkable replacement therapy to T1 and T2 tumors when medical conditions preclude curative resection [13]. As to tumors >5 cm, it is hard to deliver a large enough dose with EBRT for fear of destroying surrounding normal tissues like the medulla spinalis, stomach, as well as heart [14].

In contrast, ^{125}I seed implantation is likely to send a higher radiation dosage to a neoplastic block (100-140 Gy), and this dose decreased dramatically just in a short range. In consequence, surrounding normal tissues are in exposure to a minimum dosage of radiation, and the short-run venture of severe radiation-mediated pneumonitis is decreasing [15].

In our study, we determined that ^{125}I brachytherapy significantly inhibited NSCLC cell growth in vitro and in vivo through causing apoptosis and S phase arrest. ^{125}I brachytherapy is a useful and well tolerated approach to the treat-

ment for low grade astrocytomas. The risk is low, which is especially important for children and elder patients [16]. However, further research is needed illustrating the deep mechanism involved in the effect of ^{125}I.

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Disclosure of conflict of interest

None.

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References

[1] Tamminga M, Groen HH,Hiltermann TJ. Investigating CTCs in NSCLC-a reaction to the study of Jia-Wei Wan: a preliminary study on the relationship between circulating tumor cells count and clinical features in patients with non-small cell lung cancer. J Thorac Dis 2016; 8: 1032-1036.

[2] Planchard D, Yokoi T, McCleod MJ, Fischer JR, Kim YC, Ballas M, Shi K, Soria JC. A phase III study of durvalumab (MEDI4736) with or without tremelimumab for previously treated patients with advanced NSCLC: rationale and protocol design of the ARCTIC study. Clin Lung Cancer 2016; 17: 232-236.

[3] Wen CT, Fu JY, Wu CF, Hsieh MJ, Liu YH, Wu YC, Tsai YH, Wu CY. Survival impact of locoregional metachronous malignancy in survival of lung cancer patients who received curative treatment. J Thorac Dis 2016; 8: 1139-1148.

[4] Moumtzi D, Lampaki S, Zarogoulidis P, Porpodis K, Lagoudi K, Hohenforst-Schmidt W, Pataka A, Tsiouda T, Zissimopoulos A, Lazaridis G, Karavasilis V, Timotheadou H, Barbetakis N, Pavlidis P, Kontakiotis T, Zarogoulidis K. Prognostic factors for long term survival in patients with advanced non-small cell lung cancer. Ann Transl Med 2016; 4: 161.

[5] Wang ZM, Lu J, Liu T, Chen KM, Huang G, Liu FJ. CT-guided interstitial brachytherapy of inoperable non-small cell lung cancer. Lung Cancer 2011; 74: 253-257.

[6] Johnson M, Colonias A, Parda D, Trombetta M, Gayou O, Reitz B, Miften M. Dosimetric and technical aspects of intraoperative I-125 brachytherapy for stage I non-small cell lung cancer. Phys Med Biol 2007; 52: 1237-1245.

[7] Santos R, Colonias A, Parda D, Trombetta M, Maley RH, Macherey R, Bartley S, Santucci T, Keenan RJ, Landreneau RJ. Comparison between sublobar resection and 125Iodine brachytherapy after sublobar resection in high-risk patients with stage I non-small-cell lung cancer. Surgery 2003; 134: 691-697; discussion 697.

[8] Furhang EE, Anderson LL. Functional fitting of interstitial brachytherapy dosimetry data recommended by the AAPM radiation therapy committee task group 43. American association of physicists in medicine. Med Phys 1999; 26: 153-160.

[9] Benchimol A, Marsh CA, Desser KB. Discordant left ventricular pressure and apexcardiographic pulsus alternans. Chest 1975; 67: 477-479.

[10] Marino P, Preatoni A, Cantoni A. Randomized trials of radiotherapy alone versus combined chemotherapy and radiotherapy in stages IIIa and IIIb nonsmall cell lung cancer. A meta-analysis. Cancer-Am Cancer Soc 1995; 76: 593-601.

[11] Wang JJ, Yuan HS, Li JN, Jiang YL, Tian SQ, Yang RJ. CT-guided radioactive seed implantation for recurrent rectal carcinoma after multiple therapy. Med Oncol 2010; 27: 421-429.

[12] Reveiz L, Rueda JR, Cardona AF. Palliative endobronchial brachytherapy for non-small cell lung cancer. Cochrane Database Syst Rev 2012; 12: D4284.

[13] Fleischman EH, Kagan AR, Streeter OE, Tyrell J, Wollin M, Leagre CA, Harvey JC. Iodine125 interstitial brachytherapy in the treatment of carcinoma of the lung. J Surg Oncol 1992; 49: 25-28.

[14] Li W, Guan J, Yang L, Zheng X, Yu Y, Jiang J. Iodine-125 brachytherapy improved overall survival of patients with inoperable stage III/IV non-small cell lung cancer versus the conventional radiotherapy. Med Oncol 2015; 32: 395.

[15] Reveiz L, Rueda JR, Cardona AF. Palliative endobronchial brachytherapy for non-small cell lung cancer. Cochrane Database Syst Rev 2012; 12: D4284.

[16] Hawliczek R, Grunert P, Ferraz-Leite H, Alesch F. [The technique of stereotactic I125 brachytherapy (permanent implants) of brain tumors in Vienna]. Strahlenther Onkol 1990; 166: 647-649.