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Long-term low-dose α-particle enhanced the potential of malignant transformation in human bronchial epithelial cells through MAPK/Akt pathway

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ABSTRACT

Since the wide usage of ionizing radiation, the cancer risk of low dose radiation (LDR) (<0.1 Gy) has become attractive for a long time. However, most results are derived from epidemiologic studies on atomic-bomb survivors and nuclear accidents surrounding population, and the molecular mechanism of this risk is elusive. To explore the potential of a long-term LDR-induced malignant transformation, human bronchial epithelial cells Beas-2B were fractionally irradiated with 0.025 Gy α-particles for 8 times in total and then further cultured for 1–2 months. It was found that the cell proliferation, the abilities of adhesion and invasion, and the protein expressions of p-ERK, p-Akt, especially p-P38 were not only increased in the multiply-irradiated cells but also in their offspring 1–2 months after the final exposure, indicating high potentiality of cell malignant transformation. On opposite, the expressions of p-JNK and p-P66 were diminished in the subcultures of irradiated cells and thus may play a role of negative regulation in canceration. When the cells were transferred with p38 siRNA, the LDR-induced enhancements of cell adhesion and invasion were significantly reduced. These findings suggest that long-term LDR of α-particles could enhance the potential of malignant transformation incidence in human bronchial epithelial cells through MAPK/Akt pathway.

1. Introduction

After Rötgen's discovery of X-rays in 1895, it was demonstrated that radiation exposure could cause tissue damage and even induce cancer formation. Till now there is little doubt that most of the major types of cancers can be induced by acute radiation with doses as low as 0.2–0.5 Gy. In particular, a recent epidemiologic study on 680,000 children or adolescents received CT scans and 10,000,000 with no record of such exposures reported that the cancer risk could be heightened after pediatric CT scans so that extra cancer incidences, especially leukemia and brain cancers, were observed [1]. Accordingly, there is a pressing need in the information of the health effect of cumulative low-dose or low dose-rate exposures.

Radioactive noble gas radon and its degradation progeny are ubiquitous in the environment and the residential radon exposure is inevitable for daily life. Long term low dose radiation (LDR) of α-particle from radon was classified as a known pulmonary carcinogen in humans in 1988 by the International Agency for Research on Cancer. Since then, substantial evidence from many studies has consistently shown a significant relationship between the radon exposure and the risk of lung cancer [2]. Radon exposure has been considered the secondary cause of lung carcinoma and the first place in never smokers. Moreover, lung is also a potential target for radiation exposures during medical treatment [3], occupational radiation exposure, nuclear power accident, manned space exploration [4], and in the case of nuclear and radiological terrorism. Most importantly, lung is the primary target organ to inhalation of radionuclides. In the nuclear reactor workers and atomic-bomb survivors, approximately one third of the cancers ascribed to radiation were the lung cancer [5,6]. Therefore, the lung cancer has become the first cause of cancer mortality in the world [7]. But until now, there is no effective way to reduce the lung cancer mortality. In order to have better radiation protection efficiency, it is necessary to elucidate the pathogenesis of radiation-induced lung cancer, including the molecular mechanisms of α-particle induced lung cancer.

It was reported that human bronchial epithelial cells could be malignantly transformed by a single 0.3 Gy dose of α-particles [8] and the exposure of α-particles at a single dose of 0.6 Gy could

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induce the potential of malignant transformation in human benign prostate epithelial cells [9]. It has known that some genes participates in the progress of malignant transformation, such as p53 [9] and c-Myc [10]. But with respect to radiation with doses lower than 0.05 Gy, very few literatures are available for the effects of long-term LDR of α-particle on normal human cells, although the harmful effects of which should not be ignored. In this study, we investigated the mechanism of long-term LDR of α-particle in inducing cell malignant transformation and found that the MAPK/Akt pathway may be involved in the long-term LDR promoted cell invasion.

