RESEARCH ARTICLE

Involvement of Cdc25c in Cell Cycle Alteration of a Radioresistant Lung Cancer Cell Line Established with Fractionated Ionizing Radiation

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Abstract

  Cancer patients often suffer from local tumor recurrence after radiation therapy. Cell cycling, an intricate sequence of events which guarantees high genomic fidelity, has been suggested to affect DNA damage responses and eventual radioresistant characteristics of cancer cells. Here, we established a radioresistant lung cancer cell line, A549R , by exposing the parental A549 cells to repeated γ-ray irradiation with a total dose of 60 Gy. The radiosensitivity of A549 and A549R was confirmed using colony formation assays. We then focused on examination of the cell cycle distribution between A549 and A549R and found that the proportion of cells in the radioresistant S phase increased, whereas that in the radiosensitive G1 phase decreased. When A549 and A549R cells were exposed to 4 Gy irradiation the total differences in cell cycle redistribution suggested that G2-M cell cycle arrest plays a predominant role in mediating radioresistance. In order to further explore the possible mechanisms behind the cell cycle related radioresistance, we examined the expression of Cdc25 proteins which orchestrate cell cycle transitions. The results showed that expression of Cdc25c increased accompanied by the decrease of Cdc25a and we proposed that the quantity of Cdc25c, rather than activated Cdc25c or Cdc25a, determines the radioresistance of cells.

Keywords: Radioresistance - cell cycle checkpoint - G2-M arrest - Cdc25a - Cdc25c - lung cancer cells

Introduction

  Radiotherapy, often coupled with surgery and chemotherapy, plays a critical role in the management of non-small cell lung cancer (NSCLC). However, radioresistance, including intrinsic radioresistance before treatments and acquired radioresistance during radiotherapy, has limited the ability of radiotherapy to kill tumor cells and often leads to local recurrence and metastasis. Although tremendous progress has been made to elucidate the molecular mechanisms of radioresistance, such as p53 (Lee et al., 1993), ataxia telangiectasia mutated [ATM] (Tribius et al., 2001), epidermal growth factor receptor [EGFR] status (Liang et al., 2003), they remain largely obscure due to the complex genetic cellular response to radiation and involvement of a large number of genes.

  Cell cycle, consisting of four distinct phases: gap 1 (G1), synthesis (S), gap 2 (G2) and mitosis (M), is an intricate sequence of events which enable cells to grow and replicate without disrupting the genomic integrity. Cell cycle checkpoint is thought to prevent cells from replication or undergoing mitosis in the presence of

DNA damage caused by oxidative stress such as ionizing radiation (IR), genetoxic chemicals and ultraviolet (UV light). When cell cycle checkpoint is activated, progression through the cell cycle is halted to allow cells to repair damage. Accumulated evidence has suggested that cell cycle may function as a regulatory process in radioresistance. Krueger et al have showed that abrogation of the checkpoint by inhibition of checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2) increased low-dose radiosensitivity (Krueger et al., 2007). In metastatic brain tumors,the expression levels of phosphorylated-Chk1 proteins tend to be higher in radioresistant cancer than in radiosensitive cell lines (Seol et al., 2011). Lim et al have proved that cell-cycle checkpoint abnormalities may contribute to the radioresistance of glioma-initiating cells and may be suitable targets for therapy (Lim et al., 2012).

  The cell cycle division cycle 25 (Cdc25) protein phosphates, including three family proteins, Cdc25a, Cdc25b, Cdc25c, are critical components of cell engine that function to drive cell cycle transitions by dephosphorylating and activating cyclin-dependent kinases (Cdks) (Lee et al., 2011). Cdc25a is the master

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regulator of the G1-S transition, S-phase transition and G2-M progression, whereas Cdc25b and Cdc25c have more restricted roles in G2-M progression (Iliakis et al., 2003).