2. Materials and methods

2.1. Cell culture

Human bronchial epithelial cell line, Beas-2B cells, was an gift from Nanjing Medical University and it was maintained in DMEM (HyClone, Beijing, China) containing 5% fetal bovin serum (FBS) (Gibco Invitrogen, Grand Island, NY, USA) and supplemented with penicillin (100 U/mL), streptomycin (100 lg/mL) and cultured in a fully humidified incubator with 5% CO_2 at 37 °C.

2.2. Cell irradiation

A ^{241}Am α-particle plate source (Atom High Tech Co., Ltd., Beijing, China) was applied for the cell irradiation. The characteristic of this α-particle has been described elsewhere [11]. Because of the limited range of α-particles, cells were grown on a 2.5 μm thickness Mylar film based dish to allow the penetration of α-particles from the bottom of the dish. For the long-term radiation, cells were fractionally irradiated with 0.025 Gy of α-particles for 8 times, once every 4 days. After each irradiation, cells were reseeded to grow on a Petri-dish. Cells after the final irradiation were continuously cultured for 1 or 2 months.

2.3. Cell proliferation assay

Cell proliferation was determined with the Cell Counting Assay Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). After each treatment, cells in the logarithmic phase were seeded into a 96-well microplate at 2×10^3 cells/well. The details have been described in our previous report [12]. This CCK-8 assay was repeated triply and each trial was performed in six wells.

2.4. Cell adhesion assay

A 24-well plate was coated with 200 μl Collagen I (10 μg/ml) overnight, washed with PBS triply and blocked with 200 μl blocking buffer (3% BSA by the thermal denaturation) at room temperature for 60 min followed by washing with PBS. Then, 5×10^4 cells in 200 μl suspension were added into each well. The wells containing DMEM were used as background control. After 30 min, all wells were washed with PBS then fixed with 4% paraformaldehyde for 30 min and stained with crystal violet for 20 min. After further washing, 200 μl 10% acetic acid were added into each well and shook for 5 min. At last, the absorbance (A) at 570 nm was recorded with the plate reader. Relative absorbance rate was defined as the same method with cell proliferation assay. Experiments were performed in triplicate and repeated at least three times.

2.5. Cell invasion assay

Transwell chambers (Costar, Corning Inc., USA) with polycarbonate filters containing a plenty of 8-μm pores were used in cell Matrigel invasion assay. The upper chamber was coated with Matrigel (BD Co., USA) and then hydrated with serum-free medium as described by He [13]. Subsequently, 2×10^5 Beas-2B cells (or p38 siRNA transferred Beas-2B cells) which had been maintained in serum-free DMEM for 24 h were added into the upper chamber. The lower chamber was filled with 500 μl DMEM containing 10% FBS as chemo-attractant. With an additional 24 h of incubation at 37 °C, the total number of invaded cells was counted under a microscope [13].

2.6. Transfection of cells with p38 siRNA

Gene silencing of p38 was performed as described previously [14]. p38 siRNA duplex [5′-CAAAUUCUCCGAGGUCUAA (dTdT)-3′] was synthesized and purified by Shanghai GenePharma Co., Ltd. (Shanghai, China) [15]. In brief, 3×10^5 cells were incubated in DMEM for one day and the cells under approximately 30–40% confluence were transfected with p38 siRNA or its scrambled siRNA control using Lipofectamine 2000 (Invitrogen). One day later, the adhesion and invasion abilities of the p38-transfected cells were measured as described above.

2.7. Western blotting assay

Phospho-ERK (p-ERK), phospho-P38 (p-P38), phospho-JNK (p-JNK) and phospho-Akt (p-Akt) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Phospho-P66 (p-P66) antibody was purchased from Abcam (Abcam, MA, USA). Cell lysate was prepared and applied for protein Western-blot assay as described by He [16]. The protein bands were visualized using the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA). The protein level was measured using the Quantity One software (Bio-Rad Laboratories).