Overexpression of Cdc25 family proteins, mostly Cdc25a, has been reported in a variety of human cancers, including breast, liver, esophageal, endometrial, non–Hodgkin lymphomas and correlates with more aggressive disease and poor prognosis in some cancers and leads to genetic instability in mice (Boutros et al., 2007; Ray et al., 2008). Zhao et al. (2012) found that knockdown of Cdc25c induced a significantly more distinct hyper-radiosensitivity and prevented the development of induced radioresistance. However, few studies have been carried out to explore the different roles of Cdc25a and Cdc25c in mediating the radioresistance through the activation of cell cycle checkpoint.

In the present study, we established a radioresistant lung cancer cell line by fractionated γ-ray irradiation and found the roles of cell cycle transition in mediating the radioresistance . Moreover, the expression of Cdc25 was examined and the results revealed that Cdc25c was upregulated and could be a potentially marker of radioresistance.

Materials and Methods

Cell culture

Human lung bronchioloalveolar adenocarcinoma A549 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells are cultured in RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum, 100 UI/mL penicillin, 100 μg/mL streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO_2.

Irradiation

Exponentially growing A549 cells were exposed to γ irradiation generated by a 60Co source (60 Co therapeutic machine, GWXJ80 type, Chengdu, China) at a dose of 2Gy (dose rate 61.3 cGy/min, 35 cm×35 cm irradiation field, SSD 580 cm). Block (3-cm-thick) was placed on the top of flasks .When the irradiated cells reached an exponential growth phase, the next 2 Gy was delivered. The cells were irradiated repeatedly in the same way to a total dose of 60 Gy in 30 fractions. After the final irradiation, the radioresistant cell line, designated A549R, were isolated and maintained in culture for more than 30 passages.

Colony formation assay

Exponentially growing A549R cells and parent A549 cells were seeded into 6-well plates with 200–1000 cells/plate according to the variable doses of irradiation. On the following day the cells were irradiated and incubated for 2 weeks at 37°C in a 5% CO_2 environment to allow the colony formation. The colonies were fixed with pure ethanol and stained with 1% crystal violet. Colonies containing more than 50 cells were counted as one survived colony. Surviving fractions were calculated by normalizing to the plating efficiency of the absent control cells. The triplicate experiments were done independently.

The data were fitted into single-hit multi-target formula: $S=1-(1-e-D/D0)^N$, where S is the fraction of cells surviving a dose, D0 was used as a parameter to indicate the amount of irradiation required to reduce the survival fraction to approximately 0.37 from the survival curve. The "quasi-threshold dose" or Dq, which is the intercept of the extrapolated high dose, was also calculated. N is referred to the extrapolation number which is a parameter to measure the width of shoulder of the survival curve.

Flow cytometry analysis for cell cycle

Cells in the exponential phase of growth were irradiated at different doses (0 Gy, 4Gy) at 37°C and 12 hours after the irradiation the cells were harvested and washed twice with PBS, followed by fixation in 70% cold ethanol at 4°C overnight. The cells were then washed with PBS and incubated with propidium iodide (PI) for 30 minutes at 37°C. Cell cycles were analyzed using a CytomicsTM FC 500 (Beckman Coulter, Miami, FL, USA).

Quantitative RT-PCR

Total RNA was extracted from A549 and A549R cells using Trizol reagent (Invitrogen Carlsbad, CA, USA) according to the protocol provided by manufacturer. In brief, cDNA was synthesized from 2 μg of total RNA using Revert AidTM first strand cDNA Synthesis kit (Fermentas, Hanover, MD, USA). DNA primer sequences were designed as follows: GAPDH:5'-TGGAAGGACTCATGACCACA-3' (forward) and 5'-TTCAGCTC AGGGATGACCTT-3' (reverse).Cdc25a: 5'-TCTGAAGAATGAGGAGGAGAC-3' (forward) and 5'-AAACAGCTTGCATCGGTTGT-3' (reverse).Cdc25c: 5'-TGGTGGGC CAAACACTATCC-3' (forward) and 5'-ATCGTTGGGCTCGCAGATCACC-3' (reverse). The amplification conditions were 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s with an additional 7 min 72°C extension.Cdc25a and Cdc25c mRNA expression of different group were normalized to GAPDH. All experiments were repeated at least three times.