2.8. Statistical analysis

All results were presented as the means ± standard errors (SE) of the data obtained from at least three independent experiments. Student's *t*-test or the Mann–Whitney test of the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) were used for statistical analysis. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Long-term LDR of α-particles enhanced cell proliferation

The CCK-8 assay revealed that the proliferation of human bronchial epithelial cells was enhanced after long-term LDR. When Beas-2B cells were fractionally irradiated with 0.025 Gy/time, once every 4 days for 8 times in total, the cell proliferation rate was increased to 1.2-fold of the non-irradiated control. When the irradiated cells were continually cultured for 1 or 2 months after the final irradiation, the cell proliferation rates were still maintained at high levels compared to the non-irradiated control (Fig. 1).

3.2. Long-term LDR of α-particles enhanced cell adhesion and invasion

Over-proliferation is one characteristic of cell malignant transformation. Other two index properties of cell malignant transformation i.e., adhesion and invasion, were also measured. Fig. 1B illustrates that the cell adhesive ability was increased by 1.2-fold after the long-term LDR. With subsequent culture for 1–2 months after the final irradiation, the cell adhesive ability was still kept at this high level. Moreover, this long term LDR also enhanced the invasiveness of irradiated cells. Fig. 1C gives representative

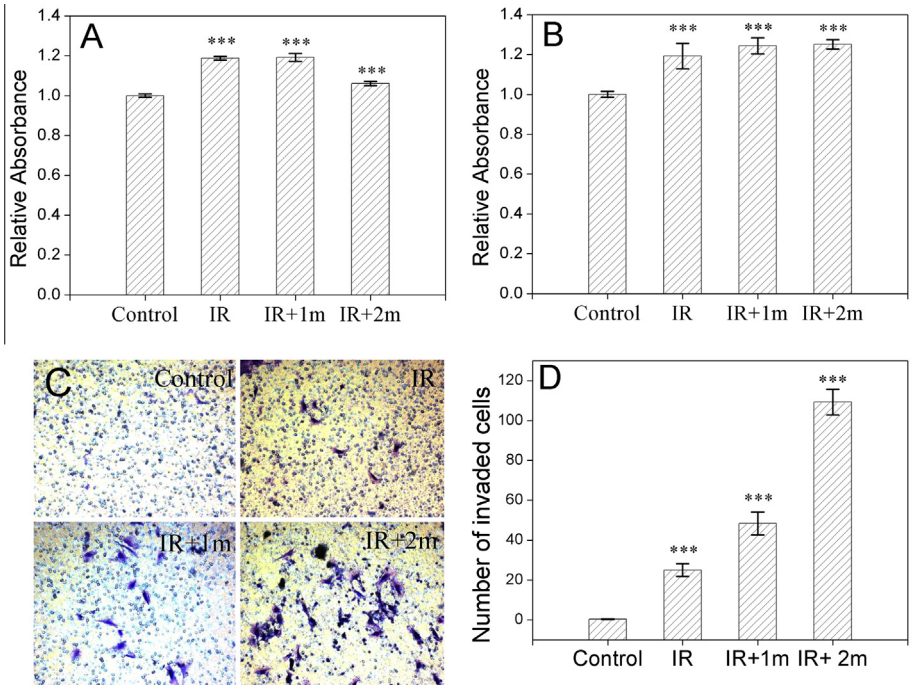

Fig. 1. Radiation effects on human bronchial epithelial cells. The cells were fractionally irradiated with 0.025 Gy for 8 times and continuously cultured for 1 and 2 months. (A) Proliferations of the irradiated cells and its offspring. (B) The adhesive abilities of the irradiated cells and its offspring. (C) Representative images of the cells passed through the Matrigel to the outside bottom of chamber. (D) The number of invaded cells from the irradiated cellєs and its offspring. $^{***}P < 0.001$ vs. control.

images of the invaded cells on the outside bottom of Matrigel chamber. It can be seen that the after irradiation the number of invaded cells increased from 0.25 on average of control to 25, indicating a significantly increase of cell invasive ability. Importantly, when the irradiated cells were continuously cultured for 1–2 months, the cell invasive ability was further enhanced along with the culture time. These results clarify obviously that the potentiality of cell malignant transformation could be significantly trigged after long-term LDR of α-particles and it was even further enhanced in the progeny of irradiated cells.

3.3. Influence of long-term LDR on protein expressions

To know what kind of signals being involved in the irradiation-enhanced transformation, the expressions of relevant proteins were examined with Western blot assay. Fig. 2A shows that after 8 × 0.025 Gy α-particle exposure, the protein expressions of p-ERK, p-Akt and p-P38increased to 2.65-, 2.95- and 2.06-fold of control, respectively. When the irradiated cells were continually cultured for 1 month and 2 months, the expressions of p-ERK and p-Akt still had a high level but did not increase with the subculture time, while the expression of p-P38 was dramatic increased to 2.83- and 4.66-fold of control, respectively. It is interesting to see that the increase tendencies of p-ERK and p-Akt are similar to that of cell proliferation and the increase tendency of p-P38 is similar to that of cell invasion shown in Fig. 1. These results indicate that the MAPK and Akt pathways may be involved in the radiation induced development of malignant transformation of human bronchial epithelial cells.

We also checked the function of JNK pathway in the cell transformation and found that the expression of p-JNK was just increased in the 8 × 0.025 Gy irradiated cells. While as time went by, the expression of p-JNK had a remarkably downward tendency compared with the control. Moreover, it was found that p-P66 was a highly expressed intrinsic protein in the Beas-2B cells. After 8 times of 0.025 Gy α-particle irradiation, the expression of p-P66 was reduced to only about 50% of the control. Especially, after 1–2 months of subculture after irradiation, the expression level of p-P66 was further decreased. It has been known that the JNK signaling pathway is interrelated with cell apoptosis and p-P66 is closely related to cell senescence, thus the low expressions of p-JNK and p-P66 in the offspring of irradiated cells suggest that the apoptosis and delayed cell senescence may contribute to the cell over-proliferation induced by long-term LDR of α-particles.

3.4. Role of p38 in the radiation induced cell malignant transformation

Compared with the expressions of p-ERK and p-Akt, the pattern of radiation-induced alteration of p-P38 was much more similar to that of cell invasion, hence p38 appears to be involved mainly in the progress of long-term LDR induced cell malignant transformation. To further verify this hypothesis, we silenced p38 in the Beas-2B cells by transferring the cells with p38 siRNA. Fig. 3A illustrates that knock down of p38 significantly suppressed the adhesive ability of the long-term α-irradiated Beas-2B cells as well as its later generations after 1–2 months of irradiation. In addition, the cell invasion assay shows that the LDR-enhanced cell invasive ability was drastically diminished by p38 siRNA transfection so that the number of invaded cells with p38-siRNA was less than that of non-transferred control (Fig. 3B). This reduction also occurred in the progeny of irradiated cells. It was observed that the p38 siRNA treatment did not completely eliminate the adhesive and invasive abilities of irradiated cells, indicating that other factors such as p-Akt are also necessary for cell transformation.

4. Discussion

The studies on Hiroshima and Nagasaki atomic bomb survivors from different ages and genders who had been exposed with a wide range of radiation doses have supplied authoritative findings