Western blotting

Cultured cells were rinsed twice with phosphate buffered saline (PBS) and mixed with 200 μl of lysis buffer (Beyotime Biotech, Nantong, Jiangsu, China). The cells in lysis buffer in the dish were removed using a scraper and transferred to an Eppendorf tube. The cells were homogenized and centrifuged 1,2000 rpm for 10 min and the supernatant were stored at -20°C. The concentration of the total protein was determined using a BCA protein assay kit (Beyotime Biotech,Nantong, Jiangsu, China). The protein extracts (50μg) were incubated in loading buffer (60 mmol/L Tris-Hcl, 25% Glycerol, 2% SDS, 14.4 mmol/L Mercaptoethanol, 0.1% Bromophenol blue) and boiled for 5 min. Proteins were separated by sodium-laurylsulfate-PAGE and transferred electrophoretically to PVDF membranes (Bio-Rad, Hercules, CA, USA). Non-specific binding sites were blocked by incubating the PVDF membrane for 1 h at 37°C with 5% nonfat dried milk in Tris-buffered saline containing 0.05%

Table 1. Survival curve Parameters Fitting the Data into Single-hit Multitarget Model

Cell line	D0	Dq	SF2	N
A549R	2.07±0.02*	0.78±0.04*	0.48±0.01*	1.37±0.02*
A549	1.64±0.06	0.40±0.06	0.35±0.01	1.24±0.04

D0, the mean lethal dose is the dose on the straight-line portion of the survival curve to decrease the survival to 37%; Dq, quasi-threshold dose is the intercept of the extrapolated high dose; SF2, survival fraction in a dose of 2 Gy; N, the extrapolation number is a measure of the width of shoulder; *$P<0.05$ is considered significant

Tween-20 (TBST). Membranes were incubated with each primary antibodies (1:1000 dilution), including anti-β-actin (Millipore,Bedford,MA,USA), anti-Cdc25a (Abcam,Cambridge, MA, USA), anti-Cdc25c (Millipore, Bedford, MA, USA), either for 1 h at room temperature or overnight at 4°C. Then, membranes were additionally washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Beyotime Biotech, Nantong, Jiangsu, China). Bands were visualized using an enhanced chemical luminescence system (ECL, Beyotime Biotech, Nantong, Jiangsu, China).

Statistical analysis

All experiments were repeated at least three times with independent samples. Results are given as means±standard deviation. Group comparisons were made by Student's t-test. Statistical analysis was performed using software SPSS 13.0 (Chicago, IL, USA) and Graphpad prism 5.0 software (San Diego, CA. USA). $P<0.05$ was considered to be statistically significant.

Results

*The effect of irradiation on cellular morphology in A549 cells*

After irradiating the parental A549 cells with a total dose of 60Gy, the irradiated A549 cells demonstrated changes in their morphologies. As shown in Figure 1, the parental A549 cells typically exhibited a spindle-like appearance and tight cell-cell junction. The A549 cells with irradiation developed a cobblestone-like morphology, disrupted cell-cell adhesion and more scattered appearance.

Identification of radioresistance of A549 cells receiving radiation

Figure 2 depicts the cell survival curves derived from colony formation assay for parental A549 cells and A549 cells with irradiation. The survival curve parameters are shown in Table1. For A549 cells receiving a total dose of 60 Gy, the radiosensitivity parameters were as follows: D0=2.07 Gy, Dq=0.78 Gy and the extrapolation number (N)=1.37. For parental A549 cells, D0=1.64Gy, Dq=0.40Gy, and N=1.24. The survival fractions at 2 Gy (SF2) were 48% for A549 cells with radiation and 35% for parental A549 cells. The radiobiological parameters in A549 cell line are significantly lower than the A549 cells with a total dose of 60Gy irradiation ($P<0.05$), thus A549
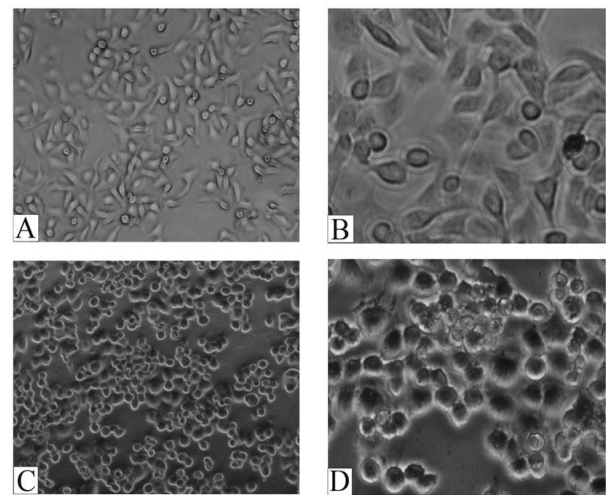
Figure 1. Morphological Changes of Parental A549 and A549 with Irradiation. The photographs were taken at a magnification of 40×(A,C) and 100×(B,D) by a light microscope. A and B depict parental A549 cells .B and D depict A549 cells with irradiation of a total dose of 60Gy
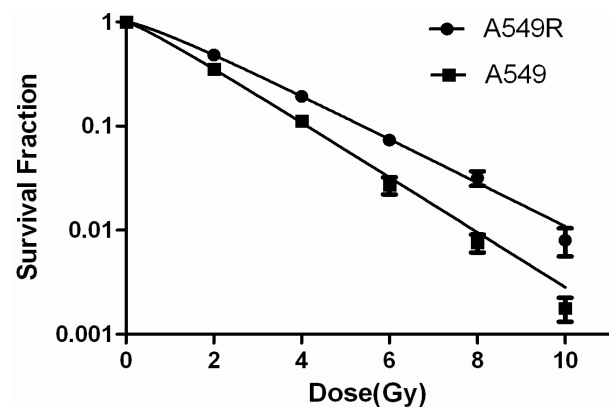

Figure 2. Survival Curves of Parental A549 Cells and A549 Cells with Radiation of a Total Dose of 60Gy. Results from three independent experiments were normalized to the controls and are shown as the mean±stand error

cells with irradiation was considered as radioresistant (designated as A549R).

Cell cycle analysis

The cell's relative radiosensitivity could be regulated by cell cycle phase with cells being most radiosensitive in the G2-M phase, less sensitive in the G1 phase, and least sensitive during the S phase (Sinclair et al., 1966). Therefore, we examined the cell cycle distribution of A549 and 549R with/without radiation of 4Gy using flow cytometry for DNA content after staining the cells with propidium iodide (Figure3A-D). The results in Figure 3E show that compared to parental A549 cells, A549R cells showed a statistically significant decrease in G1 phase and increase in S and G2/M phase. This phenomenon may suggest a strong relationship between radioresistance and the ratio of cells in S phase. As shown in Figure 3F and 3G, a single fraction of 4Gy induced a G2-M arrest in both cell lines, however, A549R showed a different reaction in G0-G1 phase in response to irradiation. A G0-G1 arrest was observed in A549 cells which was the conventional tragedies in reaction to irradiation, while in A549R the
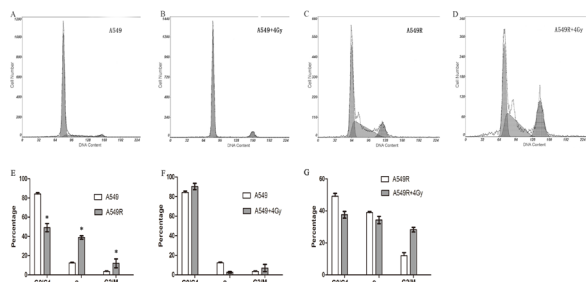
Figure 3. The Cell Cycle Distribution of the A549 Cells and the A549R Cells . A ,C: Flow cytometry analysis of A549 cells and A549R cells. B , D : Flow cytometry analysis of A549 cells and A549R cells with irradiation. Cell cycle analysis was preformed 24 hours later after 4Gy irradiation. E , F , G: Column graph showing the results from flow cytometry analysis. The y axis displayed the percentage of cells by propidium iodide staining intensities of DNA corresponding to G0/G1, S and G2/M, as shown on the x axis. Results are the mean±stand error of three independent experiments