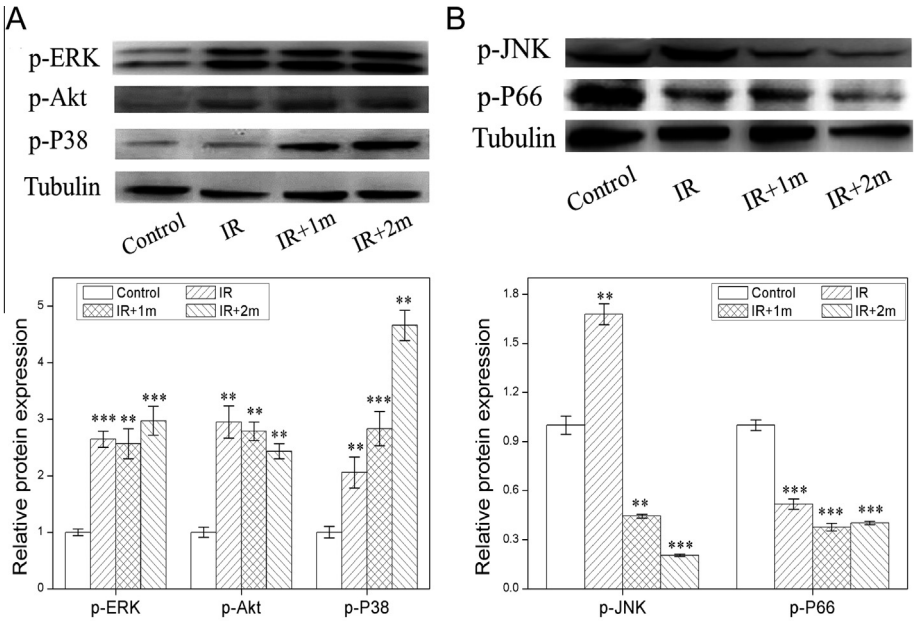

Fig. 2. Radiation effect on the protein expressions in human bronchial epithelial cells. The cells were fractionally irradiated with 0.025 Gy for 8 times and continuously cultured for 1 and 2 months. (A) Western blot images of p-ERK, p-Akt and p-P38 proteins and their relative expression levels in the irradiated cells and its offspring. (B) Western blot images of p-JNK and p-P66 proteins and their relative expression levels in the irradiated cells and its offspring. The relative protein levels were calculated by normalizing its expression to tubulin and then comparing to their corresponding control. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. corresponding control.

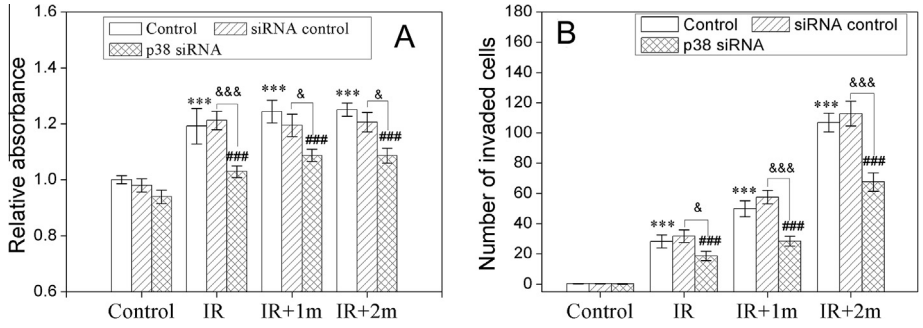

Fig. 3. Influence of p38 siRNA on cell adhesion and invasion. The cells were fractionally irradiated with 0.025 Gy for 8 times and continuously cultured for 1 and 2 months. (A) The adhesive abilities of the irradiated cells and its offspring with or without p38 siRNA treatment. (B) The number of invaded cells from the irradiated cells and its offspring with or without p38 siRNA treatment. $^{***}P < 0.001$ compared to control. $^{\#\#\#}P < 0.001$ compared to scrambled siRNA control. $^{\&}P < 0.05$ and $^{\&\&\&}P < 0.001$ between the indicated groups.

in the cancer risk of LDR with a high statistical significance [17,18]. For instance, a remarkable excess risk of solid tumor incidence was observed even at doses of 0.2–0.5 Gy [19]. Similarly, an epidemiological study by Brenner indicated that an acute exposure at doses of 10–50 mSv or a protracted exposure above 50–100 mSv of ionizing radiation could increase the risk of cancer occurrence [20]. Our present results show that the long-term fractional LDR as low as 25 mGy of α-particles could promote cell proliferation and enhance the abilities of cell adhesion and invasion. Since over-proliferation with high adhesive and invasive abilities are important features of malignant transformation, it is easy to understand that this long-term LDR of α-particles has a high incidence of cancer induction.