Figure 4. The mRNA Expression and Protein Expression of Cdc25a and Cdc25c. A, RT-PCR results for Cdc25a, Cdc25c and GAPDH derived from A549 and A549R cells. Results from three independent experiments were normalized to the controls and are shown as the mean±standard error. B,C , western blotting results for Cdc25a,Cdc25c and β-actin derived from A549 and A549R cells. Representative immunoblots are shown

cells in G0-G1 phase decreased which indicated the malfunction of G1 phase checkpoint .

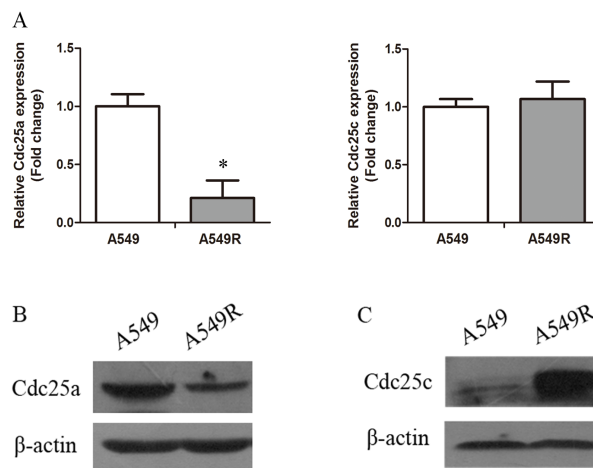*Identification of expression of Cdc25a and Cdc25c*

To examined the mechanisms underlying the impact of cell cycle in A549 and A549R cells, we checked the expression of Cdc25a and Cdc25c which orchestrate the intra-S-phase checkpoint and G2-M checkpoint . Real-time PCR was carried out to examine the gene expression and western blotting was performed to examine the protein expression. We observed a significant decrease in both gene and protein expression of Cdc25a and Cdc25c decreased only in expression level which was shown in Figure 4.

Discussion

In the present study, A549R, a radioresistant cell line, was obtained by exposing the parental A549 cells to repeated fractions of a total dose of 60 Gy . We especially focused on the influence of cell cycle redistribution on radioresistant characteristics of cancer cells. We then unveiled a possible mechanism whereby G2-M checkpoint is strongly correlated with the acquired radioresistance of A549R.The expression of Cdc25a and Cdc25c were examined and the results revealed that the Cdc25c was upregulated and could be a potentially marker of radioresistance.

It is widely accepted that cells is most radiosensitive in the G2-M phase, less sensitive in the G1 phase, and least sensitive during the S phase. In our study, A549R cells are characterized by the accumulation of cells in S phase and G2-M phase and decrease in G1 phase compared to the parental cell line.

This phenomenon indicates that a long term irradiation seems to induce a permanent intra-S-phase arrest and a G2-M phase arrest which is different from the transient arrest caused by single irradiation. However, these observations have raised concerns about the relative radiosensitivity of cells in different phase and the roles of cell cycle transition and checkpoint in mediating the radioresistance.

Nicolay et al studied the effect of pol η on radioresistance and found that loss of pol η could result in increased resistance to irradiation by accumulating the cells in S phase (Nicolay et al., 2012). Zhu et al reported that transition from the radiosensitive G1 to radioresistant S phase of the cell cycle is mediated by the decreased expression of HIF-1a and p27Kip1 in perinecrotic/pimonidazole-positive regions of malignant solid tumors and has an important role in the biological radioresistance of cancer cells (Zhu et al., 2012).Shimura et al demonstrated that acquired radioresistance to tumor cells induced by a moderate level of long-term fractionated radiation is caused by DNA-pK/AKt/GSK3β- mediated cyclinD1 overexpression, thus leads to forced progression of the cell cycle to S phase by activating Cdks and invalidating G1-S checkpoint (Shimura et al., 2010).

Although progress in the past several years has unraveled some of the underlying mechanisms of S-phase-dependent radioresistance, the specific molecular mechanism behind this resistance remains largely unknown. In our study, the cell cycle changes of A549 cells raise the contradiction about radiological dogma of cell cycle dependent radiosensitivity mentioned above. If the acquired radioresistance of A549R is strongly related to accumulation of cells in S phase, why has the cells in G2-M phase increased in A549R cells.

Here, we provide a possible explanation by considering the DNA repair pathways involved in S and G2 phase. DNA double strand breaks (DSBs) are considered the most cytotoxic lesions induced by ionizing radiation. The homologous recombination (HR) and non-homologous end-joining (NHEJ) pathways are identified as two main mechanisms involved in the repair of DSBs. HR can only be carried out during S or G2 phases of the cell cycle for the long sequence homology requirements

while NHEJ can take effect in all phases. Recently, the S-phase-dependent radioresistance has been linked to the homologous recombination and cells deficient in components of the homologous recombination pathway show different degrees of radioresistance in S phase (Tamulevicius et al., 2007). Thus, HR could play a role in cell phase dependent radioresistance and further studies are needed to illuminate the molecular mechanism behind them.

Another question arising from the present study is the relative contribution of the power to push and the power to block. If cells in G1 phase are driven to S phase due to the involvement of G1-S transition, thus increase the proportion of cells in S phase, the same result could also be achieved by the intra-S phase checkpoint. Although numerous studies focused on the importance of G1-S transition to the accumulation of cells in S phase and G2-M phase, one should not ignore the importance of cell cycle checkpoint activated by long term irradiation.

The intra-S-phase checkpoint could prevent bulk replication by largely transient, reversible inhibition of the origin firing or slowing replication fork progression in response to DNA damage (Willis et al., 2009), while G1-S checkpoint could prevent cells from traversing through G1 by inducing sustained and sometimes even permanent G1 arrest. There are two main pathways responsible for the G1-S phase checkpoint in mammalian cells, ATM (ATR)/CHEK2 (CHEK1)-P53/MDM2-P21 pathway and ATR (ATM)/CHEK1 (CHEK2)-Cdc25a pathway. The CHK1/CHK2–Cdc25a checkpoint is implemented rapidly, independently of p53, and it delays the G1/S transition only for a few hours,whereas the sustained p53-dependent mechanism prolongs the G1 arrest, even induces permanent G1 arrest (Kastan et al., 2004). The A549 is a cell line with wild-type p53 status which could trigger both pathways in response to DNA damage.

The full activation of p53/p21 pathway requires several hours after irradiation for the reason that it requires posttranslational modifications and subsequent transcriptional activation and this could allow cells enter S phase with high double-strand breaks levels, in spite of the fact that Cdc25A pathway is activated more rapidly. Several studies have demonstrated that irradiation of middle or late G1-phase cells, even with un physiologically high doses, does not abolish S-phase entry for 4–6 h after IR (Gadbois et al., 1997; Linke et al., 1997; Cann et al., 2006; Deckbar et al., 2010). Since there is a delay effect of G1-S checkpoint after irradiation, the cells with genomic lesions could easily escape from the cell cycle checkpoint .

The G2-M checkpoint prevents cells from initiating mitosis when they experience DNA damage during G2 phase or when they progress into G2 phase with some unrepaired damage inflicted during previous S and G1 phase. The similar insight into the escape mechanism of G2-M checkpoint was provided by recent studies in spite of the fact that G2-M checkpoint is activated rapidly compared to the G1-S checkpoint. The G2-M checkpoint transiently arrests heavily damaged cells in G2 phase to provide time for repair, but does not abort cell division in the presence of unrepaired DSBs, thus allowing cells harboring a significant number of DSBs to enter mitosis.