What's the mechanism of radiation-induced malignant transformation? It was demonstrated that a 75 mGy X-ray irradiation could stimulate the activation of MAPK/ERK pathway which leads to an accelerated proliferation of rat mesenchymal stem cells [21]. While a sustained activation of JNK was essential for apoptosis induction [22]. So it can be expected that when the cells have a high activation of p-MAPK or p-ERK together with a low expression of p-JNK, the cell proliferation rate could be accelerated. This deduction is consistent with our findings that, after the long-term fractional low dose of α-irradiation, the expressions of p-ERK and p-P38 were enhanced but the expression of p-JNK was decreased in the offspring of irradiated cells with an excess proliferation rate.

Our results indicate that p-P38 and p-Akt may have more important roles in radiation induced malignant transformation since the alterations of their expressions had good consistency with the multiple LDR-enhanced cell proliferation and the abilities of cell adhesion and cell invasion. It has been known that both P38/Akt and PI3K/Akt signaling pathways are associated with malignant transformation of a variety of cell types [23]. It was found that a single dose of 75 mGy or multiple 25 mGy (25 mGy daily for 3 days) of X-rays stimulated Akt phosphorylation in the kidney of mice, while single exposure of 25 mGy failed [24]. Over expression of p-Akt is probably a consequence of repetitive exposure to low dose irradiation. Indeed, PI3K/Akt signaling pathway participates in the regulation of cell proliferation, differentiation,

survival and migration. The abnormal activation of this pathway not only leads to cell malignant transformation but also closely relates to the subsequent processes of migration, adhesion, angiogenesis and extracellular matrix degradation of tumor cells and metastases [25,26].

Recent evidence suggests that p38/MAPK plays significant role in the invasion and/or motility of various cells. Kim et al. reported that the activations of p38 and ERK were both involved in H-ras-mediated cell migration and invasive phenotypes in MCF10A human breast epithelial cells, while stimulation of ERK alone is not adequate in the noninvasive/nonmigrative N-ras MCF10A cells. Thus it is p38 kinase that plays as a pivotal signaling molecule diversely regulated by H-ras and N-ras [25,27]. In addition, the latest research demonstrated that low-dose γ-radiation induced cell migration and invasion mediated by p38-MAPK could be regulated by Cx43 in the human tumorigenic cell lines, and knock-down Cx43 expression intensely inhibited cell migration and p38 activation [28]. Importantly, p38 has been found to be over-expressed in the Chernobyl cystitis patients living in the radiation contaminated areas [29], indicating a high level of p-P38 may lead to cancer susceptibility.

$P66^{Shc}$ is one member of ShcA family adapter proteins [30]. Our data showed that the level of p-P66 became much lower in the over grown cells suffered from long-term LDR of α-particles, which means that p66 negatively regulated cell proliferation and thus should contribute to cell senescence. Migliaccio et al. have given direct evidence in the relationship between $p66^{Shc}$ and cell senescence and they found that mice with knockdown of $p66^{Shc}$ gene could still survival and even with a 30% increase in life span [31]. Further efforts found that the different expression level of $p66^{shc}$ phosphorylated at Ser36 in mice correlated with age positively [32]. Recently, Ma et al. put forward that in both normal and malignant cells, the absence of $p66^{Shc}$ activated Ras expression and cause the disable of anoikis primarily [33]. Furthermore, due to the lacks of $p66^{Shc}$, retinoblastoma (pRB) and bypass anoikis, some cancer cells showed aggressive metastatic behavior, while re-expression of $p66^{Shc}$ could restore anoikis and restrain mouse Lewis lung carcinoma cells' metastases *in vivo*. Reviewing for our experimental results, $p66^{Shc}$ may negatively regulate the progress of malignant transformation to the long-term LDR exposed cells.

Accordingly, the present study provides novel evidence that the long-term α-irradiation with multiple doses of 25 mGy has high potentiality in malignant transformation of human bronchial epithelial cells. Radiation-activated MAPK/Akt pathway and radiation down-regulated p66 are crucial for the development of malignant transformation. Further deeper insight into the molecular mechanism of malignant transformation may help in the risk evaluation of fractional LDR and opens up new approaches to cell radioprotection.

Conflict of interest

The authors declare that there are no conflict of interest.

Acknowledgments

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