We provide a hypothesis that the cells harboring genomic lesions and escaping from the cell cycle checkpoint, mainly G1-S checkpoint, could evolve to the radioresistant phenotype which was similar to those observed from our study. The cells with genomic lesions will induce a more strong rebound of cell cycle checkpoint, preventing more cells from progression and finally lead to the accumulation of cells in S phase and G2-M phase. Taken together, both contribution of the escape mechanism and upgraded checkpoint are required to develop the radioresistant phenotype. However, the results observed in our study raise the question of which checkpoint plays a more important role in mediating the radioresistance, the intra S phase checkpoint? G2-M checkpoint or both?

A normal cell reacts to irradiation with the induction of G1-S arrest and G2-M arrest in order to ensure genome integrity. The different reactions of A549 and A549R to 4 Gy irradiation provides a clue to the exploration of possible mechanisms. The exposure of 4 Gy irradiation induces a significant increase of G2-M phase cells, this indicates that the G2-M checkpoint performs well when confronted with irradiation. In human leukemic cell lines the long cell cycle arrest in G2 phase is responsible for relative radioresistance of these cells, because it allows time for repair of radiation damage (Vávrová et al., 2004). In meningioma cells the radioresistance is closely correlated with the induction of G2-M arrest (Gogineni et al., 2011). Thus, we propose that the G2-M arrest could be another important factor involved in the acquired radioresistance.

On the contrary ,the malfunction of G1-S checkpoint and an insignificant change of S phase proportion lead to the exploration of the molecular mechanism behind the cell cycle change. Thus, the expression of Cdc25a and Cdc25c were carried out. Cdc25a positively regulates G1-S transition, S-phase transition and G2-M progression, whereas Cdc25c plays a more important role in G2-M phase checkpoint through the activation of cyclin B/Cdk1.

We checked the expression of Cdc25a and Cdc25c and found that compared to parental cell line the expression of Cdc25a in A549R was downregulated in both mRNA and protein level, but Cdc25c only show increase in protein level. The decrease of Cdc25a expression shows inconsistencies with the G1-S phase redistribution. If Cdc25a was downregulated, why has the proportion of cells in G1 phase decreased. Given the central role of p53/p21 pathway in regulating G1-S phase transitions, it is perhaps not surprising that there exist another pathway involved in this discrepancy.

The increase of Cdc25c expression seems inconsistent with the accumulation of cells in G2-M phase. The DNA lesion caused by irradiation or DNA-damaging agents can activate ATM, then the active ATM phosphorylates Chk2 which, in turn, inhibits phosphatase activity of Cdc25c and contributes to its cytoplasmic sequestration by an interaction with 14-3-3 proteins. The inhibition of Cdc25c maintains inhibition/inactivation of CyclinB1/Cdk1, thus arresting cells in G2-M phase.

The increase of Cdc25c expression implies that

translocation of Cdc25c plays a more important role in G2-M phase arrest and the Cdc25c which remains in cytoplasm reflects the relative radioresistance. When cells harboring a plethora of genomic lesions enter G2 phase, the cells which have more Cdc25c stand by show robust and efficient action to repair the DNA damage. Thus ,We provide a possible mode that G2-M cell cycle arrest plays a predominant role in mediating the radioresistance of A549R cells. Given the fact that the decrease of Cdc25a in mRNA and protein level shows some discrepancies with the cell cycle redistribution, we propose that the quantity of Cdc25c ,not the activated Cdc25c or Cdc25a, determines the radioresistance of cells.

Even though this studies unravel several models about the radiaresistance and the expression of Cdc25a and Cdc25c, several problems remains to be proven. First ,the Cdc25 family proteins need to be phosphorylated in order to regulate the downstream proteins. The present study focused on the importance of total expression of proteins, we should not ignore the importance of phosphorylation of Cdc25 family proteins. Second, the sub-localization and quantity of Cdc25c in A549 and A549R need to be specified and the factors which influence the translocation of Cdc25c could be investigated as a molecular target.

Acknowledgements

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